STUDIES ON THE PHYSIOLOGY OF EARLY PREGNANCY IN ALPACAS

By

ALEXIS JEAN CAMPBELL

A thesis submitted in partial fulfillment of
the requirements for the degree of

MASTER OF SCIENCE IN VETERINARY SCIENCE

WASHINGTON STATE UNIVERSITY
College of Veterinary Medicine

DECEMBER 2015

To the Faculty of Washington State University:

The members of the Committee appointed to examine the thesis of ALEXIS JEAN CAMPBELL find it satisfactory and recommend that it be accepted.

_____
Ahmed Tibary, DMV, Ph.D., Diplomate ACT, Chair

_____
James Pru, M.S., Ph.D.

_____
William Dernell, DVM, M.S., Diplomate ASVS

ACKNOWLEDGEMENTS

I would like to acknowledge the people who made my graduate work possible. First, thank you to my advisor, Dr. Ahmed Tibary, who provided invaluable mentorship, encouragement, and support throughout my training. Thank you also to the rest of my graduate committee, Dr. James Pru, Dr. William Dernell, and Dr. Thomas Spencer (honorary), for providing me the ability to perform my research and offering guidance and assistance.

Thank you to Dr. Lisa Pearson, Ms. Shirley Sandoval, and Mrs. Jamie Harden, who assisted me in the reproductive management and sample collections of the alpacas. Thank you to Dr. Josh Ramsay, who provided clinical analysis of the endometrial histology. Thank you to Mr. Gregory Burns, Ms. Kelsey Brooks, and Mrs. Cindy Pru, who provided technical assistance with the molecular studies.

Thank you to my family, especially my husband Dr. Daniel Evans and my daughter Ms. Sobella Evans, both of whom have been a constant source of strength and encouragement. I could not have completed this process without their support.

Lastly, thank you to the Washington State University College of Veterinary Medicine for supporting my program and continued education and the WSU-Qatar project for partial funding of my research.

STUDIES ON THE PHYSIOLOGY OF EARLY PREGNANCY IN ALPACAS

Abstract

by Alexis Jean Campbell, M.S.
Washington State University
December 2015

Chair: Ahmed Tibary

Camelids are economically important production animals in many areas of the world. Early pregnancy loss is a major cause of reproductive inefficiency. Pregnancy maintenance depends on a timely signaling mechanism called maternal recognition of pregnancy (MRP). This mechanism is not well characterized in camelids. The work presented in this thesis is part of a larger research program to study early embryo development and MRP, as well as factors involved in early pregnancy loss.

In a first study, the incidence and outcome of twin pregnancy in double-ovulating alpacas was evaluated by retrospective and prospective analysis. Our finding indicated that twinning is not rare and may be a cause of early pregnancy loss. The incidence of multiple ovulation approached 30%. There were more twins from bilateral ovulations than from unilateral ovulations. A high rate of spontaneous reduction of twin pregnancies (62.5% of cases within the study) to a singleton occurs early, however a significant proportion of twin pregnancies may result in loss of both embryos (37.5% of cases within the study).

The objective of the second and third study was to compare uterine histology in the left and right uterine horn in non-pregnant and early pregnant alpacas. Uterine tissue samples from pregnant and open females (n=5 per group) taken at 9 and 14 days post-ovulation were evaluated for differences in endometrial and myometrial layer thickness, glandular density and tortuosity, vascularity, and infiltration of inflammatory cells between the left and right uterine horns in pregnant and non-pregnant animals. All groups under progesterone influence showed similar histological features. There were no histological differences between the left and right uterine horns in all groups. Expressions of estrogen receptor alpha

and progesterone receptor were identified within the endometrium using immunohistochemistry. Quantification of these receptors is being conducted.

These studies showed that establishment of pregnancy in the left uterine horn in camelids cannot be explained by histologic differences. A genomic and proteomic study is planned to further investigate the MRP in this species.

TABLE OF CONTENTS

PAGE

APPENDIX

A. Estrogen receptor alpha and progesterone receptor expression in the endometrium of pregnant and non-pregnant alpacas

LIST OF TABLES

LIST OF FIGURES

Chapter 1

Introduction

1.1 Background

Camelidae (or camelids) are economically important in South America, Africa, and the Middle East. Llamas and alpacas have become increasingly popular in other parts of the world including the United States.[1] Reproductive capacity is an important component of production and profitability within the industry. Expanded knowledge on the signaling mechanisms responsible for maternal recognition of pregnancy (MRP) and the physiology of early pregnancy would allow for a better understanding of mechanisms involved in infertility and early embryonic death. In addition, our understanding of the crosstalk between the embryo and the uterus could further advance reproductive technologies, such as embryo transfer.

The luteal phase of the estrous cycle is simultaneously prepared for two conflicting events to occur. Either luteolysis will occur allowing the animal to return to estrus for another opportunity at becoming pregnant, or luteal maintenance for pregnancy will result.[2] MRP is a physiological process whereby the conceptus signals its presence to the maternal system resulting in a prolonged lifespan of the corpus luteum (CL).[2, 3] The CL is necessary for the production of progesterone, which is required to maintain a uterine environment consistent with embryo survival. In domestic large animal species the conceptus must provide a timely biochemical signal to prevent luteolysis. This is specifically important in ruminant, camelid, swine, and equine species because the pre-attachment period of the embryo within the uterus is long when compared to other species, such as primates.[4] Maternal recognition signaling and CL maintenance can be classified into one of 3 categories: luteotrophic, antiluteolytic, or luteostatic. Luteotrophic maternal recognition signaling directly promotes luteal function. Antiluteolytic signaling prevents the release of luteolytic prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and luteostatic signaling alters the distribution of $PGF_{2\alpha}$ so that it does not reach the CL in an amount necessary to result in loss of the CL.[2, 4, 5]

While some domestic species, such as pigs and ruminants,[6, 7] have well defined MRP signaling pathways, other domestic species, including camelids, are still under investigation. The reproductive anatomy and physiology of the female camelid is dramatically different compared to ruminants.[8] Alpacas are induced ovulators, with ovulation occurring equally from both the right and left ovary.[1, 8] Although fertilization can occur in either side, embryos migrate to the left uterine horn for attachment.[9] Camelids are unique in the fact that greater than 98% of pregnancies are carried in the left uterine horn.[1]

In camelids, the CL reaches a maximum size 8-9 days post-breeding and luteolysis occurs and is complete 10-11 days post-breeding if a pregnancy is not present.[10] Therefore in these species MRP has to take place prior to Day 9-10 after mating and fertilization. Embryos enter the uterus approximately 7 days after mating and rapidly elongate after migration to the left uterine horn on Day 10-11.[1, 9, 11]

In ruminants, the antiluteolytic mechanism of maternal recognition involving the secretion of interferon tau (IFNt) has been well established.[6, 12] While an interferon-like protein has not been identified [13], there is speculation that estrogen does play at least some role in MPR in camelids. In pigs, it has been well demonstrated that estrogen plays a significant role in MRP. Pig conceptuses secrete an abundance of estrogen during early pregnancy preventing the secretion of $PGF_{2\alpha}$ into the uterine vein by redirecting its release into the uterine lumen where it is metabolized to a non-luteolytic form.[6, 14] In camelids, pre-implantation blastocysts produce increasing amounts of estradiol-17β and exhibit high aromatizing activity.[15] Although this increase in estradiol has been documented, its potential role in MRP is not fully understood.

Due to the fact that the majority of pregnancy loss in camelids is experienced prior to 60 days of gestation, a more thorough understanding of the MRP signaling and the physiology of early pregnancy is paramount both scientifically and clinically.

1.2 Rationale

Early pregnancy losses can cause significant financial loss within the industry, and identification of the MRP signaling pathway would provide better case management of early pregnancy loss or infertility in camelids. The majority of studies on the physiology of early pregnancy have been performed in other domestic large animal species, specifically ruminants. One of the major recent discoveries in domestic species related to MRP and early embryonic loss is the role that genetic expression in the uterus plays in the establishment of pregnancy. There is limited information available regarding areas associated with early pregnancy in camelids and an understanding of uterine genetic expression during pregnancy in alpacas has not been established. The alpaca genome has been sequenced in recent years [16], and although still in its infancy, identification of genetic markers can further reproductive research, reproductive health, and fertility. Identification of, understanding of expression of, and localization of factors associated with MRP in camelids may provide information beneficial to advancements of reproductive veterinary medicine.

The work presented in this thesis is part of a research effort to study early embryo development and maternal recognition of pregnancy, as well as factors involved in early pregnancy loss.

1.3 Hypotheses

Two hypotheses were developed:

Multiple ovulations are relatively common resulting in twin conception followed by reduction to a singleton or complete loss of pregnancy.

Embryo migration and elongation triggers gene expression changes and maintenance of pregnancy in camelids. It is expected that there is a difference in the genetic expression and mRNA production in the pregnant and non-pregnant endometrium.

1.4 Objectives

Four studies were designed to test these hypotheses.

The first study: *Double ovulation and occurrence of twinning in alpacas (V. pacos)* (Chapter 3) was designed to link the clinical observation of multiple ovulations to the observation of multiple ovulations within the experimental design. Data from a retrospective study and the prospective study were evaluated to determine the incidence and characteristics of multiple ovulations in female alpacas.

The second, third, and fourth studies used reproductive tract samples collected from pregnant and non-pregnant alpacas during critical times of early pregnancy to further decipher factors associated with MRP signaling and the crosstalk between the embryo and the uterus during early pregnancy in camelids.

In the second study: *Histologic evaluation of uterine endometrium in the pregnant and non-pregnant alpaca* (Chapter 4) the objective was to determine if there are any significant structural differences between the left and right uterine horn. Histological comparison between the left and right uterine horns was made at 9 and 14 days post-mating or induction of ovulation in both pregnant and non-pregnant female alpacas.

In the third study: *Estrogen receptor alpha and progesterone receptor expression in the endometrium from pregnant and non-pregnant alpacas* (Appendix A), the objective was to evaluate expression and localization of estrogen receptor alpha and progesterone receptor within the endometrium at 9 and 14 days post-mating or induction of ovulation in both pregnant and non-pregnant female alpacas.

In the fourth study: *RNA sequence mapping of endometrium from pregnant and non-pregnant alpacas,* the objective was to identify specific genes that play a role in the conceptus-endometrium crosstalk during MRP, followed by proteomic analysis to identify potential conceptus signaling mechanisms involved in MRP in camelids. While the alpaca genome has been sequenced in previous years, the quality of the genome is such that it was not possible to perform sequencing of the endometrial RNA samples.

Alternatives were considered, but the timeframe with which to complete the project was beyond the scope of the current thesis.

1.5 Research design and methods to study maternal recognition of pregnancy in alpacas

Twenty healthy, non-pregnant female alpacas (Table 1.1) were used for study. Animals enrolled were assessed through an initial physical examination and breeding soundness examination. The sample size was chosen to enable detectable differences in histological changes, regulation of steroid receptors, endometrial genetic expression, and proteomics associated with pregnancy. Alpacas were maintained in stalls or outdoor paddocks and fed grass hay twice daily. Water was available ad libitum. All procedures were in accordance with Washington State University Animal Care and Use Committee guidelines.

All females were examined by transrectal ultrasonography using an Aloka SSD500 ultrasound machine (Hitachi Aloka, Medical, Ltd, Wallingford, CT, USA) equipped with a 7.5 MHz linear array transducer mounted on a polyvinyl chloride (PVC) extension rod (Figure 1.1). All females were mated to a proven fertility male within 12 hours following visualization of at least one large follicle measuring between 7 and 10 mm in diameter (Figure 1.2). All females received an injection of 50 μg of GnRH (Cystorelin®, Merial, Duluth, GA, USA) subcutaneously immediately after mating. Following mating, females were assigned randomly to 4 groups (Figure 1.3). Group I and II females (n=10) were scheduled for postmortem collection of the reproductive tract and embryos at either Day 9 (n=5) or Day 14 (n=5) post-mating respectively. Group III and IV females (n=10) were scheduled for pregnancy diagnosis on Day 14 post-mating. All females in group III and IV received an injection of 250 μg of cloprostenol (Estrumate®, Merck Animal Health, De Soto, KS, USA) intramuscularly after pregnancy diagnosis. Following luteolysis the follicular wave was monitored again and ovulation was induced with GnRH when the largest follicle reached at least 7 mm in diameter. Ovulation was induced with administration of an injection of 50 μg of GnRH

subcutaneously. Group III and IV females were scheduled for postmortem collection of the reproductive tract at either Day 9 (n=5) or Day 14 (n=5) post-administration of GnRH respectively.

Females in all groups were examined 7 days after mating and GnRH treatment or GnRH treatment alone, to determine presence and number of CL (Figure 1.4). Group II females collected at 14 days after mating were also examined for pregnancy diagnosis on Day 13 or Day 14 after mating (Figure 1.5). Blood samples from all females were collected after mating and GnRH treatment or GnRH treatment alone and prior to euthanasia by jugular venipuncture. Samples were centrifuged and serum was stored at -80°C for hormone assays to be performed. All females were euthanized using an overdose of barbiturates on Day 9 or Day 14. The uteri were collected *en bloc* immediately after euthanasia and flushed as previously described (Figure 1.6).[9]

The ovaries were evaluated for number and side of CL(s) (Figure 1.7). Embryos (Figure 1.8) and collection fluid were frozen in liquid nitrogen. Reproductive tract tissue samples collected included the ovaries, oviducts, uterotubal-junction (UTJ), endometrium, and myometrium. The endometrium and myometrium were sampled in 2 locations (near the tip and at the base) of each uterine horn. Samples were either flash frozen in liquid nitrogen and stored at -80°C or transferred to 10% buffered formalin for 48 hours and stored in 70% ethanol prior to analysis.

Uterine horn tissue samples stored in 70% ethanol following fixation were used for histological study. In cassettes the tissue sections were rinsed in 70% ethanol, 80% ethanol, and 95% ethanol for an hour at each concentration. The tissue sections were then rinsed in 100% ethanol for 3 hours, xylenes for 2 hours, and embedded in paraffin.

For histological evaluation the tissue sections were stained with haematoxylin and eosin (H&E). Sections were evaluated for differences in the ratio between endometrial and myometrial thickness, glandular density and tortuosity, vascularity, and infiltration of inflammatory cells between the left and right uterine horns in pregnant and non-pregnant animals.

An immunohistochemical technique previously described[17] was used to visualize estrogen receptor α (ERα) and progesterone receptor (PR) immunostaining after minor modifications. Please refer to the Appendix for complete details. A general observation of receptor immunostaining for ERα and PR was performed. Further analysis and evaluation to quantify estrogen and progesterone receptor expression, along with hormonal assays evaluating for estradiol-17β and progesterone serum concentrations, is warranted. Our laboratory is currently engaged in a collaborative program to continue evaluation of estrogen and progesterone receptor expression in pregnant and non-pregnant alpacas.

Endometrial tissue samples were processed and RNA was isolated, depleted of ribosomal RNA, and subjected to next-generation sequencing using the Illumina HiSeq 2000 platform (Figure 1.2). The results were to be fed into the Trinity pipeline for de novo transcriptome assembly and differential expression analysis (Figure 1.2). While the alpaca genome has been sequenced in previous years, the quality of the genome is such that it was not possible to perform sequencing of the endometrial RNA samples. Alternatively an even-toed ungulate genome was considered for RNA sequence mapping, but the timeframe with which to complete the project was beyond the scope of the current thesis. Our laboratory is currently engaged in a collaborative program to continue RNA sequence mapping of alpaca endometrium and proteomic analysis of gene regulation associated with MRP and pre-implantation embryonic development during early pregnancy.

1.6 References

1. Tibary A, Anouassi A, Sghiri A, et al. Current knowledge and future challenges in camelid reproduction. Society of Reproduction and Fertility, Volume 64. Nottingham: Nottingham University Press; 2007. p. 297-313.

2. Senger PL. Pathways to pregnancy and parturition. Pullman: Current Conceptions, Inc; 2004. 368 pp.

3. McKinnon AO, Squires EL, Vaala WE, et al. Equine reproduction, Volume 1. Ames: Wiley-Blackwell; 2011. 1648 pp.

4. Bazer FW, Spencer TE, Johnson GA, et al. Comparative aspects of implantation. Reproduction. 2009;138:195-209.

5. Brooks K, Burns G, Spencer TE. Conceptus elongation in ruminants: roles of progesterone, prostaglandin, interferon tau and cortisol. Journal of Animal Science and Biotechnology. 2014;5:(16 November 2014).

6. Bazer FW. Pregnancy recognition signaling mechanisms in ruminants and pigs. Journal of Animal Science and Biotechnology. 2013;4:(26 June 2013).

7. Spencer TE, Johnson GA, Bazer FW, et al. Pregnancy recognition and conceptus implantation in domestic ruminants: Roles of progesterone, interferons and endogenous retroviruses. Reprod Fert Develop. 2007;19:65-78.

8. Vaughan JL, Tibary A. Reproduction in female South American camelids: a review and clinical observations. Small Rumin Res. 2006;61:259-81.

9. Picha Y, Tibary A, Memon M, et al. Chronology of early embryonic development and embryo uterine migration in alpacas. Theriogenology. 2013;79:702-8.

10. Skidmore JA, Starbuck GR, Lamming GE, et al. Control of luteolysis in the one-humped camel (Camelus dromedarius). Journal of Reproduction and Fertility. 1998;114:201-9.

11. McKinnon AO, Tinson AH, Nation G. Embryo transfer in dromedary camels. Theriogenology. 1994;41:145-50.

12. Arosh JA, Banu SK, Kimmins S, et al. Effect of interferon-τ on prostaglandin biosynthesis, transport, and signaling at the time of maternal recognition of pregnancy in cattle: evidence of polycrine actions of prostaglandin E2. Endocrinology. 2004;145:5280-93.

13. Skidmore JA, Allen WR, Heap RB. Estrogen synthesis by the periimplantation conceptus of the one-humped camel (Camelus-Dromedarius). Journal of Reproduction and Fertility. 1994 Jul;101:363-7.

14. Bazer FW, Song G, Kim J, et al. Uterine biology and in pigs and sheep. Journal of Animal Science and Biotechnology. 2012;3:(16 July 2012).

15. Powell SA, Smith BB, Timm KI, et al. Estradiol production by preimplantation blastocysts and increased serum progesterone following estradiol treatment in llamas. Anim Reprod Sci. 2007;102:66-75.

16. Reed KM, Chaves LD. Simple sequence repeats for genetic studies of alpaca. Animal Biotechnology. 2008;19:243-309.

17. Bianchi CP, Meikle A, Sartore I, et al. Uterine estrogen receptor alpha and progesterone receptor during the follicular and luteal phase in llamas. Anim Reprod Sci. 2007;99:117-26.

Table 1.1 Signalment of 20 female alpacas enrolled for reproductive tract sampling and embryo collection

Animal ID	Age (Years)	Collection Group
1	7	9-day pregnant
2	5	9-day pregnant
3	6	9-day pregnant
4	9	9-day pregnant
5	4	9-day pregnant
6	5	9-day control
7	5	9-day control
8	3	9-day control
9	5	9-day control
10	9	9-day control
11	9	14-day pregnant
12	8	14-day pregnant
13	14	14-day pregnant
14	6	14-day pregnant
15	6	14-day pregnant
16	3	14-day control
17	13	14-day control
18	11	14-day control
19	7	14-day control
20	9	14-day control

Figure 1.1 Transrectal ultrasonography was used to monitor follicular development, confirmation of ovulation, and pregnancy diagnosis

Figure 1.2 A female alpaca being mated to a proven, fertile male

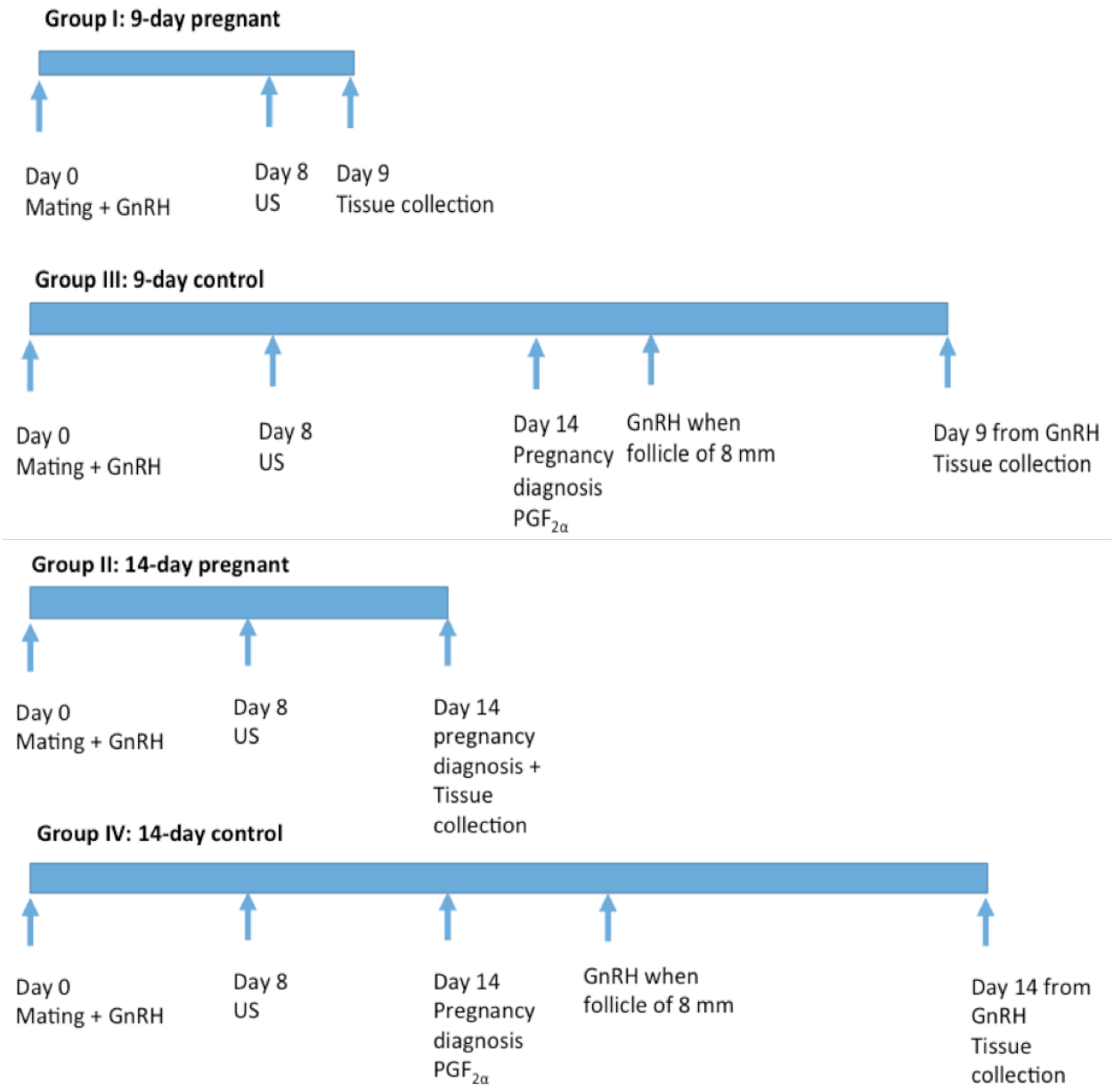

Figure 1.3 Protocol for the experimental treatment groups

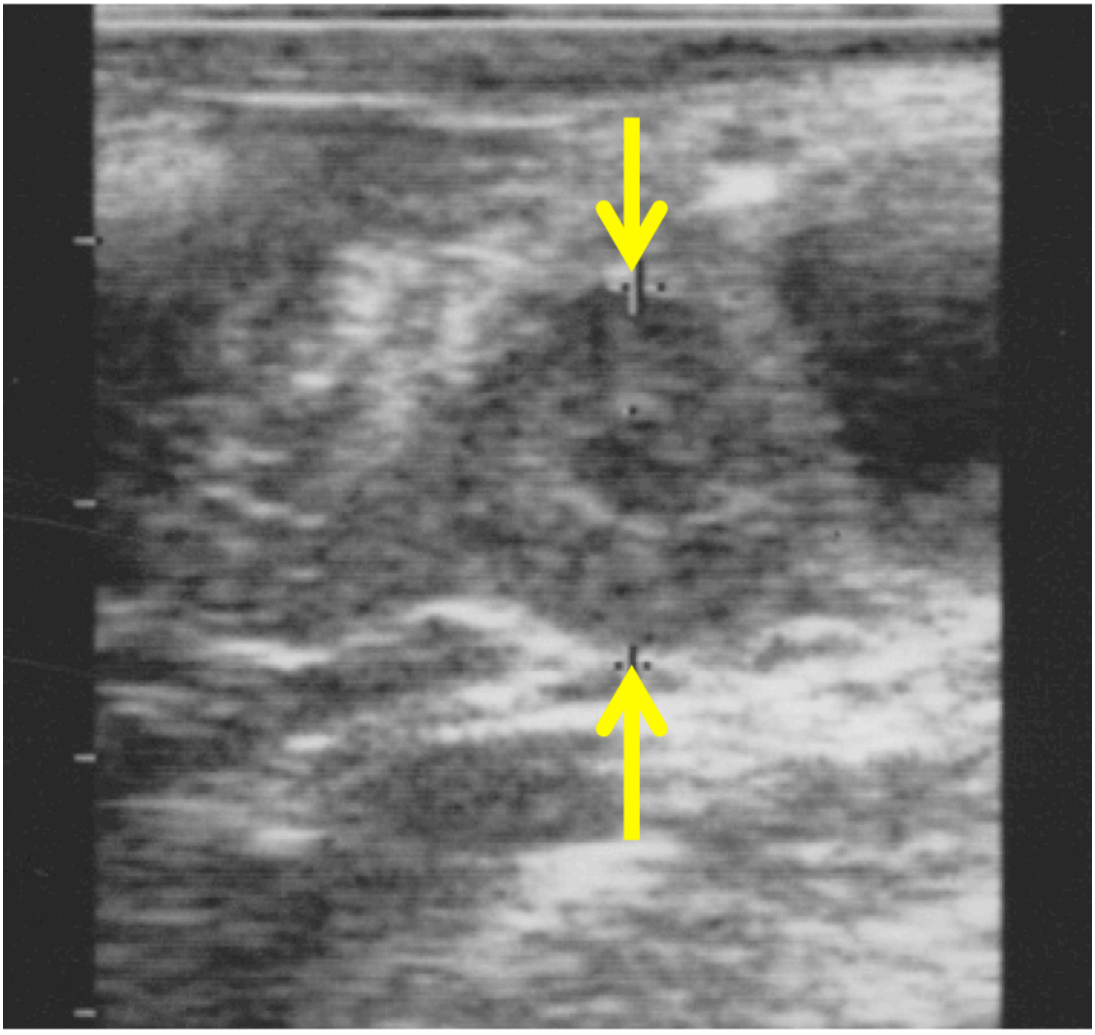

Figure 1.4 Transrectal ultrasonogram of an ovary demonstrating the presence of a corpus luteum (CL) (arrows)

