OVARIAN FOLLICULAR SYNCHRONIZATION, OVULATION AND OOCYTE DEVELOPMENT IN LLAMAS AND ALPACAS

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ABSTRACT

The purpose of the studies reported in this thesis was to increase our understanding of the reproductive physiology of South American camelids. Studies were conducted in llamas and alpacas to investigate methods to electively control ovarian follicular dynamics, to determine the effects of hormone preparations or biological factors derived from seminal plasma on ovulation induction, and to evaluate the establishment of superstimulatory protocols to induce a consistent ovarian follicular response for oocyte collection.

The first study was designed to compare the efficacy of treatments intended to induce follicular wave synchronization among llamas, and to determine the effect of these treatments on pregnancy rates after fixed-time natural mating. In the first experiment, lutenizing hormone (LH) and follicular ablation treatments were most effective for inducing follicular wave synchronization, while estradiol plus progesterone (E/P) treatment was intermediate. In the second experiment, llamas were assigned randomly to Control, (E/P), and LH groups. A single, fixed-time natural mating was permitted 10 to 12 days after treatment. The pregnancy rate was higher ($P<0.05$) for synchronized llamas (LH and E/P groups combined) than for non-synchronized llamas (Control group).

The second study was done to compare the effects of hormonal treatments and natural mating on ovulation induction, interval to ovulation, and luteal development in llamas. No differences were detected among groups (mated, LH, and GnRH) in ovulation rate (80%, 91%, 80%, respectively; $P = 0.6$), or interval from treatment to ovulation (30.0 ± 0.5, 29.3 ± 0.6, 29.3 ± 0.7 h, respectively; $P = 0.9$). Similarly, no differences were detected among groups (mated, LH, and GnRH) in maximum corpus luteum (CL) diameter.

The third study documents the existence of an ovulation-inducing factor (OIF) in the seminal plasma of alpacas and llamas. In Experiment 1, female alpacas were given

alpaca seminal plasma or saline intramuscularly (im) or by intrauterine infusion. Only alpacas that were given seminal plasma im ovulated. In Experiment 2, ovulation was detected in 9/10 (90%) llamas at a mean of 29.3 ± 0.7 hours after seminal plasma treatment. In Experiment 3, female llamas were given llama seminal plasma, GnRH, or saline im, and ovulation was detected in 6/6, 5/6, and 0/6 llamas, respectively ($P < 0.001$). Treatment was followed by a surge ($P < 0.01$) in plasma LH concentration beginning 15 minutes and 75 minutes after treatment with GnRH and seminal plasma, respectively. Plasma LH remained elevated longer in the seminal plasma group ($P < 0.05$), and plasma progesterone concentration was twice as high in the seminal plasma group ($P < 0.01$).

The fourth study describes the presence of an OIF in the seminal plasma of *Bos taurus* – a species conventionally considered to ovulate spontaneously - contains OIF. Bull seminal plasma induced ovulations in 26% (5/19) of llamas compared to 0% (0/19) in PBS group ($P < 0.001$). The ovulation rate was lower ($P < 0.01$) in bull seminal plasma group compared to that in the groups treated with alpaca or llama seminal plasma (100%).

The fifth study was conducted to determine a local versus systemic effect of ovulation-inducing factor in seminal plasma. Ovulation rate in the seminal plasma intramuscular group (93%) was higher ($P < 0.01$) than seminal plasma intrauterine group (41%), while the seminal plasma intrauterine curettage group was intermediate (67%).

The sixth study was done to determine the time required for llama oocyte to reach the maturation stage, and to establish a superstimulatory treatment for oocyte collection. Llama oocytes reached second metaphase as early as 28 h after *in vitro* culture. The FSH- and eCG-treated groups did not differ ($P = 0.85$) with respect to the number of follicles ≥6 mm at the time of cumulus-oocyte complex (COC) collection (17.9 ± 2.2 vs 17.7 ± 2.2), the number of COC collected (10.7 ± 2.1 vs 11.2 ± 2.3 per llama), or the collection rate per follicle aspirated (71 vs 74%).

Finally, in the last study, the effect of two superstimulatory treatments was evaluated on ovarian response and COC collection efficiency and oocyte maturation in alpacas. No difference (P = 0.54) was observed between FSH and eCG- treated alpacas in the number of expanded (11.5 ± 2.9 vs 8.8 ± 2.8) or compact COC collected with ≥3 layers of cumulus cells (12.5 ± 4.3 vs 14.3 ± 2.6; P = 0.72). No difference (P = 0.1) was detected between FSH and eCG groups in the number of expanded COC at first metaphase (1.2 ± 1.2 vs 1.7 ± 0.6) or second metaphase stage (8.5 ± 1.9 vs 6.0 ± 2.1) respectively.

In conclusion, these studies demonstrated that the control of ovarian follicular wave emergence and ovulation induction in llamas will contribute consistently to the establishment of fixed-time natural or artificial insemination as well as recipient synchronization in embryo transfer programs. The discovery of an ovulatory molecule in the semen of this species generates a new area of research regarding the ovulation mechanism in induced ovulators. Characterization of this factor may have important implications in the diagnosis and treatment of male and female infertility. Finally, the superstimulatory treatments and oocyte development studies will establish the baseline for the development of an in vitro embryo production system in llamas and alpacas.

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DEDICATION

I dedicate this thesis to my family, my wife, Ximena Paola, my children, Sebastian Luciano, Vicente Francisco and Jose Ignacio for supporting me with love. I also dedicate this thesis to my parents, Hector and Beatriz for the spiritual support provided during my entire career.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

.

LIST OF ABREVIATIONS

AI	artificial insemination
CL	corpus luteum
FSH	follicle stimulating hormone
GnRH	gonadotropin releasing hormone
PG	prostaglandin $F_{2\alpha}$
hCG	human chorionic gonadotropin
LH	luteinizing hormone
pFSH	porcine follicle stimulating hormone
IU	international units
ng	nanogram
ml	milliliter
h	hours
d	days
im	intramuscular
µm	micron
µg	microgram
MPA	medroxiprogesterone actetate
eCG	equine chorionic gonadotropin
CIDR	control internal drug release
MBH	medio-basal hypothalamus
OPS	open pull straw
LSD	least significant difference
SAS	statistical analysis system
PBS	phosphate buffered saline
COC	cumulus oocyte complex
OIF	ovulation-inducing factor
E/P	estradiol/progesterone
FA	Follicle ablation

KG	kilogram
GV	germinal vesicle
GVBD	germinal vesicle breakdown
MI	metaphase I
MII	metaphase II
Sperm-TALP	sperm washing -tyrode, albumin, lactate, pyruvate medium
FERT-TALP	fertilization- tyrode, albumin, lactate, pyruvate medium
BSA	bovine serum albumin
SD	standard deviation
vs	versus
ml or mL	milliliter
min	minute
SEM	standard error of the means

1.0 INTRODUCTION

The majority of reproductive studies on South American camelids have been conducted over the past 15 years using llamas and alpacas. Our understanding of the reproductive physiology of South American camelids appears miniscule when compared to our knowledge of traditional livestock species, such as cattle or sheep. Most of the existing information from wild South American camelids, vicuña and guanaco, relates to population, habitat, behavior, and biodiversity. Currently, llamas and alpacas are a valuable economic resource for Peruvian, Bolivian and Chilean people living in the high plains of the Andes Mountains, and they have become a consistent feature of North American livestock production.

Ultrasonographic studies of the reproductive organs and systematic endocrinology studies in llamas were carried out in the 1990s. These studies have contributed to a better understanding of follicular development, ovulation and corpus luteum function. However, the need to carry out further studies to better understand the reproductive physiology of South American camelids is evident.

The reproductive management of llamas and alpacas in high Andes communities is very poor or non-existent. Llama and alpaca breeding systems in the high Andes communities and in North American herds are based on sexual behavior, i.e., mating is performed when females adopt the sternal-recumbent copulatory posture in the presence of a male. However, sexual receptivity is not strictly correlated with the presence of a mature follicle capable of ovulating a competent oocyte. The control of ovarian follicular development in these species would facilitate the use of natural mating or artificial insemination (AI). Ideally, AI would be done at a fixed time with the presence of a healthy mature follicle containing a competent oocyte to maximize conception rate.

The South American camelids are induced ovulators that require natural mating to induce ovulation. Although hormone preparations have been used to induce ovulation, studies are needed to determine whether the interval to ovulation and the induced luteal phase are similar to those occurring after natural mating. The control of follicular development, ovulation and the luteal phase, either by the use of exogenous hormones or biological products, would contribute tremendously to the development and application of AI and embryo transfer. Llamas and alpacas are considered species with low reproductive efficiency due to low rates of pregnancy and birthing. An embryo mortality of up to 35% has been documented in alpacas during the first 35 days after natural mating. However, it is not clear whether embryo loss is caused by alterations in the maternal environment or because of intrinsic defects in the oocyte or embryos after ovulation and subsequent fertilization.

The application of assisted reproductive technology is in the formative stages for the domestic species of South American camelids (llama and alpaca) and is virtually non-existent for the wild species (guanaco and vicuña). Although, camelid embryos have been produced by *in vitro* fertilization and more recently by nuclear transplantation, the birth of live offspring following the use of these techniques have not been reported.

Presently, it is important to focus on the development of reliable methods for semen collection and manipulation techniques, as well as the development of superstimulatory treatments and in vitro culture systems that could improve oocyte competence and embryo development.

2.0 REVIEW OF LITERATURE

2.1 Importance of South American camelids

The family of Camelids encompasses the New World and Old World camelids. The New World camelids, which are also known as South American camelids, are the llama (*Llama glama*), alpaca (*Llama pacos*), guanaco (*Llama guanicoe*) and vicuña (*Vicugna vicugna*). The Old World camelids are the dromedary, or one-hump camel, (*Camelus dromedarius*) that inhabits the hot deserts from Asia to North Africa and the two-hump Bactrian camel (*Camelus bactrianus*) living in the deserts of China and Mongolia (Novoa, 1970). The total population of South American camelids including both domesticated (llama and alpaca) and wild species (vicuña and guanaco) is estimated to be approximately 6.8 million and is distributed throughout Argentina, Bolivia, Chile, Colombia, Ecuador, Paraguay and Peru (Raggi, 2001).

Historically, llamas and alpacas have played an important role in the development of the Inca civilization. Llamas served as transport animals, whereas alpacas were an important source of meat and fibre (Burton et al., 1969). Presently, llamas and alpacas are the primary source of income to the Aymaras communities living in the high Andes Mountains in Peru, Chile and Bolivia. These communities use the llama for meat and transportation and alpacas for meat and fibre production.

The quality of the fibre produced by South American camelids is appreciated world wide by the textile industry. The fibre obtained from Alpacas is softer, finer and lighter than llama fibre. The alpaca fiber has a diameter ranging from 12 to 28 μm whereas the fiber from llamas ranges in diameter from 20 to 80 μm. However, the finest fibre is found in the wild species, guanaco and vicuña, with fibre diameters ranging from 16 to 18 and 11 to 14 μm, respectively (Wheeler, 1995).

World production of fiber from alpacas exceeds 4 million kg and it is worth more than US $12 million (Raggi, 2001). Peru is one of the biggest alpaca fibre producers. Fibre is processed by USA textile companies in Peru and exported to other countries. Peru has over 3 million alpacas and 1 million llamas; Bolivia has more llamas (2 million) than alpacas (325,000), while Chile has the smallest number of animals (33,000 alpacas and 67,000 llamas). The high quality and commercial value of the alpaca fibre attracted the attention from textiles industries of developed countries. A massive and uncontrolled number of high genetic valued animals were exported from 1989 to 1998 from Peru and Chile to the USA, New Zealand, Australia, England, and Canada (Raggi, 2001). There are more than 150,000 registered llamas and alpacas in North America.

2.2 Overview of the reproductive physiology in South American camelids

Our knowledge of the reproductive physiology of South American camelids is largely based on data obtained from domesticated species. Llamas and alpacas are classified as induced or reflex ovulators because ovulation will only take place after copulatory stimulation (San Martin et al., 1968; England et al., 1969; Fernandez-Baca et al., 1970a). In the absence of a male, female alpacas may remain in estrus for up to 36 days with only occasional periods of anestrus that last no longer than 48 hours (San Martin et al., 1968). Similar estrus behavior has been observed in llamas (England et al., 1971). Camelids do not have an estrus cycle; therefore, follicular and luteal phases are more convenient terms that could be used in these species to characterize the sexual

cycle. Sexual behavior can be considered in two unique patterns, i.e., receptivity and non-receptivity of the female camelid.

The exact age at which llamas and alpacas reach puberty is not clearly defined. Traditionally, llamas are bred at 3 years of age, whereas alpacas are usually bred when they reach approximately 60% of adult body weight (Sumar, 1985). Early reports indicate that alpacas are sexually receptive and capable of ovulating at 12 months of age (Novoa et al., 1972). Ovulation, fertilization and embryo development rates of yearling alpacas are similar to those of adult alpacas (Fernandez-Baca et al., 1972).

Under normal conditions, sexually receptive females lie down in a prone position (copulatory position) in the presence of a sexually mature male. Sexually receptive females have been known to lie near a mating couple (San Martin et al., 1968; England et al., 1971). However, sexual receptivity is not always associated with the presence of a dominant follicle (Sumar et al., 1993; Bravo et al., 1994). The presence of an aggressive dominant male may trigger non-receptive females to adopt the copulatory response (Adams, 1997). On the other hand, sexual behavior in non-receptive females is highly correlated with the presence of a corpus luteum. Non-receptive females consistently reject male advances by spitting, kicking and screaming (Sumar, 1988; Adams et al., 1989; Fernanadez-Baca 1993; Pollard et al., 1994).

The reproductive pattern in South American camelids is influenced by conditions of the environment and management. In the Northern Hemisphere, llamas and alpacas breed throughtout the year (Schmidt 1973; Johnson, 1988). The breeding season of llamas and alpacas inhabiting the highlands of Peru, Bolivia and Argentina takes place from December to March, which corresponds to the warm, rainy months and highest availability of green forage (Fernandez-Baca 1993; Sumar, 1996). In New Zealand, alpacas breed both in the spring and autumn months (Pollard et al., 1995). The conception rate observed in females mated in spring did not differ from those mated in autumn (Knight et al., 1995). However, another report suggests that both males and females show less sexual interest during the spring months (Pollard et al., 1995).

Llamas, alpacas and vicuñas have a wave-like pattern of follicular development (Bravo and Sumar, 1989; Adams et al., 1990; Bravo et al., 1990a; Chavez et al., 2002; Vaughan et al., 2004; Miragaya et al., 2004). The pattern of follicular waves is supported by periodic increases in the number of follicles and the emergence of a dominant follicle, which may reach a diameter of 7 mm or more in both llamas and alpacas (Adams et al., 1990; Vaughan et al., 2004). It has been proposed that the ability of llamas and alpacas to ovulate in response to a mating stimulus is influenced by the developmental status of the dominant follicle at the time of mating (Adams et al., 1990; Bravo et al., 1991; Vaughan et al., 2004). Growing dominant follicles ≥6 mm are capable of ovulating (Adams et al., 1990), whereas smaller follicles or those undergoing regression are not capable of ovulating (Bravo et al., 1991).

The luteal phase following natural induction of ovulation has been well described in llamas and alpacas (Sumar et al., 1988; Adams et al., 1990, 1991a; Aba et al., 1995). The maximum corpus luteum (CL) diameter and plasma progesterone concentrations were detected at day 8 after mating (day 0 = mating). The first significant decrease in CL diameter and plasma progesterone profiles during luteolysis in non-pregnant females occurred on days 11 and 10 after mating and reached a nadir on days 15 and 14, respectively. The uterus controls CL regression in the non-pregnant llama and alpaca (Fernandez-Baca et al., 1979; Sumar et al., 1988). Partial hysterectomy prolongs the corpus luteum lifespan ipsilateral to the missing uterine horn. In addition, the right uterine horn appears to have a local luteolytic effect, while it has been hypothesized that the left uterine horn has both a local and a systemic luteolytic effect (Fernandez-Baca et al., 1979). A recent report suggests that the left horn may induce luteolysis of the CL in the right ovary via a local veno-arterial pathway mechanism (Del Campo et al., 1996). Luteolysis has been temporally associated with a pulsatile release of prostaglandin ($PGF_{2\alpha}$) from the uterus around days 8 to 10 after mating (Sumar et al., 1988; Aba et al., 1995). If conception is successful after natural mating, the CL is maintained during the entire pregnancy (Sumar, 1983). The gestation length has been reported to be 325-361 days for alpacas (San Martin et al., 1968) and 331-361 days for llamas (Sumar, 1988). Although a high rate of fertilization (70% to 80%) has been

reported in alpacas after natural mating (Fernandez-Baca et al., 1970b), 25% to 50% of alpaca embryos are lost during the first 30 days of gestation (Fernandez-Baca et al., 1970b; Knight et al., 1995). It is unknown whether the high embryo loss is due to an intrinsic defect of the gametes or alterations within the uterine environment. Interestingly, embryo migration from the right to the left uterine horn is common and apparently required in this species. Approximately 98% of the gestations are carried out in the left uterine horn in alpacas (Fernandez-Baca et al., 1973) regardless of the site of ovulation (Fernandez-Baca et al., 1973; Sumar and Leyva, 1979). This suggests that embryo development in the right uterine horn may be compromised.

2.3 Follicular growth pattern in llamas and alpacas

The wave pattern of follicular development has been documented in several domesticated and wild ruminant species (Adams, 1999). Follicular dynamics have been extensively studied in cattle (Pierson and Ginther, 1984; Pierson and Ginther, 1987; Savio et al., 1988; Sirois and Fortune, 1988; Adams et al., 1994a). It appears that the mechanisms of recruitment, selection and dominance are highly conserved across all monovulatory species.

Follicular wave activity has been well-documented in llamas under three different physiological conditions: anovulatory (non-mated), ovulatory but non-pregnant (mated by vasectomized male), and ovulatory pregnant llamas (Adams et al., 1990). The periodic growth of a cohort of follicles, the appearance of a dominant follicle that reaches at least 7 mm in diameter and the inverse relationship between the diameter of the largest follicle (dominant follicle) and the total number of follicles (subordinate follicles) support the existence of follicle waves in llamas.

In llamas, the dominant follicle of the first anovulatory wave is detected 3 days after ovulation in females mated to a vasectomized or intact male. The dominant follicle is first identified retrospectively at a diameter of 3 to 4 mm. The subordinate follicles do not exceed a diameter of 7 mm. Dominant follicles may range in diameter from 9 to 15

mm on day 15 (Adams et al., 1990). Dominant follicles with smaller diameters have been associated with the presence of the CL and with lactational stage. These factors also decrease the number of follicles in each cohort. The lifespan of the anovulatory dominant follicles is 20 to 25 days, in unmated and vasectomy-mated llamas the inter-wave interval is 19.8 ± 0.7 days and in pregnant llamas the inter-wave interval is 14.8 ± 0.6 days. Lactation was associated with an inter-wave interval that was shortened, on average, by 2.5 ± 0.5 days (Adams et al., 1990). Successive dominant follicles do not appear to follow an alternate pattern between the right and left ovaries.

Studies conducted in alpacas by laparoscopy (Bravo and Sumar, 1989) and llamas by ultrasonography (Bravo et al., 1990a) were consistent with the presence of a wave-like pattern of follicular development in camelids. However, follicular wave emergence and inter-wave interval were not defined in these studies. In addition, the lifespan of dominant follicle, the apparently inter-wave interval, and the incidence of occurrence of successive dominant follicles between left and right ovaries were substantially different to that previously reported (Adams et al., 1990). The dominant follicle spanned 12 to 14 days, and the inter-wave interval lasted approximately 11.1 and 15 days in llamas and alpacas, respectively. The occurrence of successive dominant follicles tended to alternate between ovaries in 85% of the follicle waves. In a recent study of llamas (Chavez et al., 2002), the lifespan of the dominant follicle (22.6 ± 2.5 days) and the inter-wave interval (18.0 ± 2.6 days) in non-pregnant females, were similar to that described by Adams et al. (1990). Apparently the inter-wave interval of 15 days observed in a study of alpacas (Bravo et al., 1989) was similar to that found in a recent report (Vaughan et al., 2004) in which 15/38 (39%) and 12/38 (32%) of alpacas had inter-wave intervals of 12 and 16 days, respectively. In addition, an inter-wave interval of 4.2 ± 0.3 days was reported for non-pregnant, non-lactating vicuñas (Miragaya et al., 2004).

The variability of the duration of the inter-wave interval observed in anovulatory llamas and alpacas could be attributed to different degrees of follicular dominance related to the maximum diameter reached by the dominant follicle in each species.

Considering follicular dominance as a function of follicular size, the larger the size of a dominant follicle the longer the expected lifespan in each species. This was shown in separate reports in which the maximum size of dominant follicles spanned from 7 to 12 mm in alpacas (Bravo and Sumar, 1989; Vaughan et al., 2004) and 9 to 16 mm in llamas (Adams et al., 1990). This could partially explain the different inter-wave intervals observed between alpacas (Vaughan et al., 2004) and llamas (Adams et al., 1990), However, it does not explain the shortest inter-wave interval (4.2 ± 0.3 days) described in anovulatory vicuñas (Miragaya et al., 2004), in which the maximum diameter of dominant follicles ranged from 6.2 to 11.2 mm, similar to that of alpacas.

Although follicle development in llamas and alpacas shares many of the characteristics previously reported in cattle, it appears that there are many differences in the control of the wave-like pattern between this species.

Each follicular wave in cattle is preceded by a surge in circulating FSH (Adams et al., 1992a). Suppression of the FSH surge with follicular fluid (Turzillo and Fortune, 1993) or exogenous estradiol (Bo et al., 1995a) prevented new wave emergence. However, it appears that there is no temporal association between FSH and wave emergence in llamas (Bravo et al., 1990a). The selection of the dominant follicle in cattle has been temporally associated with the nadir of FSH and a change in dominant follicle responsiveness to LH with an increased capacity to produce estradiol (Adams et. al., 1993a; Ginther et al., 2001). After selection, the dominant follicle grows in function for LH secretion, and during the luteal phase in cattle, progesterone reduces LH pulse frequency and the dominant follicle regresses (Savio et al., 1993; Campbell et al., 1995). Treatment with exogenous progesterone during early metestrus has been shown to suppress the diameter of the first-wave dominant follicle in a dose-dependent manner (Adams et al., 1992b), indicating that LH pulse frequency regulates turnover of the dominant follicle in these species (Roche et al., 1999). Although these events have not been extensively characterized in camelids, some evidence suggests the possibility that a similar mechanism may operate in this species. The concentration of plasma estradiol has been correlated with follicular size in llamas and alpacas (Bravo et al., 1991; Lasley

et al., 1989; Bravo et al., 1990b). However, these studies failed to demonstrate temporal relationships between estradiol and plasma gonadotropin concentrations.

In llamas, the diameter of the dominant follicle of the ovulatory wave may be in part associated with the pulsatile secretion of LH. Progesterone secreted by the corpus luteum resulting from mating the female with a vasectomized male appears to decrease the diameter of the dominant follicle of the first anovulatory wave (Adams, 1990) Furthermore, prolonged exposure to progesterone observed in pregnant llamas results in significant reduction in the diameter of the second anovulatory wave, and an early regression of the dominant follicle of the first wave resulting in a shorter inter-wave interval than that observed in anovulatory (non-mated) and ovulatory (sterile-mated) females. However, the regression of the dominant follicle occurs in the anovulatory female with undetectable levels of progesterone; therefore intraovarians factors may play an important role in the control of the dominant follicle lifespan in these species.

2.3.1 Control of ovarian follicular wave emergence

Methods to effectively schedule fixed-time natural mating or AI in llamas and alpacas must take into account the need to have a healthy mature dominant follicle present at the time of mating. Thus, synchronization protocols should be able to induce the emergence of a new follicular wave regardless of the stage of the cycle. The rationale of these treatment protocols is based on the removal of the suppressive effect of dominant follicles at random stages of the follicular wave either by inducing its ovulation or atresia and thereby resulting in the recruitment of a new follicular wave.

Ovarian synchronization has been achieved in cattle using physical and hormonal methods to induce follicular wave emergence. Ultrasound-guided follicle aspiration of all follicles ≥5 mm in diameter at random stages of the estrous cycle in heifers has been used to induce a transient increase in FSH and a synchronous emergence of a new wave within 1.5 days post-treatment (Bergfelt et al., 1994). This technique has been combined with prostaglandin $F_{2\alpha}$ and GnRH or LH to improve the synchrony of

ovulation in cattle (Bergfelt et al., 1994; Brogliatti et al., 1998). Ultrasound-guided follicle aspiration effectively synchronizes follicular wave emergence. However, the applicability of this technique with a large number of animals and under field conditions significantly reduces its large-scale application.

Hormonal treatments for controlling follicular wave emergence in cattle include the use of GnRH (Pursley et al., 1995; Martinez et al., 1999), LH (Macmillan and Thatcher, 1991; Twagiramungu et al., 1992; Pursley et al., 1997), and estradiol in combination with progestagens (Bo et al., 1994, 1995a, b). Ovulation has been reported to occur in 55% of GnRH-treated heifers (Pursley et al., 1995) and 78% of LH-treated heifers (Martinez et al., 1999) with an interval from treatment to the new follicular wave emergence of approximately 2 days.