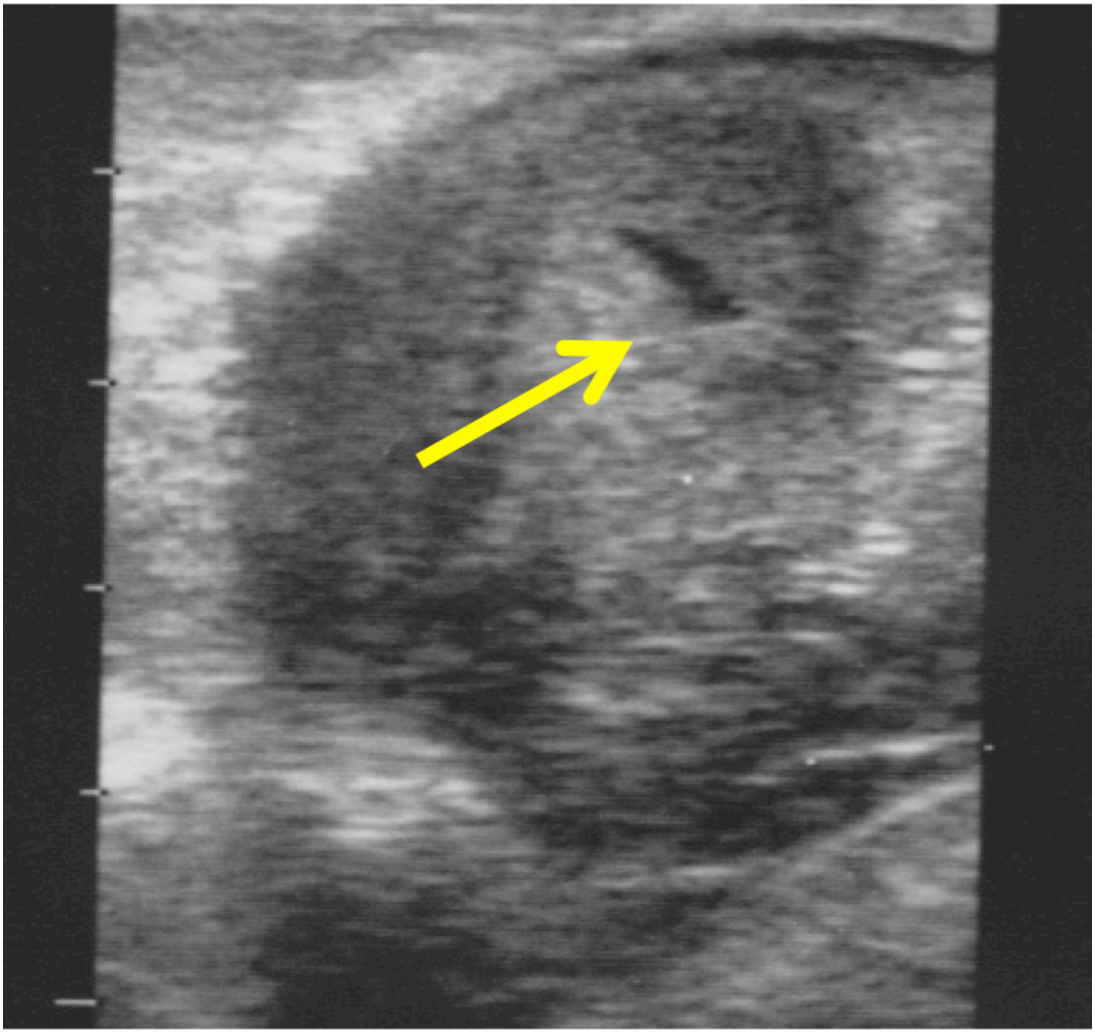

Figure 1.5 Transrectal ultrasonogram of the uterus demonstrating the presence of a 14-day pregnancy (arrow)

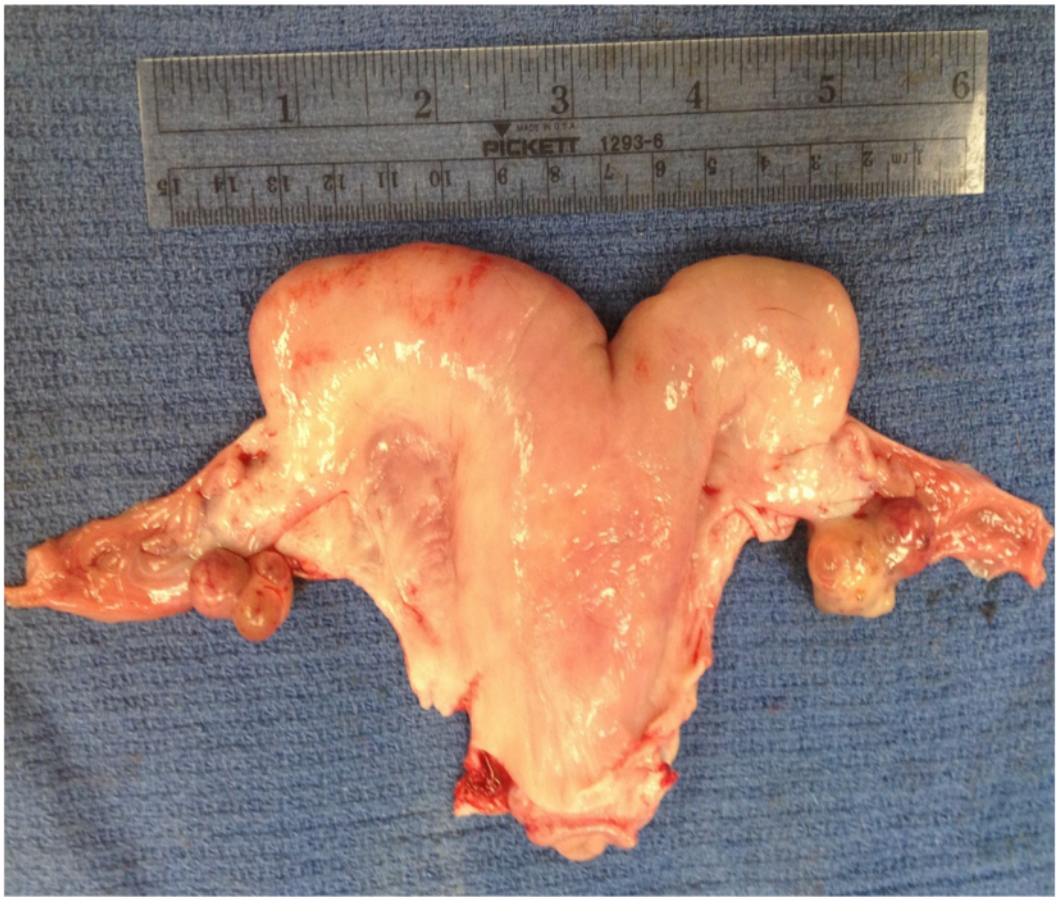

Figure 1.6 The uterus collected *en bloc* for tissue sampling

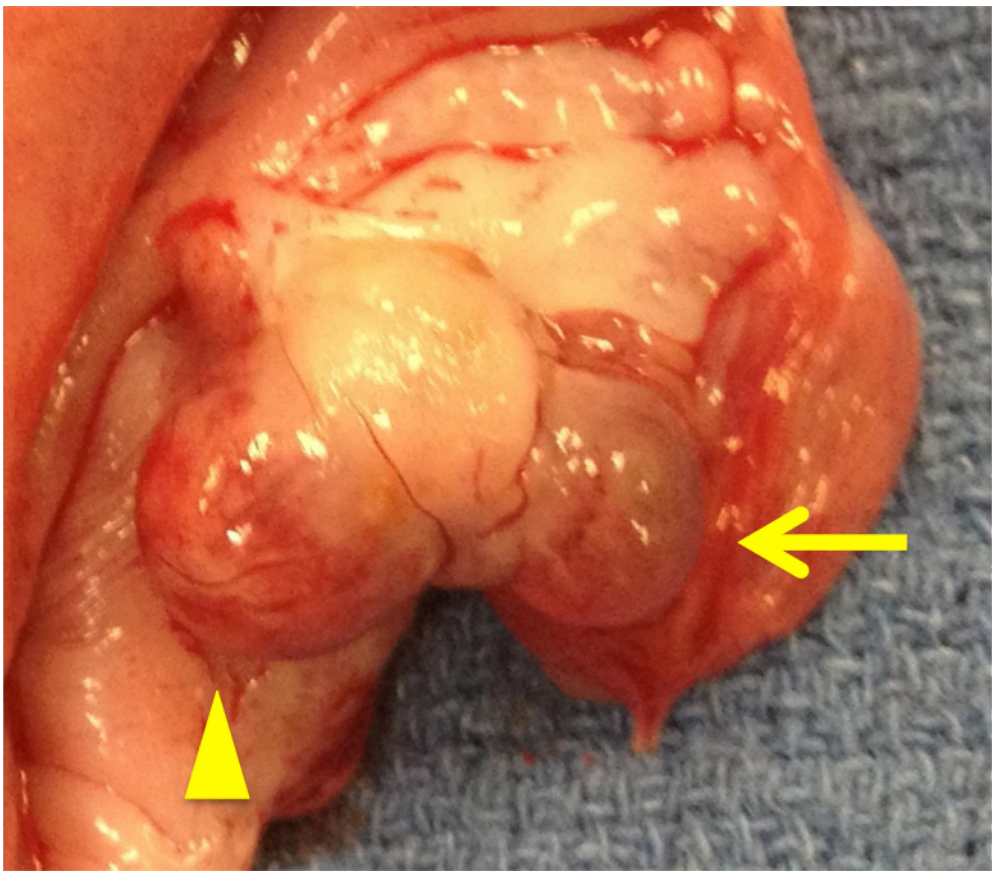

Figure 1.7 An ovary with a corpus luteum (CL) and follicle observed grossly (Follicle: arrow; CL: arrowhead)

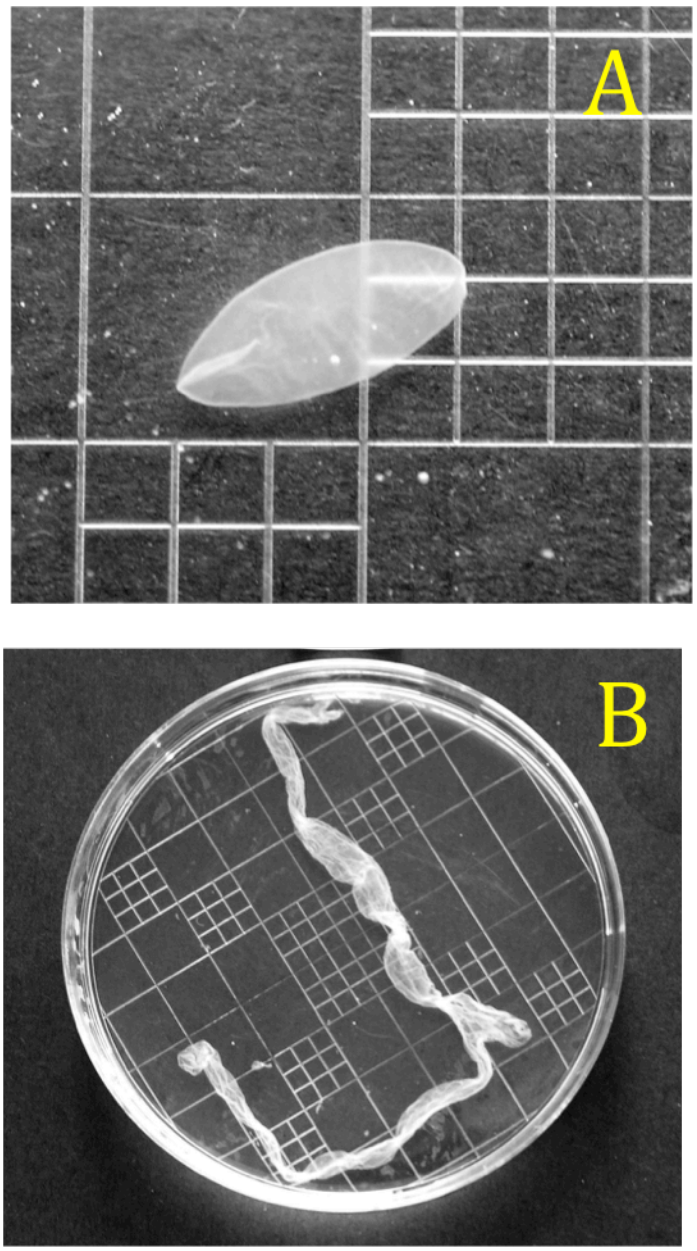

Figure 1.8 Alpaca embryos collected by ex vivo uterine flushing (A) 9-day embryo, (B) 14-day embryo (Images credited to Picha et. al, 2013; permission A. Tibary)

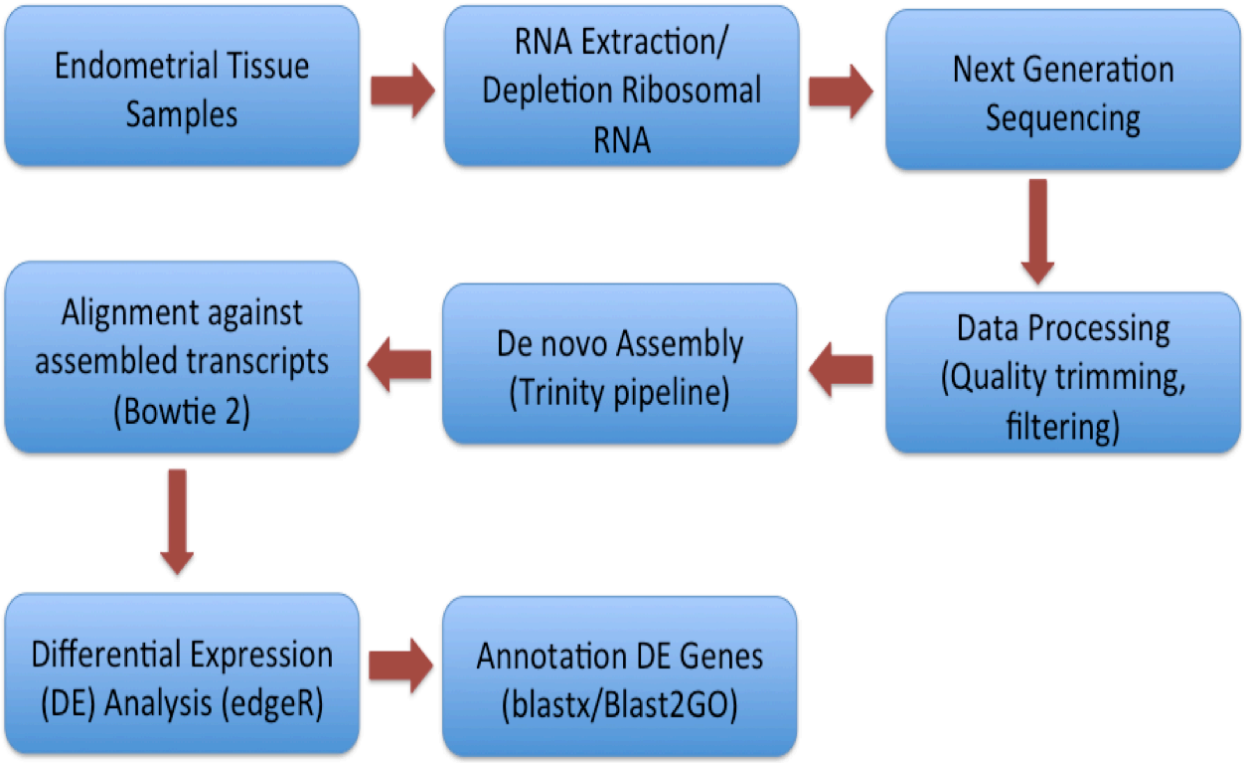

Figure 1.2 RNA sequence mapping: Transcriptome analysis of alpaca endometrium

Chapter 2

Early embryo development and maternal recognition of pregnancy: A literature review

2.1 Introduction

Maternal recognition of pregnancy (MRP) is the biological process that allows a female to recognize the presence of a conceptus and remodel the endocrine, histological, and secretory function within the reproductive tract to allow attachment/implantation and further embryo and fetal development. This process, which can be summarized as a mechanism of prevention of luteolysis, involves signaling between the embryo and the uterus leading to successful implantation. In-depth studies on MRP have been sparked by the impact of early pregnancy loss on productivity in various species (cattle[1], camelids[2-4], horses[5-8]). Additionally, early embryonic loss (loss in the pre-implantation period) is a common problem with many advanced reproductive biotechnologies involving embryo/gamete manipulations. This increased early embryo loss is generally attributed to impaired communication between the developing blastocyst and the maternal endometrium.[9] In the past three decades tremendous advances have been made in deciphering some of the MRP signaling particularly in ruminants[10-12], swine[13-15] and to a lesser extent in horses.[16-18]

Camelids (llamas, alpacas, dromedaries, Bactrian camels) are important livestock in many areas of the world. They are the only production animal species that can survive under some of the harshest climatic conditions on the planet. Studies on early embryo development and MRP in these species are scarce. The objectives of this paper are to present an overview of our knowledge on MRP in domestic large animal species and discuss early embryo development in camelids and how it may relate to MRP.

2.2 Maternal recognition or pregnancy in traditional domestic large animals

2.2.1 Ruminants

In ruminants, the blastocyst hatches from the zona pellucida around Day 8 to 10 post-fertilization and starts rapidly elongating by Day 13. In cattle, the conceptus doubles in length every day between Days 9 and 16. This process is critical for successful implantation and placentation around Day 16 in sheep and Day 19 in cattle. MRP is hallmarked by the secretion of interferon tau (IFNt) by the trophoblast cells during this elongation phase.[10] IFNt acts on the endometrium in a paracrine manner to prevent luteolysis. IFNt seems to be unique to ruminants and has potential antiviral, anti-proliferative, and immune-modulatory properties.[19] It is secreted by the elongating blastocyst at concentrations proportional to its size.[20] The mechanism of action of IFNt in prevention of luteolysis has been a subject of debate. It was first hypothesized that IFNt inhibits estrogen receptor (ER) expression in the luminal (LE) and glandular epithelia (GE), which in turn, blocks oxytocin receptor (OTR) expression. As a consequence, the episodic release of prostaglandin$F_{2\alpha}$ ($PGF_{2\alpha}$) from the endometrium is blocked because its synthesis requires oxytocin coupling with its receptor.[21] However, more recent studies have shown that inhibition of oxytocin-induced $PGF_{2\alpha}$ production does not require down regulation of ER.[22] Recently, IFNt was shown to be embryotrophic and an essential regulator of conceptus elongation. This action is predominantly paracrine. It is important to note that elongation does not occur in vitro, which confirms the important role of the endometrial glands and the histotorph during this phase of embryo development.[23, 24]

Another hypothesis is that IFNt increases prostaglandin E_2 (PGE_2), which is luteotrophic, in the endometrium. The antiluteolytic effects of IFNt inhibit transcription of the estrogen receptor alpha (ESR1) gene in sheep and the OTR gene in both sheep and cattle, specifically in the endometrial LE. In cattle, IFNt down regulates OTR and prostaglandins-generating enzyme prostaglandin-endoperoxide synthase 2 (PTGS2; formerly cyclooxygenase 2 or COX2) expression, which results in a reduced expression of PGF synthase and consequently reduced $PGF_{2\alpha}$ secretion.[25] In sheep, the expression of PTGS2 and secretion of large amounts of prostaglandins (PGs) are present in both pregnant and non-pregnant females.[26] In the

presence of pregnancy, luteolysis is prevented by inhibition of transport of endometrial prostaglandins by IFNt.[27]

In addition to antiluteolytic effects, IFNt acts in a paracrine manner on the endometrium to induce or enhance expression of IFN-stimulated genes (ISG) that are hypothesized to regulate uterine receptivity and conceptus elongation and implantation.[28-30] Conceptus-derived PGs, in coordination with IFNt or cortisol, have been found to increase expression of genes that are involved in conceptus elongation, migration, and attachment of trophectoderm cells. [31-34] PTGS2 is up-regulated by IFNt in early pregnancy.[35] Type I interferons (IFN) stimulate synthesis of PGE_2 and $PGF_{2\alpha}$. PTGS2-derived PGs from the conceptus likely have a paracrine, autocrine, and perhaps intracrine effect on endometrial function and conceptus development in early pregnancy. Conceptus elongation is also regulated by cortisol from the endometrium and the conceptus.[24, 33, 34]

In cattle, several type I IFN-stimulated genes (ISG) have been identified in the endometrium in response to IFNt, which demonstrate complex cell-specific biological effects of IFNt.[36, 37] In sheep, it appears that interferon regulatory factor 2 (IRF2) in LE restricts IFNt induction of most classical ISGs to the uterine stroma and GE. IFNt has also been shown to exert systemic effects that may affect CL function.[38-41] IFNt also stimulated expression of non-classical ISG, which are initially induced by progesterone and loss of progesterone receptors (PGR) in the epithelia. These non-classical ISG are critical for proliferation, migration, attachment and or adhesion and nutrient transport.

Another lipid mediator that has been shown to play a role in establishment of pregnancy is lysophosphatidic acid (LPA). LPA is produced by the endometrium and the conceptus and has been shown to stimulate PGE-synthase.[42]

In cattle, the major changes required to drive conceptus elongation and establish uterine receptivity to implantation occur between Days 7 and 13 in response to ovarian progesterone (reviewed by [43]). Progesterone induces the expression of many conceptus elongation and implantation-related genes

including those coding for attachment and migration factors, cell proliferation factors, adhesion protein insulin-like growth factors, prostaglandin G/H synthase, PTSG2, hdroxysteroid (11-beta) dehydrogenase, proteases, protease inhibitors, candidate cell proliferation factor, glucose transporters, and cationic amino acid transporter (reviewed by [24]). This gene expression pattern occurs in association with loss of progesterone receptors between Day 10 and 12 in endometrial LE and between Days 12 and 16 in the GE after onset of estrus; however, PGR remain present in the stroma and myometrium throughout pregnancy.

2.2.2 Swine

Estrus lasts 24 to 72 hours and ovulation occurs 44 hours after the onset of estrus. Early embryo development occurs near the ampullary-isthmic junction of the oviduct. Embryos descend into the uterine cavity at the four-cell stage around 60 to 72 hours after mating. The blastocyst stage is reached by Day 5. Hatching from the zona pellucida occurs between Day 6 and 7 and is followed by a period of expansion and then rapid elongation. By Day 16, the embryo takes a filamentous form and starts its initial attachment between the trophectoderm and uterine luminal epithelium. The swine blastocyst possesses high aromatase activity and produces significant amounts of estrogen between Days 14 and 18 of pregnancy.[44] In addition to estrogen, the swine conceptus produces several proteins and PGs including PGF and PGE.[13]

Maintenance of pregnancy in pigs relies on progesterone secretion from the corpora lutea. Estradiol plays an important role in prevention of luteolysis through inhibition of secretion of $PGF_{2\alpha}$ from the endometrium into the uterine venous drainage.[45] Administration of estradiol to gilts between Days 11 and 15 can produce a state of pseudopregnancy. The same treatment increases the chances of maintenance of pregnancy following transfer of a single embryo.[46] The exact mechanism by which estradiol prevents luteolysis is not known. It is possible that $PGF_{2\alpha}$ is converted to an inactive metabolite by the endometrium or the conceptus or both. It is important to note that both $PGF_{2\alpha}$ and PGE_2 are required for conceptus development and implantation.

An important characteristic of pregnancy in swine is embryo spacing/distribution between the 2 uterine horns. It has been hypothesized that estrogen produced by the embryo may redirect $PGF_{2\alpha}$ synthesis towards the endometrial lumen where it would stimulate uterine contraction and help the even distribution of embryos within the uterus. The shift from endocrine diffusion of $PGF_{2\alpha}$ to an exocrine one prevents it from reaching the ovaries and causing luteolysis. Histamine, released by the endometrium in response to conceptus-derived estrogen, has been suggested to be involved in the intrauterine migration of porcine embryos.[47]

Attachment and development of the conceptus is supported by the histotroph and special cellular remodeling at the uterine luminal epithelium-trophectoderm interface. These events are facilitated by a number of signaling pathways involving interlukin-1 beta, interferon delta and gamma, transforming growth factor beta, and fibroblast growth factor 7, which regulate expression of genes in trophectoderm, uterine epithelial and stromal cells.[13]. Several transcriptome changes in the endometrium have been identified during the phase of MRP and implantation.[48-50] Recently, it was shown that small noncoding RNAs may have a potential implication in the development of embryos and embryo-maternal cross-talk during early pregnancy.[51]

2.2.3 Equine

Embryonic development and early pregnancy development in the horse present some unique features including selective passage of embryos into the uterine cavity, transuterine migration, maintenance of a spherical shape of the embryo until post-fixation, presence of a capsule, and development of specialized invasive cells (endometrial cups) during implantation and initiation of placentation.[52]

The equine embryo reaches the uterus at the late morula/early blastocyst stage around 5.5 days post-fertilization. Oviductal transport is selective in this species and only viable embryos are able to pass

into the uterine lumen through the utero-tubal junction (UTJ). Relaxation of the UTJ is mediated through PGE secreted by the embryo.[53]

In the open cyclic mare, oxytocin from the hypothalamic-pituitary axis and probably from the endometrium triggers OTR to initiate the pulses of $PGF_{2\alpha}$ secretion that lead to luteolysis.[54-57] The ability of the endometrium to secret $PGF_{2\alpha}$ in response to oxytocin is suppressed between Days 10 and 14 of pregnancy.[57, 58] In addition, $PGF_{2\alpha}$ production by the endometrium is significantly reduced between Days 13 and 15 of pregnancy due to the absence of PTGS2.[59, 60] These observations lead to the hypothesis that MRP in the mare is primarily antiluteolytic rather than luteotrophic. Contrary to ruminants, the primary transmembrane $PGF_{2\alpha}$ transporter (solute carrier organic anion transporter family, member 2A1, *SLCO2A1* mRNA) gene is up-regulated in the first 2 weeks of pregnancy.[61, 62]

Recent research on MRP in mares suggests that an initial suppression of the ability of the endometrium to secrete $PGF_{2\alpha}$ is due to a combination of reduced PTGS2 gene expression accompanied by a suppression of OTR expression at the protein level. However, by Day 21 of pregnancy these mechanisms are removed and the endometrium is able to produce $PGF_{2\alpha}$ in response to $PGF_{2\alpha}$. Therefore the suppression of prostaglandin F receptors (PTGFR) at Days 14 and 21 of pregnancy plays a significant role in preventing $PGF_{2\alpha}$ luteolytic pulses by disabling both oxytocin-$PGF_{2\alpha}$ feedback and $PGF_{2\alpha}$-$PGF_{2\alpha}$ feed-forward loops. The equine conceptus produces significant quantities of $PGF_{2\alpha}$ until at least Day 32 of gestation, which may genomically down regulate PTGFR.[63] Exposure of endometrial explants to conceptus-conditioned medium decreases PTGS2 mRNA abundance.[59] During early pregnancy, both the conceptus and the endometrium secrete PGE_2, which may prevent luteolysis.[60] In addition, it was demonstrated that the presence of a conceptus on Day 15 of pregnancy inhibits COX2 and reduces the expression of $PGF_{2\alpha}$.[59, 60]

During the biosynthesis of PGs, cytosolic phospholipase A2 (PLA2) mobilizes arachidonic acid from membrane phospholipids, which in turn is converted to prostaglandin H2 (PGH2) through the

enzymatic action of either PTGS1 or PTGS2. In the mare, it appears that that PLA2 expression does not play a major role in reduction of prostaglandin release during early pregnancy.[64]

In the mare there is no production of oxytocin from the ovary and therefore luteal derived oxytocin does not seem to play a role in luteolysis. However, the involvement of oxytocin in luteolysis in the mare is evidenced by in vitro and in vivo experiments. Exposure of endometrial tissues to oxytocin increases the release of $PGF_{2\alpha}$.[59] In vivo, administration of oxytocin in the early luteal phase causes prolongation of luteal function.[55, 65, 66] Uterine-derived $PGF_{2\alpha}$ has been proposed to trigger the oxytocin secretion from the pituitary during initiation of luteolysis, which in turn establishes a positive feedback through binding to its endometrial receptor and further stimulation of endometrial prostaglandin secretion. In pregnant mares (Day 14), responsiveness to administration of oxytocin decreases due to decreased binding capacity of the endometrium and results in a decrease in $PGF_{2\alpha}$ secretion.[55, 57]

The nature of pregnancy recognition signaling in mares has been a subject of debate. It is postulated to be a prostaglandin inhibiting factor between 3 and 10 KDa proteinase K-resistant substance that may be adsorbed by dextran-coated charcoal.[67, 68]

One of the characteristics of early pregnancy in the mare is the lack of elongation. The embryo remains spherical during all of the preimplantation period and is surrounded by an acellular glycoprotein capsule up until 21 days. The capsule, which is produced primarily by the trophoblast, has been shown to be required for embryo survival.[69] It is important to note that formation of the capsule requires the uterine environment, as in vitro produced embryos secrete glycoproteins necessary for capsular formation but do not actually form a functional capsule.[70] It has been hypothesized that one of the roles of the capsule is to prevent adhesion of the embryo. The biochemical proprieties of the capsule change as pregnancy progresses and its content in sialic acid is significantly reduced around the time of embryo fixation (Day 16). The capsule disappears progressively between Days 22.5 and 24.5.[71, 72]

Transuterine migration of the conceptus between Days 9 and 16 of pregnancy is required to maintain the antiluteolytic activity.[73-75] Ultrasonographic studies on embryo motility and particularly the independent mobility of twin embryos has led to the hypothesis that the conceptus drives its own mobility through induction of uterine contractility.[76, 77] Contractility of the uterus may be induced by secretion of PGs (PGE_2, $PGF_{2\alpha}$ and PGI) produced by the conceptus and the endometrium in contact with the conceptus. Production of $PGF_{2\alpha}$ by the endometrium in the proximity of the conceptus may be the result of the effect of estrogen produced by the latter.[78] The role of $PGF_{2\alpha}$ in conceptus mobility is further demonstrated by the decrease in this phenomenon when mares are administered flunixin meglumine between Days 10 and 18 of pregnancy.[78]

Estrogen produced by the conceptus was believed to be the primary MRP signal as in pigs. However, administration of estradiol-17α during the pre-fixation period (Day 10 to 16), or until 32 days, did not result in prevention of luteolysis in open mares.[79, 80] Also, intrauterine administration of estradiol in mineral oil did not prolong the lifespan of the CL.[81] These studies suggest that conceptus-derived estradiol is not the primary MPR in the mare. However, other steroids synthesized by the equine conceptus (testosterone, androstendione, progesterone, 17α-hydroxyprogesterone, and 20α-hydroxyprogesterone) may also be involved.[82]

The role of PGE_2 in prevention of luteolysis has been demonstrated in ruminants and pigs. The equine conceptus produces significant amounts of PGE_2. Intrauterine administration of PGE_2 resulted in prolonged luteal function in one study.[79]

The role of IFNs in equine MRP has been investigated. Interferon equine α1, omega 1 and omega 2 are not expressed between Day 13 and 25 conceptuses.[83]. However, EqIFN-delta 1 and EqIFN-delta 2 are expressed in conceptuses at high levels but only later in gestation (Day 16 and day 22 conceptuses).[84, 85] There are limited studies on expression of interferon-induced genes in the equine endometrium. *Mx* mRNA is low in both pregnant and non-pregnant mares between ovulation and Day 20.[86] Expression of

ISG15 conjugated proteins does not differ between pregnant and non-pregnant mares at Day 14 and Day 50 of pregnancy.[87]

Prevention of luteolysis can be obtained by insertion of an intrauterine device (IUD) such as glass marbles or water-filled plastic balls.[88, 89] Mares with extended luteal phase showed low prostaglandin $F_{2\alpha}$ metabolites (PGFM) levels suggesting a prevention of release of PG from the endometrium by the IUD.[90] However, in this limited study, 3 out of 5 mares with IUDs still displayed moderate levels of PTGS2.

Transcriptional profiling of equine endometria from pregnant and open mares has been reported recently. Micro-array analysis failed to demonstrate any differential gene expression between pregnant and open mares on Day 8. However, significant differences were found between pregnant and non-pregnant mares on Days 12 and 13.5, particularly for genes that are regulated by estrogens.[62] Genes related to angiogenesis and vascular remodeling were significantly enriched on Day 12 pregnant endometria. In addition, genes related to steroid hormone and PG signaling and genes possibly related to the process of mechanostransuction were identified.[62] Secreted proteins and genes involved in transport and cell-cell signaling, as well as genes involved in cholesterol transport, lipid biosynthesis are up regulated in pregnant endometria.[16, 87] In addition, endometrial samples from pregnant mares showed a down regulation of proteinases (Cathepsin L and plasminogen), which is thought to prevent dissolution of the capsule and endometrial erosion.[87] Sialidase 2 mRNA level increases from Day 8 to day 14 conceptuses and may be involved in capsular changes and successful embryo fixation.

2.3 Pregnancy in camelids

The physiology of pregnancy in camelids presents several peculiarities compared to that of other domestic species. Knowledge of embryonic development, fetal development and characteristics of placentation are fundamental not only for diagnosis of pregnancy and fetal evaluation but also for clinical and pathological evaluation in cases of pregnancy loss or abortion. In addition, the understanding of early

embryo development and mechanisms of maternal recognition pregnancy are important for development of embryo transfer technologies.[2]

2.3.1 Ovulation and Fertilization

All camelid species are induced ovulators. The induced nature of ovulation in these species has long been suspected based on clinical and hormonal studies.[2] However, the major breakthroughs in defining the mechanisms of induction of ovulation came in two main groups of studies. The first demonstrated the hypothalamic response to mating in camels[91, 92] and South American camelids (SAC).[93] Luteinizing hormone was shown to increase sharply within minutes following mating when a mature follicle was present. The second group of studies led to the hypothesis of the presence of an ovulation-inducing factor (OIF) within the seminal plasma.[94-97] Recent studies in llamas and alpacas identified the OIF as β nerve growth factor (βNGF).[98, 99] Both βNGF and endometrial inflammation are required to maximize the ovulation rate (see review[98]). βNGF was also identified in dromedary camel seminal plasma.[100] It is important to note that βNGF may have a luteotrophic effect on the CL.[101]

Oocyte maturation is completed immediately following copulation and LH release and shortly after ovulation, which occurs around 30 hours after copulation. The LH surge triggers resumption of meiosis in the oocyte, disruption of cumulus cell cohesiveness and rupture of the follicular wall.[102] Cumulus dispersion is observed in cumulus-oocyte complexes (COC) recovered by aspiration from follicles 18 to 24 hours after human chorionic gonadotropin (hCG) injection.[2, 103]

Both ovaries are equally active and alternance of ovulation between ovaries occurs occasionally.[2, 104] Double ovulations are not uncommon in most domestic camelids in good health and nutritional status.[104] Triple and quadruple ovulations have also been documented in the dromedary camel.[105]

Semen is deposited deep into the uterine horns throughout the mating period and slowly forms a sperm reservoir in the uterine tube as it liquefies.[2, 106, 107] The molecular and ultrastructural aspects of

sperm oviductal attachment and reservoir formation have been investigated in llamas. These studies demonstrated the role played by the bulbourethral gland secretion in this phenomenon.[108-111] Sperm may be stored in the isthmus region for up to 5 days.[2, 112, 113]

Fertilization rates are very high (>80%) in alpacas.[104] Some authors have observed higher fertilization rates in alpacas for ovulation from the left ovary than from the right ovary.[3] However, this difference has not been observed in our laboratory in camels and alpacas.[104, 105, 114] Conception rates per mating are generally lower (50 to 75%) when females are mated based on receptivity than when mating is based on presence of a mature follicle.[2, 104, 115] However, in well-managed herd conception rates are generally higher than 90%.[104]

2.3.2 Early embryo development (oviductal stages)

Studies conducted in our laboratory on camels and alpacas show that the development of the embryo from fertilization to the hatched blastocyst stage takes about 6.5 days. Early stages of embryo segmentation take place in the uterine tube.[2, 114] Collection of camelid embryos from the uterus yields only hatched embryos and rarely embryos in the process of hatching.[2, 114, 116-119] There does not seem to be a selective transport through the UTJ as seen in the horse. Unfertilized ova degenerate or are eliminated in the uterine cavity soon after ovulation.[114]

2.3.3 Embryo hatching and elongation

The camelid embryo reaches the uterine cavity at the hatched blastocyst stage around Day 6.5 post-ovulation (7.5 days after mating).[114] The hatched blastocyst expands promptly and starts to elongate on Day 9 growing rapidly to occupy the entire uterine cavity by Day 12. By Day 15 post-ovulation, the trophoblast is in contact with the entire endometrial surface although not yet attached.[2, 114] During the process of elongation, the embryo migrates from the right uterine horn to the left uterine horn for females

that had a right-sided ovulation. In alpacas, 83.3% of the embryos resulting from right ovary ovulation were found in the left uterine horn on Day 9.[114] This suggests that embryo migration to the left horn is an important mechanism in prevention of luteolysis in camelids. These observations are supported by embryo transfer experiments. In llamas, transfer of embryos to the uterine horn ipsilateral to the ovulation side resulted in higher pregnancy rates for left ovulation (50%) than right ovulation (30%) recipients, while transfer of the embryo to the left uterine horn when the CL was on the right ovary resulted in only 20% pregnancy rate.[120, 121] In alpacas, transfer of embryos collected at 7 days and 4 hours into the left horn regardless of the side of ovulation in the recipient resulted in higher pregnancy rate (20.3%) when the CL was on the left ovary than when the CL was on the right (12.4%).[122] In the dromedary, deposition of the embryo in the right uterine horn when the CL is on the right ovary resulted in higher pregnancy rates compared to left horn transfer.[123]

2.3.4 Maternal recognition of pregnancy

In absence of pregnancy, the luteal phase in camelids is very short. The CL develops after ovulation and reaches its maximal size and function (progesterone production) by Day 8 to 9 post-mating. Luteolysis occurs in response to increased pulsatile $PGF_{2\alpha}$ secretion and is complete by Day 10 or 11 post-ovulation. Ovulating non-pregnant female camelids return to receptivity and develop a dominant follicle 14 days after mating.[2, 124, 125]

In the presence of pregnancy, luteolysis is prevented and the CL is maintained as the sole source of progesterone throughout pregnancy.[2, 126, 127] The size of the CL varies between 11 and 20 mm in SAC and 15 to 25 mm in camels. A cavitary CL is normal.[2] Recent studies have shown that a CL resulting from ovulation induced by seminal plasma (i.e. mating to vasectomized male) are larger and produce more progesterone that those emanating from induction of ovulation with GnRH.[101]

The mechanism of MRP responsible for maintenance of CL function remains poorly studied in camelids. Clinical and endocrine studies suggest that MRP in camelids has to take place relatively early after mating (between Day 8 to Day 10) in order to prevent luteolysis. Despite the presence of a conceptus, a pulsatile release of $PGF_{2\alpha}$ is observed between Day 7 and 15 post-mating. This release of $PGF_{2\alpha}$ results in a transient decrease in progesterone concentrations by Day 9 post-mating.[128, 129] During the same period, endometrial expression of COX-2 in the luminal epithelium decreases, reaching one-third the level observed during luteolysis in non-pregnant animals by Day 12.[130] It is important to note that MRP occurs at a time when the embryo undergoes a rapid elongation as observed in ruminants. However, attempts to identify in camelids a substance such IFNτ in ruminants were not successful.[131] Similarly to the equine embryo, camelid embryos have a high aromatizing ability between Days 10 and 15 of pregnancy.[131, 132] There is a temporal relationship between an increase in estradiol synthesis by the embryo and MRP. Additionally, administration of estradiol benzoate (10 mg, IM) to llamas from Day 7 to Day 15 after induction of ovulation with hCG, resulted in an extension of the CL lifespan and progesterone production.[132] In alpacas, administration of estradiol on Days 8 and 9 post-ovulation improved embryo survival by 30 to 50%.[133, 134] However, a similar protocol in llamas resulted in a decrease in pregnancy rate.[120] The involvement of estrogens in MRP in camelids is further supported by the increase in estrogen receptor α (ERα) between Days 8 to 12 post-mating in pregnant animals and a reduction in the expression of PGR by Day 12 post-mating.[130]

The time of embryo attachment is not well defined in camelids. Although early studies have suggested that the embryo remains free within the uterus for the first 30 days[135], recent studies suggest that attachment may start as early as 14 days. One of the major peculiarities of embryo fixation and pregnancy in camelids is that nearly all fetuses are located in the left horn.[136-139] This situation predominates (98 to 100%) in all camelids although the location of the CL is equally distributed between the left and right ovary (Table 2.1). A higher incidence of right horn pregnancies (40%) was reported in one study in the dromedary.[140] These authors reported an incidence of right horn pregnancies of 30 to 60%

even for stages of pregnancy beyond 95 days. The discrepancy between the results of this particular study and those of all other published studies on this topic raises some doubt as to their validity. In one study on alpacas, only one fetus out of 48 (2%) was located in the right uterine horn at 120 days of pregnancy.[136] In our clinic, only 4 out of 640 pregnancies were diagnosed in the right uterine horn between 25 and 40 days of pregnancy and all resulted in an early loss of pregnancy before 60 days. The side of ovulation does not influence pregnancy rate.[105, 141]

The preponderance of left-horn pregnancies in camelids is attributed to a difference in $PGF_{2\alpha}$ release between the two uterine horns. $PGF_{2\alpha}$ release from the right uterine horn is local whereas its release from the left horn is systemic.[2] The migration of the embryo originating from an ovulation in the right ovary to the left horn is therefore required to prevent $PGF_{2\alpha}$ release into the general circulation and may even exert a luteotrophic effect making possible the survival of the embryo.[2, 114, 139]

The exact mechanism of embryo migration is not known. It has been suggested that embryo migration is facilitated by the shortness of the uterine body, the smallness of the right horn, and the increased number of mucosal folds in the endometrium of the right horn.[142] Mobility of the embryo as observed in the horse is less likely to happen in camelids, because of the elongation of the blastocyst, the decrease in tone and contraction of the uterus during pregnancy. Estrogen receptor β (ERβ) expression was found to be greater in pregnant than in sterile-mated llamas particularly on Day 13. The authors suggested that the presence of an embryo may be influencing this expression.[143] Endometrial expression of ERα and ERβ was not affected by uterine side or days post-mating. However, the presence of a CL seems to up regulate ERβ expression in the uterus mostly in non-endometrial tissue. While endometrial ERβ are not affected by pregnancy status, the uterine ERβ is significantly increased. This led the authors to conclude that the embryo exerts a direct effect on non-endometrial tissue (myometrium and perimetrium). These effects may operate through estradiol secretion. Because all embryos migrate to the left horn, a greater expression of ER in the right horn to promote its contraction was expected but could not be

demonstrated. This suggests that a more complex mechanism with differential expression of ER subtypes may be involved in embryo migration from the right to the left horn.[143]

2.3.5 Placentation

Placentation in camelids is epitheliochorial, microcotyledonary, and diffuse.[144-149] Attachment starts around Day 14 or 15 of pregnancy and progresses slowly to form more intimate contact with the uterus.[150, 151] A pre-contact phase, where the mononuclear trophoblast cells are separated from the endometrium by a gap containing inter-areolar histotroph was identified. This phase is followed by the development of multinuclear giant cells and formation of uterine ectoplasmic pads. Finally comes the adhesion phase where the apical cell membrane of the trophoblast and the endometrium adhere closely to each other with various degrees of microvilli formation. A well-formed microvillus junction between the fetal and maternal tissues is established in the inter-areolar regions by Day 45 in alpacas[151] and Day 56 to 70 of pregnancy in the dromedary.[150, 152] Trophoblastic cells in camelids present morphological variability suggesting specialization in uptake and metabolism of important molecules.[149, 152-155] Mono and binucleated trophoblastic cells exhibit PAS-reactivity.[149, 151, 154] Acid phosphatase reactivity was found in trophoblastic cells related to the areolae in alpacas[151] and at the top of the chorionic projections in llamas.[149] A difference in intensity of this reactivity between llamas and dromedaries has been attributed to adaptation to high altitude of the former.[149] Giant multinucleated trophoblastic cells have been identified in all camelid species.[149, 151, 154] They are believed to result from the fusion of contiguous mononucleated cells.[150] Camelid trophoblastic giant cells are much more complex than those of the bovine and are the result of mitotic polyploidization.[156, 157] Morphological and histochemical characteristics of camelid areolae are similar to those of swine and serve as a reservoir of substances to support the fetus.[149, 151, 152] Uteroferrin was immunolocalized in llama glandular lumen and cytoplasm of the glandular cells. Uteroferrin acts as a transport protein for iron.[149] Although camelids present several similarities in placentation, differences do

exist in many aspects including uteroplacental glycosylation which can explain the failure of interspecies pregnancies between camels and new world camelids.[158] Failure of glycan-glycan complementation between maternal and fetal tissue during implantation may explain in part the early pregnancy loss seen in camelid interspecies pregnancies.[158, 159]

There is an adhesion between the allantoamnion and the allantochorion along the entire dorsal surface of the fetal horn. The amniotic membranes do not extend to the non-fetal horn.[142, 160] An extra-fetal membrane of epidermal origin, known as the epidermal membrane, epithelion, or 4th membrane, is found in all species of camelidae.[149, 161-163] This membrane is made up of a layer of epithelial cells that cover the entire fetus. It is connected to the mucocutaneous junction of the lips, nose, eyes, prepuce, vulva and coronary bands. It is not exactly known when this membrane first appears but in the dromedary it can be easily identified at 3 months of gestation.[140] Due to the presence of this membrane, the fetus is never in contact with the amniotic fluid, unlike in other domestic animals. The epidermal membrane is thought to play an important role as a lubricant during parturition because of its slippery nature, as well as protection from dehydration for the newborn.

The size and diameter of the umbilical cord increases with advancing gestation. At full term, the cord measures 20 to 30 cm in alpacas and llamas and 45 to 50 cm in camels.[140] The umbilical cord presents about 5 to 6 clockwise twists.

2.3.6 Fetal development

The development of the provisional organs - the amnion, yolk sac, and allantois - shows some peculiarities compared to other species. The amnion is laid down when the embryo has more than 10 somites, which is later than cattle and sheep. The yolk sac has no central swelling. Organogenesis presents the same features observed in the other ungulates. At the stages of formation of the visceral fissures and arches, the embryo has a small liver and mesonephros. Heart beats are present by Day 22 of

pregnancy. Differentiation of the major organs is completed between 45 and 60 days of pregnancy.[142, 160, 164] In alpacas and llamas, morphogenesis is completed by 60 days of pregnancy. Macroscopic fetal sex determination is not possible at 30 days but is easy at 60 days.[136]

Fetal development throughout pregnancy was described in the dromedary[137, 165], alpaca[136, 166] and llama.[167, 168] These studies led to the development of a prediction equation for pregnancy stage based on fetal biometrics. Accuracy of these formulas is variable most likely due to the great variability in pregnancy duration and other factors such as breed and nutrition. The same studies also give detailed accounts of the morphological changes of the conceptus throughout pregnancy. In all species of camelids, fetal growth is very slow during the first 6 months of pregnancy. Most of the fetal growth and weight gain (65%) occurs in the last trimester.[135-137, 166] During the last four months (from 270 to 390 days) the fetus is the major contributor to the total mass of the gravid uterus.[136, 137] In the dromedary, fetal weight doubles during the last 45 days of pregnancy.[137] Fetal weight of alpacas at 8 months, 9 months and 10 months of gestation is respectively 30%, 51% and 65% of birth weight. At 11 months, fetal weight (8.6 kg) is not significantly different from birth weight (8.8 kg).[136]

2.3.7 Endocrinology of pregnancy

Camelids depend on ovarian progesterone for maintenance of pregnancy. Progesterone levels remain above 2 ng/ml until shortly before parturition.[124, 125, 129, 169-171] In the dromedary, progesterone levels during the first 90 to 100 days were relatively constant (3 to 5 ng/ml), then decreased significantly to stabilize for the rest of the pregnancy at 2 to 4 ng/ml.[172] The decrease in plasma progesterone is observed after 3 months and may be due to a dilution effect due to an increase of total blood volume and body weight. In llamas and alpacas, serum progesterone concentrations increase 5 days after mating and remain above 2.0 ng/ml (3 to 4.5 ng/ml) throughout pregnancy.[129, 171, 173-175] A transient decrease in

progesterone was described between Days 8 and 10 in pregnant llamas[124, 129, 175] and at Day 13 in alpacas.[176]

Progesterone starts to decline about 2 weeks before parturition, drops markedly during 24 to 72 h before parturition, and is <0.5 ng/ml on the day of parturition.[173, 174] A similar decline is observed in the urinary metabolite of progesterone (pregnediol glucoronide) during the last 5 days of pregnancy.[145, 177] The regulatory mechanism(s) associated with the gradual decline in progesterone concentrations during the 2 weeks before parturition is unknown and could be due to the conversion of progesterone to estrogen by 17α-hydroxylase, synthesized in response to fetal cortisol secretion. This change in the estrogen-to-progesterone ratio could be important for myometrial activity, which is potentiated via increased synthesis of gap junctions and contractile proteins. Plasma progesterone profiles following mating and during pregnancy in the guanaco and vicuna follow closely the same trends reported in llamas and alpacas. Serum and particularly fecal progestogens may be used for pregnancy diagnosis in non-domesticated camelids.[178-180]

Estrogen levels increase by Day 18 to 20 after mating and show a great variation during the first 2 trimesters of pregnancy.[150, 172] The main source of estrogen and especially estradiol-17ß in early pregnancy is the embryonic vesicle and the placenta.[131] Conjugated estrogens are found in high levels in maternal blood in the form of estrone sulfate. The feto-placental origin of estrogen in the pregnant dromedary is also demonstrated by the sharp fall of the concentration of this hormone after expulsion of the fetus and placenta following abortion or parturition.[170, 181] Estrogen levels are also very high (2411 ± 390 pg/ml) in the allantoic fluid at parturition.[182, 183] The increase in estrogen in the maternal blood and allantoic fluid found during the last month of pregnancy could be due to an increased conversion of 17α-hydroxyprogesterone to estrogen through a 17α-hydroxylase system.[150]

Estrogen profiles during pregnancy in llamas and alpacas are similar to that of the dromedary.[173] Estrone sulfate concentration increases rapidly between Days 21 and 25. Another increase is seen in the last 60 to 80 days of pregnancy peaking at delivery.[171, 184] In pregnant llamas, estradiol-17ß is at basal concentration (10-46 pg/ml) during the majority of pregnancy and does not peak (196 ± 10 pg/ml) until the final third of pregnancy.[173] Urinary estrone sulfate levels follow closely serum estrogen variation.[177] Steroid synthesis, estrogens in particular, occurs in the trophoblast/placenta. This steroidogenesis seems to be mostly a function of the mononucleate trophoblast cell between Day 14 and day 30 of pregnancy, followed by the trophoblastic giant cell until term.[155]

In the Bactrian camel, detailed studies on the endocrinology of pregnancy are scarce. In one study limited to the last month before parturition, estradiol-17ß was high (30 ng/ml). A further increase was observed 3 days before parturition, peaking at 55 ng/ml on the day of delivery and declining within the first 4 days postpartum.[91]

Thyroid hormones are at their highest levels between 2 and 5 months and lowest between 8 and 10 months of gestation. [185] In the llama, plasma triiodothyronine concentration varies between 0.5 and 4.5 ng/ml (1.9 ± 0.1) throughout pregnancy and the peri-parturient period.[173, 186] Plasma thyroxine concentration varies between 21.3 and 91.5 ng/ml (56.5 ± 0.8) from mating until about 39 weeks of pregnancy when it begins to decline in the last 15 days of gestation. This decline in thyroxine could be the result of nutrient redistribution to the mammary gland in preparation for lactation.[173]

Secretion of $PGF_{2\alpha}$ is inhibited during pregnancy. In the dromedary, an increase in PGFM that parallels the increase in estrogen is observed starting 50 days prepartum.[172, 181] A sharp peak of this hormone occurs on the day of parturition.[172, 181] Similarly, in llamas and alpacas, PGFM increase steadily during the last 100 days of pregnancy and surges just before parturition.[171]

Contrarily to domestic ruminants (i.e. cattle, sheep and goat), camelids have been shown to produce relaxin during pregnancy.[184, 187] In the alpaca, relaxin increases significantly at 3.5 months of

pregnancy. A decrease is observed between 5.5 and 7 months followed by a steady increase until parturition.[184] In the dromedary, sources of relaxin include the ovary (CL) and placental tissue.[187, 188] The biological function of relaxin in camelids is not clear. It is suggested that it may be involved in expression of vascular endothelial growth factor (VEGF), which is essential for CL development. Relaxin is expressed in the uteroplacental tissue around 85 days in llamas and alpacas[184] and 93 days in the dromedary.[187] There are no studies on the profile of prolactin in camelids.

2.3.8 Pregnancy associated glycoproteins in camelids

Studies on camelid placental proteins thus far have failed to detect any pregnancy associated protein (PAG) that can be used for pregnancy diagnosis or feto-placental evaluation. Drew et al. failed to detect Pregnancy Specific Protein B (PSPB) in sera from llamas between 2 and 244 days of pregnancy.[189] More recently, alpaca and dromedary placental extracts showed complete absence of immunoreactivity with antiserum against molecules of the PAG-1 family to which PSPB belongs, but expressed marked immunoreactivity against anti-boPAG-II serum.[190]

Placental expression of PAGs was detected within trophectoderm cells in *C. dromedarius* and *C. bactrianus*, named CdPAGs and CbPAGs, with high structural similarities to PAG-like epitopes in pigs.[191-193] Chorionic PAGs family were identified in alpaca and named LpPAGs.[192]

2.4 Conclusion

Pregnancy loss is the second most common complaint in camelid theriogenology.[2, 4, 194, 195] Epidemiological data on the incidence of pregnancy loss in camelids is scarce.[106, 119, 196] Early embryonic death can reach up to 50% in naturally mated females and between 20 and 60% in embryo transfer recipients.[123] There are several contributing factors to early embryo loss. Season of mating was shown to be an important factor in alpacas. Pregnancy loss prior to 81 days of gestation are higher in autumn-bred

females (17.3%) that in spring-bred females (2.8%).[196] In camels, pregnancy loss can reach alarming levels particularly when nutrition in not adequate or during outbreaks of diseases such as trypanosomiasis.[105, 197] As in other species, oocyte quality, male factors, and environmental factors may be involved in occurrence of pregnancy loss. Our knowledge of MRP in camelid lags behind compared to other domestic species, particularly ruminants. Deciphering the mechanism of prevention of luteolysis in camelids is fundamental for furthering our knowledge on causes of early embryo loss and its prevention. Our laboratory is engaged in an ambitious collaborative program to answer specific questions regarding ovulation and early embryo development and embryo/uterus crosstalk. The ultimate goal of these studies is to determine the pathways of MRP in camelids.

2.5 References

1. Diskin MG, Morris DG. Embryonic and early foetal losses incattle and other ruminants. Reprod Domestic Anim. 2008;43:260-7.

2. Tibary A, Anouassi A, Sghiri A, et al. Current knowledge and future challenges in camelid reproduction. Soc Reprod Fertil Suppl. 2007;64:297-313. Epub 2007/05/12.