Synchronization programs in cattle may involve the combination of progesterone and estradiol treatments to synchronize follicle wave emergence. Progesterone administration reduced the size of the dominant follicle of the first follicular wave in cattle (Adams et al., 1992b), and in a similar fashion it reduced the size of the dominant follicle of the first follicular wave in ovulatory vasectomized-mated llamas and the size of the dominant follicle in wave 1 and 2 in pregnant llamas (Adams et al., 1990). In cattle, progesterone decreases the frequency of the LH pulses and results in a decreased diameter of the dominant follicle (Goodman and Karsch, 1980; Ireland and Roche, 1982). Although progesterone suppresses the LH pulse frequency, it has no significant effect on FSH secretion (Goodman and Karsch, 1980); thus it will induce atresia of only LH-dependent follicles. Therefore, a treatment protocol with the administration of progesterone alone will be effective only at a specific stage of the follicular wave.

Estrogens induces the atresia of FSH-dependent follicles by reducing FSH concentration through a negative feedback. A new follicular wave will emerge when estradiol concentration returns to basal levels (Bo et al., 1995b). The administration of exogenous estradiol in sheep and cattle with low progesterone concentrations resulted in

a decreased LH pulse amplitude (Goodman and Karsch, 1980; Rawlings et al., 1984). The combination of estradiol and progesterone treatment has been reported to have an additive suppressive effect on LH secretion (Bo et al., 1994). The use of estradiol 17-ß and progestagen-implanted heifers induces a new follicular wave emergence on an average of 4.3 ± 0.1 days, suggesting that this combination is able to more effectively control the ovarian follicular synchronization in cattle regardless of the stage of the follicular wave at the time of treatment.

The use of GnRH (Bravo et al., 1992; Aller et al., 1999; Cancino et al., 1999; Huanca et al., 2001), LH (Huanca et al., 2001; Taylor et al., 2000) and hCG (Adam et al., 1992; Bourke et al., 1992; Correa et al., 1997; Ratto et al., 1997) to induce ovulation in normal and superstimulated females has yielded variable results in camelids. However, these studies were not designed to systematically evaluate the development of follicular waves post-treatment. Other experiments have simply adapted the progesterone or progestagens protocols previously used in cattle and sheep to synchronize llamas and vicuñas (Bourke et al., 1992; Aba et al., 1999; Chaves et al., 2002; Aba et al., 2005).

So far, studies have not addressed the interaction between ovarian steroids and gonadotropin concentrations in camelids. Ovarian steroids have been reported to exert a negative effect on hypothalamic GnRH release and pituitary LH secretion in induced ovulators (Carroll and Baum, 1989; Johnson and Gay, 1981; Pau et al., 1986). Estradiol reduces the frequency and amplitude of the LH pulses in a dose dependent manner via a negative effect at the level of hypothalamus or pituitary gland (Pau et al., 1986; Carroll et al., 1987; Ramirez et al., 1994). The administration of exogenous estradiol benzoate slowly reduced FSH concentrations over 2 to 3 days in rabbits (Dufy-Barbe et al., 1978). Ovariectomized ferrets that received exogenous progesterone had similar LH concentrations to those of non-treated females. In addition, LH concentrations in progesterone-primed ferrets were significantly higher than those of ovariectomized estrogen-primed ferrets (Baum et al., 1986). The use of estradiol alone to control follicular wave dynamics has been reported in only one alpaca study (D'Occhio et al.,

1997). Exogenous estradiol given at doses of 0.5 or 2 mg induced follicular regression and emergence of a new follicular wave in which the new dominant follicle reached ovulatory size 10 to 12 days after treatment.

There is no clear evidence that progesterone exerts a negative feedback effect on LH secretion in induced ovulators (Bakker and Baum, 2000). An intravaginal device (CIDR) containing 0.33 g of progesterone was used in sixteen adult, non-pregnant and non-lactating llamas (Chaves et al., 2002). The maximum plasma progesterone concentration reached was 9.45 ng/ml on day 1 after treatment (day 0 = CIDR implant), and then progesterone concentration decreased to 2.2 ng/ml on day 3, and returned to pretreatment values at day 11. Although, the stage of the follicular wave at the time of the treatment was not stated, apparently progesterone affected the diameter of the dominant follicles during the growing phase and early in the static phase. However, no data was reported regarding the emergence of a new follicular wave in those females. A similar study was done in vicuñas (Aba et al., 2005) in which CIDRs containing 0.33 g of progesterone were implanted in adult females at random stages of follicular development. Similar to the llama study, maximum plasma progesterone concentration was 8.15 ng/ml at day 1 after treatment (Day 0 = CIDR implant), and progesterone concentrations remained elevated at approximately 3 ng/ml until the day of CIDR withdrawal. No follicular activity was observed during the CIDR treatment, maximum follicle diameter was 4 mm, and no information was provided regarding the emergence of a new follicular wave. Finally, in another study (Aba et al., 1999), 120 mg medroxyprogesterone acetate (MPA) sponges were inserted for 9 days in 22 adult llamas. After sponge withdrawal, females were allocated to different ovulatory treatments. Although all animals ovulated and formed a functional CL, the ovarian follicular dynamic was not characterized in this investigation.

2.4 Mechanism of ovulation in induced ovulators

The females of spontaneous ovulators have ovarian cycles at regular intervals in which the preovulatory LH surge and subsequent ovulation depend on ovarian steroid concentrations. Substantial evidence has been reported for the effects of ovarian steroids and the preovulatory LH surge in spontaneous ovulators (Karsch, 1987; Turzillo and Net, 1999). In contrast, early studies in camelids concluded that the copulation stimulus elicits ovulation. Stimuli provided by either intact or vasectomized males and administration of human chorionic hormone (hCG) induced ovulation in more than 80% of llamas and alpacas (San Martin et al., 1968; Fernandez-Baca et al., 1970a; England et al., 1969). However, the first endocrine study that correlated the levels of LH and ovulation following copulation in llamas showed that LH concentrations increased at 15 minutes, peaked at 2 hours, and declined to basal levels by 7 hours after natural mating (Bravo et al., 1990b). The rapid increase in plasma LH after mating in llamas (Bravo et al., 1990b) resembled that observed in rabbits after a single mating (Jones et al., 1976). Apparently, the number of matings did not increase either the ovulation rate in alpacas or the amplitude of the LH surge in llamas and alpacas (Fernandez-Baca, 1970a; Bravo et al., 1992), while in other induced ovulators such as cat, multiple mating increased both plasma LH amplitude and ovulation rate (Concannon et al., 1980).

Administration of exogenous estradiol with or without progesterone did not elicit LH secretion in rabbits and ferrets (Sawyer and Markee, 1959; Baum et al., 1990). Estradiol in induced ovulators was reported to act in the female hypothalamus inducing behaviors characteristic of the proceptive and receptive sexual stages (Baum and Schretlen, 1978). Although the increase of estradiol has been positively correlated to the increase of the size of the dominant follicle in llamas and alpacas, these physiological increases did not induce ovulation in these species (Bravo et al., 1990a, 1990b, 1991, 1992).

Gonadotropin releasing hormone (GnRH) is the central hypothalamic regulator of LH pulses in both spontaneous and induced ovulators. GnRH is produced by

hypothalamic neurons from a precursor polypeptide by enzymatic processing and packaged in storage granules that are transported down neural axons to the median eminence (Fink, 1988). Immunocytochemical studies have indicated that GnRH neurons are scattered throughout the medio-basal hypothalamus (MBH), rostrally and dorsally to the preoptic area and ventromedial hypothalamic nuclei (reviewed in Karsch, 1987). Although there are clear species differences in the localization of GnRH neurons, these differences are not related to the type of ovulation mechanism. In general, the terminal axons of GnRH neurons lie in the pericapillary space of the hypophyseal portal system in the median eminence.

The measurements of GnRH secretion have been reported only in the rat (Levine and Ramirez, 1982), rabbit (Ramirez et al., 1986; Pau et al., 1986), sheep (Moenter et al., 1991), and rhesus monkeys (Levine et al., 1985a, b). Studies conducted in rabbits by using a push-pull perfusion technique showed that GnRH release is pulsatile in the rabbit, and when coitus was allowed, there was a 40 to 100-fold increase in GnRH within 20-60 minutes from the MBH followed by an increase in LH concentrations (Ramirez et al., 1986; Pau et al., 1986). Spies et al. (1997) provided a detailed comparison between induced and spontaneous ovulators using the rabbit and the monkey as models. Although the initial stimulus to elicit the GnRH/LH surge is different between induced (neural) and spontaneous ovulators (hormonal), there are cases of induced ovulators undergoing spontaneous ovulation and spontaneous ovulators undergoing induced ovulation. This likely suggests that these animals are capable of responding to both types of stimulus but a single type of stimulus is predominant for a given species.

The neural pathways involved in the activation of GnRH neurons in induced-ovulators are still poorly understood. Neurotransmitter and peptidergic systems have been shown to affect GnRH secretion and subsequently LH release in mammalian females (reviewed in McEwen and Parsons, 1982). Rabbits and ferrets are the only species of induced ovulators in which neurotransmitter and neuropeptide studies have been conducted to evaluate the neuroendocrine signal(s) that control GnRH release

after mating. Although coitus and estrogen stimuli have been shown to increase norepinephrine concomitantly with increasing GnRH secretion, the latency of central activation of this neuroendocrine system by these two types of stimuli is governed by minutes or seconds in rabbits and hours in monkeys (Spies et al., 1997). Therefore, this differential speed in response could be attributed to different pathways required to elicit a GnRH/LH release in spontaneous or induced ovulators.

2.4.1 Effect of natural mating and hormonal treatment on ovulation and CL formation

In an early study designed to determine factors associated with eliciting ovulation in alpacas (Fernandez-Baca et al., 1970a), ovulation rate was compared among females that 1) were unmated, 2) were mounted only followed with or without artificial insemination, 3) had interrupted mating, 4) had sterile mating (vasectomized male) followed with or without artificial insemination, 5) had single or multiple uninterrupted mating (intact male), or 6) were given hCG. A high ovulation rate (80 to 100%) was observed in the females mated by intact or vasectomized males and when hCG was used. However, the interval from stimulus to ovulation was not reported because ovaries were collected from a slaughterhouse 3 days after treatment.

In a study involving a one-time examination of the ovaries during necropsy at 2- to 6-h intervals post-mating (n = 1 to 5 alpacas/time interval; San Martin et al., 1968), ovulation was detected as early as 26 h after mating and 24 h after hCG treatment. However, the method of detection (necropsy) precluded characterization of the mean interval and distribution of ovulations. In another llama study, ovulation was detected about 28 h after GnRH treatment (Bourke et al., 1992). Studies conducted in alpacas by laparoscopy every 12 h (Sumar et al., 1993) showed that the interval from mating to ovulation was 30 to 72 h in 50% (38/76) and approximately 30 h in 24% (18/76) of females. Based on daily ultrasonography of the ovaries in llamas (Adams et al., 1989, 1990), ovulation was detected 2.1 ± 0.1 days (mean ± SEM) after copulation.

Most of the studies that characterize the luteal phase in llamas and alpacas are based on laparotomies performed at very long intervals or measurements of plasma progesterone concentrations collected at different times after natural mating (Fernandez-Baca et al., 1970c; England et al., 1969; Sumar et al., 1988; Adams et al., 1989; Aba et al., 1995). Only one study has systematically reported the growth, regression and day-by-day diameter of CL after mating (Adams et al., 1991a). According to the other studies, the CL in alpacas reaches a maximum diameter of 14 mm around day 8-9 after mating (day 0 = mating), and regresses by day 8-12. The maximum progesterone concentration ranged from 3.6 to 6.3 ng/ml on day 7-8 post mating (Bravo et al., 1995; Sumar et al., 1988; Aba et al., 1995).

Based on daily ultrasonography of the ovaries and every-other-day blood sampling after natural mating in llamas (Adams et al., 1991a), the maximum CL diameter and plasma progesterone concentrations were detected at day 8 after mating (day 0 = mating). Although an artificial luteal phase has been induced in camelids by treatment with gonadotropins (England et al., 1969; Sumar 1985; Ratto et al., 2003) or GnRH (Sumar 1985; Adams et al., 1992), these studies were not designed to evaluate the interval to ovulation and CL dynamics.

2.4.2 Effect of semen on ovulation induction in llamas and alpacas

Seminal plasma is secreted by the testes and male accessory glands. Traditionally, the seminal plasma was regarded simply as a vehicle for sperm cells. However, it is believed that proteins, growth factors, hormones and cytokines present in the seminal plasma may also regulate important functions in the spermatozoa and in the female reproductive system.

Evidence that ovulation was induced by intravaginal semen deposition without any physical contact with the male was initially reported by investigators in China who concluded that a factor in semen, rather than mechanical stimulation during copulation, was responsible for eliciting ovulation in Bactrian camels (Chen et al., 1985). In

addition, a study of alpacas (cited by Sumar, 1994) showed that intravaginal deposition of semen in alpacas was associated with ovulation. Peruvian scientists conducted similar experiments in 1985 (cited in Sumar, 1994) but no information is available about the experimental design, methodology and analysis of the data. A later study showed that alpaca seminal plasma stimulates LH secretion from primary cultures of rat pituitary cells (Paolicchi et al., 1999). These reports suggested the presence of a factor(s) in alpaca seminal plasma that had GnRH-like activity. However, the first scientific study designed to evaluate the effect of seminal plasma on ovulation indicated the presence of an ovulation-inducing factor (OIF) in alpaca seminal plasma. Thirteen out of 14 female alpacas (93%) ovulated after an intramuscular injection of alpaca seminal plasma (Adams et al., 2001).

The presence of these ovulation-inducing substances may not be restricted to the seminal plasma of induced ovulators. In cattle, the preovulatory LH surge can be advanced if mating is conducted during the first 6-8 hours of behavioral estrus (Jochle, 1975). In pigs, the intrauterine deposition of boar seminal plasma accelerates ovulation in gilts (Wabersky et al., 1995). Early studies on human seminal plasma reported the presence of molecules that immunoreacted with GnRH antibodies (Sokol et al., 1985; Izumi et al., 1985). These substances are believed to be involved in sperm or oocyte function (Morales et al., 1994). Whether these factors are related to ovulation mechanisms in humans or whether these chemicals are closely related to those of Bactrian camels (Chen et al., 1985) remains unknown.

2.5 Assisted reproductive technology in South American camelids

The development of assisted reproductive techniques such as artificial insemination, embryo transfer and *in vitro* technologies in South American camelids, is considerably behind that of other livestock species. The genetic improvement of cattle

and sheep herds has been accelerated through the commercial application of embryo transfer and superstimulation treatments.

Very little has been published on embryo transfer in South American camelids. Relatively poor results have tempered the initial enthusiasm for embryo transfer in llamas and alpacas in the 1990s. Among the reasons for limited success in the use of embryo technologies in llamas and alpacas are the lack of effective superstimulatory treatments, low embryo recovery, and scanty knowledge of embryo physiology. The first report on llama IVF was published in 1994 (Del Campo et al., 1994), and the first alpaca embryo produced by *in vitro* technique was reported in 2002 (Gomez et al., 2002). However, there are no reports of attempts to develop or establish IVF-techniques in llamas and alpacas. Since the first update regarding the application of reproductive technologies in South American camelids published in 1995 (Del Campo et al., 1995), few reports have been published in the field of oocyte collection and morphology and embryo freezing and cloning technology.

2.5.1 Ovarian Superstimulation

Superstimulation has been attempted using equine chorionic gonadotropin (eCG) and follicle stimulating hormone (FSH) during a luteal phase (induced by eliciting ovulation, or by progesterone treatment) or during the sexually receptive phase. After superstimulatory treatment, the females were mated and given gonadotropin-releasing hormone (GnRH) or human chorionic gonadotropin (hCG) to induce ovulation. Superstimulatory treatment schemes may be summarized as follows:

A. *Luteal phase induced by ovulation (*Bourke et al., 1995a): GnRH or hCG was given when a follicle of ≥9 mm was present (day 0). At day 7, 1000 IU of eCG was administered intramuscularly. At day 9, a luteolytic dose of prostaglandin was given and finally, to induce ovulation, 750 IU of hCG was given when follicles reached a diameter of 9 to 13 mm.

B. *Luteal phase simulated by progestogen treatment* (Bourke et al., 1992, 1994; Correa et al., 1994): The luteal phase has been simulated either by the application of a CIDR, norgestomet, or daily progesterone treatment for 7 to 12 days. Gonadotropin treatments consisted of 20 mg pFSH (NIH-FSH-P1) im every 12 h for 5 days (total dose of 200 mg) or 1000 IU of eCG, starting 48 h before progestogen removal. Finally, 750 IU of hCG or 8 μg of GnRH was administered to induce ovulation.

C. *Sexually receptive phase* (Correa et al., 1997; Ratto et al., 1997): Females that displayed continuous sexual receptivity for 5 days received 20 mg pFSH (NIH-FSH-P1) im every 12 h for 5 days (total dose of 200 mg). After the last injection of FSH, females were treated with 750 IU of hCG to induce ovulation.

One study reported that doses of 500 and 1000 IU were optimal for inducing multiple follicular growth in llamas (Bravo et al., 1995). The need to induce a luteal phase prior to or during superstimulation in camelids is not clearly understood, but it may simply reflect an empirical bias to conventional methods used in other ruminants. The number of ovulations or CL varies widely among studies, ranging from 2 to more than 11 per animal with a number of transferable embryos ranging from 0 to 2 per animal. Much of the variation may be attributed to the variation in follicular status at the time superstimulation treatments were initiated. More studies are needed to determine if follicular dominance will suppress the superstimulatory response in llamas and alpacas as it does in cattle. Recently, Bravo et al. (2004) reported an ovarian response of 3 to 7 CL and a mean of 3.9 embryos after eCG treatment in alpacas. However, this study did not provide the stimulatory protocols used in the experiment.

2.5.2 Oocyte collection

Oocyte recovery rate after follicular aspiration by laparotomy in 4 alpacas treated with FSH and 7 alpacas treated with eCG was 83% (105/127) and 82% (163/198) of the total follicles aspirated, respectively (Gomez et al., 2002). The

proportion of expanded COC that had reached the second metaphase in the 2 groups was 18/45 (40%) and 16/61 (26.2%), respectively. Ultrasound-guided follicle aspiration is a standard procedure used to collect cattle oocytes, but the first report of this technique for any species of camelids appeared in a recent study in llamas (Brogliatti et al., 2000).

2.5.3 Embryo collection and transfer

The first collection of zygotes from the oviducts of alpacas after spontaneous ovulation and from superovulated females by laparatomy 3 days after ovulation was reported in 1968 (Novoa and Sumar, 1968). The flushes were carried out normograde, from the ovarian end of the oviduct to the uterine end. The authors suggested that the muscular utero-tubal junction made retrograde flushing impossible (Sumar, 1983).

The non-surgical method of embryo collection is similar to that used in cattle. Briefly, a catheter is introduced through the cervical canal and the cuff is placed just cranial to the internal cervical os. Both uterine horns are flushed simultaneously by infusing collection medium until the horns are distended and then the fluid is recovered by aspiration or gravity flow. The process is repeated several times until 500 to 1000 ml of medium are recovered (Del Campo, et al., 1995; Smith, et al., 1994, Bourke, et al., 1992; Correa, et al., 1992). Uterine flushing has been done on days 6.5 to 12 after mating, but embryo recovery has been frustratingly variable. Regardless of the method of embryo collection, zygote recovery does not exceed 50% of the expected zygotes based on CL counts, (Del Campo et al., 1995).

The application of embryo transfer techniques over the past 30 years has generated approximately 13 live offspring throughout the world (reviewed Del Campo et al., 1995). The first birth of an alpaca using surgical collection and transfer techniques was reported in 1974 (Sumar, 1983). The first llama born using non-surgical collection and transfer was reported by Wiepz and Chapman (1985). In this experiment, collection and transfer were done 7 d after GnRH treatment. In 1987, the birth of 2

alpacas was reported in Peru through the use of nonsurgical collection and transfer (Palomino et al., 1987). Six live offspring were born in the United Kindgom from 1992 to 1995 from 27 embryos transferred nonsurgically to 21 synchronized recipients (Bourke et al., 1994, 1995b). Interestingly, only recipients synchronized with GnRH became pregnant; no pregnancies resulted in those that received progestagen implants. In Chile, the birth of 1 llama offspring after 2 nonsurgical embryo transfers was reported in 1994 (Gatica, et al., 1994). More recently, Canadian scientists reported the recovery of 23 embryos from 5 superstimulated llamas (Palasz et al., 2000), and an American study reported the recovery of 37 embryos from 47 unstimulated donors (79%), 41% of which established pregnancies after transfer to recipients (Taylor et al., 2000). The first report of successful interspecies transfer in camelids appeared in 2001 after 2 alpaca offspring were born to llama recipients (Taylor et al., 2001).

2.5.4 Embryo Morphology

Camelid embryos collected from superovulated females 6 to 7 days after mating vary in size from 0.1 mm to 1 mm and are usually found as hatched expanded blastocysts. From a total of 163 llama embryos and 19 alpaca embryos, the mean diameter of the embryo was 527.1 ± 168.0 μm and 534 ± 151.4 μm, respectively (Del Campo et al., 2002). There is also great variation in the diameter of single embryos collected from the same female in successive collections (Del Campo et al., 2002). About 35% of recovered embryos were small (≤450μm in diameter), 40% were medium (451 to 650 μm) and 24% were large (≥ 651μm). The size distribution was almost identical for alpacas.

It appears that embryos develop more rapidly in llamas and alpacas than in other species. Morulae have been recovered from llama oviducts as early as day 3 after insemination. Trophoblast expansion ranged from a mean of 1.2 mm in diameter on day 6.5 to 7.5, to 83 mm in length on day 13 to 14 (Bourke et al., 1992). This accelerated rate of embryo development may be related to the apparent early maternal recognition of pregnancy in these species (Adams et al., 1991a; McEvoy et al., 1992).

2.5.5 In *vitro* Fertilization (IVF) and cloning

Growing follicles of llamas are spherical, and 85% of their surface protrudes from the surface of the ovary (Del Campo et al., 1994). Llama and alpaca cumulus oocyte complexes (COC) are dark, thus easily distinguished through the follicle wall by trans-illumination (Del Campo, et al., 1994). In llamas, the COC was located in the follicle hemisphere containing the expected ovulation point. Oocytes collected from follicles 2 to 11 mm in diameter ranged from 172 to 200μ (mean ± SD, 183±14). Immature oocytes had a very distinct and large germinal vesicle with a dark nucleolus. A greater number of oocytes were collected by mincing the ovary with a razor blade (average 27 oocytes/llama) than by aspiration of follicles between 1 and 6 mm in diameter (6.4 oocytes/llama; Del Campo et al., 1992, 1994).

The first successful *in vitro* maturation and *in vitro* fertilization in llamas were reported in 1992 and 1994, respectively, using ovaries collected from a slaughterhouse (Del Campo et al., 1992, 1994). The authors reported that 62% of oocytes achieved metaphase II after 36 hours of culture and 57% of the matured oocytes displayed signs of fertilization after *in vitro* culture with epididymal sperm. Of 234 oocytes placed in llama oviductal epithelial cells co-culture for 9 days, 32% cleaved, 5.6% reached the morula stage, 6% reached the early to expanded blastocyst stage and 4.7% reached the hatched blastocyst stage. The first interspecies embryos were produced by heterologous *in vitro* fertilization using capacitated llama epididymal sperm and alpaca oocytes (Gomez et al., 2002).

Recently, somatic cell nuclear transfer was reported in llamas (Sansinena et al., 2003). Adult female llama fibroblasts were used for donor karyoplasts and injected into enucleated llama oocytes. A total of 11 embryos (8-cell to morula stages) were transferred to synchronized recipient llamas, but no pregnancies were observed.

2.5.6 Embryo cryopreservation

The effects of 2 cryoprotectants, propylene glycol and ethylene glycol, on post-thaw re-expansion and morphology of blastocysts have been compared (Palasz et al., 2000). After 12 h of culture only blastocysts preserved in ethylene glycol re-expanded, and although transfers were not attempted, the authors concluded that ethylene glycol might be the cryoprotectant of choice in this species. Recently, vitrification of llama embryos has been attempted using an open pull straw (OPS) method (von Baer et al., 2002). Embryos ranging from 0.3 to 0.8 mm were exposed in one step to a high concentration of cryoprotectant (40% ethylene glycol) and then submerged directly into liquid nitrogen. *In vitro* re-expansion of embryos after thawing was acceptable, but no pregnancies resulted after transfer of embryos into 2 recipients. High lipid content in the cytoplasm of camelid oocytes and embryos may contribute to low survival after cryopreservation (Brogliatti et al., 2000; von Baer et al., 2002). Recently, 2 out of 4 llamas became pregnant after the transfer of 2 vitrified embryos in each female (Aller et al., 2002), but it is unknown whether these pregnancies resulted in offspring.

3.0 GENERAL OBJECTIVES

The purpose of the studies reported in this thesis was to investigate methods to electively induce follicular wave emergence followed by the development of a new dominant follicle capable of ovulating at a consistent and predictable time. In addition, studies were done to determine the effects of hormone preparations or biological factors derived from seminal plasma on ovulation induction and corpus luteum function. Finally, studies were conducted to evaluate the establishment of superstimulatory protocols to induce a consistent ovarian follicular response and to determine their effects in oocyte collection and morphology in llamas and alpacas.

4.0 SPECIFIC OBJECTIVES

Aim 1: A study conducted to manipulate ovarian follicular dynamics in llamas is presented in Chapter 5 of this thesis. This study was done to determine whether physical or hormonal treatments could control ovarian follicular wave emergence and if oocytes ovulated from the induced follicular waves were competent for fertilization and embryonic development under field conditions.

Aim 2: A study was conducted to compare the effects of natural mating versus hormonal treatments on ovulation induction, interval to ovulation, and corpus luteum function (Chapter 6).