3. Fernandez Baca S, Hansel W, Novoa C. Embryonic mortality in the alpaca. 1970.

4. Tibary A, Pearson LK. Pregnancy loss and abortion in camelids. Proc of First Latin American Animal Reproduction Congress, Buenos Aires, Argentina, Mrch 11-15, 2015, pages 67-87. 2015.

5. Vanderwall DK. Early Embryonic Loss in the Mare. Journal of Equine Veterinary Science. 2008 Nov;28:691-702.

6. Allen WR, Brown L, Wright M, et al. Reproductive efficiency of flatrace and national hunt Thoroughbred mares and stallions in England. Equine Veterinary Journal. 2007 Sep;39:438-45.

7. Ball BA, Woods GL. Embryonic Loss and Early-Pregnancy Loss in the Mare. Comp Cont Educ Pract. 1987 Apr;9:459-468.

8. Canisso IF, Beltaire KA, Bedford-Guaus SJ. Premature luteal regression in a pregnant mare and subsequent pregnancy maintenance with the use of oral altrenogest. Equine Veterinary Journal. 2013;45:97-100.

9. Farin CE, Farmer WT, Farin PW. Pregnancy recognition and abnormal offspring syndrome in cattle. Reprod Fert Develop. 2010;22:75-87.

10. Spencer TE, Sandra O, Wolf E. Genes involved in conceptus-endometrial interactions in ruminants: insights from reductionism and thoughts on holistic approaches. Reproduction. 2008;135:165-79.

11. Spencer TE, Johnson GA, Bazer FW, et al. Pregnancy recognition and conceptus implantation in domestic ruminants: Roles of progesterone, interferons and endogenous retroviruses. Reprod Fert Develop. 2007;19:65-78.

12. Sandra O, Constant F, Carvalho AV, et al. Maternal organism and embryo biosensoring: insights from ruminants. Journal of Reproductive Immunology. 2015 Apr;108:105-13.

13. Bazer FW, Johnson GA. Pig blastocyst-uterine interactions. Differentiation. 2014 Jan-Feb;87:52-65.

14. Franczak A, Bogacki M. Local and Systemic Effects of Embryos on Uterine Tissues During Early Pregnancy in Pigs. Journal of Reproduction and Development. 2009 Jun;55:262-72.

15. Jalali BM, Bogacki M, Dietrich M, et al. Proteomic analysis of porcine endometrial tissue during peri-implantation period reveals altered protein abundance. J Proteomics. 2015 Jul 1;125:76-88.

16. Klein C, Troedsson MHT. Transcriptional Profiling of Equine Conceptuses Reveals New Aspects of Embryo-Maternal Communication in the Horse. Biol Reprod. 2011 May;84:872-85.

17. Klein C, Troedsson MHT. Maternal recognition of pregnancy in the horse: a mystery still to be solved. Reprod Fert Develop. 2011;23:952-63.

18. Katila T. Maternal recognition of pregnancy in the horse. Pferdeheilkunde. 2011 May-Jun;27:261-4.

19. Roberts RM. Conceptus interferons and maternal recognition of pregnancy. Biology of Reproduction. 1989;40:449-52.

20. Lo W, Summers P. In vitro culture and interferon-tau secretion by ovine blastocysts. Anim Reprod Sci. 2002;70:191-202.

21. Spencer TE, Becker WC, George P, et al. Ovine interferon-tau regulates expression of endometrial receptors for estrogen and oxytocin but not progesterone. Biology of Reproduction. 1995;53:732-45.

22. Robinson RS, Hammond A, Wathes M, et al. Corpus luteum-endometrium-embryo interactions in the dairy cow: underlying mechanisms and clinical relevance. Reprod Domestic Anim. 2008;43:104-12.

23. Gray CA, Taylor KM, Ramsey WS, et al. Endometrial glands are required for preimplantation conceptus elongation and survival. Biol Reprod. 2001;64:1608-13.

24. Brooks K, Burns G, Spencer TE. Conceptus elongation in ruminants: roles of progesterone, prostaglandin, interferon tau and cortisol. Journal of Animal Science and Biotechnology. 2014 Nov 16;5:53.

25. Xiao CW, Murphy B, Sirois J, et al. Down-regulation of oxytocin induced Cyclooxygenase-2 and Prostaglandin F Synthase expression by Interferon-т in Bovine Endometrial Cells. Biology of Reproduction. 1999;60:656-63.

26. Charpigny G, Reinaud P, Créminon C, et al. Correlation of increased concentration of ovine endometrial cyclooxygenase 2 with the increase in PGE2 and PGD2 in the late luteal phase. Journal of Reproduction and Fertility. 1999;117:315-24.

27. Banu S, Lee J, Stephens S, et al. Interferon tau regulates PGF2alpha releasefrom the ovine endometrial epithelial cells via activation of novelJAK/EGFR/ERK/EGR-1 pathways. Mol Endocrinol. 2010;24: 2315-30.

28. Bauersachs S, Ulbrich S, Gross K, et al. Embryo-induced transcriptome changes in bovine endometrium reveal speciesspecific and common molecular markers of uterine receptivity. Reproduction. 2006;132:319-31.

29. Johnson G, Spencer T, Burghardt R, et al. Interferon-tau and progesterone regulate ubiquitin cross-reactive protein expression in the ovine uterus. Biology of Reproduction. 2000;62:622-7.

30. Johnson G, Austin K, Collins A, et al. Endometrial ISG17 mRNA and a related mRNA are induced by interferon-tau and localized to glandular epithelial and stromal cells from pregnant cows. Endocrine Journal. 1999;10:243-52.

31. Dorniak P, Bazer FW, Spencer TE. Prostaglandins Regulate Conceptus Elongation and Mediate Effects of Interferon Tau on the Ovine Uterine Endometrium. Biology of Reproduction. 2011 Jun;84:1119-27.

32. Dorniak P, Bazer FW, Wu GY, et al. Conceptus-derived prostaglandins regulate endometrial function in sheep. Biology of Reproduction. 2012;87:9.

33. Dorniak P, Welsh TH, Bazer FW, et al. Endometrial HSD11B1 and cortisol regenera-tion in the ovine uterus: effects of pregnancy, interferon tau, andprostaglandins. Biology of Reproduction. 2012;86:124.

34. Dorniak P, Welsh TH, Jr., Bazer FW, et al. Cortisol and interferon tau regulation of endometrial function and conceptus development in female sheep. Endocrinology. 2013;154:931-41.

35. Arosh J, Banu S, Kimmins S, et al. Effect of interferon-tau on prostaglandin biosynthesis, transport, and signaling at the time of maternal recognition of pregnancy in cattle: evidence of polycrine actions of prostaglandin E2. Endocrinology. 2004;145:5280-3.

36. Mansouri-Attia N, Aubert J, Reinaud P, et al. Gene expression profiles of bovine carun-cular and intercaruncular endometrium at implantation. Physiological Genomics. 2009;39:14-27.

37. Minten M, Bilby T, Bruno R, et al. Minten, M.A., et al., 2013. Effects of fertility on gene expression and func-tion of the bovine endometrium. . PLOS ONE. 2013;8:e69444.

38. Hansen T, Henkes L, Ashley R, et al. Endocrine actions of interferon-tau in ruminants. Soc Reprod Fertil. 2010;Suppl. 67:325-40.

39. Antoniazzi A, Webb B, Romero J, et al. Endocrine delivery of interferon tau protects the corpus luteum from prostaglandin F2 alpha-induced luteolysis in ewes. Biol Reprod. 2013;88:144.

40. Antoniazzi AQ, Henkes LE, Oliveira JFC, et al. The role of interferon-tau during maternal recognition of pregnancy in ruminants. Cienc Rural. 2011 Jan;41:176-85.

41. Bott RC, Ashley RL, Henkes LE, et al. Uterine Vein Infusion of Interferon Tau (IFNT) Extends Luteal Life Span in Ewes. Biology of Reproduction. 2010 Apr;82:725-35.

42. Woclawek-Potocka I, Kowalczyk-Zieba I, Skarzynski DJ. Lysophosphatidic acid action duringearly pregnancy in the cow: in vivo and in vitro studies. J ReprodDev. 2010;56:411-20.

43. Forde N, Lonergan P. Transcriptomic Analysis of the Bovine Endometrium: What is Required to Establish Uterine Receptivity to Implantation in Cattle ? Journal of Reproduction and Development. 2012 Apr;58:189-95.

44. Geisert RD, Zavy MT, Moffatt RJ, et al. Embryonic steroids and the establishment of pregnancy in pigs. Journal of Reproduction and Fertility. 1990;Suppl. 40:293-305.

45. Bazer FW, Thatcher WW. Theory of maternal recognition of pregnancy in swine based on estrogen controlled endocrine versus exocrine secretion of prostaglandin F2a by the uterine endometrium. Prostaglandins. 1977;14:397-401.

46. Kawarasaki T, Enya S, Otsu Y. The effect of estrogen administration during early pregnancy upon the survival of single implanted pig embryos. J Anim Sci. 2012 Dec;90:4781-7.

47. Pope WF, Maurer RR, Stormshak F. Intrauterine migration of the porcine embryo: influence of estradiol-17 beta and histamine. Biology of Reproduction. 1982;27:575-9.

48. Samborski A, Graf A, Krebs S, et al. Transcriptome Changes in the Porcine Endometrium During the Preattachment Phase. Biology of Reproduction. 2013 Dec;89.

49. Bauersachs S, Wolf E. Transcriptome analyses of bovine, porcine and equine endometrium during the pre-implantation phase. Anim Reprod Sci. 2012 Sep;134:84-94.

50. Bauersachs S, Wolf E. Uterine Responses to the Preattachment Embryo in Domestic Ungulates: Recognition of Pregnancy and Preparation for Implantation. Annu Rev Anim Biosci. 2015;3:489-511.

51. Krawczynski K, Najmula J, Bauersachs S, et al. MicroRNAome of Porcine Conceptuses and Trophoblasts: Expression Profile of microRNAs and Their Potential to Regulate Genes Crucial for Establishment of Pregnancy. Biology of Reproduction. 2015 Jan 1;92.

52. Meira C, Ferreira JC, Silva ESM, et al. Developmental aspects of early pregnancy in mares. Anim Reprod. 2012 Jul-Sep;9:166-72.

53. Weber J, Woods G. Influence of embryonic secretory chemical on selective oviductal transport in mares. Equine Vet J 1993;Suppl. 15:26-38.

54. Vanderwall DK, Silvia WJ, Fitzgerald BP. Concentrations of oxytocin in the cavernous sinus of mares during luteolysis:temporal relationship with concentrations of 13,14-dihydro-15-ketoprostaglandin F2 alpha. Journal of Reproduction and Fertility. 1998;112:337-46.

55. Goff AK, Pontbriand D, Sirois J. Oxytocin stimulation of plasma 15-keto-13,14-dihydroprostaglandinF-2alpha during the oestrous cycle and early pregnancy in the mare. Journal of Reproduction and Fertility. 1987;Suppl/ 35:253-60.

56. Sharp DC, Thatcher M-J, Salute ME, et al. Relationship between endometrial oxytocin receptors and oxytocin induced prostaglandin F2 alpha release during the oestrous cycle and early pregnancy in pony mares. Journal of Reproduction and Fertility. 1997;109:137-44.

57. Starbuck GR, Stout TAE, Lamming GE, et al. Endometrial oxytocin receptor and uterine prostaglandin secretion in mares during oestrous cycle and early pregnancy. Journal of Reproduction and Fertility. 1998;113:173-9.

58. Stout TA, Allen WR. Prostaglandin E(2) and F(2alpha) production by equine conceptuses and concentrations in conceptus fluids and uterine flushings recovered from early pregnant and dioestrous mares. Reproduction. 2002;123:261-8.

59. Ealy AD, Eroh ML, Sharp DC. Prostaglandin H synthase Type 2 is differentially expressed in endometrium based on pregnancy status in pony mares and responds to oxytocin and conceptus secretions in explant culture. Anim Reprod Sci. 2010 Jan;117:99-105.

60. Boerboom D, Brown KA, Vaillancourt D, et al. Expression of key prostagland in synthases in equine endometrium during late diestrus and early pregnancy. Biology of Reproduction. 2004;70:391-9.

61. Atli MO, Kurar SA, Aslan S, et al. Evaluation of genes involved in prostaglandin action in equine endometrium during estrous cycle and early pregnancy. Anim Reprod Sci. 2010;122:124-32.

62. Merkl M, Ulbrich SE, Otzdorff C, et al. Microarray analysis of equine endometrium a tDays 8 and 12 of pregnancy. Biology of Reproduction. 2010;83:874-86.

63. de Ruijter-Villani M, van Tol HTA, Stout TAE. Effect of pregnancy on endometrial expression of luteolytic pathway components in the mare. Reprod Fert Develop. 2015;27:834-45.

64. Ababneh MM, Ababneh H, Shidaifat F. Expression of Cytosolic Phospholipase A2 in Equine Endometrium during the Oestrous Cycle and Early Pregnancy. Reprod Domestic Anim. 2011 Apr;46:268-74.

65. Stout TA, Lamming GE, Allen WR. Oxytocin administration prolongs luteal function in cyclic mares. Journal of Reproduction and Fertility. 1999;116:315-20.

66. Vanderwall DK, Rasmussen DM, Woods GL. Effect of repeated administration of oxytocin during diestrus on duration of function of corpora lutea in mares. J Am Vet Med Assoc. 2007;231:1864-7.

67. Ababneh MM, Troedsson MH, Michelson JR, et al. Partial characterization of an equine conceptus prostaglandin inhibitory factor. journal of Reproduction and Fertility. 2000;Suppl. 56:607-13.

68. Sharp DC, McDowell KJ, Weithenauer J, et al. The continuum of events leading to maternal recognition of pregnancy in mares. Journal of Reproduction and Fertility. 1989;Suppl. 37:101-7.

69. Stout TA, Meadows S, Allen WR. Stage-specific formation of the equine blastocyst capsule is instrumental to hatching and to embryonic survival in vivo. Anim Reprod Sci. 2005;87.

70. Tremoleda JL, Stout TA, Lagutina I, et al. Effects of in vitro production on horse embryo morphology, cytoskeletal characteristics, and blastocyst capsule formation. Biology of Reproduction. 2003;69:1895-906.

71. Oriol JG, Betteridge KJ, Clarke AJ, et al. Mucin-like glycoproteins in the equine embryonic capsule. Molecular Reproduction and Development. 1993;34:255-65.

72. Oriol JG, Sharom FJ, Betteridge KJ. Developmentally regulated changes in the glycoproteins of the equine embryonic capsule. Journal of Reproduction and Fertility. 1993;99:653-64.

73. McDowell KJ, Sharp DC, Grubaugh W, et al. Restricted conceptus mobility results in failure of pregnancy maintenance in mares. Biology of Reproduction. 1988;39:340-8.

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

75. Ginther OJ. Fixation and orientation of the early equine conceptus. Theriogenology. 1983;19:613-23.

76. Gastal MO, Gastal EL, Kot K, et al. Factors related to the time of fixation of the conceptus in mares. Theriogenology. 1996;46:1171-80.

77. Ginther OJ. Mobility of twin embryonic vesicles in mares. Theriogenology. 1984;22:83-95.

78. Stout TA, Allen WR. Role of prostaglandins in intrauterine migration of the equine conceptus. Reproduction. 2001;121:771-5.

79. Vanderwall DK, Woods GL, Weber JA, et al. Corpus luteal function in nonpregnant mares following intrauterine administration of prostaglandin E(2) or estradiol-17beta. Theriogenology. 1994;42:1069-83.

80. Woodley SL, Burns PJ, Douglas RH, et al. Prolonged interovulatory interval after oestradiol treatment in mares. Journal of Reproduction and Fertility. 1979;Suppl. 27:205-9.

81. Wilsher S, Allen WR. Intrauterine administration of plant oils inhibits luteolysis in the mare. Equine Veterinary Journal. 2011 Jan;43:99-105.

82. Goff AK, Leduc S, Poitras P, et al. Steroid synthesis by equine conceptuses between days 7 and 14 and endometrial steroidmetabolism. Domestic Animal Endocrinology. 1993;10:229-36.

83. Baker CB, Adams MH, McDowell KJ. Lack of expression of alpha or omega interferons by the horse conceptus. Journal of Reproduction and Fertility. 1991;Suppl. 44:439-43.

84. Cochet M, Vaiman D, Lefevre F. Novel interferon delta genes in mammals: cloning of one gene from the sheep, two genes expressed by the horse conceptus and discovery of related sequences in several taxa by genomic database screening. Gene. 2009;433:88-99.

85. Budik S, Lussy H, Aurich C. Quantification of different type I interferons in equine embryos at days 10–16 of gestation. Anim Reprod Sci. 2010;121S:S307-8.

86. Hicks BA, Etter SJ, Carnahan KG, et al. Expression of the uterine Mxprotein in cyclic and pregnant cows, gilts, and mares. J Anim Sci. 2003;81:1552-61.

87. Klein C, Scoggin KE, Ealy AD, et al. Transcriptional Profiling of Equine Endometrium During the Time of Maternal Recognition of Pregnancy. Biol Reprod. 2010 Jul;83:102-13.

88. Nie GJ, Johnson KE, Braden TD, et al. Use of an intra-uterine glass ball protocol to extend luteal function in mares. Journal of Equine Veterinary Science. 2003;23:266-73.

89. Rivera del Alamo MM, Reilas T, Kindahl H, et al. Mechanisms behind intrauterine device-induced luteal persistence in mares. Anim Reprod Sci. 2008;107:94-106.

90. Rivera del Alamo MM, Reilas T, Kindahl H, et al. The luteostatic effect of intrauterine devices in mares is associated with endometrial expression of COX-2. Anim Reprod Sci. 2010;121:227-8.

91. Chen BX, Yuen ZX. Reproductive pattern of the Bactrian camel. . In: Cockrill, WR (ed) The Camelid, an all-purpose animal, Scand Inst Afric Studies, Uppsala Sweden. 1984.

92. Marie M, Anouassi A. Mating-Induced Luteinizing Hormone Surge and Ovulation. Biology of Reproduction. 1986;35:792-8.

93. Bravo PW, Stabenfeldt GH, Fowler ME, et al. Pituitary response to repeated copulation and/or gonadotropin-releasing hormone administration in llamas and alpacas. Biology of Reproduction. 1992;47:884-8.

94. Chen BX, Yuen ZX, Pan GW. Semen-induced ovulation in the bactrian camel (Camelus bactrianus). J Reprod Fert. 1985;73:335-9.

95. Chen BX, Yuen ZX, Pan GW, et al. Studies on the ovulation mechanism in the bactrian camel. II. The role of semen in induction of ovulation. Acta vet zoo-tech sin (In Chinese). 1983;14:161-6.

96. Li XL, Zhang Y, Chen BX, et al. The concentrations of LH, FSH, oestradiol-17 beta and progesterone in the blood plasma of the female Bactrian camel (Camelus bactrianus) before and after intramuscular injection of seminal plasma. Vet Res Commun. 2002 Oct;26:571-6.

97. Li XL, Zhao XX. Separation and purification of ovulation-inducing factors in the seminal plasma of the Bactrian camel (Camelus bactrianus). Vet Res Commun. 2004 Apr;28:235-45.

98. Adams GP, Ratto MH. Ovulation-inducing factor in seminal plasma: a review. Anim Reprod Sci. 2013;136:148-56.

99. Kershaw-Young CM, Druart X, Vaughan J, et al. β-Nerve growth factor is a major component of alpaca seminal plasma and induces ovulation in female alpacas. Reproduction, Fertility and Development. 2012;24:1093-7.

100. Kumar S, Sharma V, Singh S, et al. Proteomic identification of camel seminal plasma: Purification of b-nerve growth factor. Anim Reprod Sci. 2013;136:289-95.

101. Silva M, Ulloa-Leal C, Norambuena C, et al. Ovulation-inducing factor (OIF/NGF) from seminal plasma origin enhances Corpus Luteum function in llamas regardless the preovulatory follicle diameter. Anim Reprod Sci. 2014 Aug;148:221-7.

102. Vaughan JL. Ovarian function in South American camelids (alpacas, llamas, vicunas, guanacos). Anim Reprod Sci. 2011;124:237-43.

103. Del Campo MR, Del Campo CH, Adams GP, et al. The application of new reproductive technologies to South American camelids. Theriogenology. 1995;43:21-30.

104. Campbell A, Pearson L, Spencer T, et al. Double ovulation and occurence of twining in alpacas 9Vicugna pacos). Theriogenology. 2015;84:421-4.

105. Tibary A, Anouassi A. Ultrasonographic Changes of the Reproductive-Tract in the Female Camel (Camelus-Dromedarius) During the Follicular Cycle and Pregnancy. Journal of Camel Practice and Research. 1996;3:71-90.

106. Vaughan JL, Tibary A. Reproduction in female South American camelids: a review and clinical observations. Small Ruminant Research. 2006;61:259-81.

107. Bravo PW, Moscoso J, Ordoñez C, et al. Transport of spermatozoa and ova in female alpaca. Anim Reprod Sci. 1996;43:173-9.

108. Apichela SA, Valz-Gianinet JN, Schuster S, et al. Lectin binding patterns and carbohydrate mediation of sperm binding to llama oviductal cells in vitro. Anim Reprod Sci. 2010;118:344-53.

109. Apichela SA, Argañaraz ME, Giuliano S, et al. Llama oviductal sperm reservoirs: involvement of bulbourethral glands. Andrologia. 2014;46:290-5.

110. Apichela SA. Sperm reservoir in the llama oviduct and advances in the study of the proteolytic systems involved. Archivos Latinoamericanos de Producción Animal. 2011;19:55-7.

111. Apichela S, Jiménez-Díaz MA, Roldan-Olarte M, et al. In vivo and in vitro sperm interaction with oviductal epithelial cells of llama. Reprod Domestic Anim. 2009;44:943-51.

112. Stekleniov EP. Des particularites anatomo-morphologiques de la structure et des fonctions physiologiques des trompes de Fallope chez les camelides (genres Lama et Camelus). 6th ICAR. 1968;1:74.

113. Thibault C. Sperm Transport and Storage in Vertebrates. J Reprod Fert, Suppl. 1973;18:39-53.

114. Picha Y, Tibary A, Memon M, et al. Chronology of early embryonic development and embryo uterine migration in alpacas. Theriogenology. 2013 Mar 1;79:702-8.

115. Gupta AK, Choudhary MS, Barhat NK. A note on optimum time for service in camel (Camelus dromedarius). Indian Journal of Animal Sciences. 1978;48:324-5.

116. Adam CL, Bourka DA, Kyle CE, et al. Ovulation and embryo recovery in the llama. Proceedings of the First International Camel Conference, Dubai, 2nd 6th February. 1992.

117. Bourke DA, Adam CL, Kyle CE, et al. Superovulation and embryo transfer in the llama. Proceedings of the First International Camel Conference, Dubai, 2nd 6th February. 1992.

118. McKinnon AO, Tinson AH, Allen WRHAJ, et al. Embryo transfer in dromedary camels. Proceedings of the First International Camel Conference, Dubai, 2nd 6th February. 1992.

119. Tibary A. Pregnancy loss in camelids. 147th Annual Convention, Atlanta, July 31-August 3rd. 2010.

120. Trasorras V, Chaves MG, Neild D, et al. Embryo transfer technique: Factors affecting the viability of the corpus luteum in llamas. Anim Reprod Sci. 2010 Sep;121:279-85.

121. Carnero S S, Huanca L W, Cordero R A, et al. Embryo transfer ipsilateral and contralateral to the position of the corpus luteum and embryo survival in Llamas. Revista de Investigaciones Veterinarias del Perú (RIVEP). 2011;22:114-20.

122. Picha Y, Sumar J, Arellano P, et al. Effect of corpus luteum on pregnancy rate and location following embryo transfer in alpacas. Clinical Theriogenology. 2010;2010:365. (Abstract).

123. Anouassi A, Tibary A. Effect of side of embryo transfer in relationship to location of the corpus luteum on pregnancy rate in camels (Camelus dromedarius). Clinical Theriogenology. 2010;2:391.

124. Aba MA, Forsberg M, Kindahl H, et al. Endocrine changes after mating in pregnant and non-pregnant llamas and alpacas. Acta Veterinaria Scandinavica. 1995;36:489-98.

125. Marie M, Anouassi A. Induction of luteal activity and progesterone secretion in the nonpregnant one-humped camel (Camelus dromedarius). J Reprod Fertil. 1987;80:183-92.

126. Adams GP, Sumar J, Ginther OJ. Form and function of the corpus luteum in llamas. Animal Reproduction Science. 1991;24:127-38.

127. Smith BB, Timm KI, Reed PJ, et al. Use of cloprostenol as an abortifacient in the llama (Lama glama). Theriogenology. 2000;54:497-505.

128. Aba MA, Kindahl H, Forsberg M, et al. Levels of progesterone and changes in prostaglandin F-2 alpha release during luteolysis and early pregnancy in llamas and the effect of treatment with flunixin meglumine. Anim Reprod Sci. 2000 Apr 28;59:87-97.

129. Aba MA, Bravo PW, Forsberg M, et al. Endocrine changes during early pregnancy in the alpaca. Animal Reproduction Science. 1997 Jul;47:273-9.

130. Bianchi CP. Estudios sobre el control endocrino de la luteólisis y reconocimiento materno de la preñez en llamas (Lama glama). Doctoral thesis. Universidad Nacional del Centro de la Provincia de Buenos Aires, Argentina. 2011.

131. Skidmore JA, Allen WR, Heap RB. Estrogen synthesis by the periimplantation conceptus of the one-humped camel (Camelus-Dromedarius). Journal of Reproduction and Fertility. 1994 Jul;101:363-7.

132. Powell SA, Smith BB, Timm KI, et al. Estradiol production by preimplantation blastocysts and increased serum progesterone following estradiol treatment in llamas. Animal Reproduction Science. 2007 Nov;102:66-75.

133. Chipayo Y. Efecto del estradiol en el periodo de reconocimiento materno de la preñez sobre la supervivencia embrionaria en alpacas. Rev Inv Vet Perú 2003;14:111-8.

134. Palomino CJM, Huanca WL, Cervantes FMP. Efecto de la aplicaci6n de estradiol sobre la sobrevivencia embrionaria en llamas (Lama glama). . Proc of IV Congreso Mundial sobre Camelidos Catamarca (Argentina), 11-15 Oct 2006, 18. 2006.

135. Bravo WM, Sumar JK. Desarrolo fetal de la alpaca. Proc 4th Conv Int Camelidos Sudam, Punte Arenas, Chile. 1981:14-5.

136. Bravo PW, Varela MH. Prenatal development of the alpaca (Lama pacos). Anim Reprod Sci. 1993;32:245-52.

137. El Wishy AB, Hemeida NA, Omar MA, et al. Functional Changes in the Pregnant Camel with Special Reference to Foetal Growth. Br Vet J. 1981;137:527-37.

138. Fernandez-Baca S, Sumar J, Novoa C, et al. Localizacion del cuerpo luteo y la ubicacion del embrion en la alpaca. Mem ALPA, Guadalajara, Mexico. 1974;9:87-8.

139. Fernandez-Baca S, Sumar J, Novoa C, et al. Relacion entre la ubacacion del cuerpo luteo y la localizacion del embrion en la alpaca. Rev Inv Pec (IVITA) Univ Nac S Marcos. 1973;2:131-5.

140. Hussein FM, Nosier MB, El Bawab IE, et al. Prenatal assessment of weight and dimensions of the camel conceptus (Camelus dromedarius). Anim Reprod Sci. 1991;26:1-2.