Aim 3: Three studies were conducted to determine whether alpaca or llama seminal plasma influences ovulation mechanisms in these species. The aim of the first study was to determine the presence of an ovulation-inducing factor in the seminal plasma of llamas and alpacas, and to evaluate their effect on ovulation rate and corpus luteum function; this study is presented in Chapter 7. A second study was designed to determine if the ovulation-inducing factor is present in the semen of spontaneous ovulating species, e.g., bull (Chapter 8). Finally, the third study evaluated the effect of seminal plasma delivery routes on ovulation rate and corpus luteum formation in alpacas (Chapter 9).

Aim 4: Studies were conducted to determine whether superstimulatory treatments given during follicular wave emergence would influence the ovarian follicular response and oocyte maturation (*in vivo* maturation) in alpacas and llamas, and to evaluate the effect of *in vitro* culture time on maturation of llama oocytes (*in vitro* maturation). These studies are presented in Chapters 10 and 11, respectively.

5.0 OVARIAN FOLLICULAR WAVE SYNCHRONIZATION AND PREGNANCY RATE AFTER FIXED-TIME NATURAL MATING IN LLAMAS

5.1 Abstract

The study was designed to compare the efficacy of treatments intended to induce follicular wave synchronization among llamas (Experiment 1), and to determine the effect of these treatments on pregnancy rates after fixed-time natural mating (Experiment 2). In Experiment 1, llamas were treated with either 1) saline (Control, n = 20), 2) estradiol and progesterone (E/P, n = 20), 3) LH (LH, n = 20), or 4) transvaginal ultrasound-guided follicle ablation (FA, n = 20). The ovarian response was monitored daily by transrectal ultrasonography. The intervals from treatment to follicular wave emergence and to the day on which the new dominant follicle reached ≥7 mm, respectively, did not differ between LH (2.1 ± 0.3 days and 5.2 ± 0.5 d) and FA groups (2.3 ± 0.3 days and 5.0 ± 0.5 d), but both were shorter ($P<0.05$) and less variable ($P<0.01$) than in the Control group (5.5 ± 1.0 days and 8.4 ± 2.0 d), while the E/P group (4.5 ± 0.8 days and 7.7 ± 0.5 d) was intermediate. In Experiment 2, llamas at unknown stages of follicular development were assigned randomly to Control, E/P, and LH groups (n = 30 per group). A single, fixed-time natural mating was permitted 10 to 12 days after treatment. Ovulation rates did not differ among groups (Control, 93%; E/P, 90%; LH, 90%; $P = 0.99$), but the pregnancy rate was higher ($P<0.05$) for synchronized

llamas (LH and E/P groups combined, 41/54) than for non-synchronized llamas (Control group, 15/28). In conclusion, LH and FA treatments were most effective for inducing follicular wave synchronization, while E/P treatment was intermediate. Synchronization treatments did not influence ovulation rate subsequent to fixed-time natural mating, but a higher pregnancy rate in synchronized than non-synchronized llamas warrants critical evaluation of the effects of follicular status on the developmental competence of the contained oocyte.

5.2 Introduction

Female llamas and alpacas ovulate in response to copulation (England et al., 1969; San Martin et al., 1968). Hence, unmated females remain in a follicular phase characterized by more-or-less constant sexual receptivity (San Martin et al., 1968; England et al., 1971) and regular emergence of anovulatory follicular waves (Adams et al., 1990; Bravo et al., 1990a). Despite virtually constant sexual receptivity, the ability to ovulate in response to a mating stimulus was influenced by the developmental status of the dominant follicle at the time of mating (Bravo et al., 1991). Growing dominant follicles ≥6 mm were capable of ovulating (Adams et al., 1990), but smaller follicles or those that were regressing were not (Bravo et al., 1991). To be able to effectively schedule mating or insemination in llamas and alpacas, a method of controlling follicular wave status must be developed to ensure that mating coincides with the presence of a healthy mature dominant follicle.

In one study (Adams et al., 1990), the interval between the emergence of successive dominant follicles was 17 to 20 days in unmated and sterile-mated llamas, and lactation was associated with an abbreviation of 2.5 days in the interval. The interval during which the dominant follicle was ≥6 mm was approximately 19 days, of which 8 days were in the growing phase, 7 days were in the static phase, and 4 days were in the regressing phase (Adams et al., 1990). Assuming that the dominant follicle is responsive to a mating stimulus only when it is growing and ≥6 mm, then approximately half of the females may be expected to ovulate in response to mating on any given day. However, it is assumed that the dominant follicle remains viable for half the static phase, then about two thirds of the females may be expected to ovulate in response to mating on any given day. To achieve a response approaching 100%, a protocol must be devised to induce the proper follicular phase at the desired time of mating.

Ovarian synchronization has been reported in cattle using physical and hormonal methods to elicit follicular wave emergence. Ultrasound-guided follicle aspiration of all

follicles ≥5 mm in diameter induced the emergence of a new wave within 1.5 days after treatment (Bergfelt et al., 1994; Baracaldo et al., 2000). Hormonal treatments for controlling follicular wave emergence in cattle included the use of GnRH, LH (Macmillan and Thatcher, 1991; Pursley et al., 1995, 1997; Twagiramungu et al., 1992; Martinez et al., 1999), and estradiol in combination with progestogens (Bo et al., 1994, 1995a, 1995b). Similar to follicle ablation, the effectiveness of a hormonal method is based on removal of the suppressive effects of the dominant follicle, either by inducing its ovulation (GnRH, LH) or atresia (estradiol and progesterone). The final effect is elective induction of a new follicular wave followed by the development of a new dominant follicle capable of ovulating at a consistent and predictable time (Martinez et al., 2000).

In camelids, GnRH (Bravo et al., 1992; Aller et al., 1999; Cancino et al., 1999; Huanca et al., 2001), LH (Huanca et al., 2001; Taylor et al., 2000) and hCG (Adam et al., 1992; Bourke et al., 1992; Correa et al., 1997; Ratto et al., 1997) have been used to induce ovulation in normal and superstimulated females, with variable results. These studies, however, were focused on ovulation rate and were not designed to systematically evaluate follicular wave development after treatment. Other experiments involving the empirical use of progesterone have been reported in llamas and alpacas (Bourke et al., 1992; Aba et al., 1999; Chaves et al., 2002), based on studies done in cattle and sheep. The rationale for using progesterone alone to synchronize follicular development in llamas, however, is unclear since regular luteal phases are not a characteristic of the ovarian pattern in camelids (i.e., induced ovulators) and follicular waves continue to emerge at regular intervals during progestational states (i.e., after sterile mating or during pregnancy, Adams et al., 1990). Effective control of ovarian function will require a method of inducing emergence of a new follicular wave, regardless of the stage of the wave at the time of treatment, so that mating may be scheduled at a time when the dominant follicle is mature and contains a competent oocyte. Conventional breeding management practices for llama and alpaca producers require a substantial investment of time and labor for detecting sexual receptivity. Establishment of a protocol that will permit elective control of follicular growth in

camelids will dramatically improve breeding management by providing a means of pre-scheduling inseminations.

The objective of the study was to compare the efficacy of treatments intended to induce follicular wave synchronization among animals. Experiments were designed to determine the effects of steroids (estradiol plus progesterone), gonadotropin (LH), and ultrasound-guided follicular ablation on follicular wave dynamics in lactating and non-lactating llamas (Experiment 1), and to determine the effects of these treatments on pregnancy rates after fixed-time natural mating (Experiment 2).

5.3 Materials and Methods

Two experiments were conducted during the rainy season (January-March) at the high altitude research station at Quimsachata (4600 m above sea level) in the Department of Puno, Peru.

5.3.1 Experiment 1

Llamas, >2 years of age and weighing 75 to 150 kg, were classified as lactating (n = 40) or non-lactating (n = 40) and assigned randomly to the following treatment groups: 1) 2 ml phosphate buffered saline given im (Control, n = 20), 2) 1 mg estradiol-17β and 25 mg of progesterone (Sigma chemical Co, St.Louis, MO, USA), in a total volume of 2 ml of safflower oil given im (E/P, n = 20), 3) 5 mg Armour Standard luteinizing hormone (Lutropin, Vetrepharm Canada Inc, London, ON, Canada) given im (LH, n = 20), and 4) transvaginal ultrasound-guided ablation of all follicles ≥5 mm in diameter (FA, n = 20). Transrectal ultrasonography was done using a B-mode scanner with a 7.5 MHz linear-array transducer (Aloka, SSD500). Ovarian structures were examined once daily as described (Adams et al., 1989) from 3 days before to 19 or 20 days after treatment. Individual follicles ≥3 mm were monitored serially during this period and sketches were made of each ovary noting the number and the relative size and position of ovarian structures (Knopf et al., 1989). The ovarian response to

treatment was evaluated by determining the interval to new wave emergence, the daily diameter profile of the dominant follicle, the day on which the new dominant follicle reached ≥7 mm in diameter, and the number of follicles ≥4 mm in diameter. A follicular wave was defined as the simultaneous growth of a cohort of follicles. A dominant follicle was defined as one that grew to at least 7 mm and exceeded the diameter of all other follicles in the cohort (Adams et al., 1990). The day of wave emergence was defined as the day on which the dominant follicle was first detected, retrospectively, at a diameter of 4 mm (Adams et al., 1990).

5.3.2 Experiment 2

Lactating llamas, >2 years of age and weighing 75 to 150 kg, and of unknown (i.e., random) follicular wave status were assigned randomly to 3 treatment groups: 1) 2 ml phosphate buffered saline given im (Control, n = 30), 2) 1 mg estradiol-17β and 25 mg of progesterone, in a total volume of 2 ml of safflower oil given im (E/P, n = 30), and 3) 5 mg Armour Standard luteinizing hormone given im (LH, n = 30). Natural mating was scheduled on day 10 to 12 (day 0 = day of treatment). Each female was mated only once, and a different male was used for each female (i.e., 90 males). Matings were monitored and transrectal ultrasound examinations were conducted on the day before mating, the day of mating and 3, 8 and 25 days later to evaluate follicular size, ovulation, presence of a corpus luteum, and pregnancy, respectively. Ovulation was assumed by the disappearance of an apparent dominant follicle identified before mating and the subsequent examination, and was confirmed by the subsequent detection of a corpus luteum (Adams et al., 1990). Pregnancy was defined as the ultrasonic detection of an embryo proper (Adams, 1997).

5.3.3 Statistical Analyses

Single point measurements (i.e., interval from treatment to wave emergence, maximum size of the dominant follicle, interval from treatment to the day on which the new dominant follicle reached ≥7 mm) were performed by two-way analyses of

variance to determine the effects of hormonal treatment, lactation, and their interaction. Differences between lactating and non-lactating llamas in the interval to wave emergence and to the day on which the new dominant follicle reached ≥7 mm in diameter were not significant; therefore, data were analyzed for a treatment effect irrespective of lactational status. The degree of variability in the intervals from treatment to wave emergence and to the day the dominant follicle reached ≥7 mm was estimated by calculating the group mean and subtracting it from each data point; the resulting values were then compared among treatment groups by analysis of variance. When main effects or their interaction were significant (i.e., $P \leq 0.05$), means were compared by least significant difference (LSD). Cook's value was used to detect outliers (Rawlings, 1988). For statistical analyses of serial data and preparation of figures, follicular data were centralized to the day of wave emergence. Serial data were compared by Proc-mixed in SAS (SAS, 1985) to determine main effects of status, treatment, day, and their interactions. The Compound Symmetry model provided the best fit and was, therefore, used to interpret the results of analyses of variance. Ovulation and pregnancy rates were compared by Chi-square analyses, and follicular size before mating was compared among treatment groups by analysis of variance.

5.4 Results

5.4.1 Experiment 1

Data from 1 non-lactating llama in the Control group and 1 non-lactating llama in the E/P group were excluded from analyses because emergence of a new follicular wave was not detected during the observational period (i.e., > 20 days). The interval to wave emergence for these 2 animals were statistical outliers compared with others in the respective groups. One lactating and 2 non-lactating llamas from the Control group ovulated spontaneously, but their data were included in the analyses. By the same token, 2 lactating and 2 non-lactating llamas in the LH group did not ovulate, but their data were also included in the analyses.

The effects of treatment and lactational status on the interval from treatment to wave emergence are summarized in Table 5.1. There was an effect of treatment ($P = 0.01$) but no effect of lactation ($P = 0.8$), or treatment-by-lactation interaction ($P = 0.06$). The mean interval did not differ between LH and FA groups, but both were shorter ($P < 0.05$) than the Control group, while the E/P group was intermediate.

The effects of treatment and lactational status on the interval from treatment to the day on which the new dominant follicle reached ≥7 mm in diameter are shown in Table 5.2. There was an effect of treatment ($P = 0.01$) on the mean interval, but no effect of lactation ($P = 0.1$) or treatment-by-lactation interaction ($P = 0.07$). The mean interval did not differ between LH and FA groups, and both were shorter ($P < 0.05$) than the Control group, while the E/P group was intermediate.

Intervals from the day of treatment to the day of wave emergence and to the day on which the new dominant follicle reached ≥7 mm did not differ between lactating and non-lactating llamas; therefore, data were analyzed for degree of synchrony (variability) after combining lactating and non-lactating groups (Table 5.3). The variability in the intervals to wave emergence for the LH and FA groups were less ($P < 0.01$) than the Control group, and the E/P group was intermediate. The variability in interval to the day on which the new dominant follicle reached ≥7 mm in diameter did not differ among the E/P, LH and FA groups, but all 3 were less ($P < 0.01$) than the Control group.

Lactation was associated with a decrease ($P < 0.01$) in maximum diameter of the dominant follicle, but there was no effect of treatment or interaction (Table 5.4). The effects of treatment and lactational status on the number of follicles ≥4 mm detected on the day of wave emergence are summarized in Table 5.5. In the FA group, the number of follicles was numerically lower than in other groups, but differences did not reach significance among treatment groups ($P = 0.07$).

No effect of treatment was detected on the day-to-day profile of follicle numbers and diameter of the new wave; therefore, data were combined among treatment groups

(Figure 5.1). The dominant follicle diameter profile was smaller in lactating llamas than non-lactating llamas (lactation-by-day interaction, $P < 0.01$). There was a day effect but no lactation effect or interaction on the profile of the number of follicles ≥4 mm detected per day (Figure 5.1).

Table 5.1. Interval (mean ± SEM) from treatment to follicular wave emergence in llamas after estradiol-17β plus progesterone (E/P), luteinizing hormone (LH), or ultrasound-guided follicle ablation (FA) treatments for the purpose of ovarian synchronization.

	Control	E/P	LH	FA	Overall
Lactating (days)	7.2 ± 1.7	4.2 ± 0.6	1.9 ± 0.4	1.6 ± 0.3	3.8 ± 0.6
(n)	(10)	(10)	(10)	(10)	(40)
Non-lactating (days)	3.7 ± 1.0	5.0 ± 1.6	2.4 ± 0.3	3.1 ± 0.9	3.3 ± 0.5
(n)	(9)	(9)	(10)	(10)	(38)
Overall (days)	5.5 ± 1.0[a]	4.5 ± 0.8[ab]	2.1 ± 0.3[b]	2.3 ± 0.3[b]	3.6 ± 0.4
(n)	(19)	(19)	(20)	(20)	(78)

Treatment (P = 0.01), Lactation (P = 0.8), Treatment-by-Lactation interaction (P= 0.06).
[a,b] Values with no common superscripts are different (P < 0.05).

Table 5.2. Interval (mean ± SEM) from treatment to the day on which the dominant follicle reached ≥7 mm in diameter in lactating and non-lactating llamas after estradiol-17β plus progesterone (E/P), luteinizing hormone (LH), or ultrasound-guided follicle ablation (FA) treatments for the purpose of ovarian synchronization.

	Control	E/P	LH	FA	Overall
Lactating (days)	10.5 ±1.7	8.3 ± 0.7	5.2 ± 0.5	4.8 ± 0.5	7.2 ± 0.6
(n)	(10)	(10)	(10)	(10)	(40)
Non-lactating (days)	6.3 ± 1.0	7.2 ± 1.0	5.3 ± 0.6	5.8 ± 1.0	6.1 ± 0.5
(n)	(9)	(9)	(10)	(10)	(38)
Overall (days)	8.4 ± 2.0[a]	7.7 ± 0.5[ab]	5.2 ± 0.5[b]	5.0 ± 0.5[b]	6.7 ± 0.4
(n)	(19)	(19)	(20)	(20)	(78)

Treatment (P = 0.01), Lactation (P = 0.1), Treatment-by-Lactation interaction (P= 0.07).
[a,b] Values with no common superscripts are different (P < 0.05).

Table 5. 3. Variability (mean ± SEM)* in intervals to follicular wave emergence and the day on which the dominant follicle reached ≥7 mm in diameter in llamas after estradiol-17β plus progesterone (E/P), luteinizing hormone (LH), or ultrasound-guided follicle ablation (FA) treatments for the purpose of ovarian synchronization.

	Control	E/P	LH	F/A
Wave emergence (days)	3.4 ± 0.5^{a}	2.4 ± 0.5^{ab}	0.9 ± 0.1^{b}	1.3 ± 0.4^{b}
Range	1 - 15	1 - 14	0 - 5	0 - 11
(n)	(19)	(19)	(20)	(20)
≥ 7mm follicle (days)	3.6 ± 0.5^{a}	1.5 ± 0.4^{b}	1.4 ± 0.3^{b}	1.9 ± 0.3^{b}
Range	2 - 19	5 - 14	2 - 9	2 - 13
(n)	(19)	(19)	(20)	(20)

[a,b] Values within rows with no common superscripts are different ($P < 0.01$).
*Variability was estimated by calculating the absolute difference between the treatment group mean and individual animal value. Mean differences were compared among groups by analysis of variance.

Table 5. 4. Maximum diameter (mean ± SEM) of the dominant follicle in lactating and non-lactating llamas after estradiol-17β plus progesterone (E/P), luteinizing hormone (LH), or ultrasound guided follicle ablation (FA) treatments for the purpose of ovarian synchronization.

	Control	E/P	LH	F/A	Overall
Lactating (mm)	10.0 ± 0.8	10.8 ± 0.8	10.8 ± 0.4	11.8 ± 0.9	10.9 ± 0.8[a]
(n)	(10)	(10)	(10)	(10)	(40)
Non-lactating (mm)	13.4 ± 1.0	13.1 ± 0.7	14.0 ± 1.0	13.7 ± 1.1	13.6 ± 0.5[b]
(n)	(9)	(9)	(10)	(9)	(38)
Overall (mm)	11.7 ± 1.7	11.9 ± 1.1	12.4 ± 1.6	12.7 ± 0.9	12.2 ± 0.3
(n)	(19)	(19)	(20)	(20)	(78)

Treatment (P = 0.8), Lactation (P = 0.01), Treatment-by-Lactation interaction (P = 0.66).
[a,b] Values with different superscripts are different (P < 0.01).

Table 5.5. Number (mean ± SEM) of follicles ≥4 mm detected on the day of wave emergence in lactating and non- lactating llamas after estradiol-17β plus progesterone (E/P), luteinizing hormone (LH), or ultrasound-guided follicle ablation (FA) treatments for the purpose of ovarian synchronization.

	Control	E/P	LH	F/A	Overall
Lactating	11.3 ± 2.6	9.3 ± 0.9	5.7 ± 1.5	4.1 ± 1.0	7.6 ± 0.9
(n)	(10)	(10)	(10)	(10)	(40)
Non-lactating	11.4 ± 1.9	8.3 ± 1.5	11.2 ± 2.5	8.0 ± 2.0	9.7 ± 1.0
(n)	(9)	(9)	(10)	(10)	(38)
Overall	11.4 ± 1.6	8.8 ± 1.0	8.4 ± 1.5	6.0 ± 1.1	8.6 ± 0.7
(n)	(19)	(19)	(20)	(20)	(78)

Treatment (P = 0.07), Lactation (P = 0.2), Treatment-by-lactation interaction (P = 0.1)

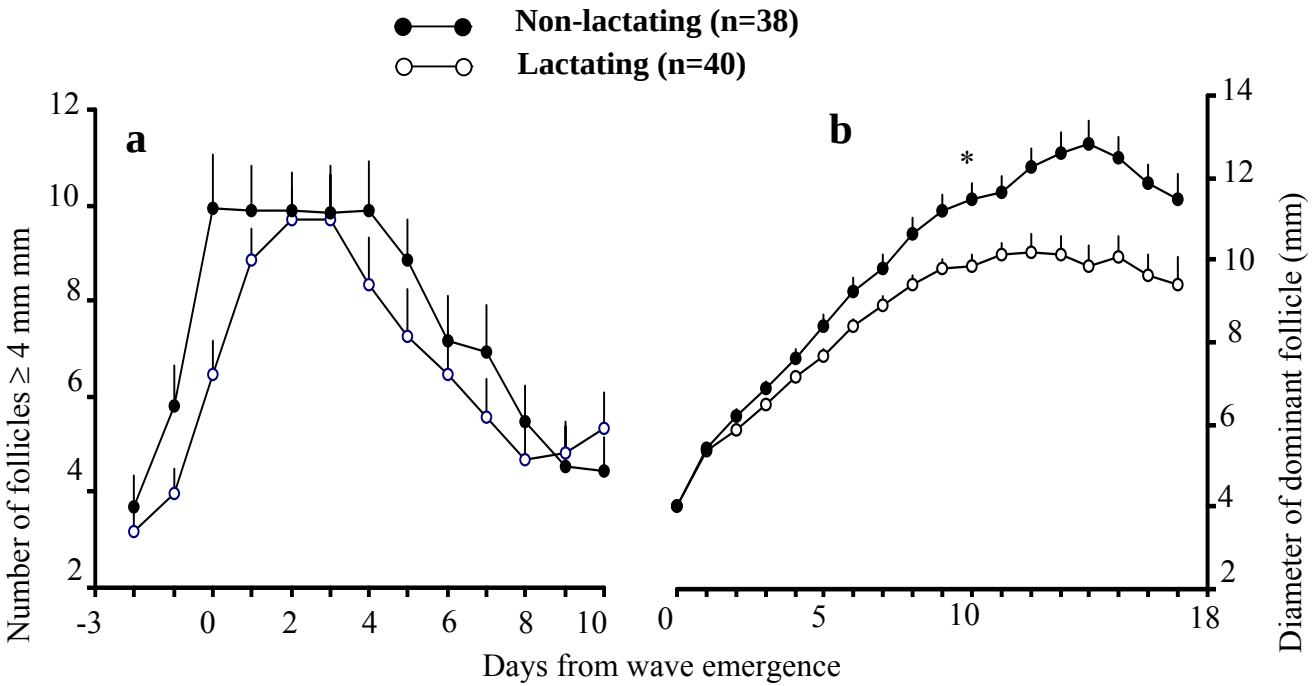

Figure 5.1. The effect (mean ± SEM) of lactational status on follicular dynamics in llamas after synchronization treatment. No effect of treatment on number of follicles ≥ 4 mm (P = 0.3) or on dominant follicle diameter (P = 0.6) was detected, therefore, data were combined among treatments groups. a) Profiles of follicles number. Effect of day (P<0.01), Lactation (P = 0.1), Treatment-by-status (P = 0.4), Treatment-by-day (P = 0.1), Status-by-day (P = 0.1), Treatment-by-status-by-day (P = 0.1). b) Profiles of follicle diameter. Lactation-by-day interaction (P<0.01), Treatment-by-status (P = 0.9), Treatment-by-day (P = 1.0), Treatment-by-status-by-day (P = 0.6). * Day 10: first day of significance (P<0.01).

5.4.2 Experiment 2

All the females were receptive to mating, regardless of group. The diameter of the largest follicle at the time of mating did not differ ($P = 0.94$) among treatment groups. Ovulation rates ranged from 90% to 93% and did not differ among groups ($P = 0.5$; Table 5.6). Eight days after mating, a CL was detected in all females in which ovulation had been detected. Differences in pregnancy rates among groups did not reach significance; however, the pregnancy rate was higher ($P < 0.05$) in synchronized animals (LH and E/P groups combined) than in those that were not synchronized (Control group; Table 5.6).

Table 5.6. Follicular size (mean ± SEM), and ovulation and pregnancy rates in lactating llamas treated with estradiol-17β plus progesterone or LH and permitted a single fixed-time natural mating (Experiment 2).

End Point	Control	E/P	LH	E/P and LH Combined
Number	30	30	30	60
Follicular diameter (mm)*	9.8 ± 0.4	9.9 ± 0.5	9.7 ± 0.4	9.8 ± 0.3
Ovulation rate	28/30	27/30	27/30	54/60
	(93%)	(90%)	(90%)	(90%)
Pregnancy rate**	15/28	21/27	20/27	41/54
	(54%)[a]	(78%)	(75%)	(76%)[b]

[a,b] Values with different superscripts are different ($P < 0.05$).
*Follicular diameter recorded before mating.
**Number pregnant over the number that ovulated.

5.5 Discussion

The ability to manipulate follicular waves in cattle has resulted in a degree of estrus and ovulation synchrony sufficient to permit fixed-time insemination (Bo et al., 2002). Consistency in estrus and ovulation synchronization protocols depends largely on the effectiveness of treatment to induce the development of a new dominant follicle among animals regardless of follicular and luteal status at the time of treatment. The present study was designed to compare the efficacy of treatments, developed initially for use in cattle, to induce follicular wave synchronization in llamas, and to evaluate the effect of such treatments on ovulation and pregnancy rate after a single, fixed-time natural mating. LH treatment and the use of ultrasound-guided follicle ablation were most effective for inducing follicular wave synchronization, while treatment with estradiol-17β plus progesterone was intermediate in efficacy. Although synchrony of wave emergence was affected by the treatments in Experiment 1, treatment-induced wave synchrony did not influence ovulation rate subsequent to natural mating in the Experiment 2. This result was unexpected and calls into question the notion that only growing- and early static-phase dominant follicles are capable of ovulating (Bravo et al., 1991).