141. Ratto M, Cervantes M, Norambuena C, et al. Effect of location and stage of development of dominant follicle on ovulation and embryo survival rate in alpacas. Anim Reprod Sci. 2011;127:100-5.

142. Musa BE, Abu Sineina ME. Development of the conceptus in the camel (Camelus dromedarius). 1976.

143. Powell SA, Smith BB, Timm KI, et al. Expression of estrogen receptors alpha and beta in the corpus luteum and uterus from non-pregnant and pregnant llamas. Molecular Reproduction and Development. 2007 Aug;74:1043-52.

144. Berg R, Taher ES, Moustafa MSEDM. Comparison of prenatal growth of some organs in the camel (Camelus dromedarius) and the Egyptian water buffalo (Bos/Bubalus/ bubalis L.). Zentralblatt fur Veterinarmedizin. 1968;15A:438-47.

145. Bustinza J, Matusita A, Gallegos M. Contribucion al patron embriologico de la alpaca. Anales Ira Convencion sobre Camelidos Sudamericanos (Auquenidos). 1970:148-9.

146. Ghazi SR, Oryan A, Pourmirzaei H. Some aspects of macroscopic studies of the placentation in the camel (Camelus dromedarius). Anatomia Histologia Embryologia. 1994;23:337-42.

147. Moniem KA. Some histological and histochemical studies on the placenta of the camel (Camelus dromedarius). Master Thesis, University of Khartoum. 1968.

148. Vasil'eva LP, Shapilov AF, Baimukhambetov K. Fetal membranes of the camel. Dokl Vses Akad Skh Nauk (Vet Bull, 54: 797, 1984). 1984;5:31-2.

149. Iturrizaga DM, Verechia FT, Santos TC, et al. The materno-fetal interface in llama (Lama guanicoe glama). Pesquisa Veterinária Brasileira. 2007;27:221-8.

150. Skidmore JA, Wooding FBP, Allen WR. Implantation and early placentation in the one-humped camel (Camelus dromedarius). Placenta. 1996 May;17:253-62.

151. Olivera L, Zago D, Leiser R, et al. Placentation in the alpaca Lama pacos. Anatomy and Embryology. 2003;207:45-62.

152. Abd-Elnaeim MM, Pfarrer C, Saber AS, et al. Feto-maternal attachment and anchorage in the early diffuse epitheliochorial placenta of the camel (Camelus dromedarius): Light, transmission-, and scanning-electron microscopical study. Cells Tissues Organs. 1999;164:141-54.

153. Gorokhovskii NL, Shmidt GA, Shagaeva VG, et al. Giant cells in the placenta of the Bactrian camel in the fetal period of development. Arkh Anat Gistol Embriol 1975;89:41-6.

154. Jones CJP, Wooding FBP, Abd-Elnaeim MM, et al. Glycosylation in the near-term epitheliochorial placenta of the horse, donkey and camel: a comparative study interbreeding and noninterbreeding species. J Rep Fert. 2000;118:397-405.

155. Wooding FBP, Ozturk M, Skidmore JA, et al. Developmental changes in localization of steroid synthesis enzymes in camelid placenta. Reproduction. 2003;126:239-47.

156. Klisch K, Mess A. Evolutionary differentiation of cetartiodactyl placentae in the light of the viviparity-driven conflict hypothesis. Placenta. 2007;28:353-60.

157. Klisch K, Bevilacqua E, Olivera LVM. Mitotic polyploidization in trophoblast giant cells of the alpaca. Cells Tissues Organs. 2005;181:103-8.

158. Jones CJP, Abd-Elnaeim M, Bevilacqua E, et al. Comparison of uteroplacental glycosylation in the camel (Camelus dromedarius) and alpaca (Lama pacos). Reproduction. 2002;123:115-26.

159. Skidmore J, Billah M, Binns M, et al. Hybridizing old and new world camelids: Camelus dromedarius 3 Lama guanicoe. Proceedings of the Royal Society, London, Series B. 1999;266:649-56.

160. Musa BE, Abu Sineina ME. Studies on the allantoic and amniotic fluids of the camel (Camelus dromedarius). 1976.

161. Fowler ME, Olander HJ. Fetal membranes and ancillary structures of llamas (Lama glama). Am J Vet Res. 1990;51:1495-500.

162. Merkt H, Boer M, Rath D, et al. The Presence of an Additional Fetal Membrane and its Function in the Newborn Guanaco (Lama guanacoe). Theriogenology. 1988;30:437-9.

163. Musa BE. A new epidermal membrane associated with the fetus of the camel (Camelus dromedarius). Anat Histol Embryol. 1977;6:355-8.

164. Schmidt P, Baptidanova L. Peculiarities of embryonic and gestation periods during the prenatal development of the two-humped camel. Reports of the Academy of Science of the USSR. 1966;169.

165. Ali A, Al-Sobayil F, Derar R, et al. Ultrasonographic fetometry and prenatal fetal sex assessment in camelids (Camelus dromedarius). Theriogenology. 2013;80.

166. Ferrer MS, Jones M, Anderson DE, et al. Ultrasonographic parameters of fetal well-being and development in alpacas. Theriogenology. 2013;79:1236-46.

167. Haibel GK, Fung ED. Real-time ultrasonic biparietal diameter measurement for the prediction of gestational age in llamas. Theriogenology. 1991;35:683-7.

168. Herrera EA, Riquelme RA, Sanhueza EM, et al. Use of fetal biometry to determine fetal age in late pregnancy in llamas. Anim Reprod Sci. 2002;74:101-9.

169. Agarwal SP, Khanna ND. Endocrine profiles of the Indian camel under different phases of reproduction. Etudes et Syntheses de l'IEMVT. 1993:77-101.

170. Agarwal SP, Khanna ND, Agarwal VK, et al. Circulating levels of estrogen and progesterone in female camel (Camelus dromedarius) during pregnancy. Theriogenology. 1987;28:849-59.

171. Aba MA, Sumar J, Kindahl H, et al. Plasma concentrations of 15-ketodihydro-PGF2α, progesterone, oestrone sulphate, oestradiol-17β and cortisol during late gestation, parturition and the early postpartum period in llamas and alpacas. Anim Reprod Sci. 1998;50:111-21.

172. Skidmore JA, Billah M, Allen WR. Patterns of hormone secretion throughout pregnancy in the one-humped camel (Camelus dromedarius). Reprod Fertil Dev. 1996;8:863-9.

173. Leon JB, Smith BB, Timm KI, et al. Endocrine changes during pregnancy, parturition and the early post- partum period in the llama (Lama glama). J Reprod Fertil. 1990;88:503-11.

174. Raggi LA, Ferrando G, Parraguez VH, et al. Plasma progesterone in alpaca (Lama pacos) during pregnancy, parturition and early postpartum. Anim Reprod Sci. 1999;54:245-9.

175. Adam CL, Moir CE, Shiach P. Plasma progesterone concentrations in pregnant and non-pregnant llamas (Lama glama). Vet Rec. 1989;125:618-20.

176. Fernandez Baca S. Luteal function and the nature of reproductive failures in the alpaca1970.

177. Bravo PW, Stabenfeldt GH, Fowler ME, et al. Urinary steroids in the periparturient and postpartum periods through early pregnancy in llamas. Theriogenology. 1991;36:267-78.

178. Riveros JL, Urquieta B, Bonacic C, et al. Endocrine changes during pregnancy, parturition and post-partum in guanacos (Lama guanicoe). Anim Reprod Sci. 2009;116:318-25.

179. Schwarzenberger F, Speckbacher G, Bamberg E. Plasma and fecal progestagen evaluations during and after the breeding season of the female vicuna (Vicuna vicuna). Theriogenology. 1995;43:625-34.

180. Volkery J, Gottschalk J, Sobiraj A, et al. Progesterone, pregnanediol-3-glucuronide, relaxin and oestrone sulphate concentrations in saliva, milk and urine of female alpacas (Vicugna pacos) and their application in pregnancy diagnosis. Vet Rec. 2012;171:195.

181. El Belely MS. Endocrine changes, with emphasis on 13,14-dihydro-15-keto- prostaglandin F2 alpha and corticosteroids, before and during parturition in dromedary camels (Camelus dromedarius). J agric sci. 1994;122:315-23.

182. Cristofori F, Quaranta G. Steroids Serum Levels During Sexual Cycle and Pregnancy in the Female One-Humped Camel. PROCEEDINGS OF THE WORKSHOP 'Is it Possible to Improve the Reproductive Performance of the Camel?' - Paris. 1990:65-75.

183. Elias E, Bedrak E, Yagil R. Estradiol Concentration in the Serum of the One-Humped Camel (Camelus dromedarius) during the Various Reproductive Stages. General and Comparative Endocrinology. 1984;56:258-64.

184. Bravo PW, Stewart DR, Lasley BL, et al. Hormonal indicators of pregnancy in llamas and alpacas. Journal of the American Veterinary Medical Association. 1996;208:2027-30.

185. Agarwal SP, Khanna ND, Agarwal VK, et al. Circulating concentrations of thyroid hormones in pregnant camels (Camelus dromedarius). Theriogenology. 1989;31:1239-47.

186. Smith BB, Pearson EG, Leon J. Evaluation of normal triiodothyronine and tetraiodothyronine concentrations in llamas (Lama glama). Am J Vet Res. 1989;50:1215-9.

187. Hombach-Klonisch S, Abd-Elnaeim M, Skidmore JA, et al. Ruminant relaxin in the pregnant one-humped camel (Camelus dromedarius). Biol Reprod. 2000;62:839-46.

188. Osman AHK, Ahmed AAM, Caceci T. Immunolocalization of relaxin in the corpus luteum of the dromedary camel. Kobe Journal of Medical Sciences. 2000;46:245-63.

189. Drew ML, Alexander BM, Sasser GR. Pregnancy determination by use of pregnancy-specific protein B radioimmunoassay in llamas. Journal of the American Veterinary Medical Association. 1995;207:217-9.

190. Bella A, Sousa NM, Dehimi ML, et al. Western analyses of pregnancy-associated glycoprotein family (PAG) in placental extracts of various mammals. Theriogenology. 2007;68:1055-66.

191. Majewska M, Panasiewicz G, Klisch K, et al. Pregnancy-associated glycoprotein (PAG) family: transcripts and gene amplicons in camelids. Reproductive biology. 2009 Jul;9:127-50. Epub 2009/09/08.

192. Majewska M, Panasiewicz G, Szafranska B. Pregnancy-associated glycoprotein (PAG) family localized in chorionic cells within the epitheliochorial/diffuse placenta of the alpaca (Lama pacos). Acta histochemica. 2011 Sep;113:570-7. Epub 2010/07/27.

193. Majewska M, Panasiewicz G, Szafranska S. Expression of pregnancy-associated glycoprotein family in the epitheliochorial placenta of two Camelidae species (C. dromedarius and C. bactrianus). Acta Histochemica. 2012;DOI:10.1016/j.acthis.2013.02.003

194. Verdier Kd, Bornstein S. Alpacas in Sweden - a new challenge. Svensk Veterinärtidning. 2010;62:19-23.

195. Pearson LK, Rodriguez JS, Tibary A. Chapter 23 - Disorders and Diseases of Pregnancy. In: Cebra C, Anderson DE, Tibary A, et al., editors. Llama and Alpaca Care. St. Louis: W.B. Saunders; 2014. p. 256-73.

196. Knight TW, Ridland M, Scott I, et al. Foetal mortality at different stages of gestation in alpacas (Lama pacos) and the associated changes in progesterone concentrations. Anim Reprod Sci. 1995;40:89-97.

197. Tibary A, Abdelhaq A, Abdelmalek S. Factors affecting reproductive performance of camels at the herd and individual level. NATO Science Series: Life and Behavioural Sciences, Vol362. Amsterdam: IOS Press; 2005. p. 97-114.

198. El Wishy AB, Allen WR, Higgins AJ, et al. Functional morphology of the ovaries of the dromedary camel. Proceedings of the First International Camel Conference, Dubai, 2nd 6th February. 1992.

199. El wishy AB, Elsawaf SA. Functional activity of the ovaries and uterine horns in fat-tailed sheep and camels (Camelus dromedarius). 1971.

200. Musa BE, Abu Sineina ME. Some observations on reproduction in the female camel (Camelus dromedarius). 1976.

201. Shalash MR. Some reproductive aspects in the female camel. World Rev Anim Prod. 1965;4:103-8.

202. Shawki H, Verma SK, Chandolia RK. Studies on corpus luteum of pregnancy in she-camel (Camelus dromedarius) throughout the year. Indian Journal of Animal Reproduction. 1992;13:80-2.

203. Ghoneim IM. The ovary of the pregnant camel with special reference to the corpus luteum. M V Sc Cairo University. 1985:68 pp.

204. Sumar J. Reproductive physiology in South American Camelids. 1984.

Table 2.1 Distribution of ovulation between left and right ovary and incidence of left horn pregnancies in camelids

Species	Left ovary (%)	Right ovary (%)	Pregnancies in the left horn (%)	Reference
C. dromedarius	52.7	47.3	100	198
	53.1	46.9	100	199
	50.2	49.8	99.	142, 200
	56.5	43.5	99.24	201
	47.3	52.7	100	202
	54.7	45.3	98.2	203
V. pacos	50	50	98	136
L. glama	50	50	98.4	204

Chapter 3

Double ovulation and occurrence of twinning in alpacas (*V. pacos*)

Abstract

Twin births are rare in alpacas despite the high incidence of double ovulation and are undesirable because they contribute to early and late pregnancy loss, abortion, and birth of non-viable neonates. The objective of the present study was to determine the incidence and outcome of twin pregnancy in double ovulating alpacas by retrospective and prospective analysis. Data from double ovulating females (n=41) presented for pregnancy diagnosis were analyzed to determine pregnancy status at 3 stages post-mating (14 to 16, 25 to 30, and 45 to 53 days). In a prospective study, adult reproductively sound alpacas (n=21) were examined by ultrasonography to determine the incidence of multiple ovulations. A subset of those alpacas (n=10) were euthanized either at 9 days (n=5) or 14 days (n=5) following a single mating and administration of GnRH to determine presence and number of embryos. A total of 31 cycles were included in the study to determine the incidence of multiple ovulations.

In the retrospective study, twin pregnancies were identified between 25 and 30 days in 47.1% of double-ovulating females. There were more twins from bilateral ovulations (62.5%) than from unilateral ovulations (37.5%). Twin pregnancies were either reduced to a singleton (62.5%) or lost completely (37.5%). One set of twins remained viable until Day 52.

In the prospective study, double ovulation occurred in 18.8% of the cycles. Two embryos were collected from 2 of the 4 double-ovulating females.

In conclusion, twin conception is not rare in alpacas. A high rate of spontaneous reduction of twin pregnancies occurs before Day 45. However, a significant proportion of twin pregnancy may result in loss of both embryos. Determination of the number of ovulations (CL) at the time of early pregnancy diagnosis (14 to 16 days) should be an integral part of any pregnancy evaluation.

Keywords: Camelid, Pregnancy, Embryo loss, Ultrasonography, Diagnosis

3.1 Introduction

Reproductive physiology in the female camelid presents striking differences compared to other domestic species. It is well established that all camelids are induced ovulators.[1] Ovulation is induced by the combined inflammatory response to mating and the intrauterine deposition of ß-nerve growth factor present in seminal plasma.[2, 3] Follicular dynamics are therefore variable depending on whether the female has been induced to ovulate or not. Follicular wave patterns have been studied in all species of camelids.[1] The incidence of follicular co-dominance and incidence of multiple ovulations are relatively common and have been reported to be 6% to 10% in alpacas[4, 5], 12% in guanacos[6], 25% in vicunas[7] and up to 45% in dromedaries[8, 9]. Double, triple and even quadruple ovulations have been reported[8, 10-12]. In Sudan, incidence of double and triple ovulations in camels was 13.7% and 1.2%, respectively, in 416 cases[12].

Camelid embryos enter the uterus at the hatched blastocyst stage approximately 7 days post-mating (dpm) and start elongating at 9 dpm. By Day 14, the elongated trophoblast covers the entire uterus.[13] Although the embryonic vesicle can be detected by transrectal ultrasonography as early as 9 dpm[14], a high accuracy of diagnosis is only achieved between 14 dpm (82.5%) and 16 dpm (100%).[15] In practice, it is highly recommended that the first pregnancy diagnosis be performed between 14 and 16 days of pregnancy to distinguish between failure of fertilization and early embryo loss. In addition, non-pregnant females will be at the peak of receptivity at this time and can be rebred.

Despite reports of the high incidence of follicular co-dominance and multiple ovulations, little is known about the incidence of twin pregnancies in camelids. In dromedaries, data from slaughterhouse studies showed an incidence of twins between 0.13% and 13% in the first 3 months of pregnancy.[12, 16-18] In alpacas, a study of 40 females reported 5 sets of twin pregnancies (12.5%) between Days 21 and 33 of pregnancy.[15] Reports from embryo transfer programs show that incidence of multiple ovulations and

fertilization is high in bred females. Collection of 2 or 3 embryos from non-stimulated females (n=667) occurred in 14.1% and 1%, respectively, of uterine flushes performed on Day 7 or 8 after mating.[11]

Knowledge of the fate of twin pregnancies during the course of gestation is important to the clinician. Twinning may be an important cause of early pregnancy loss. In one study, all twin conceptuses diagnosed between 21 and 33 days reduced to a singleton pregnancy with the conceptus in the right uterine horn non-detectable between Days 28 and 33 of pregnancy.[15] Midterm to late-term twin pregnancy often results in abortion or stillbirth. In one study on 85 abortion submissions from llamas and alpacas, twinning was the cause of 4 cases (4.7%). Abortion is likely due to placental insufficiency. Common placental lesions associated with twin abortion in that study included increased avillous areas and mineralization.[19] Although twin births are extremely rare in camelids, the outcome for the dam and neonates is often poor due to increased rate of dystocia, prematurity, and poor viability of the neonates.[20 21] Additionally, females that are born or were conceived with a co-twin male are prone to XX/XY chimerism and freemartinism.[22]

The aims of this research were to (1) to evaluate the outcome of twinning in double-ovulating alpacas and the outcome of twin pregnancy in the first 50 days through a retrospective study and (2) determine the incidence of double ovulation and twinning in alpacas in a prospective experiment.

3.2 Material and methods

3.2.1 Retrospective study

Pregnancy diagnosis records between September 1999 and September 2012 from clients of the Veterinary Teaching Hospital at Washington State University were reviewed. Records on animals that had been diagnosed with 2 corpora lutea (CL) at the time of the first pregnancy diagnosis (14 to 16 dpm) and underwent further pregnancy evaluations between 25 and 30 dpm and 45 to 52 dpm were included. A total of 41 cases met the inclusion criteria. Females that were still carrying twins at 52 dpm received an

intramuscular injection of 250 µg of cloprostenol (Estrumate, Merck Animal Health, De Soto, KS, USA) to terminate the pregnancy.

3.2.2 Prospective study

Adult reproductively sound female alpacas (n=21) that were scheduled for uterine and ovarian sampling as part of a larger experiment on maternal recognition of pregnancy were included in the study. All females were examined by transrectal ultrasonography using an Aloka SSD500 ultrasound machine (Hitachi Aloka, Medical, Ltd, Wallingford, CT, USA) equipped with a 7.5 MHz linear-array transducer mounted on a polyvinyl chloride (PVC) extension rod. All females were mated to a proven fertility male within 12 hours following visualization of at least one large follicle measuring between 7 and 10 mm in diameter. All females received an injection of 50 µg of GnRH (Cystorelin®, Merial, Duluth, GA, USA) subcutaneously immediately after mating. After mating, females were assigned randomly to 2 groups. Group I females (n=10) were scheduled for postmortem collection of embryos from the uterus at either day 9 (n=5) or day 14 (n=5) after mating. Group II females (n=11) were scheduled for pregnancy diagnosis on Day 14 after mating. All females in group II received an injection of 250 µg of cloprostenol (Etrumate®, Merck Animal Health, De Soto, KS, USA) intramuscularly after pregnancy diagnosis. After luteolysis, the follicular wave was monitored again and ovulation was induced with GnRH when the largest follicle reached at least 7 mm in diameter. One female in group II was observed for only one cycle. Females in both groups were examined 7 days after mating and GnRH treatment or GnRH treatment alone, to determine presence and number of CL. Group I females were euthanized using an overdose of barbiturates on either Day 9 or Day 14 after mating. The uteri were collected *en bloc* immediately after euthanasia and flushed as described previously.[13]

3.2.3. Statistical analysis

Descriptive statistics were derived using commercial analytical software (Statistix 10, Tallahasee, FL, USA). For the retrospective data set, pregnancy rates and frequency of twin pregnancies were determined for each period of evaluation. Pregnancy loss rates were compared at each stage of pregnancy using chi-square analysis. For the prospective study, ovulation rate, incidence of single and multiple ovulations as well as side of ovulation were determined. Significance was set at $p < 0.05$.

3.3 Results

Results from the retrospective study on 41 pregnant alpacas with 2 CL are summarized in Table 3.1. Only 9 females (21.9%) were suspected to be carrying twins at the first pregnancy diagnosis between 14 and 16 dpm. The number of twin pregnancies diagnosed on the second examination (25 to 30 dpm) was significantly higher ($p<0.05$). Pregnancy attrition from the initial diagnosis was 17.1%. On the third examination, between 45 and 52 days, the pregnancy rate further declined to 68.3% and only 2 of the pregnant females (7.1%) were still carrying twins. The overall pregnancy attrition was 31.7% by the third pregnancy examination.

The CL were located on both ovaries in 60.9% (n=25) of the cases and on the same ovary in 39.1% (n=16) of the cases. All suspected twin pregnancies at 16 dpm were from bilateral ovulations. At the second evaluation, twins were diagnosed in 62.5% of bilateral ovulations (n=10) and in 37.5% of unilateral ovulations (n=6). Only two females remained pregnant with twins by 45 to 52 days of pregnancy, and both were from bilaterally occurring ovulations. Pregnancy was reduced to a singleton fetus in 6 out of 8 bilaterally ovulating females and in 2 out of 6 unilaterally ovulating females between the second and third pregnancy diagnosis.

In the second study, there was a 100% response to mating and GnRH treatment or GnRH treatment alone in terms of induction of ovulation. Of a total of 31 cycles, single ovulation occurred in 80.6

% (n=25), double ovulations in 16.2% (n=5) and triple ovulations in 3.2% (n=1). In cases of multiple ovulations, 83.3% (5 out of 6 cases) of the females had bilateral ovulation. Double ovulations were found on the same ovary (right) in only one case. In the case of triple ovulation, 2 ovulations occurred on the left ovary and one on the right ovary.

All females (100%) mated under the conditions of this study conceived at least one embryo as determined by embryo flushing at 9 or 14 dpm or by transrectal ultrasonography at 14 dpm. Two embryos were recovered from 2 of the 4 double-ovulating females in group I while the other 2 females each produced one embryo. Transrectal ultrasonography findings were consistent with the postmortem findings in 95% (19 of 20) of the cases as far as the number and side of ovulation. All multiple ovulations diagnosed by ultrasonography were confirmed by visualization of 2 or 3 CL on postmortem examination. The case of triple ovulation was diagnosed by ultrasonography and confirmed on postmortem examination. However, one out of the 5 double ovulations was missed on ultrasonography. No twin pregnancies were suspected in double-ovulating females when transrectal ultrasonography was performed at 14 days.

3.4 Discussion

The data from the retrospective study clearly show that twin conception is not rare in double-ovulating female alpacas. This corroborates findings in a large commercial alpaca embryo transfer program where 2 and 3 embryos were collected from 14.7% and 1%, respectively, of 667 females without hormonal ovarian stimulation.[11] It is interesting to note that the number of suspected twin pregnancies at the first examination (14 to 16 dpm) is low compared to confirmed twin pregnancies at the second examination (25 to 30 dpm). Camelid embryos undergo a rapid elongation after 9 dpm and migrate to the left uterine horn. It has been shown that pregnancy diagnosis based on visualization of intrauterine fluid is highly accurate starting on Day 16 of pregnancy.[15] However, this is not sufficient to establish a diagnosis of twin pregnancy with high accuracy. After 25 days of pregnancy, twins are more accurately identified on the basis of

visualization of 2 embryos and identification of 2 distinct heartbeats. The embryo proper becomes clearly visible by ultrasonography at 22 days, and a heartbeat is initially detected at 24 days.[15]

The discrepancy between the incidence of multiple ovulations and conception and the incidence of twin births in camelids suggests the presence of a very efficient mechanism for embryonic reduction of twin pregnancies. In alpacas, pregnancy loss during the first 35 days varies between 45.5% and 58%.[4] Another study demonstrated a pregnancy loss of 35% in the first 45 dpm.[23] Embryonic mortality rate was found to be 29.6%, 29.0% and 47.7% in maiden, lactating, and barren females, respectively. The majority of losses occurred between 12 and 27 dpm. [23, 24] Risk factors for pregnancy loss include body condition, lactation status, and weight at breeding for maiden alpacas.[4, 24] The role of twinning in pregnancy loss has not been determined. In one study, twins were identified in 5 out of 40 (12.5%) pregnancies between 21 and 33 days. In all cases, the embryos were bilateral, with one located in each uterine horn. All cases reduced to single left horn pregnancy between Days 28 and 33.[15] In the present study, twins identified between Days 25 and 30 of pregnancy were predominantly from bilateral ovulations (62.5%). Reduction to a singleton by Day 45 of pregnancy was observed in 75% of females with bilateral ovulations and in 33.3% of the unilateral ovulations. Complete pregnancy loss was observed in 2 out of 8 females with bilateral ovulation and in 4 out of 6 females with unilateral ovulations. This suggests that spontaneous twin reduction occurs early in pregnancy and can result in either reduction to a singleton or complete loss of pregnancy. Although the number of observations in the present study is small, it appears that unilaterally ovulating females carrying twins may be at higher risk of losing both embryos.

Pregnancy was terminated by administration of cloprostenol in the only set of twins that was carried up to 52 days. Factors involved in the maintenance of twin pregnancy to later stages of gestation remain speculative but establishment of the pregnancies in different horns and the role of uterine size or capacity are plausible. As suggested by the pathological studies on twin abortions, the starvation theory (placental insufficiency) is most likely the cause of reduction.[19]

In the second experiment, we attempted to determine the incidence of double ovulations and twins in early pregnancy. Ovulation and conception rates obtained (100%) are higher than those reported by others. In one study, the ovulation rate and the pregnancy rate following a single mating were 96.5% and 60.2% respectively, for right-ovary ovulations and 96.3% and 56.7% respectively, for left-ovary ovulations.[24] The high pregnancy rate observed in our study may be due to the fact that only reproductively sound females and a proven high-fertility male were used. Also, the fact that all females received GnRH after mating may have played a role. However, the incidence of multiple ovulations in our study (22.3%) is comparable to that reported by others.[11, 25] Two embryos were collected from only 2 of the 4 double-ovulating females (one at 9 dpm and one at 14 dpm). Collection of embryos was performed at 9 dpm and 14 dpm, because our earlier research found that embryo migration and elongation starts at 9 days and that the embryo occupies the entire uterine cavity by 14 days of pregnancy.[13] The sample size in the present study is too small to draw conclusions about the role of elongation in embryo reduction but this is something worth considering in future research.

Another important aspect of twinning that warrants further examination is the repeatability of multiple ovulations within the same female. In the present study, only one female double ovulated on two consecutive cycles. It has been observed in one alpaca that twin conception occurred on 3 consecutive cycles (D. Volkmann,BVSc, MMed Vet, Diplomate ACT, University of Missouri, personal communication, 2015).

In conclusion, the present study showed that multiple ovulations and conception of twins is a common phenomenon in alpacas. Factors involved in the incidence of multiple ovulations merit further research. Presence of multiple of ovulations may be missed by ultrasonography if the examination is performed rapidly or if the examiner lacks experience. Double ovulations may be difficult to visualize by ultrasonography if the CL are on the same ovary. Although the majority of twin pregnancies are reduced to a singleton pregnancy, a significant proportion of females lose both embryos. Therefore, early pregnancy

diagnosis should include examination of the ovaries for presence of multiple ovulations (multiple CL). Females with multiple CL on the first pregnancy diagnosis (14 to 15 dpm) should be monitored at least until Day 45 or 50 of pregnancy for evidence of twin pregnancy, reduction to a singleton, or complete loss of the pregnancy. Elimination of both twins after this stage, by administration of a luteolytic dose of cloprostenol, may be the best course of action to avoid later abortion or complications.

3.5 References

1. Tibary A, Anouassi A, Sghiri A, et al. Current knowledge and future challenges in camelid reproduction. Society of Reproduction and Fertility, Volume 64. Nottingham: Nottingham University Press; 2007. p. 297-313.

2. Kershaw-Young CM, Druart X, Vaughan J, et al. β-Nerve growth factor is a major component of alpaca seminal plasma and induces ovulation in female alpacas. Reprod Fertil Dev. 2012;24:1093-7.

3. Ulloa-Leal C, Bogle OA, Adams GP, et al. Luteotrophic effect of ovulation-inducing factor/nerve growth factor present in the seminal plasma of llamas. Theriogenology. 2014;81:1101-7.

4. Fernandez-Baca S, Hansel W, Novoa C. Corpus luteum function in the alpaca. Biology of Reproduction. 1970;3:252-61.

5. Vaughan JL, Macmillan KL, D'Occhio MJ. Ovarian follicular wave characteristics in alpacas. Anim Reprod Sci. 2004;80:353-61.

6. Riveros JL, Schuler G, Bonacic C, et al. Ovarian follicular dynamics and hormonal secretory profiles in guanacos (Lama guanicoe). Anim Reprod Sci. 2010;119:63-7.

7. Miragaya MH, Aba MA, Capdevielle EF, et al. Follicular activity and hormonal secretory profile in vicuna (Vicugna vicugna). Theriogenology. 2004;61:663-71.

8. Tibary A, Anouassi A. Ultrasonographic changes of the reproductive tract in the female camel (Camelus dromedarius) during the follicular cycle and pregnancy. Journal of Camel Practice and Research. 1996;3:71-90.

9. Manjunatha BM, Al-Bulushi S, Pratap N. Ultrasonographic characterization of follicle deviation in follicular waves with single dominant and codominant follicles in dromedary camels (Camelus dromedarius). Reprod Domestic Anim. 2014;49:239-42.

10. Manjunatha BM, Pratap N, Al-Bulushi S, et al. Characterization of ovarian follicular dynamics in dromedary camels (Camelus dromedarius). Theriogenology. 2012;78:965-73.

11. Vaughan J, Mihm M, Wittek T. Factors influencing embryo transfer success in alpacas-a retrospective study. Anim Reprod Sci. 2013;136:194-204.

12. Musa BE, Sineina MEA. Some observations on reproduction in the female camel (Camelus dromedarius). Acta Veterinaria, Yugoslavia. 1976;26:63-7.

13. Picha Y, Tibary A, Memon M, et al. Chronology of early embryonic development and embryo uterine migration in alpacas. Theriogenology. 2013;79:702-8.

14. Parraguez VH, Cortéz S, Gazitúa FJ, et al. Early pregnancy diagnosis in alpaca (Lama pacos) and llama (Lama glama) by ultrasound. Anim Reprod Sci. 1997;47:113-21.

15. Bravo PW, Mayta MM, Ordoñez CA. Growth of the conceptus in alpacas. Am J Vet Res. 2000;61:1508-11.

16. Aria G, Bianco S, Cristofori F, et al. Ovarian changes in single and twin pregnancies in the dromedary. Atti della Societá Italiana delle Scienze Veterinarie. 1982;36:322-3.

17. Cristofori P, Aria G, Seren E, et al. Somalia. Endocrinological aspects of reproduction in the female camel. World Animal Review. 1986:22-5.

18. Shalash MR, Nawito M. Some reproductive aspects in the female camel. 5th International Congress Animal Reproduction AI, 1964. 1964;2:263-73.

19. Schaefer DL, Bildfell RJ, Long P, et al. Characterization of the microanatomy and histopathology of placentas from aborted, stillborn, and normally delivered alpacas (Vicugna pacos) and llamas (Lama glama). Vet Pathol. 2012;49:313-21.

20. Agab H. Conjoined monster in a dromedary: a case report. Journal of Camel Practice and Research. 2001;8:227.

21. Tibary A, Rodriguez JS, Anouassi A, et al. Management of dystocia in camelids. Stillwater: American Association of Bovine Practitioners; 2008. p. 166-76.

22. Hinrichs K, Buoen LC, Ruth GR. XX/XY chimerism and freemartinism in a female llama co-twin to a male. Journal of the American Veterinary Medical Association. 1999;215:1140-1.

23. Bravo PW, Diaz D, Alarcón V, et al. Effect of the reproductive state of female alpacas on embryonic mortality rate. Am J Vet Res. 2010;71:1096-9.

24. Ratto M, Cervantes M, Norambuena C, et al. Effect of location and stage of development of dominant follicle on ovulation and embryo survival rate in alpacas. Anim Reprod Sci. 2011;127:100-5.

25. Vaughan J. Ovarian function in South American camelids (alpacas, llamas, vicunas, guanacos). Anim Reprod Sci. 2011;124:237-43.

Table 3.1 Proportion of twins diagnosed in 41 double-ovulating alpaca females

Pregnancy diagnosis stage (days from mating)	**14 to 16 days**	**25 to 30 days**	**45 to 52 days**
Overall number of pregnant females (%)	41 (100)[a]	34 (82.9)[b]	28 (68.3)[b]
Number of twin pregnancies (%)	9 (21.95)	16 (47.1)	2 (7.1)
Number of singleton pregnancies (%)	-	18 (52.9)	26 (92.8)
Number of open females (%)	0	7 (17.1)	13 (31.7)

[a,b] Significance was set at $P < 0.05$

Chapter 4

Histologic evaluation of uterine endometrium in the pregnant and non-pregnant alpaca

4.1 Introduction

Development and maintenance of pregnancy requires a uterine environment that is healthy and adaptive to the needs of the growing conceptus. In domestic large animal species, the conceptus must provide a timely biochemical signal, as part of the biological process of maternal recognition of pregnancy (MRP), to prevent luteolysis of the corpus luteum (CL) and maintenance of pregnancy. This is important because the pre-implantation period of the embryo within the uterus is long in domestic large animal species.[1] Following MRP, the endometrium must change histologically in order to allow for remodeling of the endocrine and secretory functions necessary for pregnancy maintenance. The presence of a healthy, functional endometrium is vital throughout gestation, especially prior to attachment/implantation when the conceptus acquires its sustenance entirely from the histotroph produced by the endometrial glands.[1, 2]

The reproductive anatomy and physiology of the female camelid is dramatically different compared to other large animal domestic species.[3] All camelids are induced ovulators, with ovulation occurring equally from both the right and left ovary.[3, 4] Fertilization rates are high in alpacas, often exceeding 80% in well managed breeding programs.[5] While fertilization can occurs on either side, embryos that descend into the right uterine horn will migrate to the left uterine horn for attachment.[6] In alpacas, 83.3% of the embryos resulting from right ovarian ovulation were found in the left uterine horn on Day 9.[6]

Camelids are unique in the fact that greater than 98% of pregnancies are carried in the left uterine horn, suggesting that embryo migration to the left uterine horn is important to prevent luteolysis.[4, 7, 8] This is attributed to a difference in $PGF_{2\alpha}$ release between the two uterine horns. $PGF_{2\alpha}$ release from the right uterine horn is local, whereas its release from the left horn is systemic.[4] While the exact mechanism of embryo migration is not known, it is postulated that embryo migration is facilitated by the shortness of the

uterine body, the smallness of the right horn, and the increased number of mucosal folds in the endometrium of the right horn.[9] Gross examination of the uterus from fetal, pre-pubertal, and non-pregnant female camelids has demonstrated that the left uterine horn is consistently larger when compared to the right uterine horn (Figure 4.1). [3, 10-12]

Attachment of the embryo begins around Day 14 of pregnancy.[13] A well-formed microvillus junction between the fetal and maternal tissues is established by Day 45 in alpacas.[14] Placentation in camelids is epitheliochorial, microcotyledonary, and diffuse.[15]

While numerous histological studies evaluating the endometrium during pregnancy have been performed in other species[16-18 2, 19], information regarding histological changes associated with early pregnancy in alpacas is lacking. To further investigate the mechanisms associated with MRP in camelids a sound understanding and thorough evaluation of the histology of the reproductive tract during early pregnancy in camelids is necessary. Evaluating for potential histological changes between the left and right uterine horn during early pregnancy is an initial step in deciphering the mechanisms associated with MRP and embryo migration in camelids.

The objective of the present study was to compare uterine histology in non-pregnant and early pregnant alpacas. Another aim was to determine any histological differences that may be present between the left and right uterine horn.

4.2 Materials and methods

4.2.1 Experimental design

Adult reproductively sound female alpacas (n=20) that were scheduled for uterine and ovarian sampling as part of a larger experiment on MRP were included in the study.

All females were examined by transrectal ultrasonography using an Aloka SSD500 ultrasound machine (Hitachi Aloka, Medical, Ltd, Wallingford, CT, USA) equipped with a 7.5 MHz linear array

transducer mounted on a polyvinyl chloride (PVC) extension rod. All females were mated to a proven fertility male within 12 hours following visualization of at least one large follicle measuring between 7 and 10 mm in diameter. All females received an injection of 50 µg of GnRH (Cystorelin®, Merial, Duluth, GA, USA) subcutaneously immediately after mating. Following mating, females were assigned randomly to 4 groups. Group I and II females (n=10) were scheduled for postmortem collection of the reproductive tract and embryos at either Day 9 (n=5) or Day 14 (n=5) post-mating respectively. Group III and IV females (n=10) were scheduled for pregnancy diagnosis on Day 14 post-mating. All females in group III and IV received an injection of 250 µg of cloprostenol (Estrumate®, Merck Animal Health, De Soto, KS, USA) intramuscularly after pregnancy diagnosis. Following luteolysis the follicular wave was monitored again and ovulation was induced with GnRH when the largest follicle reached at least 7 mm in diameter. Ovulation was induced with administration of an injection of 50 µg of GnRH subcutaneously. Group III and IV females were scheduled for postmortem collection of the reproductive tract at either Day 9 (n=5) or Day 14 (n=5) post-administration of GnRH respectively.

Females in all groups were examined 7 days after mating and GnRH treatment or GnRH treatment alone, to determine presence and number of CL (Figure 1.4). Group II females collected at 14 days after mating were also examined for pregnancy diagnosis on Day 13 or Day 14 after mating (Figure 1.5). Blood samples from all females were collected after mating and GnRH treatment or GnRH treatment alone and prior to euthanasia by jugular venipuncture. Samples were centrifuged and serum was stored at -80°C for hormone assays to be performed. All females were euthanized using an overdose of barbiturates on Day 9 or Day 14. The uteri were collected *en bloc* immediately after euthanasia and flushed as previously described (Figure 1.6).[9]

The ovaries were evaluated for number and side of CL(s) (Figure 1.7). Embryos (Figure 1.8) and collection fluid were frozen in liquid nitrogen. Reproductive tract tissue samples collected included the ovaries, oviducts, uterotubal-junction (UTJ), endometrium, and myometrium. The endometrium and

myometrium were sampled in 2 locations (near the tip and at the base) of each uterine horn. Samples were either flash frozen in liquid nitrogen and stored at -80°C or transferred to 10% buffered formalin for 48 hours and stored in 70% ethanol prior to analysis.

Uterine horn tissue samples stored in 70% ethanol following fixation were used for histological study and immunohistochemistry. In cassettes the tissue sections were rinsed in 70% ethanol, 80% ethanol, and 95% ethanol for an hour at each concentration. The tissue sections were then rinsed in 100% ethanol for 3 hours, xylenes for 2 hours, and embedded in paraffin.

4.2.2 Histological analysis

Sections were stained with haematoxylin and eosin (H&E). Sections were subjectively assessed by a pathologist for differences in glandular density and tortuosity, vascularity, and infiltration of inflammatory cells between the left and right uterine horns in pregnant and non-pregnant animals. The endometrium and myometrium were measured for the left and right uterine horn of each animal. Two measurements were acquired and averaged to provide adequate representation of each section. Measurements were representative at the widest and narrowest areas of the endometrium and myometrium in an attempt to compensate for any obliqueness in the sections. Measurements were taken at 25x magnification using a Leica microscope connected to a Leica Application Suite (LAS) version B4.6 camera and image software (Leica Microsystems Ltd., Buffalo Grove, IL, USA).

4.2.3 Statistical analysis

Differences in measurements of endometrium and myometrium were evaluated by ANOVA using a general linear model with day of sampling and pregnancy status as main factors. In addition, these measurements were compared between the left and right uterine horn for each sampling day and pregnancy status using a paired t test.

4.3 Results

Subjective evaluation did not show any difference in glandular density and tortuosity, vascularity, or infiltration of inflammatory cells between the left and right uterine horn for females in each experimental group. There were differences in glandular tortuosity and infiltration of inflammatory cells between 14-day pregnant sections (group II) and 14-day open sections (group IV). The endometrial glands were more tortuous for 14-day pregnant sections (group II) when compared to 14-day open sections (group IV). There was a marked infiltration of inflammatory cells for 14-day open sections (group IV) compared to 14-day pregnant sections (group II).

The luminal and glandular epithelium was primarily simple columnar. Large numbers of uterine glands were scattered in the lamina propria, which connected with the luminal epithelium. The myometrium had both a thick inner circular and thinner outer longitudinal section of smooth muscle. Endometrial and myometrial vasculature appeared larger and more extensive than what is represented in other large animal domestic species. Areas of hyperemia were observed within the lamina propria along the luminal epithelium.

The epithelium was similar in the 9-day open (group III) and 9-day pregnant sections (group II), but was noticeably proliferated in the 14-day pregnant sections (group IV). The luminal epithelium in the 14-day pregnant sections (group IV) exhibited development of microvilli (Figure 4.2). Evaluation of the 14-day open sections (group IV) revealed a reduction in size of the glandular epithelial cells and sloughing of some areas of luminal epithelial cells. While there did not appear to be a difference in glandular numbers or glandular content between any of the groups, increased tortuosity with greater numbers of longitudinal sections and increased luminal diameter was visualized in the 14-day pregnant sections (group IV) (Figure 4.3). Vascularity within the endometrium and myometrium appeared similar within and between groups.

Mean thickness of the endometrium and myometrium in the uterine horns is presented in Table 4.1. The ratio of endometrium to myometrium was calculated for each section (Table 4.2). Day 14 open

females (group IV) had significantly ($P<0.05$) thinner endometrium and myometrium than the other groups (Day 9 pregnant, Day 9 open, and Day 14 pregnant females).

There were numerous lymphocytes and neutrophilic granulocytes among the luminal and glandular epithelial cells within the 14-day open sections (group IV). Lymphocytes, neutrophilic granulocytes, and macrophages were observed in the lamina propria. This was consistent among all females within this group. Fewer lymphocytes and neutrophilic granulocytes were seen scattered among the luminal epithelial cells and lamina propria for females in group I, II, and III.

No differences in histological evaluation were observed between the left and right uterine horn in all experimental groups.

4.4 Discussion

Although the vast majority of camelid pregnancies attach within the left uterine horn, histologic structure of the uterus in bilateral horns was similar. These results agree with previously reported observations in camels.[20] Additionally, histologic structure of the uterus was similar in the two uterine locations that were sampled (near the tip and at the base of the uterine horn). This is suggestive that there is uniformity in endometrial and myometrial structure throughout the uterus in alpacas. There is evidence of uniformity in endometrial structure throughout the uterus of the non-pregnant[21, 22] and pregnant mare in early gestation[2]. On subjective evaluation, there are no glandular differences associated with embryo migration to the left uterine horn in alpacas. Glandular tortuosity and increased luminal diameter was observed occurring between Day 9 and Day 14 of pregnancy, and is likely attributed to the continuing influence of luteal progesterone and increased histotroph production. These changes are consistent with the development of an agreeable environment for embryo survival during early pregnancy.

Further objective evaluation of these aspects is in progress using image analysis (ImageJ, National Institute of Health, USA) of surface density to quantify endometrial glandular density and further clarify

potential endometrial and myometrial differences between the left and right uterine horn. Evaluation of endometrial gland surface density of the endometrium during the estrous cycle and early pregnancy in the horse has been performed using computer-assisted morphometric analysis.[2, 22] Similar studies are warranted in the alpaca to quantify surface glandular density of the endometrial glands as a means for further comparison between the left and right uterine horn during different stages of early pregnancy.

The endometrium and myometrium were significantly thinner at 14 days post-induction of ovulation. This can be attributed to luteolysis having occurred by 14 days post-induction of ovulation and the uterus being under an estrogen influence. At 9 days post-induction of ovulation (luteal phase), 9 days of pregnancy, and 14 days of pregnancy the CL is active and the uterus is under a progesterone influence.

Findings of increased inflammation at 14-days open were consistent with previous findings in the camel[20], and suggest that the mucosal immunity of the female reproductive tract in alpacas is very active. It has been demonstrated that ovulating non-pregnant female camelids return to receptivity and develop a dominant follicle 14 days after mating.[4, 23] Semen is deposited deep into the uterine horns throughout the mating period, which lasts up to 30 minutes, and mechanical irritation to the endometrium is an important component for induction of ovulation.[4, 24] The large influx of inflammatory cells observed in the sections from the 14-day control females (group IV) could be initiated in preparation for the process of mating.

Pregnancy loss is most common during early pregnancy. This can especially be true in females with age-related or pathologic changes to the endometrium. Histological evaluation of endometrial biopsies has long been recognized as an important method for evaluation of infertility and breeding soundness in mares.[21, 25-28] Although a grading system for histological evaluation has been proposed in camelids,[29] it is not widely used in a clinical setting. Further evaluation and understanding of normal histological changes associated with the development of early pregnancy will improve our understanding of pathologic conditions of the reproductive tract that may lead to infertility or early embryonic loss.

Although no histological differences were observed between the left and right uterine horns in the present study, it is possible that significant changes may be occurring at the molecular level. Genomic and proteomic studies are necessary for further understanding the mechanism of MRP in this species.

4.5 References

1. Bazer FW, Spencer TE, Johnson GA, et al. Comparative aspects of implantation. Reproduction. 2009;138:195-209.

2. Lefranc AC, Allen WR. Endometrial gland surface density and hyperaemia of the endometrium during early pregnancy in the mare. Equine Veterinary Journal. 2007;39:511-5.

3. Vaughan JL, Tibary A. Reproduction in female South American camelids: a review and clinical observations. Small Rumin Res. 2006;61:259-81.

4. Tibary A, Anouassi A, Sghiri A, et al. Current knowledge and future challenges in camelid reproduction. Society of Reproduction and Fertility, Volume 64. Nottingham: Nottingham University Press; 2007. p. 297-313.

5. Campbell A, Pearson L, Spencer T, et al. Double ovulation and occurence of twining in alpacas 9Vicugna pacos). Theriogenology. 2015;84:421-4.

6. Picha Y, Tibary A, Memon M, et al. Chronology of early embryonic development and embryo uterine migration in alpacas. Theriogenology. 2013;79:702-8.

7. Bravo PW, Varela MH. Prenatal development of the alpaca (Lama pacos). Anim Reprod Sci. 1993;32:245-52.

8. El Wishy AB, Hemeida NA, Omar MA, et al. Functional Changes in the Pregnant Camel with Special Reference to Foetal Growth. Br Vet J. 1981;137:527-37.

9. Musa BE, Abu Sineina ME. Development of the conceptus in the camel (Camelus dromedarius). 1976.

10. Porjoosh A, Raji AR, Nabipour A, et al. Gross and histological study on the uterus of camels (Camelus dromedarius). Journal of Camel Practice and Research. 2010;17:91-4.

11. Srikandakumar AJ, E.H.; Mahgoub, O.; Kadim, I.T.; Al-Ajmi, D.S. Anatomy and histology of the female reproductive tract of the Arabian camel. Journal of Agricultural Sciences. 2001;13:23-6.

12. Arthur GH, Rahim ATA, Hindi ASa. Reproduction and genital diseases of the camel. British Veterinary Journal. 1985;141:650-9.

13. Skidmore JA, Wooding FBP, Allen WR. Implantation and early placentation in the one-humped camel (Camelus dromedarius). Placenta. 1996 May;17:253-62.

14. Olivera L, Zago D, Leiser R, et al. Placentation in the alpaca Lama pacos. Anatomy and Embryology. 2003;207:45-62.

15. Iturrizaga DM, Verechia FT, Santos TC, et al. The materno-fetal interface in llama (Lama guanicoe glama). Pesquisa Veterinária Brasileira. 2007;27:221-8.

16. Eulenberger KH, Neubauer K, Elze K. Histological studies of the endometrium and chorioepithelium of sows during early pregnancy. Monatshefte fur Veterinarmedizin. 1977;32:333-6.

17. Hradecky P, Mossman HW, Stott GG. Comparative development of ruminant placentomes. Theriogenology. 1988;29:715-29.

18. Keenan LR, Forde D, McGeady T, et al. Endometrial histology of early pregnant and non-pregnant mares. Journal of Reproduction and Fertility. 1987:499-504.

19. Paul S, Ghosh RK, Das P, et al. Studies on gross anatomy and histology of the non-pregnant and pregnant (up to 90 days) uterus in the non-descript cow. Indian Journal of Animal Health. 2003;42:178-81.

20. Chen Q, Liu Z, Chen B, et al. Histologic structure of female reproductive duct in Bactrian camel. Chinese Journal of Veterinary Science. 2003;23:408-11.

21. Ricketts SW. Endometrial biopsy as a guide to diagnosis of endometrial pathology in the mare. Journal of Reproduction and Fertility. 1975:341-5.

22. Lefranc AC, Allen WR. Influence of breed and oestrous cycle on endometrial gland surface density in the mare. Equine Veterinary Journal. 2007;39:506-10.

23. Aba MA, Forsberg M, Kindahl H, et al. Endocrine changes after mating in pregnant and non-pregnant llamas and alpacas. Acta Veterinaria Scandinavica. 1995;36:489-98.

24. Adams GP, Ratto MH. Ovulation-inducing factor in seminal plasma: a review. Anim Reprod Sci. 2013;136:148-56.

25. Kenney RM. Cyclic and pathologic changes of the mare endometrium as detected by biopsy, with a note on early embryonic death. Journal of the American Veterinary Medical Association. 1978;172:241-62.

26. Kenny RM, Doig, P.A. Equine endometrial biopsy. Current Therapy in Theriogenology 2. Philadelphia: WB Saunders; 1986.

27. Shideler RK, McChesney AE, Voss JL, et al. Relationship of endometrial biopsy and other management factors on fertility of broodmares. Journal of Equine Veterinary Science. 1982;2:5-10.

28. Snider TA, Sepoy C, Holyoak GR. Equine endometrial biopsy reviewed: observation, interpretation, and application of histopathologic data. Theriogenology. 2011;75:1567-81.

29. Powers BE, Johnson LW, Linton LB, et al. Endometrial biopsy technique and uterine pathologic findings in llamas. Journal of the American Veterinary Medical Association. 1990;197:1157-62.