Camelids exhibit a follicular wave pattern similar to other species, and share similar features such as recruitment, selection and dominance during follicular development (Adams, 1997). Similar to other species then, FSH and LH may play important roles in recruitment and dominance, respectively. An association between FSH surges and follicle recruitment has been described in cattle (Adams et al., 1992a), sheep (Picton et al., 1990), and swine (Driancourt et al., 1995), but in the only study of its kind in llamas (Bravo et al., 1990a), no relationship between FSH and follicular recruitment was detected. However, follicular waves and the inter-wave interval were not strictly defined, and FSH surges may have been obscured without the use of wave emergence as a reference point (Adams, 1997).

Several reports have documented the efficacy of estradiol plus progesterone treatment for synchronizing wave emergence and ovulation in cattle (Pursley et al., 1997; Martinez et al., 1999; Bo et al., 1994, 1995a, 1995b). The synchronizing effect has been attributed to treatment-induced atresia of the dominant follicle through suppression of circulating gonadotropins (Bo et al., 2000). Estradiol alone was found to suppress the growth of the dominant follicle in cattle, but the effect was more consistent when combined with progestogen treatment (Martinez et al., 1999). The dose of estradiol use in the present study was similar to that of a preliminary study (D'Occhio et al., 1997) in which a single administration of 0.5 or 2 mg of estradiol without progesterone induced follicular regression and new wave emergence in alpacas regardless of the stage of follicular development. Although the effect of steroid treatment was consistently intermediate between that of the Control and other treatment groups, a distinct synchronizing effect of estradiol/progesterone treatment was not apparent in the present study. The lack of a clear effect may have been related to the dose or duration of treatment of estradiol and progesterone. In this regard, progesterone treatment is commonly given for 6 to 9 days in cattle (Bo et al., 2002).

In one study (Bourke et al., 1992), a subcutaneous norgestomet ear implant (Crestar®) for 7 days or an intravaginal progesterone-releasing device of (CIDR®) for 9 days was used in llamas prior to ovarian superstimulation. Authors reported a high number of corpora lutea after superovulatory treatment, but a comparison with non-progestogen treated animals was not included. Recently, the use of a CIDR for 8 days in two groups of llamas with a follicular size of 6 to 9 mm and 10 to 14 mm, respectively, resulted in a decrease in follicular size in both groups 7 days after treatment (Chaves et al., 2002). Administration of progesterone (150 mg per day for 14 days) had a suppressive effect on large follicles in camel, however, progesterone treatment alone did not effectively synchronize wave emergence (Skidmore, 1994). In another brief report in camels, the use of a CIDR for 14 days, with or without an initial injection of estradiol plus progesterone, induced the development of a pre-ovulatory follicle 10 days after the CIDR was removed (Niasari-Naslaji et al., 2002). In most of these studies, however, the lack of information regarding follicular dynamics subsequent to treatment and the lack

of a clear definition of ovarian status before treatment makes interpretation difficult, particularly when success of synchrony was reported in terms of ovulation rate. Results of the present study suggest that it is not valid to make inference about follicular synchrony based on ovulatory response; i.e., ovulation rates did not differ between synchronized and unsynchronized llamas, but pregnancy rates did. The ovarian effects of dose and duration of estradiol/progesterone treatment in llamas remain to be investigated.

Follicular wave emergence after GnRH or LH treatment was variable in studies conducted in cattle (Martinez et al., 1999; Martinez et al., 2000). Wave synchronization occurred only if ovulation was induced; i.e., only when treatment coincided with the presence of a large viable dominant follicle. However, in the Experiment 1, a large proportion of llamas did ovulate (80%) after LH treatment given at random stages of follicular development, resulting in a large proportion of llamas with new follicular wave emergence. Inherent ovulatory responsiveness in combination with a strategic waiting period of 10 to 12 days, made LH treatment particularly effective for inducing follicular synchrony among llamas in the present study.

The treatment-induced follicular wave was similar to the spontaneous wave based on the observation that the daily profile of the number of follicles ≥4 mm and the diameter of the dominant follicle of the new wave were not influenced by synchronization treatments. The suppressive effect of lactation on the diameter of the dominant follicle was consistent with previous findings (Adams et al., 1990).

The ovulation rate observed in the Control group in Experiment 2 was unexpectedly high (93%). We anticipated an ovulation rate of about 66% based on the presence of a growing- or early static-phase follicle of ≥7 mm within the ovaries in 66% of the llamas at any given time (Adams et al., 1990). However, results suggest that follicles in growing, static, and regressing phases were capable of ovulating, contrary to the results of a previous study (Bravo et al., 1991). Although ovulation rate was not affected by treatment, the pregnancy rate was higher in synchronized than non-

synchronized females (Experiment 2). The disconnection between ovulation rate and pregnancy rate suggests that ovulatory capability is not directly related to the capability of the oocyte to become fertilized and develop into an embryo. Assuming that a growing- or early static-phase dominant follicle ≥7 mm is present in about 66% of llamas at any given time, and that such follicles have a high ovulation rate (85-90%, Adams et al., 1990) and contain a viable oocyte, we expected a pregnancy rate of about 57% in the control group (i.e., 66% x 87%). Despite the unexpectedly high ovulation rate in the Control group (93%), the observed pregnancy rate (54%) was very close to the expected rate. Results provide rationale for the hypothesis that oocytes from follicles outside the mid-growing to early-static phase are immature or post-mature, and not competent to develop into embryos.

In conclusion, LH and FA treatments were most effective for inducing follicular wave synchronization, while E/P treatment was intermediate. The ovulation rate after a single LH treatment or a single mating was unexpectedly high (~90%) and suggests that ovulatory capability extends through a greater proportion of the growing, static and regressing phases of dominant follicle than previously thought. Although synchronization treatments did not influence ovulation rate subsequent to natural mating, treatment was associated with a higher pregnancy rate. Differences in pregnancy rate after fixed-time natural mating between synchronized vs non-synchronized llamas warrant critical evaluation of the effects of follicular status on the developmental competence of the contained oocyte. Synchronization treatment followed by a single pre-scheduled mating resulted in acceptable pregnancy rates and may form the basis of new breeding management schemes that obviate the labor-intensive need for testing behavioral receptivity in llamas.

6.0 COMPARISON OF THE EFFECT OF NATURAL MATING, LH, AND GNRH ON INTERVAL TO OVULATION AND LUTEAL FUNCTION IN LLAMAS

6.1 Abstract

Gonadotropins and GnRH have been used to electively induce ovulation in llamas and alpacas, but critical evaluation of the natural interval to ovulation after mating has not been performed nor has a direct comparison of the effects of natural mating versus hormone treatments on this interval and subsequent luteal development. The objectives of this study were to compare the effects of hormonal treatments and natural mating on ovulation induction, interval to ovulation, and luteal development in llamas. The ovaries of llamas were examined by transrectal ultrasonography once daily. Llamas with a large follicle were assigned randomly to be: 1) mated with an intact male (mated; n = 10); 2) given 5mg of LH im (LH; n = 11); or 3) 50μg of GnRH im (GnRH; n = 10). Ultrasound examinations were performed every 4 h from treatment (day 0) to ovulation and thereafter once daily for 15 consecutive days to monitor CL growth and regression (n = 5 per group). Plasma progesterone concentrations were measured at days 0, 3, 6, 9, and 12 after treatment to evaluate CL function. The size of the largest preovulatory follicle at the time of treatment did not differ among groups (11 ± 0.6, 10.5 ± 0.8, 11.8 ± 0.9 mm, for mated, LH, and GnRH groups, respectively; P = 0.6). No

differences were detected among groups (mated, LH, and GnRH) in ovulation rate (80%, 91%, 80%, respectively; P = 0.6), or interval from treatment to ovulation (30.0 ± 0.5, 29.3 ± 0.6, 29.3 ± 0.7 h, respectively; P = 0.9). Similarly, no differences were detected among groups (mated, LH, and GnRH) in maximum CL diameter (14.2 ± 0.3, 13.2 ± 0.5, and 13.0 ± 0.7 mm, respectively; P = 0.5), the day of maximum CL diameter (7.6 ± 0.2, 7.6 ± 0.2, and 7.4 ± 0.4 mm, respectively; P = 0.6), or the day on which the CL began to regress (12.3 ± 0.3 [non-pregnant, n = 3], 11.8 ± 0.6, 12.2 ± 0.4, respectively; P = 0.4). The diameter of the CL and plasma progesterone concentrations changed over days (P < 0.0001) but the profiles did not differ among groups. In summary, ovulation rate, interval to ovulation, and luteal development were similar among llamas that were mated naturally or treated with LH or GnRH. We conclude that both hormonal treatments are equally reliable for inducing ovulation and suitable for synchronization for artificial insemination or embryo transfer program.

6.2 Introduction

Camelids are considered induced ovulators; a copulatory stimulus is necessary to elicit ovulation in females (England et al., 1969; Fernandez-Baca et al., 1970a). In an early study designed to determine factors associated with eliciting ovulation in alpacas (Fernandez-Baca et al., 1970a), ovulation rate was compared among females that 1) were unmated, 2) were mounted only followed with or without artificial insemination, 3) had interrupted mating, 4) had sterile mating (vasectomized male) followed with or without artificial insemination, 5) had single or multiple uninterrupted matings (intact male), or 6) were given hCG. It was concluded that mounting with penile intromission is necessary to induce ovulation regardless of whether the male is intact or vasectomized, and that ovulation rate can be increased with hCG treatment. Support for the hypothesis that South American camelids are induced ovulators was provided in a later study in which a rise in plasma LH concentration was detected 15 minutes after natural mating in llamas (Bravo et al., 1990b).

In a study involving one-time examination of the ovaries during necropsy at 2- to 6-hour intervals post-mating (n = 1 to 5 alpacas/time interval; San Martin et al., 1968), ovulation was detected as early as 26 h after mating and 24 hours after hCG treatment. However, the method of detection (necropsy) precluded characterization of the mean interval and distribution of ovulations. Based on daily ultrasonography of the ovaries in llamas (Adams et al., 1989, 1990), ovulation was detected 2.1 ± 0.1 days (mean ± SEM) after copulation. In another study involving ultrasonographic examination of the ovaries of llamas at 2-hour intervals (Adam et al., 1992), ovulation was detected at 27.2 ± 0.3 h (mean ± SEM) after hCG treatment and 28.6 ± 0.4 h after GnRH treatment. Although the latter study was not designed to compare the effects of treatment with natural mating, the authors reported a mating to ovulation interval of 2 d, similar to that previously described but substantially longer than the response to hormone treatment (Adam et al., 1992).

The characteristics of the luteal phase after natural induction of ovulation have been well described in llamas and alpacas (Sumar et al., 1988; Adams et al., 1990,1991a; Aba et al., 1995). Based on daily ultrasonography of the ovaries and every-other-day blood sampling after natural mating in llamas (Adams et al., 1991a), maximum CL diameter and plasma progesterone concentration were detected at day 8 after mating (day 0 = mating). The first significant decrease in CL diameter and plasma progesterone profiles during luteolysis in non-pregnant females occurred on days 11 and 10 after mating and reached nadir on days 15 and 14, respectively. Similarly, maximum plasma progesterone concentrations occurred on day 8 after hCG treatment or mating, followed by a decrease beginning on day 13 in non-pregnant alpacas (Fernandez-Baca et al., 1970c). Luteolysis was temporally associated with pulsatile release of $PGF_{2\alpha}$ from the uterus around days 8 to 10 after mating (Sumar et al., 1988; Aba et al., 1995). In pregnant llamas, a transient drop in plasma progesterone concentration was detected between days 10 and 12 after mating, and was followed by a rebound on day 14 (Adams et al., 1991). Luteal diameter and plasma progesterone in pregnant llamas continued to increase until maximum on days 23 and 27, respectively.

An artificial luteal phase has been induced in camelids by treatment with gonadotropins (England et al., 1969; Sumar 1985; Ratto et al., 2003) or GnRH (Sumar 1985; Adam et al., 1992). Hormonal treatments (GnRH, hCG or LH) were also used to synchronize ovulations in llama recipients for embryo transfer programs (Bourke et al., 1995a,b; Correa et al., 1997; Taylor et al., 2001) and to induce ovulation after artificial insemination in alpacas (Bravo et al., 1997). However, direct comparison of the effects of natural mating versus hormonal treatments on ovulation induction and corpus luteum function in camelids has not been reported.

Knowledge of the effects of hormonal preparations inducing ovulation and luteal function will provide insight on the mechanism of ovulation in these species and is critical for successful development of fixed-time insemination protocols and for recipient synchronization in embryo transfer programs. Furthermore, the apparent disparity in interval to ovulation between mating-induced versus hormone-induced

ovulation may have important implications in the design of protocols intended to control ovarian function in llamas and alpacas. Hence, the objectives of this study were to compare the effects of hormonal treatments and natural mating on: 1) ovulation induction; 2) interval to ovulation; and 3) luteal development in llamas.

6.3 Materials and methods

Mature non-pregnant female llamas (n = 31), ≥2 years of age (4.5 ± 0.2) and weighing 85-130 kg (114 ± 2 kg) were used during the breeding season (January-February) at the Quimsachata Research Station in the Department of Puno, Peru (15° S latitude, 71° W longitude, and 4,500 m above the sea level). Ovaries were examined once daily by transrectal ultrasonography (Adams et al., 1989) using a B-mode scanner with a 7.5 MHz linear-array transducer (Aloka, SSD500, Instruments for Science and Medicine Inc., Vancouver, Canada). Llamas with a follicle ≥8 mm in diameter that had grown for three consecutive days (Adams et al., 1990) were assigned randomly to be: 1) mated with an intact male (mated: n = 10); 2) given 5 mg Armour Standard LH im (LH: n = 11, Lutropin, Bioniche Animal Health Canada Inc., Belleville, Ontario, Canada); or 3) 50 µg of GnRH im (GnRH: n = 10, Cystorelin, Merial Canada Inc., Victoriaville, Quebec, Canada). In the mated group, each female was mated only once and a different male (n = 10) was used for each female.

The ovaries were examined by transrectal ultrasonography every 4 h from treatment (day 0) to ovulation. Ovulation was defined as the sudden disappearance of a large follicle (≥8 mm) that was detected during the previous examination, and was confirmed by subsequent CL formation (Adams et al., 1989). Thereafter, ultrasonographic examination was performed once daily for 15 consecutive days in 5 llamas per group to monitor CL growth and regression. Blood samples were collected from the same 5 llamas in each group into heparinized tubes by jugular venipuncture on days 0, 3, 6, 9, and 12 after treatment. Samples were centrifuged at 1700 x g for 20 min and the plasma was stored at -20°C. Plasma progesterone concentration was determined by solid-phase radioimmunoassay (Adams et al., 1991a). All samples were analyzed in a

single assay. The intra-assay coefficients of variations were 1.8%, 4.9% and 1.8%, respectively, for reference plasma progesterone concentrations of 1.8, 2.9, and 14.6 ng/ml. The sensitivity of the assay was 0.02 ng/ml.

6.3.1 Statistical Analyses

Single-point measurements (i.e., follicle size at the time of treatment, maximum CL diameter, day on which the CL was first detected) were compared among groups by analysis of variance. For statistical analyses of serial data and preparation of figures, CL diameter and progesterone data were centralized to the day of treatment (day 0). Serial data were compared by analysis of variance for repeated measure (mixed model procedure of SAS, Statistical Analysis System Institute Inc., Cary, NC, USA) to determine the effects of treatment, day, and treatment-by-day interaction. When main effects or the interaction were significant (i.e., $P \leq 0.05$), means on a given day were compared by least significant difference (LSD). Ovulation rate was compared by Chi-Square analysis.

6.4 Results

The diameter of the largest follicle at the time of treatment was similar among groups ($P = 0.6$), and no differences were detected among groups in ovulation rate ($P = 0.6$), interval to ovulation ($P = 0.9$, Table 6.1), or day on which the CL was first detected after treatment ($P = 0.8$, Table 6.2). The diameter of the CL remained elevated and plasma progesterone concentration exceeded 1ng/ml on day 12 in only 2 llamas from the mated group. Pregnancy was confirmed in these 2 llamas by ultrasonographic detection of an embryo proper on day 25.

In the mated group, luteal diameter and plasma progesterone profiles were affected by reproductive status (pregnant vs non-pregnant, $P = 0.05$, Figure 6.1ab). However, CL diameter did not differ ($P = 0.5$) between pregnant ($n = 2$) and non-pregnant ($n = 3$) llamas from days 0 to 11 after mating, and progesterone concentrations

were not different (P = 0.2) from days 0 to 9 (Figure 6.1a,b). Therefore, comparisons of CL diameter and plasma progesterone profiles among groups (Figure 6.2a,b) included data from all llamas to day 11 and to day 9, respectively; thereafter, data from the 2 pregnant llamas were omitted. Corpus luteum diameter and plasma progesterone concentration changed over days (P < 0.0001) but the profiles did not differ among groups (Figure 6.2). Maximum CL diameter and the day on which maximal diameter was detected did not differ among groups (Table 6.2). Similarly, no difference was detected among groups on the day when CL diameter began to decrease (Table 6.2). Plasma progesterone concentrations were elevated (P < 0.05) by day 6 and were maximal by day 9. Plasma progesterone concentrations decreased sharply between days 9 and 12 (Figure 6.2).

Table 6.1. Effect (mean ± S.E.M.) of mating and hormonal treatment on ovulation induction in llamas.

End Point	Mating (n = 10)	LH (n = 11)	GnRH (n = 10)	P-value
Follicle size at treatment (mm)	11.0 ± 0.6	10.5 ± 0.8	11.8 ± 0.9	0.6
Ovulation rate	8/10 (80 %)	10/11 (91 %)	8/10 (80 %)	0.6
Interval from treatment to ovulation (h)	30.0 ± 0.5	29.3 ± 0.6	29.4 ± 0.7	0.9

Table 6.2. Effect (mean ± S.E.M.) of mating and hormonal treatment on luteal development in llamas (Day 0 = day of mating or hormonal treatment).

End Point	Mating (n = 5)	LH (n = 5)	GnRH (n = 5)	P-value
1st day corpus luteum detected	2.4 ± 0.4	2.2 ± 0.3	2.6 ± 0.2	0.8
Maximum CL diameter (mm)	14.2 ± 0.3	13.2 ± 0.5	13.0 ± 0.7	0.5
Day of maximum CL diameter (mm)	7.6 ± 0.2	7.6 ± 0.2	7.4 ± 0.4	0.6
Onset of CL regression*	12.3 ± 0.3+	11.8 ± 0.6	12.2 ± 0.4	0.4

* Defined as the first day on which the corpus luteum began a progressive decrease in diameter leading to a minimum on the last day of the observational period.
+ non-pregnant (n = 3).

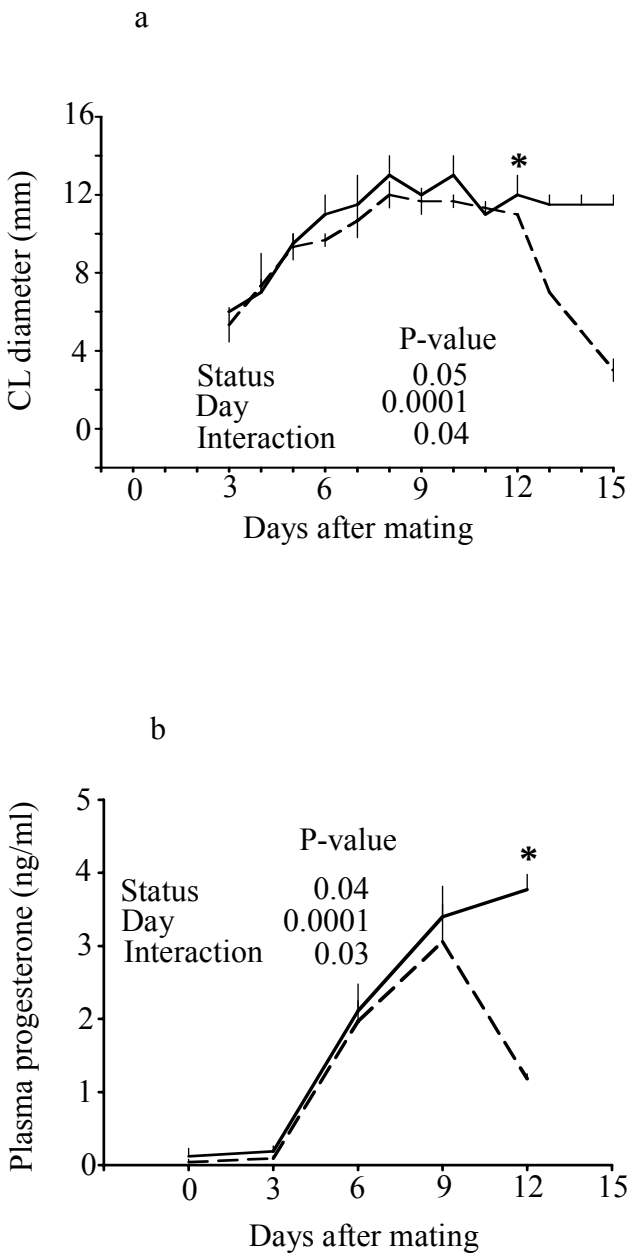

Figure 6.1. Corpus luteum diameter (a) and plasma progesterone (b) profiles (mean ± S.E.M.) in (pregnant ——— , n = 2, non-pregnant– – – , n = 3) llamas mated to an intact male. An asterisk (*) indicates the first day on which values differed between pregnant and non-pregnant llamas ($P < 0.05$).

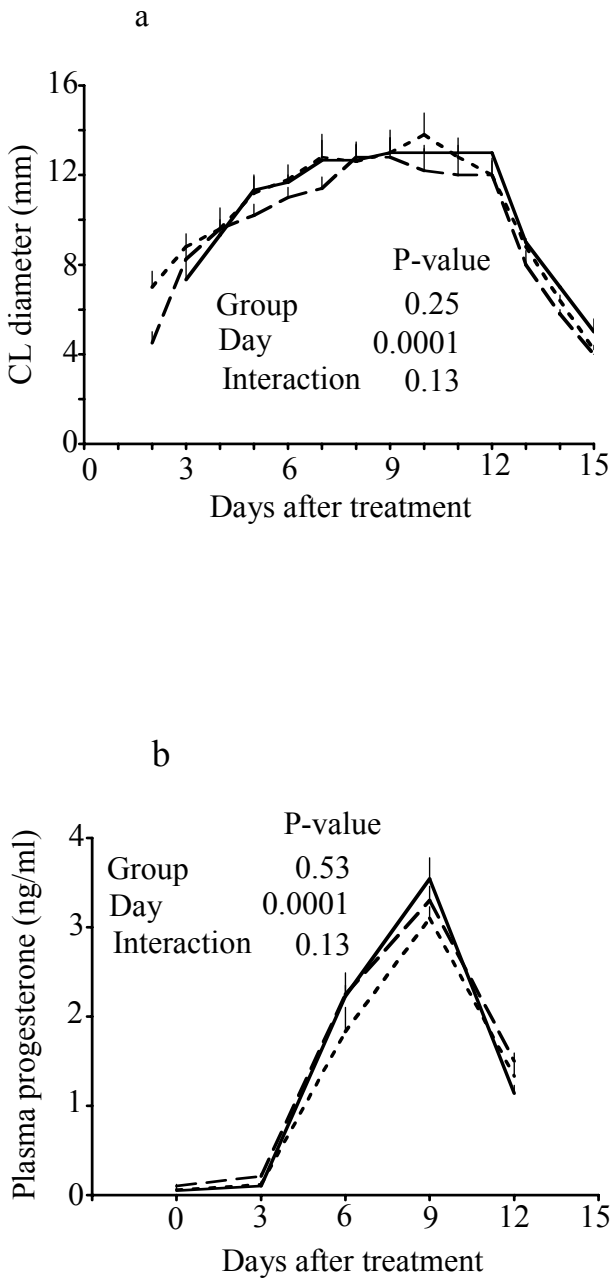

Figure 6.2. Corpus luteum diameter (a), and plasma progesterone (b) profiles (mean ± S.E.M.) in llamas after mating (——; n*), or being treated with LH (– – –, n = 5), or GnRH (·····, n = 5) to induce ovulation. Day 0 = day of mating or hormonal treatment.
*n = 5 for Days 0 to 11 for CL diameter and Days 0 to 9 for progesterone;
*n = 3 thereafter for CL diameter and progesterone.

6.5 Discussion

Direct comparison between the effects of mating and hormonal treatment on ovulation and luteal kinetics in llamas and alpacas has not been reported previously. The use of mating or gonadotropin hormone administration to induce ovulation has been reported in several studies in alpacas (Fernandez-Baca et al., 1970a) and llamas (England et. al., 1969; Sumar et al., 1988; Adams et al., 1990, Bravo et al., 1991, Adam et al., 1992), but there is a paucity of critically derived data characterizing the ovulatory response interval and the developmental dynamics of the corpus luteum (Adams et al., 1990, 1991). Results of the present study provide detailed characterization of the interval to ovulation after natural mating, and show that the effects of mating and hormonal treatment on ovulation and luteal function are similar.

In the present study, the interval from mating or LH/GnRH treatment to ovulation did not differ among groups (29-30 h), and was similar to the interval previously reported for llamas after hCG or GnRH administration (27-29 h; Adam et al., 1992). However, the interval from mating to ovulation was nearly 1 day shorter than previously reported (2 days; Adams et al., 1991a; Adam et al., 1992). This difference was attributed to examination frequency. Once daily examinations in previous studies resulted in detection of ovulation on the second day after mating in the majority of females. No difference in interval to ovulation between mating, LH, or GnRH groups (given a 4-hour examination frequency) suggests that the mechanism responsible for transducing copulatory stimulation into secretory stimulation of the hypothalamus and pituitary is rapid. This is consistent with the observation that LH begins to increase by 15 minutes after mating and peaks at 2 hours after mating (Bravo et al., 1990b).

The developmental kinetics of the CL induced by hormonal treatment in the present study were similar to that induced by natural mating; i.e., no differences were detected in the day-to-day profiles of CL diameter or plasma progesterone concentration. The maximum CL diameter and onset of regression were consistent with those observed in previous studies (England et al., 1969; Adams et al., 1990, 1991a).

Maximum plasma progesterone concentration was detected 1 day later than previously reported (Sumar et al., 1988, Adams et al., 1991a), but the disparity may be attributed to differences in blood sampling frequency. The CL decreased in diameter to 4 mm by the last day of the observational period (day 15), but was still detectable by ultrasound examination. This is a longer period of detection than previously described (Adams et al., 1991a).

In summary, ovulation rate, interval to ovulation, and luteal development were similar among llamas that were mated naturally or treated with LH or GnRH. We conclude that the hormonal preparations (LH and GnRH) are equally reliable for inducing ovulation and normal luteal function, and both are suitable for use in synchronization for artificial insemination protocols or embryo transfer programs.

7.0 OVULATION-INDUCING FACTOR IN THE SEMINAL PLASMA OF ALPACAS AND LLAMAS

7.1 Abstract

Studies were conducted to document the existence of an ovulation-inducing factor in the seminal plasma of alpacas (Experiment 1) and llamas (Experiment 2), and to determine if the effect is mediated via the pituitary (Experiment 3). In Experiment 1, female alpacas (n = 14 per group) were given alpaca seminal plasma or saline intramuscularly or by intrauterine infusion. Only alpacas that were given seminal plasma im ovulated (13/14, 93%; $P < 0.01$). In Experiment 2, ovulation was detected in 9/10 (90%) llamas at a mean of 29.3 ± 0.7 hours after seminal plasma treatment. Plasma progesterone concentrations were maximal by Day 9 and were at nadir by Day 12 post-treatment. In Experiment 3, female llamas were given llama seminal plasma, GnRH, or saline im, and ovulation was detected in 6/6, 5/6, and 0/6 llamas, respectively ($P < 0.001$). Treatment was followed by a surge ($P < 0.01$) in plasma LH concentration beginning 15 minutes and 75 minutes after treatment with GnRH and seminal plasma, respectively. Plasma LH remained elevated longer in the seminal plasma group ($P < 0.05$), and had not yet declined to pretreatment levels after 8 hours. Compared to the GnRH group, CL tended to grow longer and to a greater diameter ($P = 0.1$), and plasma progesterone concentration was twice as high in the seminal plasma group ($P < 0.01$). Results document the existence of a potent factor in the seminal plasma of alpacas and

llamas that elicited a surge in circulating concentrations of LH, and induced an ovulatory and luteotropic response.

7.2 Introduction

Ovulation in mammals involves pulsatile release of GnRH from the medio-basal nuclei of the hypothalamus into the hypophyseal portal system with subsequent release of LH from the gondotrophs of the anterior pituitary into the systemic circulation (Karsch, 1987, Karsch et al., 1997). Elevated circulating concentrations of LH elicit a cascade of events within the mature follicle culminating in follicle wall rupture and evacuation of its fluid and cellular contents (Richards et al., 2002). The broad classification of species as either spontaneous or induced ovulators is based on the type of stimulus responsible for eliciting GnRH release from the hypothalamus (Baker and Baum, 2000). In spontaneously ovulating species (e.g., human, sheep, goats, cattle, horse, pigs), release of GnRH from the hypothalamus is triggered when, in the absence of progesterone, systemic estradiol concentrations exceed a certain threshold (Knobil, 1980; Jaffe and Keys, 1974; Chenault et al., 1975; Turzillo and Nett, 1999; Kelly et al., 1988). As a consequence of regularly occurring luteolysis and development of one or more estrogen-producing follicles, a preovulatory surge in circulating concentrations of LH occurs at regular intervals. In induced ovulators (e.g., rabbits, ferrets, cats, camelids), however, neural signals from copulatory stimulation trigger GnRH secretion from the hypothalamus, followed by the preovulatory release of LH from the pituitary (Baker and Baum, 2000). Similar to spontaneous ovulators, a surge in the circulating concentration of LH appears to be requisite for ovulation in induced ovulators, but its occurrence is contingent upon copulatory stimuli; hence, ovulation is not a regular cyclic event.

The phenomenon of induced ovulation has been demonstrated in llamas (England et al., 1969), alpacas (San Martin et al., 1968), and old world camelids (Chen et al., 1980; Shalash and Nawito, 1964), but very few studies have been conducted to determine the factors responsible for eliciting ovulation in camelids. In the only study of its kind in new world camelids (Bravo et al., 1990b), ovulation induced by natural mating in llamas was associated with a rise in plasma LH concentration beginning

within 15 minutes of mating. In an early, classic study in alpacas (Fernandez-Baca et al., 1970a), the ovulation rate was compared among females that 1) were non-mounted, 2) were mounted only, followed with or without artificial insemination, 3) had interrupted mating, 4) had sterile mating (vasectomized male), followed with or without artificial insemination, 5) had a single uninterrupted mating (intact male), or 6) had multiple uninterrupted matings. It was concluded that mounting accompanied by penile intromission was necessary to stimulate ovulation. From these early studies, the concept that physical stimulation of the genitalia during copulation is the primary trigger for inducing ovulation in alpacas and llamas has become dogma.

This dogma, however, is being challenged by the suggestion that a chemical substance may be present in the semen that mediates the ovulatory cascade. The existence of such a substance was initially reported by investigators in China who concluded that some factor in the semen was responsible for eliciting ovulation in Bactrian camels, rather than the mechanical stimulation of copulation (Chen et al., 1985). Ovulation occurred after intravaginal (Chen et al., 1985; Xu et al., 1985) or intramuscular/intrauterine (Pan et al., 1992) administration of Bactrian seminal plasma to female Bactrian camels. In this regard, results of one study in alpacas (cited in Sumar, 1994) appear contradictory to the initial alpaca study (Fernandez-Baca et al., 1970a) in that artificial insemination (intravaginal deposition of alpaca semen) was associated with ovulation in 6/10 alpacas and 5/8 llamas. In a more recent report (Paolicchi et al., 1999), alpaca seminal plasma stimulated LH secretion from primary culture of rat pituitary cells in vitro. The authors suggested that the putative ovulation-inducing factor in seminal plasma had GnRH-like activity but was not GnRH because its biological activity on rat pituitary cells was not suppressed when anti-GnRH antibodies were added to the culture media.

The objective of this study was to document the existence of an ovulation-inducing factor in the seminal plasma of alpacas and llamas. Experiment 1 was designed specifically to document the existence of OIF in alpacas and to compare the effects of

intrauterine versus intramuscular administration. Experiment 2 was designed to determine the existence of OIF in llamas prior to a controlled study (Experiment 3) designed to determine if the effect of OIF is associated with a preovulatory surge in LH. These studies were designed to determine the effects of seminal plasma on ovulation rate, interval to ovulation, and corpus luteum function, and to compare the effect of seminal plasma with that of GnRH.

7.3 Materials and Methods

7.3.1 Experiment 1

The study was conducted during January to March at the Quimsachata Research Station in the Department of Puno, Peru (15° S, 71° W, and 4,500 m above sea level). Semen was collected from 8 male alpacas using an artificial vagina (Bravo et al., 1997) over a period of 2 months (16 ejaculates per animal). Ejaculates were diluted 1:1 (v/v) with phosphate buffered saline (PBS, Gibco, Grand Island, N.Y., USA) and centrifuged for 30 minutes at 1500 x g. The supernatant was decanted from the spermatozoa and a drop was evaluated by microscopy to confirm the absence of cells. If spermatozoa were detected, the sample was centrifuged again in like manner. Sperm-free seminal plasma was stored at –20°C before the experiment. Upon thawing, the diluted seminal plasma samples from all 8 males were pooled and kanamycin sulfate (Sigma Chemical Co., St Louis, MO, USA) was added to a final concentration of 25 μg/ml.

Mature non-lactating female alpacas (n = 70), ≥3 years of age and weighing an average of 70 kg, were examined daily by transrectal ultrasonography (Aloka SSD 500, Instruments for Science and Medicine Inc., Vancouver, BC, Canada) using a 7.5 MHz linear-array transducer attached to a rigid probe extension (Adams et al., 1991b, Adams, 1995). Alpacas were selected (n = 58) when a growing follicle of ≥8 mm in diameter was detected (i.e, capable of ovulating; Adams et al., 1989), and then assigned randomly to 4 groups in a 2 x 2 factorial experiment (n = 14 per group): 1 ml of diluted alpaca

seminal plasma given intramuscularly or by intrauterine infusion, or 1 ml of PBS given intramuscularly or by intrauterine infusion (negative control). Although not part of the original experimental design, an additional group of 6 mature non-lactating female alpacas from the same herd became available during the experimental period and were treated by intrauterine infusion of 5 mg of LH (Lutropin-V, Bioniche Animal Health Canada Inc., Belleville, Ontario, Canada) when a growing follicle ≥8 mm was detected by transrectal ultrasonography (Ratto et al., 2003). Intramuscular injections were given in the semimembranosus muscle using a 20-gauge 40 mm long needle, and intrauterine infusions were accomplished by passing a pipette through the cervix via transrectal manipulation.

Alpacas were examined daily by transrectal ultrasonography until Day 3 (Day 0 = treatment) to detect ovulation, and again on Day 8 to detect the presence of a corpus luteum (CL). Ovulation was defined as the sudden disappearance of a large follicle (≥8 mm) that was detected during the previous examination, and was confirmed by subsequent detection of a CL (Adams et al., 1989).

7.3.2 Experiment 2

The study was conducted from May to August at the University of Saskatchewan, Saskatchewan Canada (52° N, 106° W and 500 m above sea level) to determine if llama seminal plasma would induce ovulation in llamas, as it did in alpacas (Experiment 1), and to provide necessary information for the design of a controlled comparison of the effects of GnRH versus seminal plasma (Experiment 3), with adequate numbers for statistical interpretation (i.e., ovulation rate, interval to ovulation, CL and progesterone profiles). Semen was collected from 4 mature (5 to 7 years old) male llamas using an artificial vagina over a period of 2 months (24 ejaculates per animal). As in Experiment 1, samples were diluted 1:1 (v/v) with PBS, and centrifuged for 30 minutes at 1500 x g. Sperm-free seminal plasma was stored at –20°C. Upon thawing, the seminal plasma from all 4 males was pooled and kanamycin sulfate was added to a final concentration of 25 µg/ml.

Mature non-lactating female llamas (n = 15), ≥4 years of age and weighing 90-150 kg, were given 5 mg Armour Standard luteinizing hormone (Lutropin-V, Bioniche Animal Health, Belleville, ON, Canada) im to synchronize follicular wave emergence among animals (Ratto et al., 2003). Twelve days after LH treatment, llamas with a follicle ≥8 mm in diameter (n = 10) were given 1.5 ml of diluted llama seminal plasma im. The ovaries were examined by transrectal ultrasonography every 4 hours from the time of seminal plasma treatment until ovulation or 48 hours, whichever came first. Ultrasonographic examination was performed once daily thereafter for 15 days to monitor CL growth and regression. As in Experiment 1, ovulation was defined as the sudden disappearance of a large follicle (≥8 mm) that was detected during the previous examination. The onset of luteal regression was defined as the first day on which the corpus luteum began a progressive decrease in diameter leading to a minimum on the last day of the observational period. Blood samples were collected into heparinized tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes, New Jersey, USA) by jugular venipuncture on Day 0, 3, 6, 9, 12, and 15 (Day 0 = day of seminal plasma treatment). Blood samples were centrifuged at 1700 x g for 25 minutes and the plasma was stored at -20°C.

Plasma progesterone concentrations were determined using a commercial, double-antibody radioimmunoassay kit (Coat-a-Count total progesterone, DPC, Diagnostic Products Corporation, Los Angeles, CA, USA), as described previously (Adams et al., 1991a). All samples were analyzed in a single assay. The intra-assay coefficients of variations were 1.8%, 4.9% and 1.8%, respectively, for reference plasma progesterone concentrations of 1.8, 2.9, and 14.6 ng/ml.

7.3.3 Experiment 3

Results of Experiment 2 were used to design a study to determine if the ovulatory effects of seminal plasma treatment are associated with pituitary release of LH, by comparison with the effects of GnRH treatment. The study was conducted in the autumn (October to November) after Experiment 2 using a different group of llamas

from the same herd at the University of Saskatchewan. Mature non-lactating female llamas (n = 25), ≥4 years of age and weighing 90-150 kg, were given 5 mg Armour Standard LH (Lutropin-V) to synchronize follicular wave emergence among animals, as described in Experiment 2. Twelve days after LH administration, llamas with a follicle ≥8 mm in diameter were assigned randomly to 3 groups (n = 6 per group) in which 1.5 ml of llama seminal plasma, 1.5 ml of PBS (negative control group), or 50 μg of GnRH (Cystorelin, Merial Canada Inc., Victoriaville, Quebec, Canada; positive control group) were given by intramuscular injection. The same pool of llama seminal plasma collected during Experiment 2 was used in Experiment 3. The ovaries were examined by transrectal ultrasonography once daily from the day of treatment (Day = 0) to Day 15 to detect ovulation and CL development, as described in Experiment 1.

Blood samples for measurement of plasma LH concentration were collected in heparinized tubes by jugular venipuncture every 15 minutes for 8 hours starting immediately before treatment (Time 0 = treatment). A jugular catheter (inner and outer diameters of 1.0 and 1.5 mm, respectively) was fixed in place one day before frequent blood sampling to minimize the effects of handling stress on plasma LH concentrations. Blood samples were centrifuged at 1700 x g for 25 minutes and the plasma was stored at -20°C. Plasma LH concentrations were measured using a double-antibody radioimmunoassay (Rawlings et al., 1984). Concentrations of LH are expressed in terms of NIAMDD-oLH-24. The minimum detectable limit of the assay was 0.026 ng. The range of the standard curve was 0.026 ng (80% ligand labeled LH) to 0.19 ng (20% ligand labeled LH). The intra- and inter-assay coefficients of variation were 6.3% and 6.0%, respectively, for the high reference plasma LH concentration (0.79 ng/ml). The intra- and inter-assay coefficients of variation were 17% and 15%, respectively, for the low reference plasma LH concentration (0.17 ng/ml).

Blood samples were collected every 2 days from Day 3 (Day 0 = treatment) to Day 17 for measurement of plasma progesterone concentration and assayed as described in Experiment 2. All samples were analyzed in a single assay. The intra-assay

coefficients of variations were 5.8%, 7.6% and 2.9%, respectively, for reference plasma progesterone concentrations of 1.4, 2.3, and 11.6 ng/ml.

7.3.4 Statistical Analyses

Non-serial data (i.e., follicle size at the time of treatment, maximum CL diameter, day on which the CL was first detected, onset of CL regression) were compared between groups by analyses of variance. For statistical analyses of serial data and preparation of figures, CL diameter, LH and progesterone data were centralized to the day of treatment (Day 0). Serial data were compared by analysis of variance for repeated measures (Proc-mixed in SAS, Statistical Analysis System Institute Inc., Cary, NC, USA) to determine the effects of treatment, day, and treatment-by-day interaction. When main effects or their interaction were significant (i.e., $P \leq 0.05$), means on a given day were compared by the method of least significant difference. Ovulation rates were compared among groups by Chi-Square analysis.

The experimental protocols were approved by the University of Saskatchewan Committee on Animal Care and Supply under the guidelines of the Canadian Council on Animal Care.

7.4 Results

7.4.1 Experiment 1

Two alpacas assigned to the intrauterine PBS group and 2 assigned to the intrauterine seminal plasma group were excluded from the study because their small size precluded transrectal manipulation for intrauterine infusion.

The diameter of the largest follicle at the time of treatment did not differ among groups (P = 0.65). Ovulation was observed only in the group treated by intramuscular administration of seminal plasma (Table 7.1). Ovulations were detected on Day 1 in 6 alpacas, and on Day 2 in the remaining 7 alpacas (Day 0 = day of treatment). A corpus luteum was detected on Day 8 in all 13 ovulatory alpacas. Ovulation and luteal development were not detected in the negative control groups, nor in any of the intrauterine treatment groups (Table 7.1).

Table 7.1. Effect of intramuscular or intrauterine administration of alpaca seminal plasma, phosphate buffered saline (PBS), or luteinizing hormone (LH) on ovulation and corpus luteum formation in alpacas (mean ± SEM; Experiment 1).

	Intramuscular		Intrauterine		
	Seminal plasma (n = 14)	PBS (n = 14)	Seminal plasma (n = 12)	PBS (n = 12)	LH (n = 6)
Follicle diameter (mm) on day of treatment (Day 0)*	10.9 ± 0.3	11.1 ± 0.4	11.1 ± 0.4	10.6 ± 0.4	10.5 ± 0.4
Ovulation rate	13/14 [a] (93%)	0/14[b] (0%)	0/12[b] (0%)	0/12[b] (0%)	0/6[b] (0%)
CL diameter on Day 8 (mm)	12.2 ± 0.4	---	---	---	---

* No significant difference among groups (P = 0.6)
[a,b] Proportions with different superscripts are different (P < 0.0001).

7.4.2 Experiment 2

The diameter of the largest follicle at the time of treatment was 9.7 ± 0.4 mm (mean ± SEM). Ovulation was detected in 9/10 (90%) llamas after intramuscular administration of llama seminal plasma. The mean interval from treatment to ovulation was 29.3 ± 0.7 hours; 6 llamas ovulated at 28 hours and the remaining 4 ovulated at 32 hours. The corpus luteum was first detected on Day 2.3 ± 0.2, it reached a maximum diameter of 11.5 ± 0.5 mm on Day 6.9 ± 0.3, and began to regress on Day 9.7 ± 0.3 (Day 0 = treatment). Plasma progesterone concentrations were elevated by Day 6, were maximal by Day 9, and were at nadir by Day 12 (Figure 7.1).

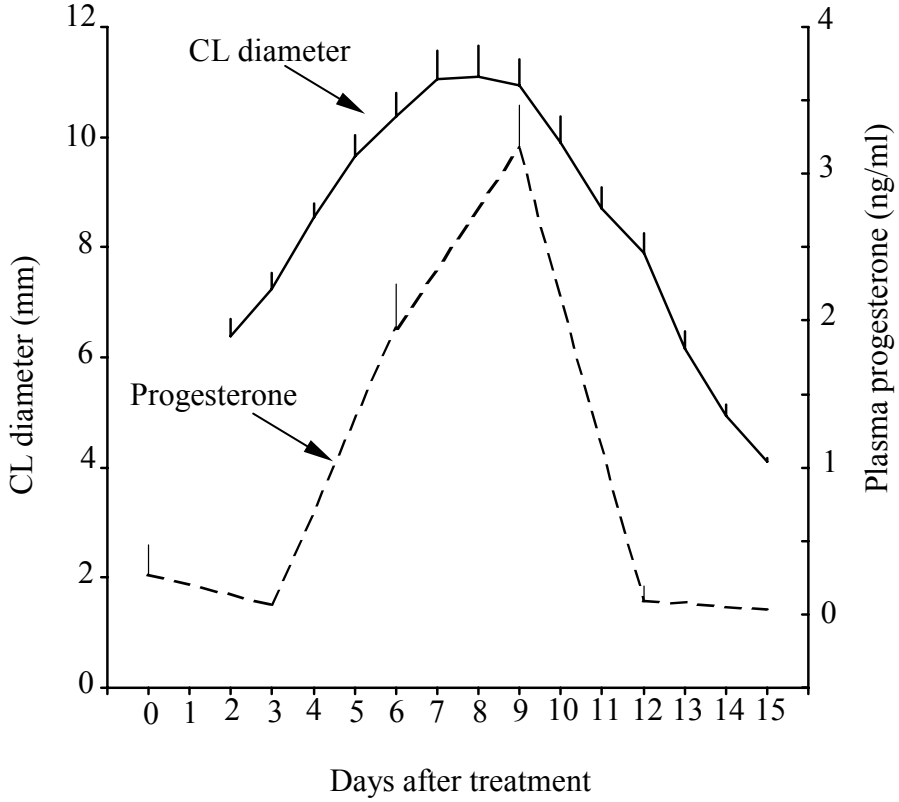

Figure 7.1. Corpus luteum diameter and plasma progesterone concentration (mean ± SEM) in llamas (n = 9) after intramuscular administration of llama seminal plasma.

7.4.3 Experiment 3

The diameter of the largest follicle at the time of treatment did not differ among treatment groups and ovulation occurred in all llamas treated with seminal plasma, all but one treated with GnRH, and none treated with PBS (Table 7.2). During the 8-hour period following treatment, plasma LH concentration increased and decreased ($P < 0.01$) in the GnRH (positive control) and seminal plasma groups, but not in the PBS (negative control) group (Figure 7.2). Plasma LH concentration in the GnRH group began to increase ($P < 0.05$) and was higher ($P < 0.05$) than in the other groups by 15 minutes after treatment. The first significant increase in LH in the seminal plasma group was detected 1 hour after treatment. At 2 hours after treatment, plasma LH concentrations were similarly elevated in the GnRH and seminal plasma groups (Figure 7.2). Within individuals, maximum plasma LH concentration occurred at 1.4 ± 0.2 and 1.9 ± 0.2 hours after GnRH and seminal plasma treatment, respectively ($P = 0.06$). Plasma LH concentration remained elevated for a longer period in the seminal plasma group than in the GnRH group ($P < 0.05$), and had not yet declined to pretreatment levels by the end of the sampling period (8 hours). In the GnRH group, plasma LH began to decrease ($P < 0.05$) at 5 hours after treatment and was similar to pre-treatment levels by 5.5 hours after treatment.

The CL tended to grow for a longer period and to a greater diameter in the seminal plasma group compared to the GnRH group (group-by-day interaction, $P = 0.1$; Figure 7.3). On an individual-animal basis, differences between GnRH and seminal plasma groups in maximum CL diameter and the day of maximum diameter were not significant (Table 7.2). Plasma progesterone concentrations were highest ($P < 0.05$) in the seminal plasma group, intermediate ($P < 0.05$) in the GnRH group and remained basal in the PBS group. In both seminal plasma and GnRH groups, progesterone concentrations increased sharply to peak values by Day 7 and decreased sharply to nadir by Day 11 (Figure 7.4).

Table 7.2. Effect (mean ± SEM) of intramuscular administration of llama seminal plasma, GnRH, or phosphate buffered saline (PBS) on ovulation and luteal development in llamas (Day 0 = day of treatment; Experiment 3).

	Seminal plasma (n= 6)	GnRH (n= 6)	PBS (n= 6)
Follicle size at treatment (mm)*	10.9 ± 0.2	10.6 ± 0.2	9.8 ± 0.2
Ovulation rate	6/6 [b] (100%)	5/6 [b] (83%)	0/6 [a] (0%)
1st day corpus luteum detected*	2.5 ± 0.2	2.4 ± 0.2	---
Maximum CL diameter (mm)*	13.5 ± 0.2	11.5 ± 0.5	---
Day of maximum CL diameter* (mm)	7.6 ± 0.2	7.4 ± 0.4	---
Onset of CL regression*	9.5 ± 0.2	9.6 ± 0.2	---

*****No significant difference among groups ($P \geq 0.3$)
[a,b] Proportions with different superscripts are different ($P < 0.001$).

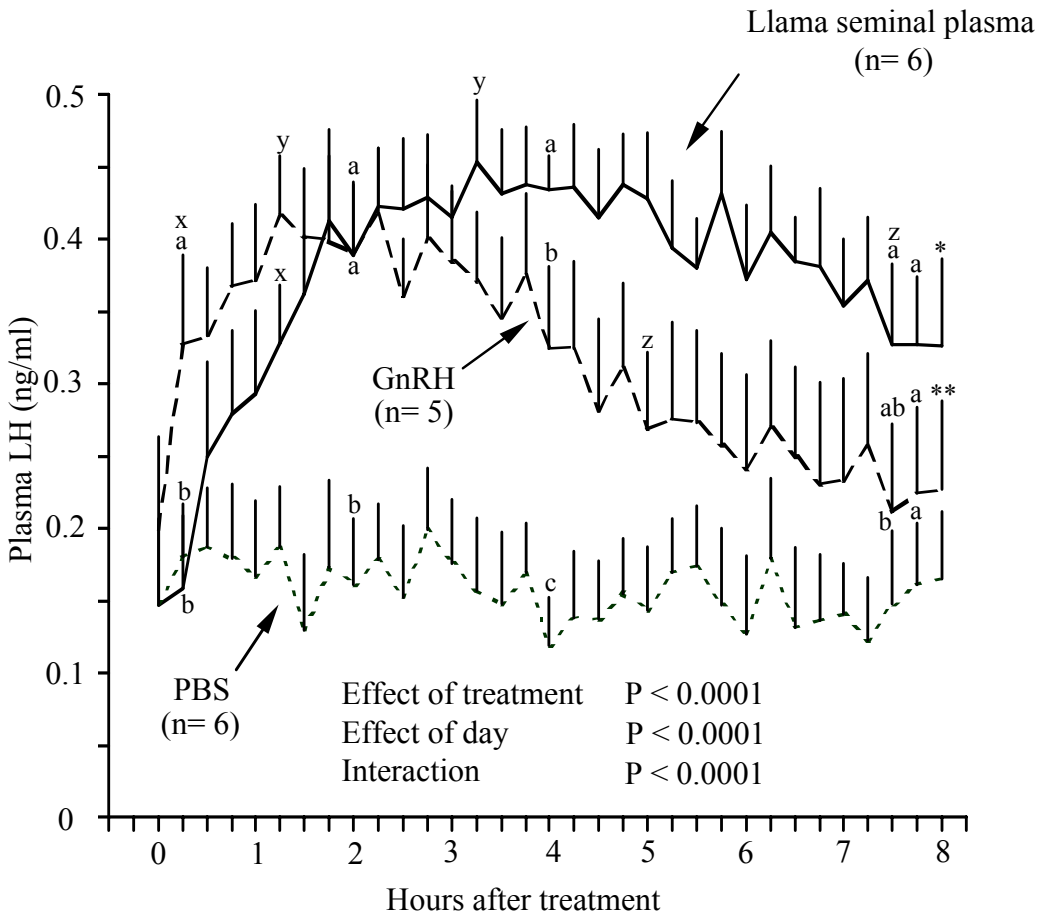

Figure 7.2. Plasma LH concentrations (mean ± SEM) in female llamas after intramuscular treatment with llama seminal plasma, GnRH or phosphate buffered saline (PBS; Experiment 3).