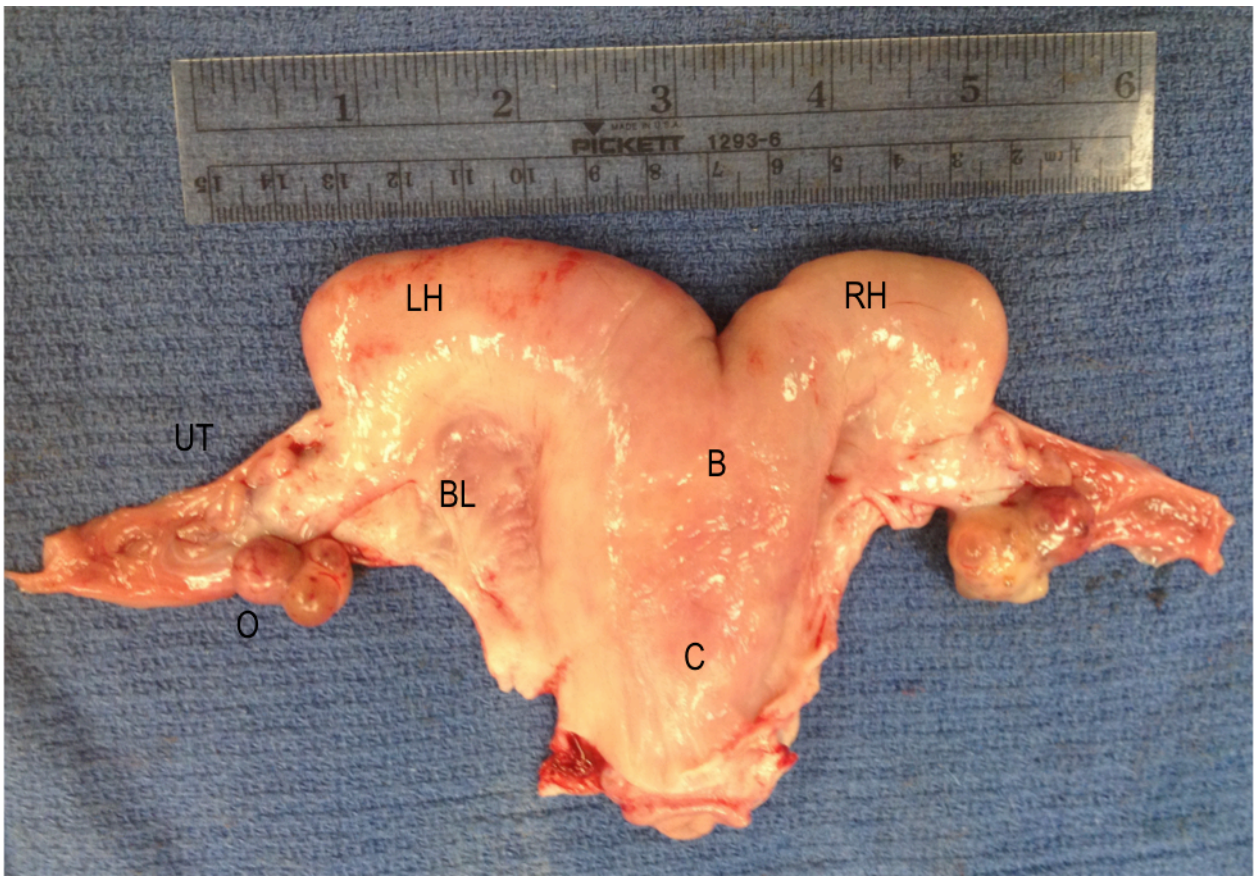

Figure 4.1 The gross anatomy of the uterus from an adult alpaca: right horn (RH), left horn (LH), body (B), cervix (C), uterine tube (UT), O (ovary), and broad ligament (BL)

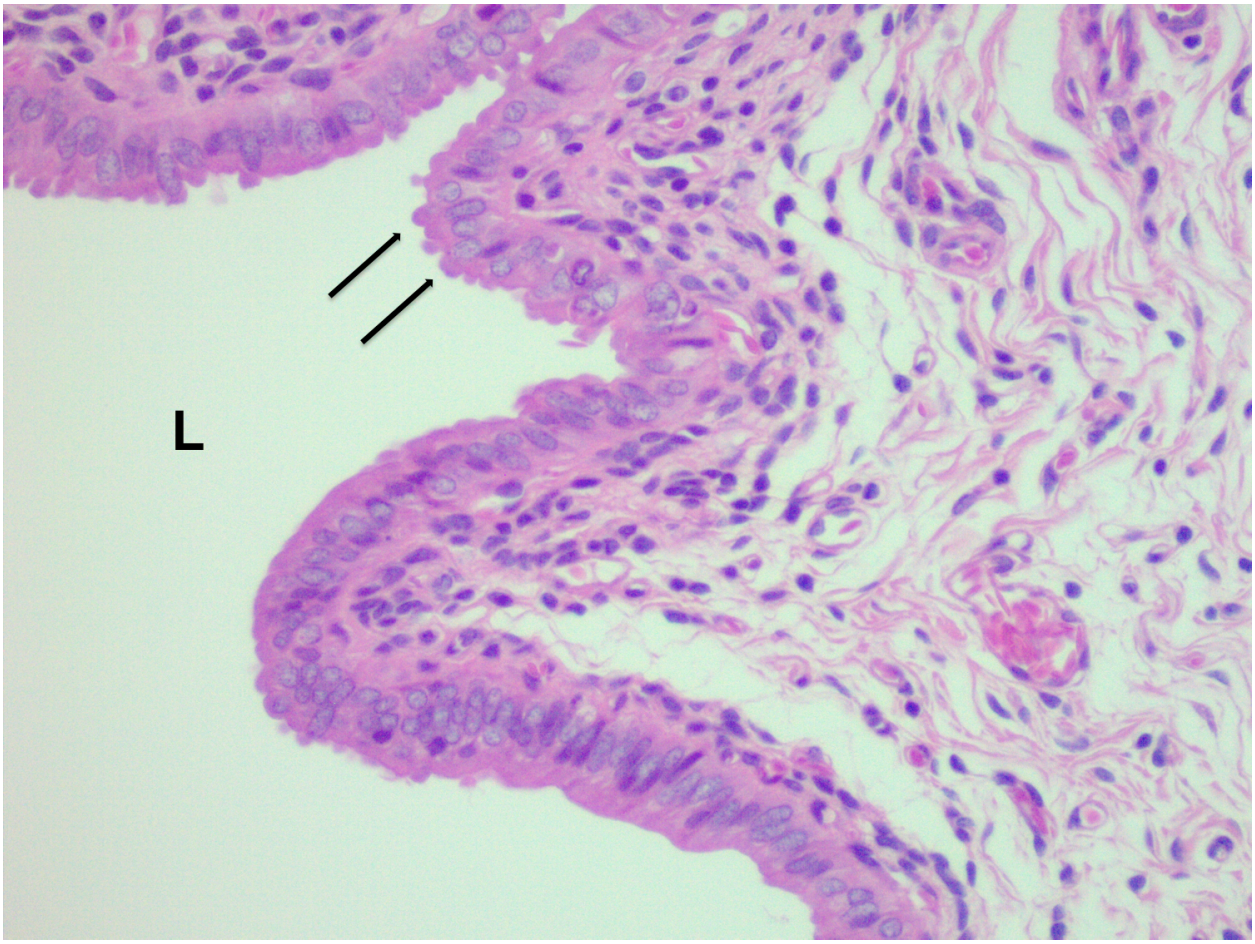

Figure 4.2 Day 14 pregnant alpaca endometrium exhibiting proliferation of luminal epithelium and development of microvilli (arrows). L= lumen (H&E; 400x)

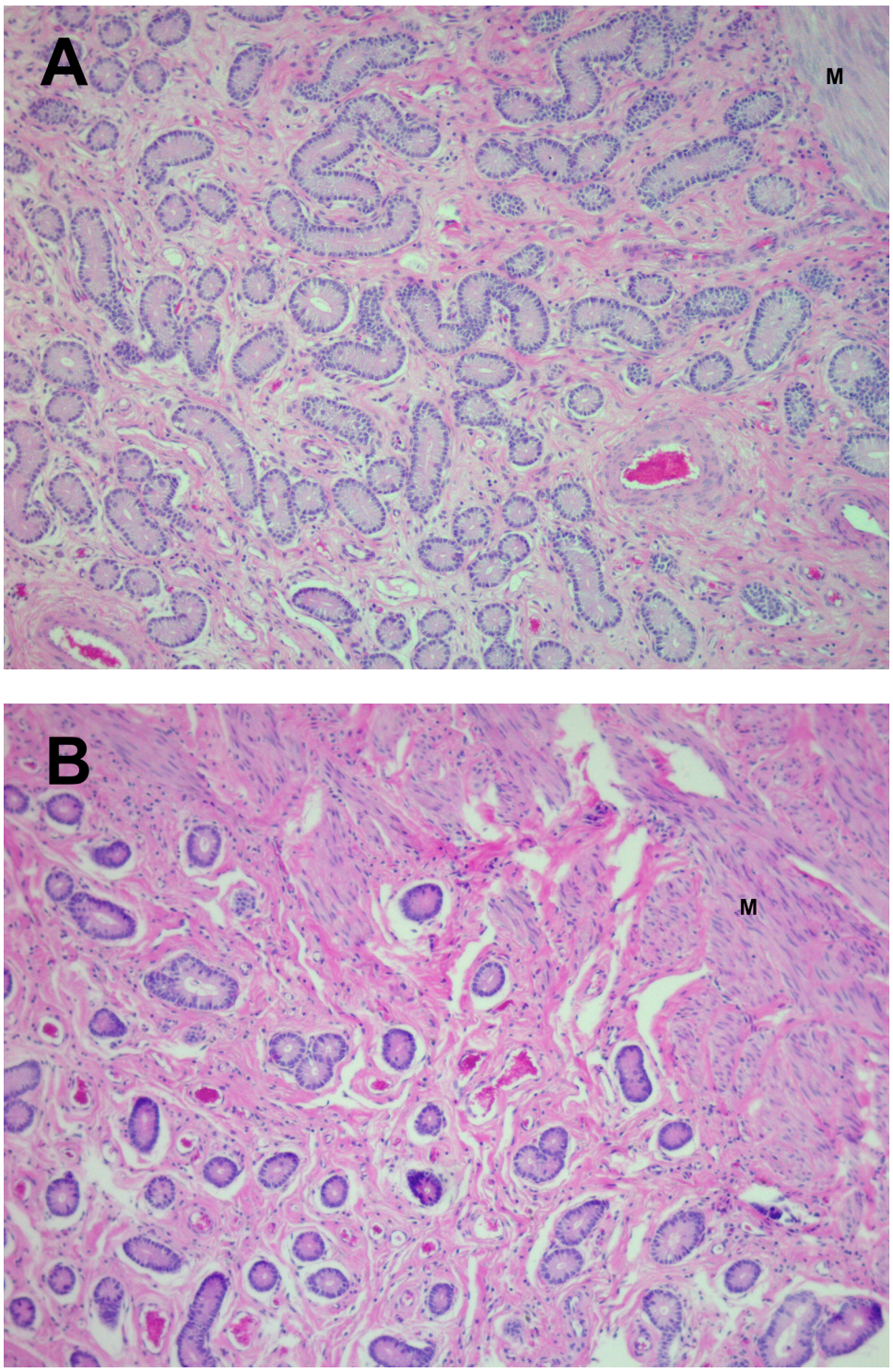

Figure 4.3 Differences in endometrial glandular tortuosity and luminal diameter A) Day 14 pregnant alpaca, B) Day 14 open alpaca. M=myometrium (H&E; 400x)

Table 4.1 Mean (Mean±SE) of the endometrial and myometrial diameter of the left (LH) and right (RH) uterine horn in 10 pregnant and 10 non-pregnant alpacas

Diameter (mm)	Pregnant Day 9	Open Day 9	Pregnant Day 14	Open Day 14
RH Endometrium	2.91±0.20[a]	2.91±0.25[a]	3.06±0.17[a]	2.17±0.19[b]
LH Endometrium	3.46±0.38[a]	3.27±0.37[a]	3.13±0.44[a]	2.53±0.13[b]
RH Myometrium	3.36±0.41[a]	2.58±0.37[a]	3.76±0.44[a]	2.75±0.19[b]
LH Myometrium	3.44±0.34[a]	2.78±0.17[a]	4.11±0.46[a]	2.58±0.40[b]

[a,b] Significance was set at $P < 0.05$

Table 4.2 Mean (Mean±SE) of the endometrial to myometrial (E:M) ratio of the left and right uterine horn in 10 pregnant and 10 non-pregnant alpacas

E:M Ratio	Pregnant Day 9	Open Day 9	Pregnant Day 14	Open Day 14
Left Uterine Horn	0.50±0.10	0.54±0.44	0.43±0.03	0.51±0.04
Right Uterine Horn	0.47±0.10	0.52±0.05	0.45±0.04	0.44±0.13

Chapter 5

Conclusions

Research on maternal recognition of pregnancy (MRP) is driven by the impact of early pregnancy loss on productivity in various species. Pregnancy loss is a common complaint in camelid theriogenology. The majority of these losses occur prior to 60 days gestation. A thorough understanding of the MRP signaling and the physiology of early pregnancy is an important step in deciphering factors affecting pregnancy loss. Our knowledge of MRP and early physiology of pregnancy in camelids lags behind the current situation in other domestic species.

The research herein aimed to expand the knowledge regarding the physiology of early pregnancy and embryonic loss in camelids. A link between the clinical observation of multiple ovulations and the observation of multiple ovulations in an experimental design was established. Data analysis from a retrospective study and prospective study demonstrated that multiple ovulations are relatively common resulting in twin conception followed by reduction to a singleton or complete loss of pregnancy. Bilateral double ovulations were more common than unilateral double ovulations. Factors involved in the incidence of multiple ovulations merit further research. Although the majority of twin pregnancies are reduced to a singleton pregnancy, a significant proportion of females lose both embryos. Therefore, early pregnancy diagnosis should include examination of the ovaries for presence of multiple ovulations (multiple corpora lutea or CL's). Females with multiple CL's at the first pregnancy diagnosis (14 to 15 days post mating) should be monitored at least until Day 45 or 50 of pregnancy for evidence of twin pregnancy, reduction to a singleton, or complete loss of the pregnancy. Elimination of both twins after this stage, by administration of a luteolytic dose of cloprostenol, may be the best course of action to avoid later abortion or complications.

In a second study, histological differences between the left and right uterine horn in pregnant and non-pregnant alpacas were investigated. Numerous histological studies evaluating the endometrium during

pregnancy have been performed in other species. Similar studies are lacking in alpacas. Evaluating for potential histological changes between the left and right uterine horn during early pregnancy is an initial step in deciphering the mechanisms associated with MRP and embryo migration in camelids. This study is important because nearly all pregnancies in camelids are established in the left uterine horn. In addition, studies in our laboratory have shown that the early embryo migrates to the left uterine horn around Day 9 of pregnancy. Our study did not show any difference histologically (glandular density and tortuosity, vascularity, inflammatory cells infiltration) between the two uterine horns. There were no glandular differences associated with embryo migration to the left uterine horn in alpacas. A more objective evaluation of these aspects is in progress using image analysis (ImageJ, National Institute of Health, USA) of surface density to quantify endometrial glandular density and further clarify potential endometrial and myometrial differences between the left and right uterine horn.

The endometrium and myometrium thickness was similar in both uterine horns for each alpaca whether open or pregnant. However, the endometrium and myometrium were significantly thinner at 14 days post-induction of ovulation in non-pregnant females. This can be attributed to lack of progesterone and an influence from estrogen as these female should have experienced luteolysis. Endometrial and myometrial measurements were similar in all groups that had a CL (high progesterone) (9 days post-induction of ovulation, 9 days of pregnancy, and 14 days of pregnancy).

Despite lack of evidence of histologic differences, it is possible that significant changes may be occurring at the molecular level. Immunohistochemistry was performed on uterine tissue sections and expression of estrogen receptor alpha and progesterone receptor within the endometrium was localized, but has not yet been quantified. We hypothesized that embryo migration and elongation triggers gene expression changes and maintenance of pregnancy in camelids as seen in other species. We expect to see a difference in the genetic expression and mRNA production in the pregnant and non-pregnant endometrium. An experiment is under way to continue RNA sequence mapping of alpaca endometrium

and proteomic analysis of gene regulation associated with MRP and pre-implantation embryonic development during early pregnancy.

APPENDIX

Appendix A

Estrogen receptor alpha and progesterone receptor expression in the endometrium of pregnant and non-pregnant alpacas

A.1 Introduction

Ovarian steroid hormones, estrogen and progesterone, have important roles in controlling uterine function in both cyclic and pregnant animals. The effects of both hormones on target cells are regulated through their respective nuclear receptors. Two subtypes of estrogen receptors (ER) have been described: estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ). ERα is the pre-dominant subtype in the uterus of most species because it mainly mediates the classical estrogen actions on the uterus.[1] Progesterone receptor (PR) has 2 major isoforms: A and B. Expression for ERα, ERβ, PRA, and PRB has been demonstrated in the epithelial and stromal cells of the endometrium in llamas.[2] [3, 4]

ER and PR populations are thought to be critical in determining cell responsiveness to steroids.[4] The regulation of steroid hormone receptor expression in the endometrium has been described to be cell type specific in sheep[5], cows[6], mares[7], sows[8], and llamas[3]. Therefore, various cell types within the endometrium can display differential sensitivities to estrogens and progestogens and the overall response of the uterus to steroid stimulation is the product of combined responses of the various cell types. [9]

The distribution of receptors in different uterine compartments varies during the estrous cycle in relation to plasma steroid hormone concentrations.[9] Estradiol has a stimulatory effect on the expression of ERα and PR, whereas progesterone down regulates both receptors. [10, 11] In ruminants, the content of ERα and PR in the uterus are greater at estrus and less during the luteal phase. [5, 6, 9, 11, 12] Recently, a study on llamas showed that ERα and PR populations in the endometrium are high during the follicular phase when peripheral plasma concentrations of estrogens are elevated and decreased during the luteal phase (Day 9 post-induction of ovulation) when plasma progesterone concentrations are still high. [3] Uterine ERβ was

found to be greater in pregnant than in sterile-mated llamas particularly on Day 13. The authors suggested that the presence of an embryo might influence this expression.[2] Endometrial expression of ERα and ERβ in this study was not affected by uterine side or days post-mating. This suggests that a more complex mechanism with differential expression of ER subtypes may be involved in embryo migration from the right to the left uterine horn. [2]

The aim of the current study was to characterize ERα and PR in different endometrial cells types and evaluate the changes in ERα and PR populations in the endometrium of the left and right uterine horn from alpacas at Day 9 of pregnancy, Day 14 of pregnancy, Day 9 post-induction of ovulation, and Day 14 post-induction of ovulation.

A.2 Materials and methods

A.2.1 Experimental design

Adult reproductively sound female alpacas (n=20) that were scheduled for uterine and ovarian sampling as part of a larger experiment on MRP were included in the study.

All females were examined by transrectal ultrasonography using an Aloka SSD500 ultrasound machine (Hitachi Aloka, Medical, Ltd, Wallingford, CT, USA) equipped with a 7.5 MHz linear array transducer mounted on a polyvinyl chloride (PVC) extension rod. All females were mated to a proven fertility male within 12 hours following visualization of at least one large follicle measuring between 7 and 10 mm in diameter. All females received an injection of 50 μg of GnRH (Cystorelin®, Merial, Duluth, GA, USA) subcutaneously immediately after mating. Following mating, females were assigned randomly to 4 groups. Group I and II females (n=10) were scheduled for postmortem collection of the reproductive tract and embryos at either Day 9 (n=5) or Day 14 (n=5) post-mating respectively. Group III and IV females (n=10) were scheduled for pregnancy diagnosis on Day 14 post-mating. All females in group III and IV received an injection of 250 μg of cloprostenol (Estrumate®, Merck Animal Health, De Soto, KS, USA)

intramuscularly after pregnancy diagnosis. Following luteolysis the follicular wave was monitored again and ovulation was induced with GnRH when the largest follicle reached at least 7 mm in diameter. Ovulation was induced with administration of an injection of 50 µg of GnRH subcutaneously. Group III and IV females were scheduled for postmortem collection of the reproductive tract at Day 9 (n=5) or Day 14 (n=5) post-administration of GnRH respectively. Females in all groups were examined 7 days after mating and GnRH treatment or GnRH treatment alone, to determine presence and number of CL. Group II females collected at 14 days post-mating were also examined for pregnancy diagnosis on Day 13 or Day 14 after mating. Blood samples from all females were collected after mating and GnRH treatment or GnRH treatment alone and prior to euthanasia by jugular venipuncture. Samples were centrifuged and serum was stored at -80°C for hormone assays to be performed.

All females were euthanized using an overdose of barbiturates on Day 9 or Day 14. The uteri were collected *en bloc* (Figure 4.1) immediately after euthanasia and flushed as previously described.[13] The endometrium and myometrium were sampled in 2 locations (near the tip and at the base) of each uterine horn. Samples were transferred to 10% buffered formalin and fixed for 48 hours. Samples were then transferred to 70% ethanol for storage prior to histological analysis. In cassettes the tissue sections were rinsed in 70% ethanol, 80% ethanol, and 95% ethanol for an hour at each concentration. The tissue sections were then rinsed in 100% ethanol for 3 hours, xylenes for 2 hours, and embedded in paraffin.

A.2.2 Immunohistochemistry

An immunohistochemical technique previously described[3] was used to visualize ERα and PR immunostaining after minor modifications. After the paraffin tissue sections (8 µm) were dewaxed and rehydrated, an antigen retrieval procedure was performed. Non-specific endogenous peroxidase activity was blocked by treatment with 3% hydrogen peroxide in methanol for 10 minutes at room temperature (RT). Additional rehydration was performed in a graded ethanol series. Sections were pre-treated in a

microwave oven at 700 watts power, in 0.01 M sodium citrate buffer (pH 6.0) until boiling, allowed to stand in the hot water for 10 minutes, and then allowed to cool for 20 minutes. After a 5-minute wash in buffer sections were exposed to a 45-minute non-immunoblock using diluted Normal Horse Serum (Vectastain®; Vector Laboratories, Burlingame, CA, USA) in buffer in a humidified chamber at RT. Details of the antibodies used for detection of each receptor and conditions are specified in Table A.1. Negative controls for each receptor were obtained by replacing the primary antibody with normal mouse IgG (Cat N° sc-2025, Santa Cruz, CA, USA) at equivalent concentrations. After primary antibody binding with overnight incubation at 4°C, the sections were incubated for 30 minutes at RT with a biotinylated IgG (Vectastain®, Vector Laboratories, Burlingame, CA, USA) diluted in Normal Horse Serum. Thereafter, the tissue sections were incubated for 30 minutes with a horseradish peroxidase-avidin-biotin complex (Cat N° PK-6100, Vectastain®, Elite ABC-kit, Vector Laboratories). The site of the bound enzyme was visualized by the application of 3,3'-diaminobenzidine (Cat N° SK-4100, DAB kit, Vector Laboratories), a chromogen that produces brown, insoluble precipitate when incubated with the enzyme. The sections were counterstained with methyl green and dehydrated before they were cover slipped with mounting medium. Test dilutions of primary antibody were performed prior to assay.

A.2.3 Image analysis

A general observation of receptor immunostaining for ERα and PR was performed. Further analysis and evaluation to quantify estrogen and progesterone receptor expression, along with hormonal assays evaluating for estradiol-17β and progesterone serum concentrations, is warranted.

After a general inspection of each slide, a subjective image analysis will be performed to estimate the expression of ERα and PR in different cell types as previously reported.[4] The evaluation will be performed by two independent observes who will not be aware of assignment to groups of animals. Ten fields will be analyzed for each cell type (luminal and glandular epithelia) at a magnification of 1000x in all

sections. The staining of the nuclei will be scored as negative (-), faint (+), moderate (++), or intense (+++) and the staining of each cell type will be in proportion on a scale of 0-10. If staining is adequate color discrimination software could be used to measure the area of positively stained cell nuclei (brown reaction product) per field and be expressed as a ratio of the total area of cell nuclei (brown reaction product + methyl green).

A.3 Results

A.3.1 General observations of ERα and PR immunostaining

Estrogen receptor alpha (ERα) and progesterone receptor (PR) immunoreactivity were observed almost exclusively in the nuclei (not cytoplasm) (Figure A.1) of the luminal and glandular epithelium (Figure A.2A-B, Figure A.3A-B, Figure A.4A-B, Figure A.5A-B, Figure A.6A-B, Figure A.7A-B, Figure A.8A-B, and Figure A.9A-B). When monoclonal specific antibodies were substituted by a nonimmune mouse IgG, the reduction of staining demonstrated the specificity of both receptors immunostaining (Figure A.2C, Figure A.3C, Figure A.4C, Figure A.5C, Figure A.6C, Figure A.7C, Figure A.8C, and Figure A.9C). There was variation between alpacas in intensity of staining and uniformity of staining across cut sections within groups. When non-uniformly stained slides were performed in duplicate, similar results were observed.

A.4 Directions and procedures of immunohistochemistry

One finding in the present study was that immunostaining of PR was inconsistent across tissue sections, even when performed in replicate. Inconsistent staining and significant background noise may be due to specificity of the primary antibody binding. Variable dilutions of both the primary and secondary antibodies were attempted with limited improvement. A different progesterone receptor antibody may be considered to achieve improved results. Other reasons for variability across samples may be due to individual animal variability, amount of tissue sample fixation, or immunohistochemistry processing error.

Subjective analysis to estimate the expression of ERα and PR in different cell types has been previously reported. [3] Evaluation is performed by independent observers who are not aware of assignment to group of animals. Ten fields are analyzed for each cell type (luminal and glandular epithelia and stroma) at a magnification of 1000x in all samples. The staining of the nuclei are scored as being negative (-), faint (+), moderate (++), or intense (+++) and the staining of each cell type is in proportion on a scale of 0-10. This grading system has been previously described. [3, 14] If staining is adequate color discrimination software could be used to measure the area of positively stained cell nuclei (brown reaction product) per field and be expressed as a ratio of the total area of cell nuclei (brown reaction product + methyl green). Alternatively western blot or real time polymerase chain reaction analytical techniques could be employed for quantitative analysis.

A.5 References

1. Meikle A, Tasende C, Sosa C, et al. The role of sex steroid receptors in sheep female reproductive physiology. Reprod Fertil Dev. 2004;16:385-94.

2. Powell SA, Smith BB, Timm KI, et al. Expression of estrogen receptors α and β in the corpus luteum and uterus from non-pregnant and pregnant llamas. Molecular Reproduction and Development. 2007;74:1043-52.

3. Bianchi CP, Meikle A, Sartore I, et al. Uterine estrogen receptor alpha and progesterone receptor during the follicular and luteal phase in llamas. Anim Reprod Sci. 2007;99:117-26.

4. Bianchi CP, Sahlin L, Meikle A, et al. Endometrial population of oestrogen receptors alpha and beta and progesterone receptors A and B during the different phases of the follicular wave of llamas (Lama glama). Reprod Domestic Anim. 2010;45:872-80.

5. Spencer TE, Bazer FW. Temporal and spatial alterations in uterine estrogen receptor and progesterone receptor gene expression during the estrous cycle and early pregnancy in the ewe. Biology of Reproduction. 1995;53:1527-43.

6. Boos A, Meyer W, Schwarz R, et al. Immunohistochemical assessment of oestrogen receptor and progesterone receptor distribution in biopsy samples of the bovine endometrium collected throughout the oestrous cycle. Anim Reprod Sci. 1996;44:11-21.

7. Watson ED, Skolnik SB, Zanecosky HG. Progesterone and estrogen receptor distribution in the endometrium of the mare. Theriogenology. 1992;38:575-80.

8. Sukjumlong S, Dalin AM, Sahlin L, et al. Immunohistochemical studies on the progesterone receptor (PR) in the sow uterus during the oestrous cycle and in inseminated sows at oestrus and early pregnancy. Reproduction (Cambridge). 2005;129:349-59.

9. Cherny RA, Salamonsen LA, Findlay JK. Immunocytochemical localization of oestrogen receptors in the endometrium of the ewe. Reprod Fertil Dev. 1991;3:321-31.

10. Ing NH, Tornesi MB. Estradiol up-regulates estrogen receptor and progesterone receptor gene expression in specific ovine uterine cells. Biology of Reproduction. 1997;56:1205-15.

11. Wathes DC, Hamon M. Localization of oestradiol, progesterone and oxytocin receptors in the uterus during the oestrous cycle and early pregnancy of the ewe. Journal of Endocrinology. 1993;138:479-91.

12. Meyer HHD, Mittermeier T, Schams D. Dynamics of oxytocin, estrogen and progestin receptors in the bovine endometrium during the estrous cycle. Acta Endocrinologica. 1988;118:96-104.

13. Picha Y, Tibary A, Memon M, et al. Chronology of early embryonic development and embryo uterine migration in alpacas. Theriogenology. 2013;79:702-8.

14. Thatcher WW, Guzeloglu A, Meikle A, et al. Regulation of embryo survival in cattle. Reproduction Supplement Series Supplement 61. Cambridge: Society for Reproduction and Fertility; 2003. p. 253-66.