[abc] On a given day, values with no common superscript are different among groups ($P < 0.05$)

[x] Within group, the first increase from pre-treatment (Time 0) concentration ($P < 0.05$)

[y] Within group, the maximum concentration ($P < 0.05$)

[Z] Within group, the first decrease from maximum concentration ($P < 0.05$)

[*] Within group, the last value is higher than the pre-treatment value ($P < 0.05$).

[**] Within group, the last value is not different from the pre-treatment value ($P = 0.9$).

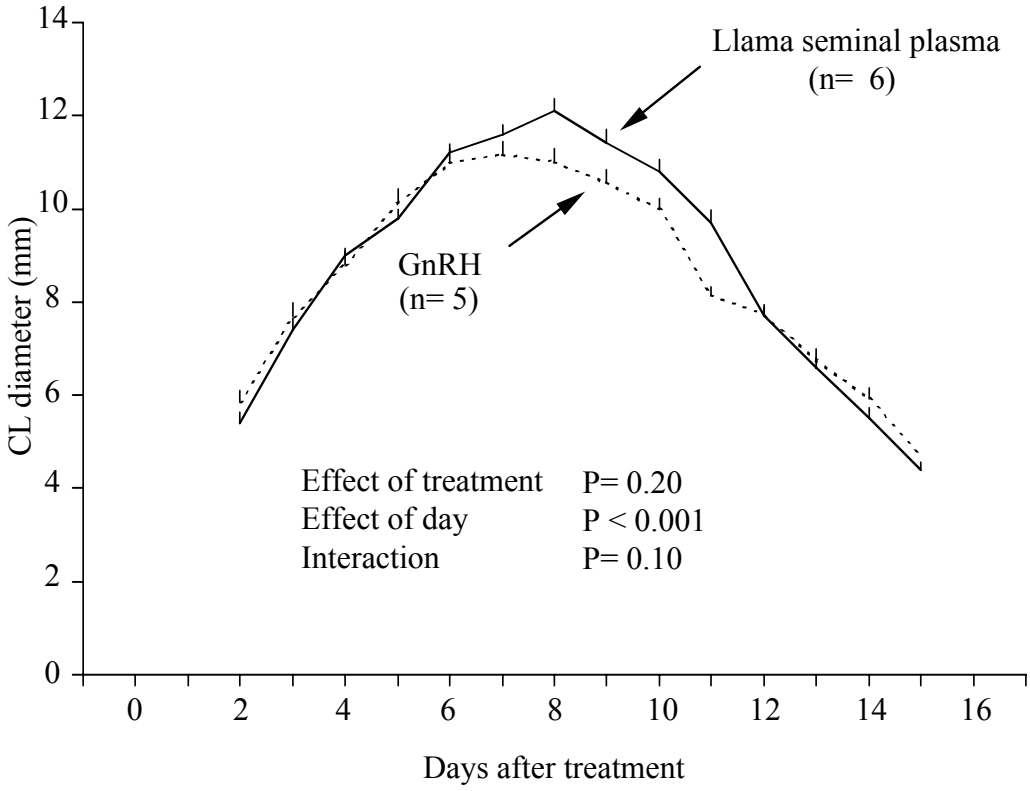

Figure 7.3. Diameter profile (mean ± SEM) of the corpus luteum in female llamas after intramuscular treatment with llama seminal plasma or GnRH (Experiment 3).

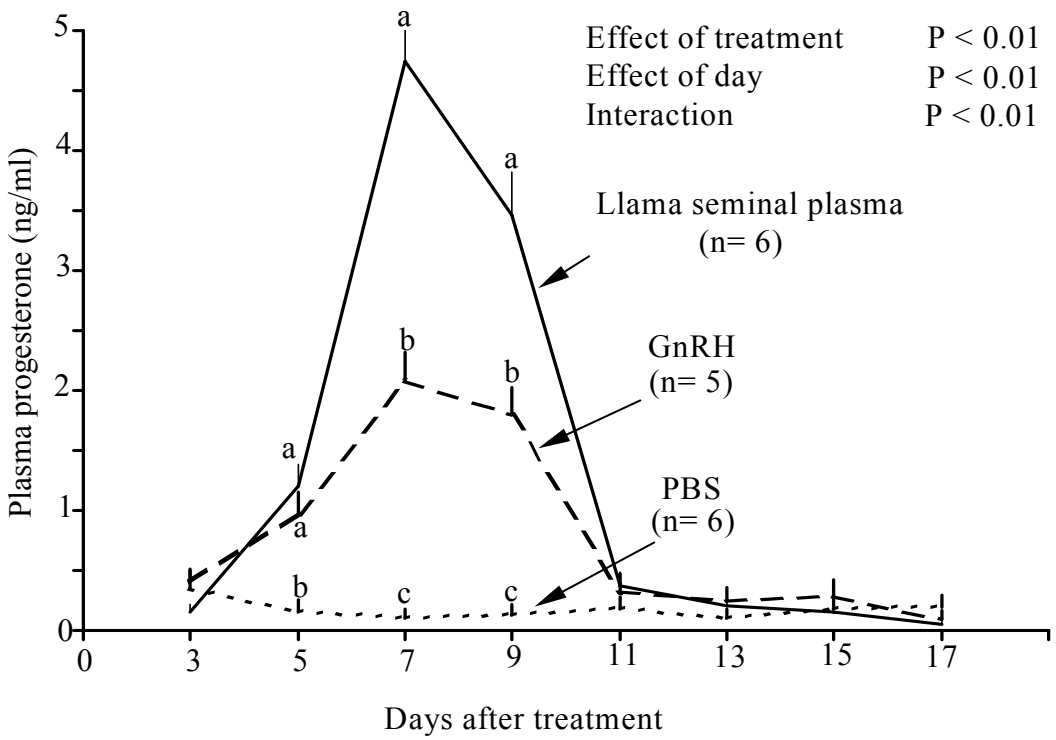

Figure 7.4. Plasma progesterone concentrations (mean + SEM) in female llamas after intramuscular treatment with llama seminal plasma, GnRH, or phosphate buffered saline (PBS; Experiment 3).
[abc] On a given day, values with no common superscript are different ($P < 0.05$).

7.8 Discussion

Copulation in llamas and alpacas is an unusually protracted event, often lasting 30 to 50 minutes (England et al., 1971; Fernandez-Baca et al., 1970a), and involves constant gutteral humming by the male, clasping and treading of the male's fore- and hind-limbs while mounted on a receptive female in sternal recumbency. While the role of physical, visual, auditory and olfactory stimuli remains to be elucidated, results document the existence of an ovulation-inducing factor (OIF) in the seminal plasma of alpacas (Experiment 1) and llamas (Experiment 2), and that the mechanism of action of OIF involves a preovulatory surge in circulating concentrations of LH (Experiment 3). These results in alpacas and llamas support earlier observations made in Bactrian camels (Chen et al., 1985; Xu et al., 1985; Pan et al., 1992), and challenge the notion that physical stimuli associated with copulation are causative of ovulation in camelid species (Fernandez-Baca et al., 1970a).

One of the most important findings in this study was the potency of the effect of seminal plasma treatment. A relatively conservative dose of seminal plasma was chosen in the present study (0.5 to 1 ml of raw seminal plasma) based on the reported average volume of the ejaculate in alpacas and llamas (2 to 3 ml; Lichtenwalner et al., 1996; Neely and Bravo, 1997). Despite the modest dose used in this study, the effects of seminal plasma were profound. Collectively, 28 of 30 (93%) alpacas and llamas given seminal plasma intramuscularly in the present series of experiments ovulated compared to 5 of 6 (83%) given GnRH and 0 of 20 (0%) given PBS. The duration of the surge in plasma concentration of LH was significantly greater after treatment with seminal plasma than with GnRH, and progesterone secretion from subsequent CL was double that of the GnRH group.

Interestingly, the post-treatment surge in LH was later in the seminal plasma group compared to the GnRH group; i.e., the first significant increase occurred 1 hour later, the maximum concentration occurred 2 hours later, and the first significant decrease occurred 2.5 hours later than in the GnRH group. In addition, post-treatment

plasma LH concentrations remained elevated above pretreatment levels in the seminal plasma group for at least 8 hours (end of sampling period), whereas LH had returned to basal levels by 5.5 hours post-GnRH treatment. These observations provide rationale for the hypothesis that OIF and GnRH effect pituitary LH release differently and are different molecules. This is consistent with the observation that the LH-secreting effect of alpaca seminal plasma on rat pituitary cells in vitro was not suppressed when anti-GnRH antibodies were added to the primary culture (Paolicchi et al., 1999).

While the disparity in amplitude and duration of the LH surge between GnRH- and seminal plasma-treated llamas did not influence the ovulation rate or interval to ovulation, it was associated with a subtle increase in CL diameter profile and a striking increase in plasma progesterone concentration in the seminal plasma group. This observation provides rationale for the hypothesis that the degree of luteogenesis is directly proportional to the duration of the preovulatory LH surge. Results of studies done in primates (Chandrasekher et al., 1994) are consistent with this hypothesis; a prolonged surge of LH (48-50 h) during the periovulatory phase was necessary to achieve normal luteinization of granulosa cells, expression of progesterone receptors, and development of a functional CL. Shorter endogenous LH surges (14 h) elicited by exogenous GnRH given after ovarian stimulation protocols in primates were associated with deleterious effects on luteinization of granulosa cells and CL development and function (Chandrasekher et al., 1994). Studies conducted in rabbit (Bomsel-Helmreich et al., 1989) and rats (Peluso, 1990; Ishikawa, 1992) documented that changes in concentration and duration of gonadotropins during the periovulatory period can influence changes in oocyte maturation, granulosa cell luteinization and corpus luteum formation.

The ovulation rate and interval to ovulation after seminal plasma treatment in llamas (Experiment 2) were similar to those reported previously in llamas after natural mating or hormonal treatment (Ratto et al., 2005a). In the latter study, a direct comparison of natural mating, LH treatment (5 mg Lutropin-V), and GnRH treament (50 μg Cystorelin), revealed no differences in ovulation rate (80%, 91%, 80%, respectively),

interval to ovulation (30.0 ± 0.5, 29.3 ± 0.6, 29.4 ± 0.7 h, respectively), CL diameter profiles, or plasma progesterone profiles. Relatively infrequent sampling in Experiment 2 limits the ability to interpret the effects of seminal plasma treatment on subsequent progesterone production.

The difference in the effects of seminal plasma given by intramuscular injection versus intrauterine infusion was unexpected and supports the notion that the effect of seminal plasma involves a systemic rather than a local pathway. None of the alpacas given seminal plasma by intrauterine infusion in the present study ovulated. This result is consistent with the finding that ovulation rate was not increased by artificial insemination of female alpacas that were allowed to be mounted only (i.e., no intromission; Fernandez-Baca et al., 1970a), but is in contrast to the findings of another study in which ovulation was observed in 6/10 alpacas and 5/8 llamas after intravaginal deposition of alpaca semen (cited in Sumar, 1994). In 3 separate studies in Bactrian camels, ovulation was induced by intravaginal or intrauterine infusion of whole semen or seminal plasma in ≥75% of females (Chen et al., 1985; Xu et al., 1985; Pan et al., 1992). The reason for the disparity in results is not clear, but differences appear too great to be due to chance alone. It is interesting to note that in the present study, alpacas given an intrauterine infusion of LH also failed to ovulate. The dose of LH used was the same as that used in previous studies (Ratto et al., 2003; 2005) in which ovulation was induced in 27/30 and 10/11 llamas after intramuscular administration, respectively. Differences may be attributed to differential adsorption from the genital mucosa compared to the muscle. In this regard, copulation in alpacas and llamas is prolonged (30 to 50 minutes) and ejaculation is intrauterine (Bravo et al., 1996). A normal sequela of copulation is acute, transient inflammation of the endometrium as a result of repeated abrasion by the penis (Bravo et al., 1996). Perhaps absorption of OIF in seminal plasma subsequent to natural mating is facilitated by the hyperemia of the excoriated endometrium. Test of this hypothesis might include curettage of the endometrium at the time of intrauterine infusion of seminal plasma.

The categorical distinction between induced and spontaneous ovulators is not as clear as the label implies. Authors of early studies suggested that camels are spontaneous ovulators (Asdell, 1964; Bodenheimer, 1954; Leonard, 1894), and even in llamas and alpacas there are conflicting reports on whether spontaneous as well as induced ovulations can occur (Fernandez-Baca et al., 1970b; Sumar, 1983). Based on laparotomy or necropsy examinations, the ovulation rate of unmated alpacas (spontaneous ovulation) was reported to be about 5% (Fernandez-Baca et al., 1970a). In a critical ultrasound study (Adams et al., 1990), the incidence of spontaneous ovulation was 8% in unmated llamas, and ovulation failure rate was 10% in mated llamas. Rodents appear to occupy an intermediate position between induced and spontaneous ovulators. Ovulation in mice and rats occurs spontaneously, but CL development and function is contingent upon mating (Suckow, 2001; Maeda et al., 2000). The distinction between spontaneous and induced ovulators is further blurred by the finding that ovulation was hastened in gilts by intrauterine application of a pronase-sensitive fraction of boar seminal plasma (Waberski et al., 1995). Further, the existence of a GnRH-like molecule was detected in studies on human seminal plasma (Sokol et al., 1985). Although the nature and function of these molecules remains unknown, they may represent an evolutionary vestige of a common ancestry or indeed play a pivotal role in the ovulatory mechanism or a role in gamete interaction (Morales, 1998).

In summary, results clearly document the existence of a potent factor in the seminal plasma of alpacas and llamas that elicited a surge in circulating concentrations of LH and inducing ovulation in more than 90% of animals treated. The presence of a potent ovulation-inducing factor in seminal plasma would seem an evolutionary asset and one upon which natural selection pressure would be brought to bear. The existence of OIF in camelids begs the question of its existence and its effect in other species. The discovery of this novel factor may have broad implications on our understanding of ovulation and on diagnosis and treatment of ovulatory perturbations in this and other species.

8.0 INTERSPECIES COMPARISON OF THE EFFECT OF OVULATION-INDUCING FACTOR (OIF) IN SEMINAL PLASMA

8.1 Abstract

We have recently reported the presence of an ovulation-inducing factor (OIF) in the seminal plasma of llamas and alpacas – a species characterized as induced ovulators. The study was designed to test the hypothesis that OIF is well conserved among species by comparing the effects of seminal plasma from conspecific versus hetero-specific males using the llama ovulation model as a bio-assay. The seminal plasma of alpacas, a closely related species (*Lama pacos*), and cattle, a distantly related ruminant species (*Bos taurus*) considered to be spontaneous ovulators, was compared with that of the llama (*Lama glama*). Ovulation and corpus luteum development were compared by ultrasonography among female llamas (n = 19 per group) treated intramuscularly with phosphate buffered saline (PBS, negative control) and those treated with the seminal plasma of bulls, alpacas, or llamas (conspecific control). The diameter of the pre-ovulatory follicle did not differ among groups at the time of treatment. Bull seminal plasma induced ovulations in 26% (5/19) of llamas compared to 0% (0/19) in PBS group ($P < 0.001$). The ovulation rate was lower ($P < 0.01$) in bull seminal plasma group compared to that in the groups treated with alpaca or llama seminal plasma (100%). A corpus luteum was detected on Day 8 in all llamas in which ovulation was

detected earlier (Day 2) by ultrasonography. The diameter of the CL did not differ among groups. Results documented the presence of OIF in the seminal plasma of *Bos taurus*. The interspecific effects of seminal plasma on ovulation and luteal development provide support for the hypothesis that OIF is a highly conserved molecule among mammals.

8.2 Introduction

Llamas and alpacas require a copulatory stimulus to induce ovulation (England et al., 1969, Fernandez-Baca et al., 1970a). Despite elevated circulating concentrations of estradiol during the period of maturation of the dominant follicle, a spontaneous preovulatory LH surge is not triggered in camelids (Bravo et al., 1990b; Homeida et al., 1988) as it is in other species (e.g. cattle and sheep; Karsch 1987, Karsch et al., 1997). Although a number of different stimuli (tactile, olfactory, visual) have been associated with eliciting ovulation in induced ovulators, the physical stimulation of penile intromission has been ascribed the dominant role in triggering the preovulatory LH surge (Baker and Baum 2000). Authors of studies in llamas and alpacas showed that hCG administration effectively induced ovulation and concluded that the only the physical act of coitus, and not just mounting (no intromission) with or without artificial insemination, elicited a similar response in these species (Fernandez-Baca et al., 1970a; England et al., 1969). The first significant increase in circulating LH concentration was detected at 15-30 minutes after mating in llamas and alpacas, and peaked at 2 hours (Bravo et al., 1990b; Bravo et al., 1991). A similar increase in LH was observed in camels 20-30 minutes after mating (Xu et al., 1985; Marie and Anouassi 1986). The rapid increase in plasma LH concentration after mating in camelids is similar to that observed in rabbits and cats after mating (Jones et al., 1976; Concannon et al., 1980).

In contrast, others have suggested that penile penetration of the vagina and cervix is not the only stimulus responsible for eliciting ovulation in camelids. Ovulation was detected in alpacas (cited in Sumar 1994) and Bactrian camels (Chen et al., 1985; Xu et al., 1985) after intravaginal deposition of semen from the same species. Results of a more recent study documented the presence of an ovulation-inducing factor (OIF) in the seminal plasma of alpacas and llamas (Adams et al., 2005). Collectively, ovulation was induced in 28/30 (93%) females after intramuscular administration of alpaca or llama seminal plasma in the respective species, but none of the females (0/32) ovulated after intramuscular or intrauterine administration of phosphate buffered saline (negative control groups). Seminal plasma treatment was followed by a surge in circulating

concentrations of LH that peaked at 3 hours, and ovulation at 29 hours after treatment. In addition, seminal plasma treatment elicited a potent luteotrophic response. In light of these recent findings, the classical concept of a physical neuro-endocrine reflex as the mechanism by which ovulation is induced is in question.

Induced (reflex) ovulation and spontaneous ovulation are not discrete species-specific traits in mammals (Zarrow and Clark 1968; Conaway 1971; Jochle 1975; Martin 1990). In rabbits, ovarian steroids can facilitate the release of LH in response to vaginal stimulation (Sawyer and Markee, 1959). In cattle and pigs, coital activity was associated with enhanced LH secretion and a higher ovulation rate (Marion 1950; Signoret et al., 1972; Jochle 1975). Therefore, the presence of an OIF may not be restricted to the seminal plasma of species traditionally classified as induced ovulators. In a more recent study (Wabersky et al., 1995), a pronase-sensitive protein in the seminal plasma of pigs accelerated ovulation in gilts after intrauterine deposition. In addition, studies on human seminal plasma suggest the presence of a GnRH-like substance (Sokol 1985; Izumi et al., 1985) that may be involved in sperm/oocyte interaction (Morales et al., 1994). It is unknown whether these factors in spontaneous ovulators are involved in the mechanism of ovulation or if they are related to OIF in the semen of alpacas and llamas.

In preliminary studies of the interspecies effects of seminal plasma, ovulation was detected by transrectal palpation in 3 of 7 Bactrian camels after intravaginal deposition (Chen et al., 1985) and 1 of 3 after intramuscular administration of bull semen (Pan et al. 1992). In another preliminary study, however, no ovulations (0/5) were observed in llamas after intravaginal administration of bull semen (cited by Sumar 1994). The camelid species represents a good model for the study of ovulation-inducing factors because they are amenable to ovarian ultrasonography (Adams et al., 1989) and because their follicular, ovulatory, and luteal dynamics have been well characterized (Adams et al., 1990; Ratto et al., 2005).

The study was designed to test the hypothesis that OIF is well conserved among species by comparing the effects of seminal plasma from conspecific versus heterospecific males using the llama ovulation model as a bio-assay. The effect of seminal plasma of alpacas - a closely related species (*Lama pacos*), and cattle - a distantly related ruminant species (*Bos taurus*) considered to be spontaneous ovulators, was compared with that of the llama (*Lama glama*) using the llama ovulation model as an *in vivo* bio-assay.

8.3 Materials and methods

8.3.1 Semen collection and handling

Semen was collected from male llamas (n = 4) and alpacas (n = 8) twice per week over a period of 2 months prior to the start of the experiment. Semen was collected with the use of an artificial vagina designed for use in sheep that was fitted into a phantom mount built of wood and covered with alpaca or llama hide (Bravo et al., 1997). Semen from Hereford bulls (n = 6) was obtained by electro-ejaculation (Pulsator III; Lane Manufacturing, Denver, CO, USA) using a 75 mm in diameter rectal probe with three ventrally oriented electrodes. A total of 10 ejaculates were obtained per animal.

The seminal plasma from each species was prepared using procedures described previously (Adams et al., 2005). In brief, ejaculates were diluted 1:1 (v/v) with phosphate buffered saline (PBS, Gibco, Grand Island, N.Y., USA) and centrifuged for 30 minutes at 1500 x g. The supernatant was decanted to remove spermatozoa and a drop was evaluated by microscopy to confirm the absence of cells. If spermatozoa were detected, the sample was centrifuged again in like manner. Sperm-free seminal plasma was stored at –70°C. Upon thawing, the diluted seminal plasma was pooled by species (i.e., a pool for each species) and kanamycin sulfate (Sigma Chemical Co., St Louis, MO, USA) was added to a final concentration of 25 μg/ml.

8.3.2 Animals and treatments

The study was conducted during February and March at the Quimsachata Research Station in the Department of Puno, Peru (15° S, 71° W, and 4,500 m above sea level). Mature non-lactating female llamas (n = 120), ≥4 years of age and weighing 120-150 kg were used. To facilitate data collection, ovarian follicular development was synchronized among females by giving 5 mg Armour Standard LH (Lutropin-V®, Bioniche Animal Health, Belleville, ON, Canada) to induce ovulation. We expected approximately 85% to 90% of the llamas to ovulate after LH treatment, resulting in synchronous emergence of a new follicular wave 2 days after treatment (Ratto et al., 2003). Llamas were examined by transrectal ultrasonography 12 days after LH treatment; sufficient time to permit complete luteal regression after ovulation and growth of a dominant follicle to an ovulatory diameter (Ratto et al., 2003; 2005). Females with an ovarian follicle ≥8 mm in diameter (i.e., mature enough to ovulate) were assigned randomly to 4 groups (n = 19 per group). Llamas in the respective groups were given an intramuscular (semimembranosus or semitendinosus muscle) injection of 2.0 ml of phosphate buffered saline (PBS, negative control group), or 2 ml of diluted seminal plasma of llamas, alpacas, or bulls. Based on a mean interval from stimulus to ovulation of 29 hours (Ratto et al., 2005), llamas were examined by transrectal ultrasonography at Day 2 (Day 0 = treatment) to detect ovulation. Ovulation was defined as the sudden disappearance of a large follicle (≥8 mm) that was detected during the previous examination (Adams et al., 1989). To confirm ovulation and assess corpus luteum development, transrectal ultrasonography was repeated on Day 8; i.e., expected time of maximum CL diameter (Adams et al., 1989; Ratto et al., 2005).

8.3.3 Statistical Analyses

Single-point measurements (i.e., follicle size at the time of treatment, maximum CL diameter) were compared among groups by analyses of variance. If the overall effect was significant ($P<0.05$), specific comparisons were made between groups using Tukey

multiple comparisons. Ovulation rates were compared among groups by chi-square analysis.

8.4 Results

The diameter of the largest follicle at the time of treatment did not differ among groups ($P = 0.8$; Table 8.1). Ovulations were observed in all groups except in the group treated with PBS (negative control group, Table 8.1). Bull seminal plasma induced ovulations in 26% (5/19) of llamas compared to 0% (0/19) in PBS group ($P < 0.001$). The ovulation rate was lower ($P < 0.01$) in bull seminal plasma than llama or alpaca seminal plasma groups (100%). A corpus luteum was detected on Day 8 in all llamas in which ovulation was detected by ultrasonography on Day 2. Luteal development was not detected in the negative control group and CL diameter did not differ among llamas that ovulated after bull, llama and alpaca seminal plasma administration (Table 8.1).

Table 8.1. Effect of intramuscular treatment with phosphate buffered saline (PBS, negative control), or with the seminal plasma of llamas, alpacas, or bulls on ovulation and corpus luteum development in female llamas (mean ± SEM).

	Phosphate buffered saline	Seminal Plasma		
		Llama	Alpaca	Bull
Follicle diameter at treatment (mm)*	10.0 ± 0.7 (n = 19)	8.9 ± 0.3 (n = 19)	9.0 ± 0.3 (n = 19)	8.7 ± 0.2 (n = 19)
Ovulation rate (%)	0/19[a] (0%)	19/19[b] (100%)	19/19[b] (100%)	5/19[c] (26%)
CL diameter (mm) on Day 8 (Day 0 = treatment)*	----	10.4 ± 0.4 (n = 19)	10.1 ± 0.3 (n = 19)	9.6 ± 0.7 (n = 5)

* No differences among groups ($P \geq 0.2$).
[a,b,c] Proportions with different superscripts are different ($P < 0.001$).

8.5 Discussion

The present study was designed to determine the ovulation-inducing effect of semen of like-species (llama), related species (alpaca) and unrelated species (bovine) using the female llama as a bio-assay; i.e., an induced ovulating species in which follicle dynamics and ovulation have been well characterized, and in which ovulation can be readily monitored by ultrasonography (Adams et al., 1990; Bravo et al., 1990b, Ratto et al., 2003, 2005). The species used represent the 2 broad categories of ovulation type – spontaneous (bovine) and induced (camelid). Results document clearly the existence of an ovulation-inducing factor (OIF) in the seminal plasma of bulls, and confirm the results of a previous study in which OIF was identified in the seminal plasma of llamas and alpacas (Adams et al., 2005).

We infer from the results of the present study that OIF is a potent and highly conserved molecule. Similar to our earlier study (Adams et al., 2005), a relatively conservative dose of seminal plasma was used in the present study (1 ml of raw seminal plasma) based on the reported average volume of the ejaculate in alpacas and llamas (2 to 3 ml; Lichtenwalner et al., 1996, Neely and Bravo 1997). Despite the modest dose used in this study, the effects of seminal plasma were profound. Collectively, 38 of 38 (100%) llamas given camelid seminal plasma and 5 of 19 (26%) given bovine seminal plasma ovulated. The ovulation rate was lower with bull seminal plasma than alpaca or llama seminal plasma, suggesting that bull OIF is somewhat altered or present in a lower concentrations compared to that in camelid semen. Results are consistent with those of a preliminary study of Bactrian camels monitored by transrectal palpation (Pan et al., 1992) in which ovulation was detected in 9 of 10 camels after intramuscular treatment with camel seminal plasma and 1 of 3 camels after treatment with bull seminal plasma. The relative effects of diluting bovine and camelid ejaculates will require more critical evaluation to determine the total dose of OIF being administered.

Some have postulated that induced ovulation may be a primitive trait from which spontaneous ovulation has evolved (Conaway 1971; Jochle 1975). This hypothesis is

consistent with evidence that induced ovulation appeared early phylogenetically in Orders *Lagomorpha* and *Rodentia* (Jochle 1975), and prevailed later in other closely related orders such as *Insectivora* and *Carnivora* (Zarrow and Clarke 1968). However, the presence of induced ovulation in a more distant Order such as *Artyodactyla* (camelids) challenges the hypothesis that induced ovulation is a primitive mechanism. According to one view, induced ovulators originated as a result of drastic changes in the environment that necessitated a change in reproductive behavior to maximize the possibility of conception (Lariviere and Ferguson 2003). The progenitor of present-day camelids originated on the North American continent but was forced through the Panamanian isthmus or across the Bering land bridge during the last glacial advance and evolved into what are now known as old world camelids (Bactrian and dromedary) in Asia and Africa, and new world camelids (llama, alpaca, vicuna, and guanaco) in South America (Wheeler 1995). Whether the ancestral species was a spontaneous or induced ovulator is unknown, but both old and new world camelids of today inhabit harsh, arid and isolated environments such as deserts and high altitudes. Perhaps environmental changes associated with the surging ice age, or the harshness of the present interglacial environment resulted in an evolutionary strategy for constant sexual receptivity to ensure survival of the species. Evidence of OIF in the bovine species, however, supports the hypothesis that acquisition of the characteristic of induced ovulation is a more ancient phenomenon that preceded the division of *Tylopoda* from other *Artiodactyls*. In this vein, the presence of an attenuated form or quantity of OIF in the seminal plasma of bulls may represent an evolutionary vestige of a common ancestry between ruminants and camelids. The existence of OIF in cattle demonstrated herein provides a plausible explanation for previous studies (Jochle 1975) in which mating was associated with an enhanced LH secretion and ovulation in cattle and sheep.

We conclude that bull semen contains an ovulation-inducing factor. The inter-species effect of seminal plasma provides rationale for the exciting hypothesis that OIF is a conserved molecule among all mammals and raises the immediate questions of the breadth of species in which OIF is present and the role of OIF in spontaneous ovulators.

9.0 LOCAL VERSUS SYSTEMIC EFFECT OF OVULATION-INDUCING FACTOR IN SEMINAL PLASMA

9.1 Abstract

Camelids are induced (reflex) ovulators. We have recently documented the presence of an ovulation-inducing factor (OIF) in the seminal plasma of alpacas and llamas. The objective of this study was to test the hypothesis that OIF exerts its effect via a systemic rather than a local route and that endometrial curettage will enhance the ovulatory response to intrauterine deposition of seminal plasma in alpacas.

Female alpacas were assigned randomly to 6 groups (n = 15 to 17 per group) in a 2 x 3 factorial design to test the effect of seminal plasma versus phosphate-buffered saline (PBS) given by intramuscular injection, by intrauterine infusion, or by intrauterine infusion after endometrial curettage. Specifically, alpacas in the respective groups were given 1) 2 ml of alpaca seminal plasma intramuscularly, 2) 2 ml of PBS intramuscularly (negative control group), 3) 2 ml of alpaca seminal plasma by intrauterine infusion, 4) 2 ml of PBS by intrauterine infusion (negative control group), 5) 2 ml of alpaca seminal plasma by intrauterine infusion after endometrial curettage, or 6) 2 ml of PBS by intrauterine infusion after endometrial curettage (negative control

group). The alpacas were examined by transrectal ultrasonography to detect ovulation and measure follicular and luteal diameters. Intramuscular administration of seminal plasma resulted in a higher ovulation rate than intrauterine administration of seminal plasma (93% versus 41%; $P < 0.01$), while intrauterine seminal plasma after endometrial curettage was intermediate (67%). None of the saline-treated controls ovulated. The diameter of the CL after treatment-induced ovulation was not affected by the route of administration of seminal plasma.

We conclude that 1) OIF in seminal plasma effects ovulation via a systemic rather than a local route, 2) disruption of the endometrial mucosa by curettage facilitated the absorption of OIF and increased the ovulatory effect of seminal plasma, and 3) ovulation in alpacas is not associated with a physical stimulation of the genital tract, and 4) the alpaca represents an excellent biological model to evaluate the bioactivity of OIF.

9.2 Introduction

Early studies of South American camelids documented that copulatory stimulation is responsible for inducing ovulation in these species (Fernandez-Baca et al., 1970a; England et al., 1969). The first significant increase in plasma LH concentrations occurred 15-40 minutes after the initiation of mating in llamas and alpacas (Bravo et al., 1990b; Bravo et al., 1991). A similar LH increase was observed in Bactrian and dromedary camels (related camelid species) beginning 20-30 min after mating (Xu et al., 1985; Marie and Anouassi, 1986). The rapid increase in plasma LH concentration after mating in camelids resembles that observed in rabbits (Jones et al., 1976) and cats (Concannon et al., 1980) – also classified as induced ovulators. A 40-fold increase in GnRH secretion from the medio-basal hypothalamus was detected within 20-60 minutes of mating in rabbits (Spies et al., 1997), followed by a preovulatory LH surge and ultimately ovulation about 10 hours after mating (Milligan, 1982).

The primary mechanism responsible for ovulation induction in these species is thought to involve a neuro-endocrine response to physical stimulation of the vagina and cervix by the penis during mating (Baker and Baum, 2000). The results of recent studies in llamas and alpacas, however, provide support for the hypothesis that a chemical substance in the semen is responsible, in whole or in part, for inducing ovulation (Adams et al., 2005). The existence of a potent ovulation-inducing factor (OIF) was demonstrated by intramuscular administration of cell-free llama and alpaca seminal plasma to females of the respective species. Collectively, 28 of 30 (93%) females ovulated after seminal plasma treatment compared to 0 of 32 (0%) saline-treated controls (Adams et al, 2005).

The discovery of OIF in llamas and alpacas is consistent with an early study in which intrauterine or intramuscular administration of Bactrian semen induced ovulation in Bactrian females (Chen et al., 1985; Xu et al., 1985). However, conflicting results have been reported about the effect of local versus systemic administration of semen. In female alpacas that were mounted by a male (intromission prevented) and those that

were mounted (intromission prevented) followed by artificial insemination, ovulation (detected at necropsy 3 days later) occurred in 2/15 and 3/9, respectively (Fernandez-Baca et al. 1970a). Since ovulation occurred in 36/44 females after natural copulation, the authors concluded that the physical act of coitus was responsible for eliciting ovulation in alpacas. In contrast, ovulation was detected in 6/10 alpacas and 5/8 llamas inseminated intravaginally with conspecific semen (cited in Sumar, 1994). In Bactrian camels, ovulation was detected by rectal palpation after intravaginal or intrauterine infusion of whole semen or seminal plasma in ≥75% of females (Chen et al. 1985, Xu et al. 1985; Pan et al., 1992). In a recent ultrasonographic study (Adams et al., 2005), ovulation was detected in 13 of 14 alpacas given seminal plasma intramuscularly, but in 0 of 12 given seminal plasma by transcervical intrauterine deposition.

The reason for the disparity in results is not clear, but authors of the latter study (Adams et al., 2005) speculated that differences may be attributed to attenuated absorption of OIF from the genital mucosa compared to the muscle. In this regard, copulation in alpacas and llamas is a prolonged event (30 to 50 minutes; San Martin et al., 1968; Bravo et al., 1990b) and ejaculation is intrauterine (Bravo et al., 1996). A normal sequela of copulation in these species is acute, transient inflammation of the endometrium as a result of repeated abrasion by the penis (Bravo et al., 1996). Perhaps absorption of OIF in seminal plasma subsequent to natural mating is facilitated by the hyperemia of the excoriated endometrium.

The objective of the present study was to test the hypothesis that OIF exerts its effect via a systemic rather than a local route and that endometrial curettage will enhance the ovulatory response to intrauterine deposition of seminal plasma in alpacas. A 2-by-3 factorial design was used to compare the ovulatory effects of alpaca seminal plasma versus phosphate-buffered saline (control) administered by intramuscular injection, by intrauterine deposition, or by intrauterine deposition after endometrial curettage.

9.3 Material and Methods

9.3.1 Seminal plasma collection

Semen was collected from male alpacas (n = 8) by artificial vagina (Bravo et al., 1997) over a period of 2 months prior to the start of the experiment (10 ejaculates per animal) and processed as previously described (Adams et al., 2005). Briefly, ejaculates were diluted 1:1 (v/v) with phosphate buffered saline (PBS, Gibco, Grand Island, N.Y., USA) and centrifuged for 30 minutes at 1500 x g. The supernatant was decanted to remove spermatozoa and a drop was evaluated by microscopy to confirm the absence of cells. If spermatozoa were detected, the sample was centrifuged again in like manner until all spermatozoa were removed. Sperm-free seminal plasma was stored at –70°C. Upon thawing, the diluted seminal plasma was pooled and kanamycin sulfate (Sigma Chemical Co., St Louis, MO, USA) was added to a final concentration of 25 μg/ml.

9.3.2 Animals & Treatments

The study was conducted during February to March at the Quimsachata Research Station in the Department of Puno, Peru (15º S, 71º W, and 4,500 m above sea level) using mature non-lactating female alpacas ≥4 years of age and weighing an average of 75 kg. To facilitate data collection, ovarian follicular development was synchronized among females (n = 100) by giving 5 mg Armour Standard LH (Lutropin-V®, Bioniche Animal Health, Belleville, ON, Canada) to induce ovulation. We expected approximately 85% to 90% of the alpacas to ovulate after LH treatment, resulting in synchronous emergence of a new follicular wave 2 days after treatment (Ratto et al., 2003). Alpacas were examined by transrectal ultrasonography (Aloka 500 with a 7.5 MHz linear-array probe, Instruments for Science & Medicine Inc., Vancouver, BC, Canada) 12 days after LH treatment - sufficient time to permit complete luteal regression and growth of a new dominant follicle (Ratto et al., 2003, 2005). Alpacas with a follicle ≥8 mm in diameter (n = 92) were assigned randomly to 6 groups and given: 1) 2 ml of alpaca seminal plasma intramuscularly (n = 15), 2) 2 ml of PBS

intramuscularly (control; n = 15), 3) 2 ml of alpaca seminal plasma by intrauterine infusion (n = 17), 4) 2 ml of PBS by intrauterine infusion (control; n = 15), 5) 2 ml of alpaca seminal plasma by intrauterine infusion after endometrial curettage (n = 15), or 6) 2 ml of PBS by intrauterine infusion after endometrial curettage (control; n = 15). Intramuscular injections were given in the semimembranosus muscle using a 20-gauge 40 mm long needle. Intrauterine infusions were accomplished by passing a plastic pipette through the cervix via transrectal manipulation and depositing 1 ml of alpaca seminal plasma or PBS into each uterine horn. To mimic the transient inflammation of the endometrium caused by the penis during natural mating (Bravo et al., 1996), both uterine horns were curettaged before intrauterine infusion by repeatedly scraping the tip of the plastic infusion pipette back and forth over the surface of the endometrial of both uterine horns for 3 minutes. Curettage was accomplished by transrectal manipulation of the uterus with one hand and manipulation of the pipette with the other.

Alpacas were examined by transrectal ultrasonography on Day 2 (Day 0 = treatment) to detect ovulation. Ovulation was defined as the sudden disappearance of a large follicle (≥8 mm) that was detected during the previous examination (Adams et al., 1989). To confirm ovulation and assess corpus luteum (CL) development, transrectal ultrasonography was repeated on Day 8; i.e., expected time of maximum CL diameter (Adams et al., 1989, Ratto et al., 2005).

9.3.3 Statistical Analyses

Single-point measurements (i.e., follicle size at the time of treatment, maximum CL diameter) were compared among groups by analyses of variance. If the overall effect was significant ($P<0.05$), specific comparisons were made between groups using Tukey multiple comparisons. Ovulation rates were compared among groups by chi-square analysis.

9.4 Results

The diameter of the largest follicle at the time of treatment did not differ among groups ($P = 0.9$). Ovulations were observed in groups treated by intramuscular administration or intrauterine deposition of seminal plasma (Table 1). Ovulation and luteal development were not detected in females that were given PBS by intramuscular or intrauterine administration (control groups). The ovulation rate in the intramuscular group (93%) was higher ($P < 0.01$) than in the intrauterine group (41%), while the endometrial curettage group was intermediate (67%). Of the alpacas that ovulated, the diameter of the CL did not differ among groups.

Table 9.1. Effect of administration of alpaca seminal plasma administered intramuscularly or by intrauterine infusion with or without endometrial curettage on ovulation and corpus luteum formation (mean ± SEM) in female alpacas.

	Intramuscular		Intrauterine		Intrauterine with curettage	
	Seminal plasma	Phosphate buffered saline	Seminal plasma	Phosphate buffered saline	Seminal plasma	Phosphate buffered saline
Follicle diameter at treatment (mm)*	8.0 ± 0.3 (n = 15)	8.2 ± 0.3 (n = 15)	8.1 ± 0.3 (n = 17)	8.0 ± 0.3 (n = 15)	8.3 ± 0.2 (n = 15)	8.4 ± 0.3 (n = 15)
Ovulation rate (%)	14/15[a] (93%)	0/15[c] (0%)	7/17[b] (41%)	0/15[c] (0%)	10/15[ab] (67%)	0/15[c] (0%)
CL diameter (mm) on Day 8 (Day 0 = treatment)*	9.3 ± 0.4 (n = 14)	----	9.5 ± 0.3 (n = 7)	----	9.4 ± 0.4 (n = 10)	----

* No difference among groups ($P \geq 0.9$)

[a,b,c] Proportions with different superscripts are different ($P < 0.01$)

9.5 Discussion

The results of the present study provide support for the hypothesis that the ovulation-inducing effect of seminal plasma is mediated via a systemic rather than a local route. A higher ovulation rate in alpacas treated by intrauterine infusion would have provided evidence to the contrary, but the ovulation rate was significantly lower in the intrauterine infusion group than in the intramuscular group. These results are consistent with those of a previous study (Adams et al., 2005) in which intramuscular administration of llama seminal plasma was followed by a surge in plasma LH concentration and ovulation. However, results do not unequivocally rule out a potential local contribution of seminal plasma to ovulation induction. In this regard, results of a study of the effects of boar seminal plasma deposited into different segments of the uterine horn in gilts were suggestive of a local unilateral mechanism influencing the interval to ovulation (Wabersky et al., 1999). Ovulation was advanced in the ovary ipsilateral to the side of semen deposition, but interestingly, only when deposited near the utero-tubal junction; no effect was found when seminal plasma was deposited in the middle of the uterine horn between two ligatures. No information has been reported regarding circulating gonadotropin concentrations subsequent to intrauterine or intravaginal deposition of semen.

The results are also consistent with the concept that systemic absorption of OIF from the uterus is facilitated by endometrial curettage. The ovulation rate in the curettage group was intermediate between that of the intramuscular group and the intrauterine group without curettage. Endometrial curettage in the present study was mild and was accomplished by rubbing a smooth, round-tipped plastic infusion pipette against the endometrium for 3 minutes. Perhaps more aggressive curettage would induce sufficient endometrial inflammation to increase absorption of OIF and result in an ovulation rate more typical of natural mating during the period of follicular readiness (i.e., 90%; Adams et al., 1990)

The disparity between the present study and our previous study (Adams et al., 2005) in the effect of intrauterine treatment in non-curettaged alpacas (ovulation rate of 41% versus 0%, respectively) may be attributed to the dose and site of deposition of seminal plasma. A total of 2 ml of seminal plasma was infused in the uterine horns (1 ml in each horn) in the present study, while only 1 ml of seminal plasma was infused into the uterine body in the previous study (Adams et al., 2005). Regarding local versus intramuscular absorption, the addition of a GnRH analogue (Buserelin) to the semen induced ovulation in rabbits after intravaginal artificial insemination (Quintela et al., 2004), but the dose of GnRH required for ovulation induction by intravaginal deposition was ten times higher than that used by intramuscular administration in the control group (8 μg versus 0.8 μg per inseminated female). This is consistent with the results from our previous experiment (Adams et al., 2005) in which no ovulations were detected in alpacas after intrauterine deposition of 5 mg of LH (Lutropin), a dose that caused ovulation in more that 80% of the females when given intramuscularly (Ratto et al., 2003, 2005). Hence, higher systemic concentrations of OIF may have been achieved in the present study by using larger dose and causing greater dispersion of seminal plasma throughout the endometrial surface. No mention was made regarding uterine manipulations in previous studies in llamas and alpacas (Fernandez-Baca et al., 1970a; Sumar, 1994) or Bactrian camels (Chen et al. 1985; Xu et al. 1985; Pan et al., 1992), and it is unclear if semen was deposited into the vagina, the cervix, or the uterus.

Results did not support the notion that physical stimulation of the vagina, cervix and uterus is involved in a neuro-endocrine system for ovulation induction, nor was there any evidence that OIF is produced by tissues of the female reproductive tract. Despite purposeful manipulation and irritation of the genitalia in the present study, none of the 45 females treated with saline alone ovulated.

We conclude that 1) OIF in seminal plasma effects ovulation via a systemic rather than a local route, 2) disruption of the endometrial mucosa by curettage facilitated the absorption of OIF and increased the ovulatory effect of seminal plasma, and 3)

ovulation in alpacas is not associated with a physical stimulation of the genital tract, and 4) the alpaca represents an excellent biological model to evaluate the bioactivity of OIF.

10.0 IN VITRO AND IN VIVO MATURATION OF LLAMA OOCYTES

10.1 Abstract

Cumulus-oocyte complexes (COC) were collected from abbatoir-derived llama ovaries and cultured *in vitro* for 28, 30, or 36 hr at 39°C in 5% CO_2 to determine the time required for maturation. The majority of COC (n = 298, 87%) were classified as Categories 1 and 2 (COC with ≥5 layers or 2 to 4 compact layers of cumulus cells, respectively) and homogeneous ooplasm, and the proportion that underwent nuclear maturation (MII) was 78, 81 and 80%, after 28, 30 and 36 hr, respectively (P = 0.65). To compare the effectiveness of FSH versus eCG for inducing *in vivo* maturation, in Experiment 2, llamas (n = 20 per group) were treated with: 1) 25 mg FSH bid for 4 d, plus 5 mg armour of LH at the end of FSH treatment; or 2) 1000 IU of eCG, plus 5 mg armour of LH 4 d after eCG treatment. The FSH- and eCG-treated groups did not differ (P = 0.85) with respect to the number of follicles ≥6 mm at the time of COC collection (17.9 ± 2.2 vs 17.7 ± 2.2), the number of COC collected (10.7 ± 2.1 vs 11.2 ± 2.3 per llama), or the collection rate per follicle aspirated (71 vs 74%). As well, no difference (P = 0.49) was detected between the FSH and eCG groups in the number of expanded COC collected (8.3 ± 2.1 vs 10.6 ± 2.2) or the number of COC at the MII stage (6.9 ± 1.8 vs 8.9 ± 1.9). In conclusion, llama oocytes reached MII as early as 28 h after in vitro culture and both FSH and eCG were equally effective in inducing ovarian superstimulation. Treatment with LH after either FSH or eCG superstimulation permitted the recovery of a preponderance of expanded COC in metaphase II that may be suitable for *in vitro* fertilization without *in vitro* maturation.

10.2 Introduction

Embryo transfer technology and superstimulatory protocols have been applied in camelids with limited success (Sumar, 1983; Bourke et al., 1995a; Correa et al., 1997; Ratto et al., 1997). Ovarian superstimulation protocols used to date have resulted in extremely variable follicular responses and low embryo recovery rates. A second limitation to the application of embryo transfer in llamas and alpacas is that most embryos recovered are at the hatched blastocyst stage, which makes embryo handling and cryopreservation difficult. Development of an *in vitro* embryo production system in camelids may circumvent some of the problems associated with embryo transfer, but issues related to ovarian superstimulation, oocyte collection, and *in vitro* culture conditions remain to be addressed.

Initial studies using abbatoir-derived ovaries from non-stimulated llamas provided preliminary information regarding oocyte collection, quality, and maturational state (Del Campo et al., 1992, 1994). In the first study (Del Campo et al., 1992), COC were collected by aspiration from follicles 1 to 12 mm in diameter (mean collection rate of six oocytes/llama) and 62% reached the second metaphase (MII) after 36 hr of *in vitro* culture. In the second study by the same authors (Del Campo et al., 1994), COC were collected by mincing the ovaries with a razor blade (mean collection rate of 27 oocytes/llama) and an *in vitro* maturation time of 30 hr was used; however, the maturation rate was low (30%). These initial studies were not designed to account for the effects of follicular size and status; this may account for the variability in the rate of maturation after prolonged culture *in vitro*. In a study in dromedary camels (Abdoon, 2001), oocyte yield and quality was influenced by season and ovarian physiological status; the number and quality of oocytes recovered increased when collection was done during the breeding season and in the absence of a corpus luteum. In another camel study (Torner et al., 2003), oocytes of non-pregnant females matured faster *in vitro* (32 hr) than those of pregnant females (36 hr). In a detailed study on the interval to ovulation in llamas, ovulation was detected by ultrasonography at 29.8±2.1 hr after an

ovulatory stimulus (Ratto et al., 2004). This interval may have important implications for determining the optimal time required for *in vitro* oocyte maturation.

In more recent studies, oocyte quality and morphology were examined following superstimulatory treatment and ultrasound-guided transvaginal follicle aspiration in llamas (Brogliatti et al., 2000) or follicular aspiration at laparotomy in alpacas (Gomez et al., 2002). In the llama study, gonadotropin treatments were conducted at a random stage of follicular development whereas in the alpaca study, treatment was initiated only when the largest follicle was <7 mm (i.e., ostensibly in the absence of a dominant follicle). In the llama study (Brogliatti et al., 2000), 42% of the COC recovered were classified as compact, 29% were degenerated, and despite treatment with gonadotropins, none were expanded. In contrast, 43% of alpaca COC recovered were expanded and 40% of the expanded COC had reached the second metaphase (Gomez et al., 2002). No data are available regarding oocyte quality, morphology and nuclear maturation in llamas and alpacas after superstimulatory treatment at known stages of follicular development.

Ovarian status at the time of superstimulatory treatment has been shown to influence the magnitude and variability of the follicular response in cattle (Adams, 1999). A greater and more consistent ovarian response resulted when treatment was initiated near the time of follicular wave emergence than when initiated later or at random stages of the estrous cycle (Nasser et al., 1993; Adams, 1994b; Adams et al., 1994c. The effect has been attributed to the suppressive effect of the dominant follicle on the growth of its subordinates and the emergence of the next follicular wave (Adams et al., 1993ab; Hagemann, 1999). In addition, follicular status has been shown to influence the developmental competence of its contained oocyte (Salamone et al., 1999; Vassena et al., 2003). In both studies, bovine oocytes recovered from subordinate follicles in the mid- to late-static phase (5 d after wave emergence), were more competent to develop to the morula-blastocyst stage than growing-phase follicles.