Table A.1 List of antibodies used for the determination of endometrial ERα and PR expression by immunohistochemistry

Receptor	Laboratory	Antibody	Dilutions with PBS
ERα	Santa Cruz Laboratories (C-311). Cat # sc-787, CA, USA	Monoclonal Mouse	1:50
PR	Zymed Laboratories Inc. Cat # ZS18-0172, San Francisco, USA	Monoclonal Mouse	1:200

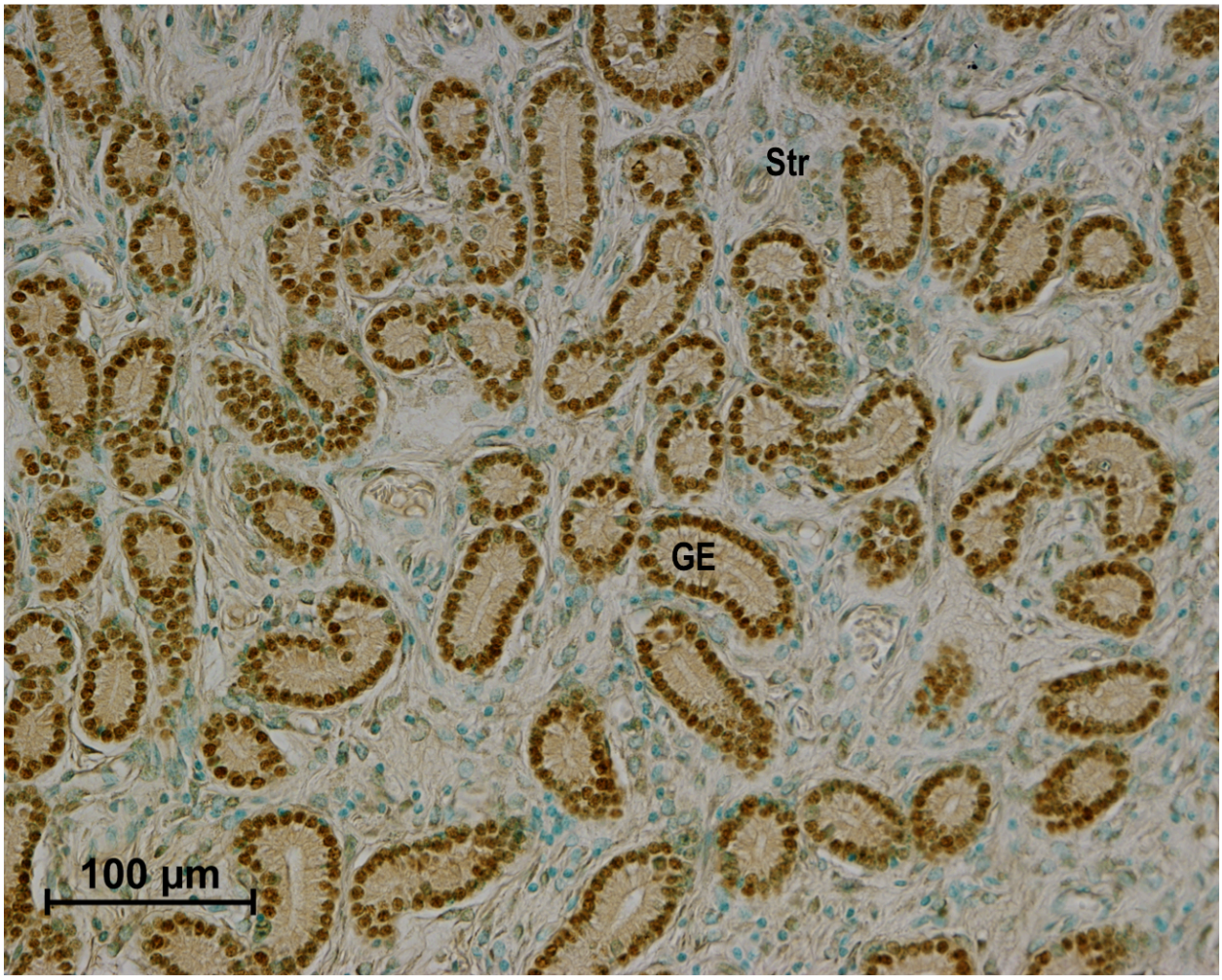

Figure A.1 Representative microphotograph of immunohistochemical localization of ERα almost exclusively in the nuclei. GE = glandular epithelium; Str = Stroma (200x)

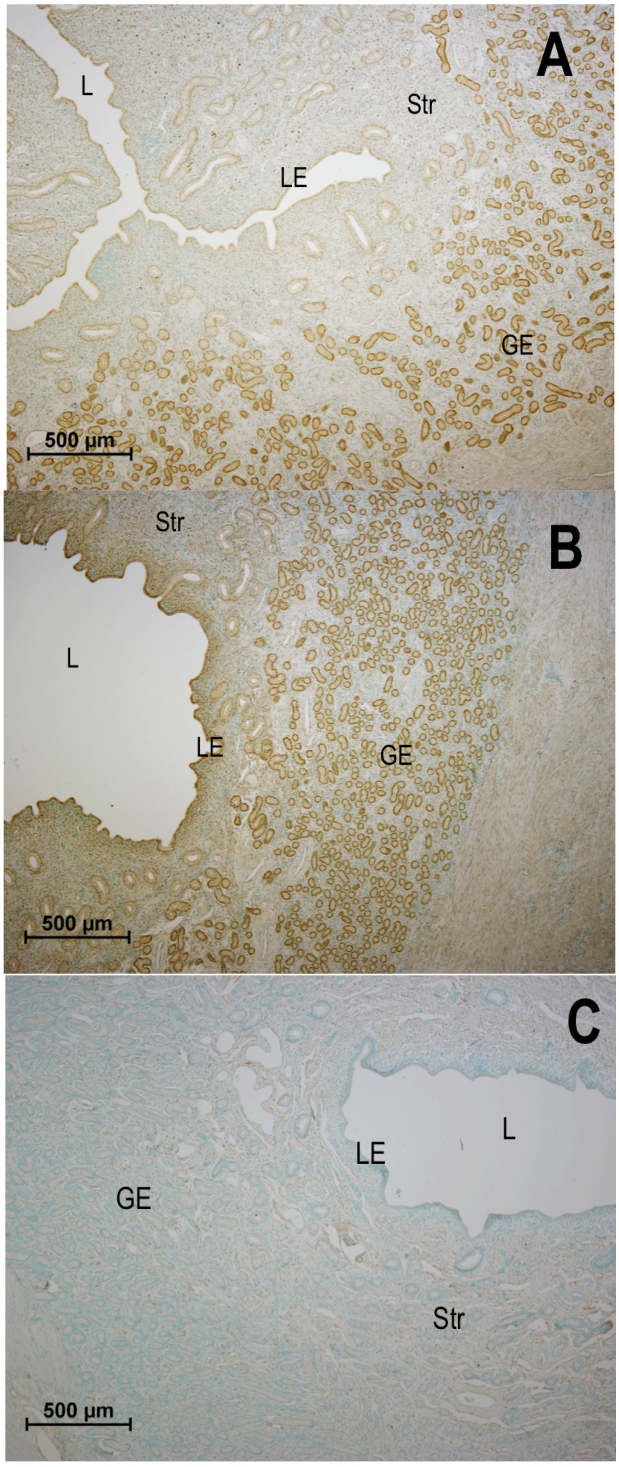

Figure A.2 Immunohistochemical localization of ERα in the endometrium of Day 9 pregnant alpacas: A) left horn, B) right horn, C) negative control. L = lumen; LE = luminal epithelium; GE = glandular epithelium; Str = Stroma (40x)

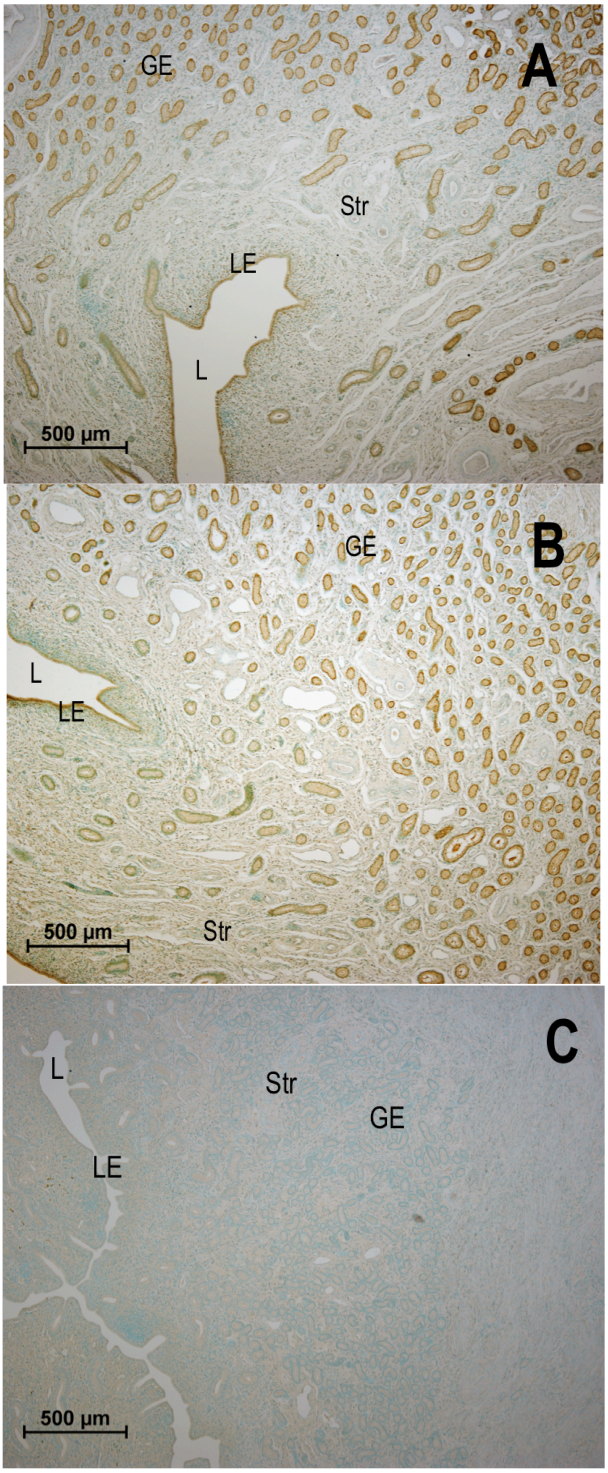

Figure A.3 Immunohistochemical localization of ERα in the endometrium of alpacas on Day 9 post-induction of ovulation: A) left horn, B) right horn, C) negative control. L = lumen; LE = luminal epithelium; GE = glandular epithelium; Str = Stroma (40x)

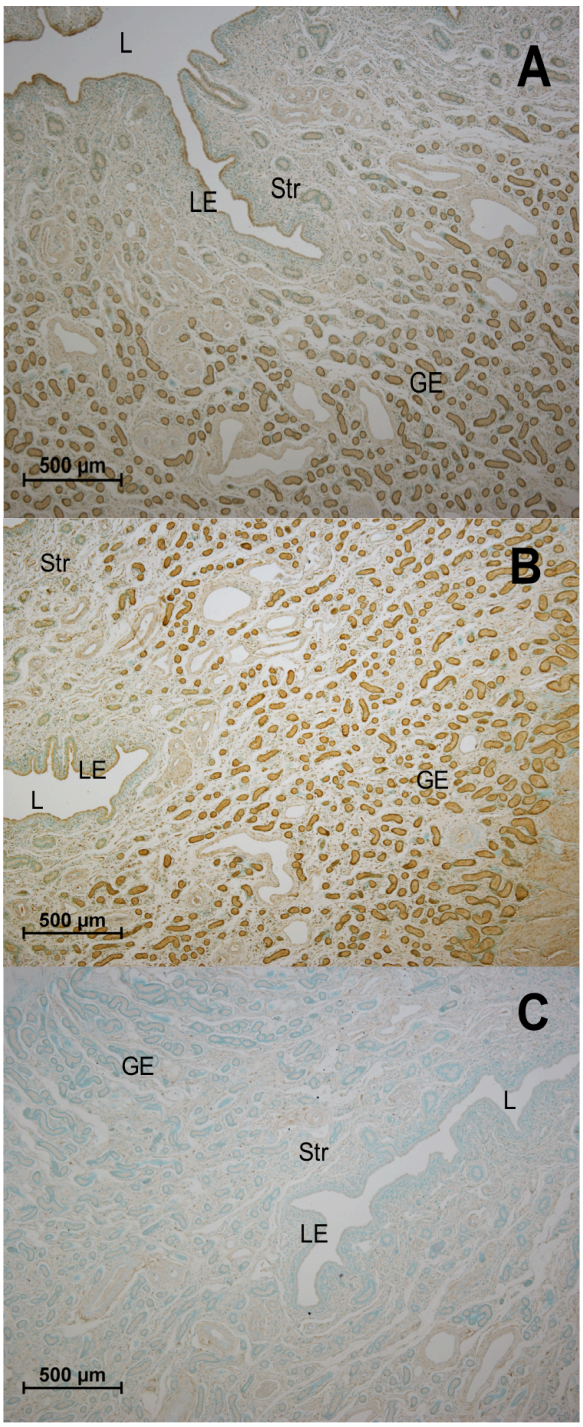

Figure A.4 Immunohistochemical localization of ERα in the endometrium of Day 14 pregnant alpacas: A) left horn, B) right horn, C) negative control. L = lumen; LE = luminal epithelium; GE = glandular epithelium; Str = Stroma (40x)

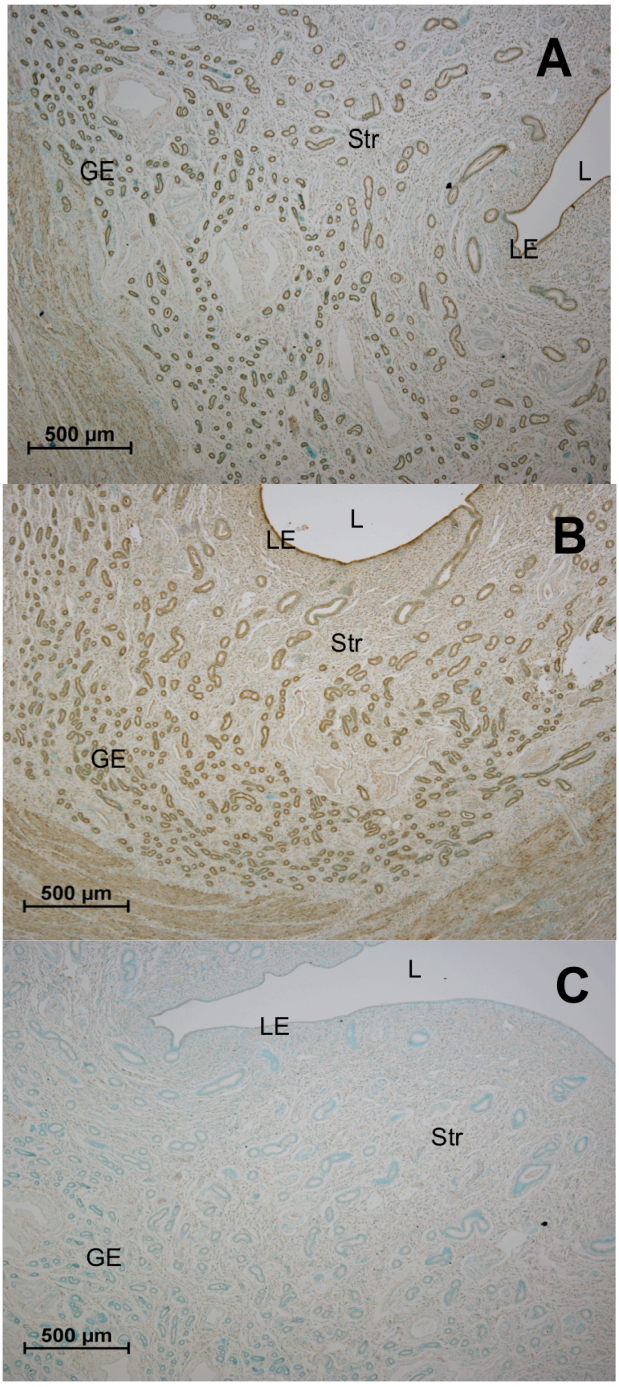

Figure A.5 Immunohistochemical localization of ERα in the endometrium of alpacas on Day 14 post-induction of ovulation: A) left horn, B) right horn, C) negative control. L = lumen; LE = luminal epithelium; GE = glandular epithelium; Str = Stroma (40x)

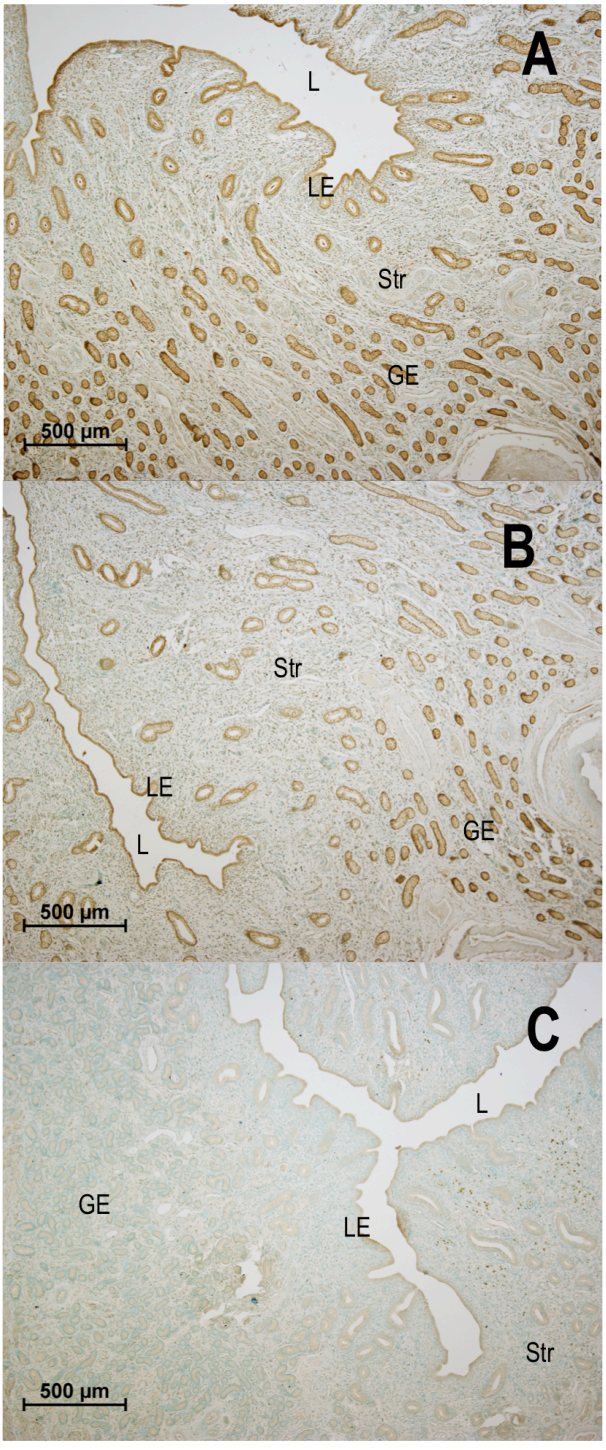

Figure A.6 Immunohistochemical localization of PR in the endometrium of Day 9 pregnant alpacas: A) left horn, B) right horn, C) negative control. L = lumen; LE = luminal epithelium; GE = glandular epithelium; Str = Stroma (40x)

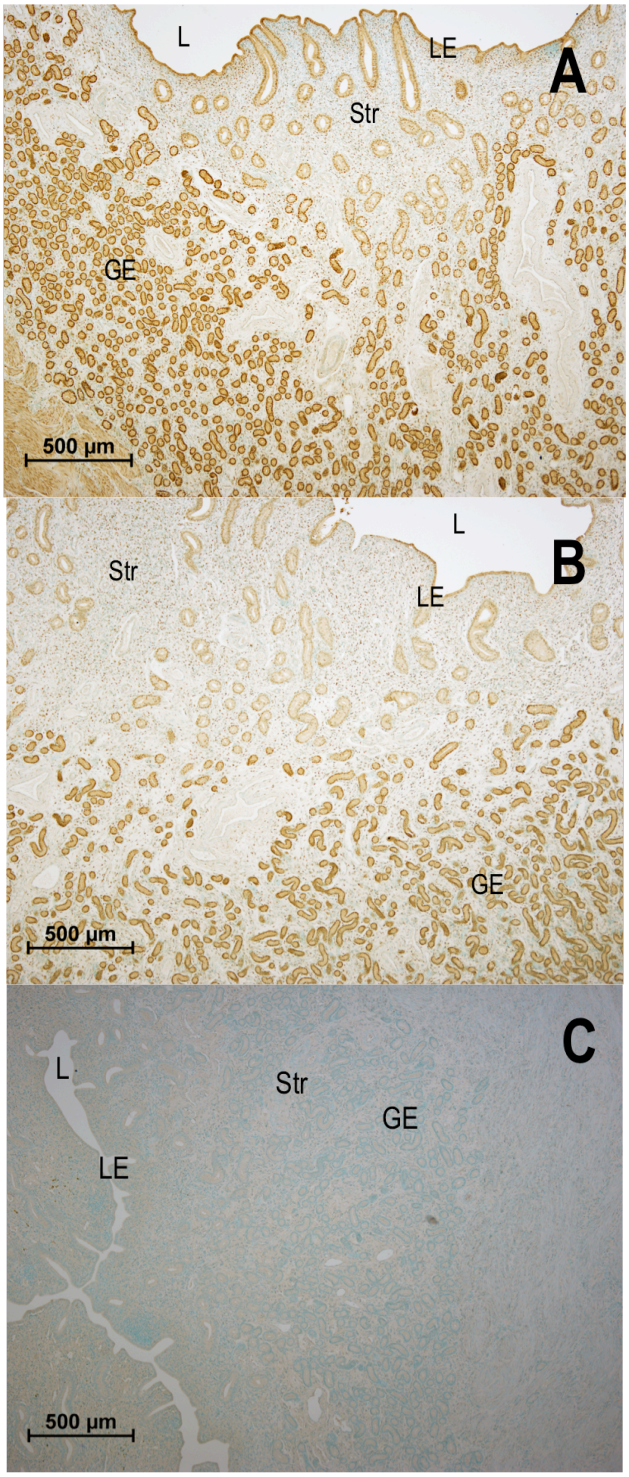

Figure A.7 Immunohistochemical localization of PR in the endometrium of alpacas on Day 9 post-induction of ovulation: A) left horn, B) right horn, C) negative control. L = lumen; LE = luminal epithelium; GE = glandular epithelium; Str = Stroma (40x)

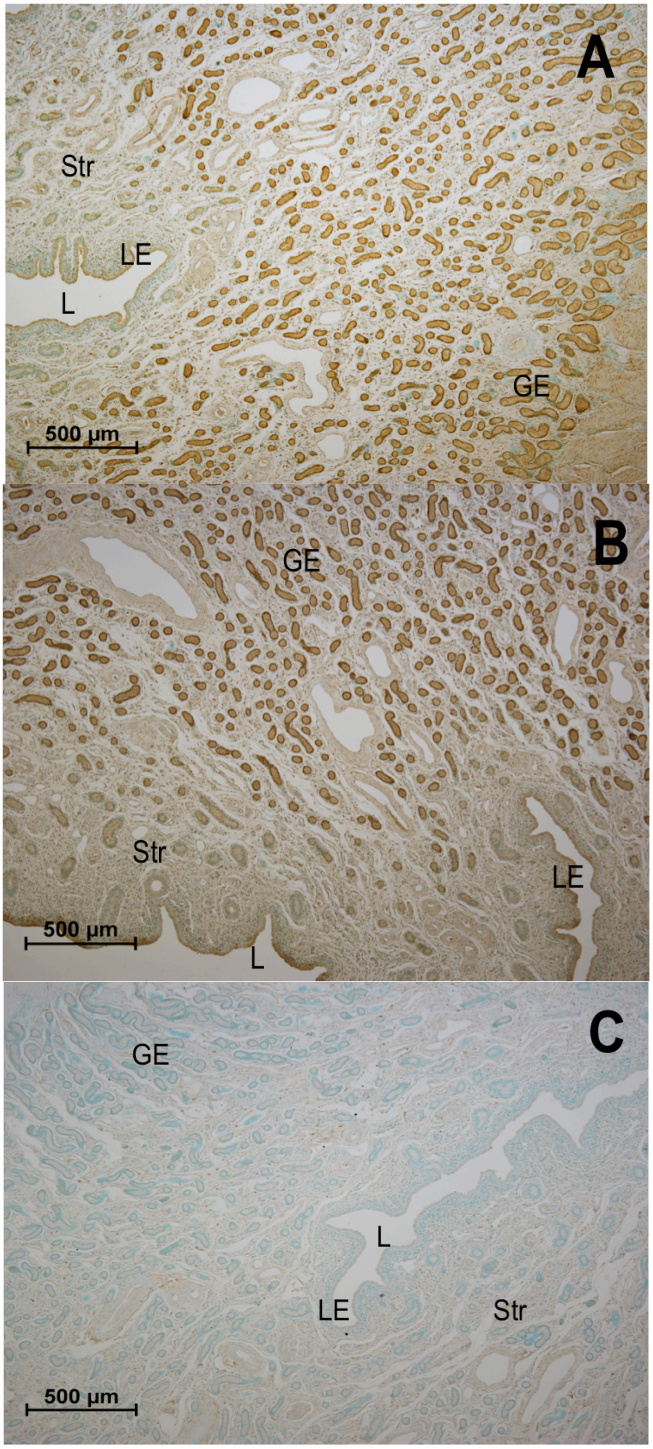

Figure A.8 Immunohistochemical localization of PR in the endometrium of Day 14 pregnant alpacas: A) left horn, B) right horn, C) negative control. L = lumen; LE = luminal epithelium; GE = glandular epithelium; Str = Stroma (40x)

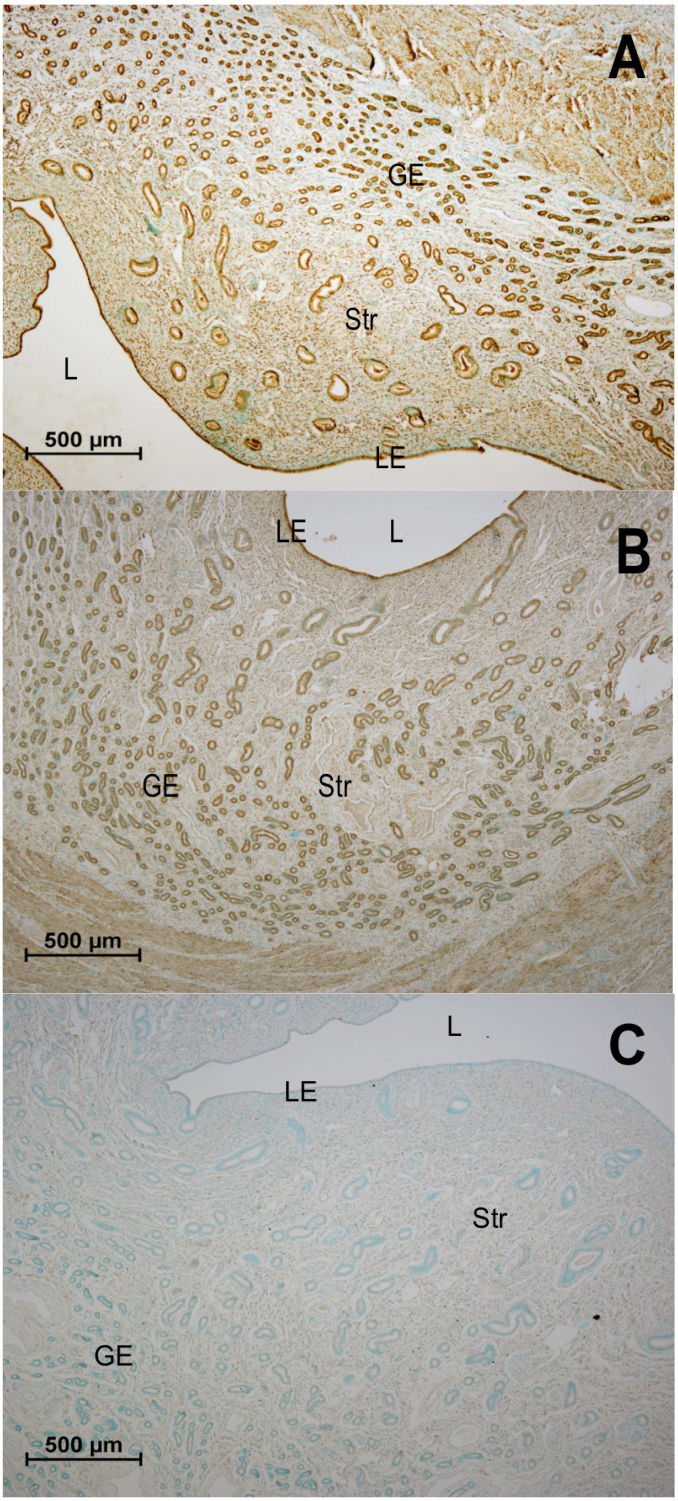

Figure A.9 Immunohistochemical localization of PR in the endometrium of alpacas on Day 14 post-induction of ovulation: A) left horn, B) right horn, C) negative control. L = lumen; LE = luminal epithelium; GE = glandular epithelium; Str = Stroma (40x)