The objectives of the present study were to determine the effect of *in vitro* culture time on nuclear maturation of oocytes from non-stimulated ovaries (Experiment 1 - in vitro maturation), and to determine the effect of superstimulatory gonadotropin treatment after follicular wave synchronization on oocyte collection, quality, and maturational stage (Experiment 2 - *in vivo* maturation) in llamas.

10.3 Materials and methods

10.3.1 Experiment 1

Llama ovaries were obtained from mature non-pregnant females during the breeding season (November-January) from an abbatoir in Arica, Chile (15° S latitude, 70° W longitude, at sea level) and transported in a thermos containing phosphate buffered saline (PBS, Gibco, Invitrogen Corporation, Grand Island, NY, USA) supplemented with penicillin (100 IU/mL) and streptomycin (100 μg/mL) to the laboratory (Catholic University of Temuco; 38º S Latitude, 72º W longitude, 100 meters altitude) by air within 5 h of recovery. The temperature of the ovaries upon arrival was approximately 25 °C. COC were aspirated from follicles 3- 6 mm in diameter using a 21-gauge needle attached to a sterile 10 mL syringe containing PBS supplemented with penicillin (100 IU/mL), streptomycin (100 μg/L) and 0.3% bovine serum albumin (BSA, Sigma Chemical Co., St. Louis, MO, USA). Follicular aspirates were transferred to a 10 mL conical tube and allowed to settle for 10 min. The sediment was aspirated with a Pasteur pipette and transferred into a 60 mm petri dish containing PBS to search for COC. The COC were examined using a stereomicroscope at a magnification of 15x and categorized according the number of cumulus cell layers and the appearance of the oocyte cytoplasm: Category 1 - COC with ≥5 layers of compact cumulus cells, and homogeneous cytoplasm; Category 2 - COC with 2 to 4 compact layers of cumulus cells, and homogeneous cytoplasm; Category 3 - ≤1 layer of granulosa cells or partly denuded, and vacuolated cytoplasm; and Category 4 - denuded oocyte, and granular cytoplasm (De Loose et al., 1989). Only COC from Categories 1 and 2 were used for *in vitro* culture.

Follicles were aspirated in the laboratory and COC were placed in culture within 3 to 5 hr after arrival (i.e., 8-10 hr after slaughter). The COC were matured *in vitro* using the technique previously described (Del Campo et al., 1992, 1994). Briefly, COC were cultured in groups of 10 in 50 μL drops of maturation medium consisting of TCM-199 (Gibco) supplemented with 10% heat-treated fetal calf serum (Gibco), 0.2 mM sodium pyruvate, 0.5 μg/mL FSH, 1 μg/mL estradiol-17β, and 25 μg/mL gentamycin (the latter four products were from Sigma Chemical Co.). Drops were covered with mineral oil and cultured for 28, 30, or 36 h at 39 °C with 5% CO_2 and high humidity.

Nuclear morphology was assessed after denuding the COC by repeated aspiration and expulsion through a Pasteur pipette and fixing them in 1:3 acetic acid/ethanol for at least 24 h. The COC were assessed after 28, 30, 36 h of in vitro culture (103, 67, and 128 COC, respectively). Denuded oocytes were stained with 1% orcein (w/v) in 45% acetic acid (v/v) and evaluated using phase-contrast microscopy (400 x). Oocytes were classified as GV (oocytes with an intact germinal vesicle), GVBD (oocytes with no distinct nuclear membrane or nucleolus), MI (oocytes with a metaphase plate and no polar body), MII (oocytes with a metaphase plate and a polar body), abnormal or degenerated (i.e., oocytes with fragmented ooplasm, no distinct nuclear membrane or nucleolus, chromosomes dispersed in clumps within the ooplasm, or chromatin not recognizable at any stage of the meiosis).

10.3.2 Experiment 2

Mature, non-pregnant female llamas (n = 40), ≥3 yr of age and weighing an average of 120 kg, were used during the breeding season (February to March) at the Quimsachata Research Station, in the Department of Puno, Peru (15° S latitude, 71° W longitude, and 4,500 m above sea level). Ovarian follicles ≥5 mm were ablated by transvaginal ultrasound-guided follicle aspiration using a 5.0 MHz convex-array ultrasound transducer (Aloka SSD-500, Instruments for Science and Medicine Inc., Vancouver, BC, Canada) and a 19-gauge needle to synchronize follicular wave emergence among animals (Bergfelt et al., 1994, Ratto et al., 2003).

Llamas were assigned randomly into two groups (n = 20 per group) and treated 48 hr after follicle ablation (i.e., expected time of follicular wave emergence; Ratto et al., 2003) with: 1) 25 mg FSH (Folltropin, Bioniche Animal Health Canada Inc., Belleville, ON, Canada) im, twice daily for 4 d, plus 5 mg Armour Standard LH (Lutropin, Bioniche Animal Health Canada Inc.) im, 36 hr after the last FSH treatment; or 2) 1000 IU eCG (Novormon, Bioniche Animal Health Canada Inc) as a single dose i.m., plus 5 mg LH im, 4 d after eCG treatment.

The ovarian response was assessed by transrectal ultrasonography using a 7.5 MHz linear-array transducer (Aloka SSD-500) immediately before oocyte collection at 20 to 22 hr after LH treatment in both groups. COC were collected by transvaginal ultrasound-guided follicle aspiration as previously described (Brogliatti et al., 2000). Caudal epidural anesthesia was induced with 2.5 mL of 2% lidocaine (Bimeda-MTC Animal Health Inc, Cambridge, ON, Canada) and the perineal region was washed with surgical scrub. A 19-gauge, 55 cm single-lumen needle was placed in the needle guide of the ultrasound probe and advanced through the vaginal fornix and into the follicular antrum. Follicular fluid was aspirated using a regulated vacuum pump set at a flow-rate of 22 mL/min. The contents of all follicles ≥6 mm were aspirated into a 50 mL conical tubes containing PBS with 0.3% BSA, heparin (10,000 IU/L PBS) and 50 μg/L of gentamycin. Aspirates were transferred to petri dishes to search for and evaluate the COC using a stereomicroscope. COC were examined as described in Experiment 1, and were classified by stereomicroscopy as expanded, compact (≥2 layers of granulosa cells tightly surrounded the oocyte), denuded, or degenerated (pyknotic granulosa cells and vacuolated ooplasm). All COC were then denuded by repeated aspiration and expulsion through a Pasteur pipette, and nuclear status was classified after orcein staining and examination by phase contrast microscopy.

10.3.3 Statistical Analyses

Parametric data were compared between groups using Student's t-tests and proportional data were compared by Chi-square analyses.

10.4 Results

10.4.1 Experiment 1: *In vitro* maturation

A total of 344 COC were collected after aspiration of 3 to 6 mm follicles of 148 abbatoir-derived ovaries (average, 2.3 COC per ovary). Categories 1 and 2 COC comprised 87% (n = 298) of the total, while Category 3 and 4 COC comprised 13% (n = 46; Figure 10.1 A,B). After in vitro maturation of Categories 1 and 2 COC, there were no differences among culture times (28, 30 and 36 h) in the proportion of oocytes in each maturational stage (GV, GVBD, MI, MII, degenerated; P = 0.65; Table 10.1). Overall, 77.7 % (80/103), 80.6 % (54/67), and 80.4 % (102/128) of the oocytes reached MII after 28, 30 and 36 h of *in vitro* maturation culture respectively.

The unstained stereoscopic morphology of the COC was characterized by remarkably and invariably dark ooplasm, both before and after in vitro culture (Figure 10.1A-D). Varying degrees of cumulus expansion were evident after the respective periods of in vitro maturation (Figure 10.1C, D). Incomplete cumulus expansion was marked by the presence of dark clumps of what appeared to be degenerate cumulus cells (Figure 10.1C). Complete cumulus expansion was marked by the presence of spherical clusters of cumulus cells of varying size within the expanding matrix around the oocyte (Figure 10.1D). The first polar body was distinct and was clearly detectable by stereomicroscopy at 20 x to 40 x magnification (i.e., before nuclear staining) in 80% of oocytes after *in vitro* maturation (Figure 10.1E).

Table 10.1 Effect of *in vitro* maturation time on the percentages of nuclear maturation of COC collected from 3 to 6 mm follicles of slaughterhouse-derived llama ovaries.

In vitro culture time (hours)*	No. of COC	Oocyte morphology (%)				
		GV	GVBD	MI	MII	Degenerated
28	103	0	5.8	9.2	77.7	6.8
30	67	0	---	10.4	80.6	8.9
36	128	0	3.1	11.7	80.4	4.7

*No differences among maturation times for any end point (P = 0.65).
GV: Germinal Vesicle, GVBD: Germinal vesicle break down, MI: Metaphase I, MII: Metaphase II, Degenerated.

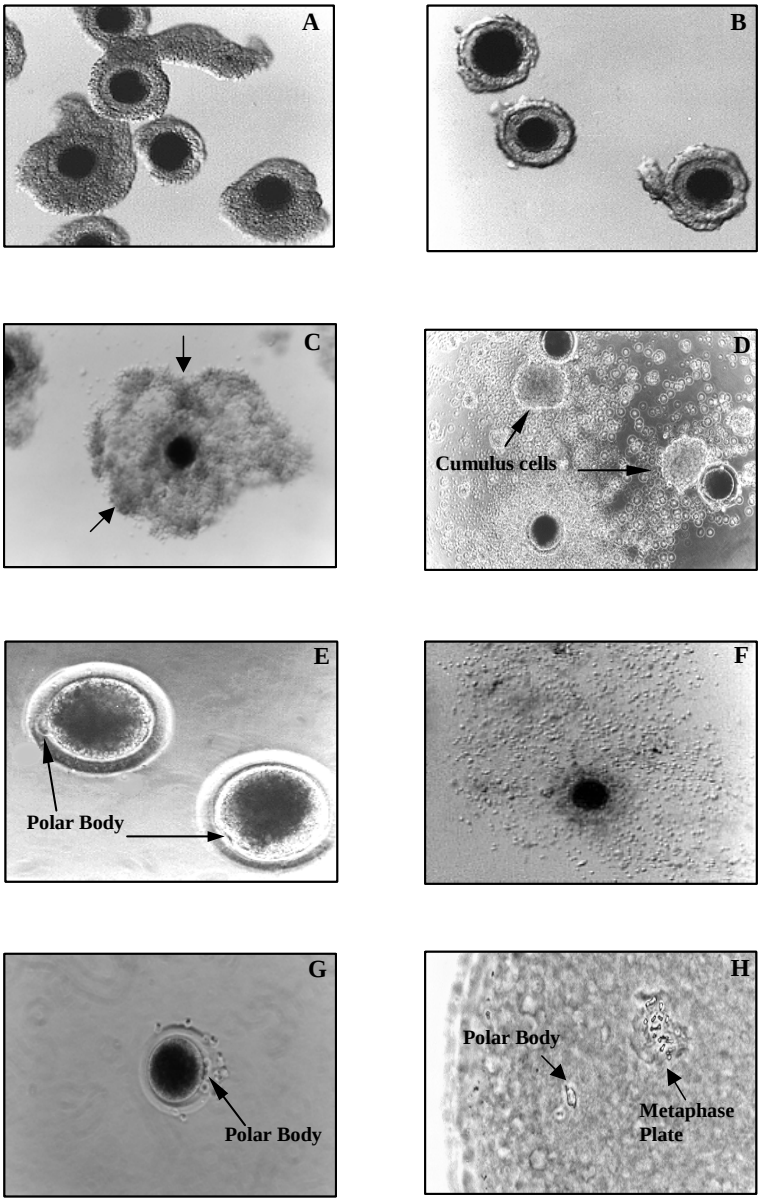

Figure 10.1. Morphological features of llama cumulus oocyte complexes (COC) during *in vitro* or *in vivo* maturation. A) Compact COC with ≥5 layers of cumulus cells (Category 1). B) Compact COC with two layers of cumulus cells (Category 2). C) COC matured *in vitro* for 30 hr, showing partial cumulus expansion and with dark changes of cumulus cells (arrows). D) COC matured *in vitro* for 30 hr showing fully expanded and clustered cumulus cells. E) Oocytes with a polar body detectable by stereomicroscopy after 30 hr of *in vitro* maturation. F) Expanded COC collected by transvaginal ultrasound-guided follicular aspiration 22 hr after gonadotropin treatment. G) Denuded oocyte with a polar body detectable by stereomicroscopy 22 hr after gonadotropin treatment. H) Llama oocyte in meiosis II with a polar body and metaphase plate.

10.4.2 Experiment 2: *In vivo* maturation

Ovarian follicles did not exceed 5 mm by the day of COC collection in two llamas treated with FSH, and in three llamas treated with eCG. Their data were not included in statistical analyses.

There were no differences between FSH- and eCG-treated groups in the number of follicles ≥6 mm at the time of COC collection, the number of follicles aspirated, the number of COC collected per llama, or the proportion of COC recovered ($P = 0.85$; Table 10.2). There was no difference between groups in the number of expanded COC collected per llama ($P = 0.44$), but a higher proportion of expanded COC was collected from llamas treated with eCG ($P < 0.01$). In addition, a higher number and proportion of compact COC were collected from llamas treated with FSH ($P < 0.01$; Table 10.3).

Differences between groups in the mean number of COC that reached the respective nuclear stages were not significant ($P = 0.12$; Table 10.4), except for a tendency for a higher mean number ($P = 0.07$) of COC at the GV stage in the FSH group. Of the total COC recovered, the proportion of COC that reached MII was lower ($P < 0.01$) in the FSH group (124/193, 64%) than in the eCG group (152/192, 79%). Of the total expanded COC recovered from the FSH and eCG treatment groups, the proportion that reached MII (Fig. 1H) was 82% (124/151) and 84% (152/180), respectively ($P = 0.57$).

Similar to the stereoscopic morphology of *in vitro* matured COC (Experiment 1), the ooplasm of in vivo matured COC was dark, but cumulus expansion was more complete and neither dark areas nor spherical clusters of cumulus cells were observed around the oocyte (Figure 10.1F). Similar to observations made in Experiment 1, the first polar body was very distinct and was clearly detectable by stereomicroscopy without staining in most of the oocytes (Figure 10.1G).

Table 10.2. Ovarian follicular response (mean ± SEM per llama) and COC recovery rate from llamas superstimulated with FSH or eCG at the time of follicular wave emergence followed by LH treatment (Experiment 2).

Treatment Group	No. of follicles ≥6 mm	No. of follicles aspirated	No. of COC collected	COC recovery rate
FSH (n=18)	17.9 ± 2.2	15.1 ± 2.0	10.7 ± 2.1	193/273 (71%)
eCG (n=17)	17.7 ± 2.2	15.1 ± 2.2	11.2 ± 2.3	192/258 (74%)

* No differences between groups for any end point (P = 0.85).

Table 10.3. Morphologic characteristics (mean number ± SEM per llama) and proportion (%) of COC collected from llamas superstimulated with FSH or eCG at the time of follicular wave emergence followed by LH treatment (Experiment 2).

Treatment group	COC collected	Expanded COC	Compact COC > 3 layers	Denuded	Degenerated
FSH (n=18)	10.7 ± 2.1 (100%)	8.3 ± 2.1 (78%) [x]	2.1 ± 0.7 [a] (19.7%)[x]	0.2 ± 0.1 (1.5%)	0 ± 0 (0%)
eCG (n=17)	11.2 ± 2.3 (100%)	10.6 ± 2.2 (93.7%) [y]	0.5 ± 0.1 [b] (4.7%) [y]	0.05 ± 0.05 (0.5%)	0.1± 0.1 (1.5%)

[ab] Mean values within columns are different ($P < 0.01$)
[xy] Proportions within columns are different ($P < 0.01$)

Table 10.4. Number (mean ± SEM per llama) and proportion (%) of oocytes at each stage of nuclear maturation at the time of collection from llamas given superstimulatory treatment of FSH or eCG followed by LH (Experiment 2).

Treatment group	No of COC collected	Morphology of oocyte nucleus				
		GV	GVBD	MI	MII	Degenerated
FSH	10.7 ± 2.1	2.0 ± 0.7[a]	0.4 ± 0.2	1.1 ± 0.3	6.9 ± 1.8	0.3 ± 0.2
(n = 18)	(100%)	(19%) [x]	(4.1%) [x]	(10.4%)	(64.2%) [x]	(2.6%)
ECG	11.2 ± 2.3	0.5 ± 0.2[b]	0.06 ± 0.6	1.4 ± 0.4	8.9 ± 1.9	0.3 ± 0.04
(n = 17)	(100%)	(5.2%) [y]	(0.5%) [y]	(12.5%)	(79.2%) [y]	(2.6%)

[ab] Mean values within columns tended to differ ($P < 0.07$)
[xy] Proportions within columns are different ($P < 0.01$)

GV: Germinal vesicle, GVBD: Germinal vesicle break down, MI: metaphase I, MII: metaphase II, Degenerated.

10.5 Discussion

The kinetics of *in vitro* oocyte maturation has not been critically studied in camelids, but has been examined in some detail in cattle (Sirard et al., 1989; Lonergan et al., 1997). Although maturation of the oocyte is not required for sperm penetration or for sperm nuclear decondensation under *in vitro* conditions (Chian et al., 1992), exposure of the sperm to immature oocytes was associated with decreased embryo development. In addition, the period of *in vitro* culture required for an oocyte to undergo nuclear maturation is reflective of its subsequent developmental competence. Bovine oocytes that extruded the first polar body as early as 16 h of *in vitro* culture were more competent to develop to the blastocyst stage than those that matured more slowly (Dominko and First, 1997).

In the present study, the proportion of COC in the second metaphase was high at the shortest time interval (i.e., 78% at 28 h), in contrast to an earlier study (Del Campo et al., 1992) in which a culture time of 36 hr was required for the majority (62%) of oocytes to mature. The proportion of COC in the second metaphase at 30 hr of *in vitro* culture in the present study was higher than that reported in a previous study (Del Campo et al., 1994) for the same period of culture (i.e., (80.6 versus 30.4%). Differences between studies may be related to the source of COC. In the early study, ovarian mincing using a razor blade likely resulted in the collection of a heterogeneous population of oocytes from preantral and antral follicles of all stages of development (Hyttel et al., 1997). In cattle, oocytes from follicles < 2 mm were developmentally incompetent (Lonergan et al., 1994); perhaps oocytes from immature llama follicles are also developmentally incompetent.

In addition to a greater proportion of matured COC in a shorter culture period, the proportion of degenerated oocytes observed after *in vitro* maturation appeared to be lower in the present study (4.7 to 8.9%) compared to earlier reports (30%; Del Campo et al., 1992, 1994), and may be attributed to the time interval between ovarian collection and COC aspiration (18 to 20 hr), or to the method of COC collection (razor blade).

Interestingly, the *in vitro* maturation time in the present study (28 hr) was similar to the time interval between mating and ovulation in this species. Based on ultrasonographic examination every 4 h, ovulation occurred 29.8±2.1 h after an ovulatory stimulus (Ratto et al., 2004). It appears that *in vivo* and *in vitro* maturation time is similar under the conditions of the present study. However, additional study is required to determine if maturation occurs earlier than 28 h of *in vitro* culture. It is also noteworthy that most camel COC (85%) reached MII after 36 h of *in vitro* culture (Abdoon, 2001), which is consistent with the reported time interval between mating and ovulation in camels (36 h; Abdoon, 2001). The importance of determining optimal *in vitro* oocyte maturation time is illustrated by the results of studies in cattle that show that oocyte aging may be the cause of reduced fertility if *in vivo* insemination is delayed (Hunter, 1989). In addition, delayed insemination *in vitro* has been associated with oocytes that are capable of being fertilized but unable to develop into embryos as a result of deranged cortical granules and microtubules (Long et al, 1994).

The dark appearance of the cytoplasm of llama oocytes was consistent with that previously described (Del Campo et al., 1994) and may be attributed to the prevalence of lipid droplets (Brogliatti et al., 2000). Cumulus expansion in COC matured *in vivo* (i.e., those collected by transvaginal ultrasound-guided follicle aspiration after gonadotropin treatment) was more complete than *in vitro* matured COC. Furthermore, *in vitro* maturation was associated with apparent aberrations in cumulus expansion (i.e., dark clumping and spherical clusters of cumulus cells). The significance of these apparent aberrations has been documented in a previous study as well (Del Campo et al., 1994) and it is unknown, but *in vitro* maturation conditions have been shown to affect the level of maternal mRNA polyadenylation, and alter the storage of mRNA necessary for the early embryo development (Pocar et al., 2001). In addition, COC morphology (cumulus cells and ooplasm) associated with competence to reach the second metaphase and blastocyst stage has been correlated with the expression of several specific transcripts (De Souza et al., 1998). In cattle, *in vivo* matured oocytes are more competent to develop to the blastocyst stage than *in vitro* matured oocytes (Van de Lemput et al., 1999; Dieleman et al., 2002; Bordignon et al., 1997; Rizos et al., 2002).

Specific differences in the morphologic characteristics of *in vitro* and *in vivo* matured llama COC will provide clues about factors responsible for imbuing developmental competence of an oocyte.

Superstimulatory treatments (FSH or eCG) were equally efficacious in inducing multiple follicle growth, consistent with a similar comparison made in alpacas (Gomez et al., 2002). Gonadotropin treatment effectively increased the number of follicles accessible for oocyte collection, as reported in cattle (Pieterse et al., 1988; Looney et al., 1994; Goodhand et al., 1999; Brogliatti and Adams, 1996). The superstimulatory response was relatively consistent among animals in both groups. Of 40 llamas, only five (12%) failed to respond to gonadotropin treatment (i.e., no follicles >5mm), and of those that responded, all had >3 follicles ≥6 mm. The consistency in response was attributed to the emergent stage of follicular wave development at the time treatment was initiated (Nasser et al., 1993; Adams, 1994b). Based on previous work in llamas (Ratto et al., 2003), follicular wave emergence was expected 2.3±0.3 d after follicular ablation; hence, treatment was initiated 2 d after ablation in the present study.

The number of COC collected after superstimulatory gonadotropin treatment in the present study (~11 per llama) was approximately half that previously reported in alpacas (~24 per alpaca) (Gomez et al., 2002), a difference that may be attributed to a greater ovarian response and to the laparotomy technique used to collect COC in the alpaca study. The COC recovery rate was similar between treatment groups (about 72%) and exceeded the expected recovery rate based on a previous study in which transvaginal ultrasound-guided follicle aspiration was used (56%) (Brogliatti et al., 2000). The COC collection rate also appeared to be higher than that described in cattle using a similar technique (52% and 38%) (Looney et al., 1994; Goodhand et al., 1999). Further investigation is required to determine whether LH-induced maturational changes improve COC collection rate by weakening the attachment to the follicular wall.

The number of expanded COC observed after FSH or eCG treatment in this study was higher than that reported in superstimulated alpacas (Gomez et al., 2002). Due

to large individual variation, differences observed in the mean number of oocytes per llama that reached respective maturational stages did not reach significance. However a higher proportion of expanded and matured COC were collected from llamas after eCG treatment. Regardless, over 80% of the expanded COC in both treatment groups were in metaphase II. In general, the low number of degenerated oocytes in the Experiment 2 may be attributed to the timing of collection; i.e., following gonadotropin treatment of a newly emerged follicular wave (Goodhand et al., 1999; Lonergan et al., 1993; Arlotto et al., 1996).

In conclusion, llama oocytes collected from non-stimulated 3 to 6 mm follicles reached the MII stage as early as 28 h after *in vitro* culture. Treatments with FSH and eCG were equally effective in inducing ovarian superstimulation when administered during follicular wave emergence. Treatment with LH after ovarian superstimulation permitted the recovery of a preponderance of expanded COC (80 to 90% of total collected), most of which were in MII (64 to 79% of the total collected). Superstimulation with eCG was associated with a slightly higher proportion of expanded COC and COC in MII compared to superstimulation with FSH. Therefore, either treatment may be used to collect oocytes that are suitable for immediate *in vitro* fertilization (i.e., *in vitro* maturation not required).

11.0 EFFECT OF OVARIAN SUPERSTIMULATION ON COC COLLECTION AND MATURATION IN ALPACAS

11.1 Abstract

The objectives of the study were to evaluate the ovarian response to 2 superstimulatory treatments in alpacas and to determine the effect of these treatments on cumulus-oocyte complex (COC) collection efficiency and maturation. Alpacas (n = 7 per group) were treated with 1) 200 mg of FSH im divided bid for 3 d, plus a single iv dose of 1000 IU hCG 24 hours after the last FSH treatment, or 2) 1200 IU of eCG as a single im dose, plus a single iv dose of 1000 IU of hCG on Day 3 after eCG treatment (Day 0 = Start of superstimulatory treatment). At 20-24 h post-hCG treatment, the ovaries were surgically exposed and COC were collected by needle aspiration of all follicles ≥6 mm. The FSH and eCG treatment groups did not differ with respect to the number of follicles ≥6 mm at the time of COC collection (20.0 ± 7.5 versus 27.0 ± 3.3; P = 0.5), the number of COC collected (26.2 ± 8.4 versus 23.3 ± 3.7; P = 0.7), or the collection rate per follicle aspirated (89% versus 87%; P = 0.7). No difference was detected between FSH- and eCG-treated alpacas in the number of expanded COC (11.5 ± 2.9 versus 8.8 ± 2.8; P = 0.54) or compact COC with ≥3 layers of cumulus cells (12.5 ± 4.3 versus 14.3 ± 2.6; P = 0.72). As well, no difference (P = 0.1) was detected between FSH and eCG groups in the number of expanded COC MII stage (8.5 ± 1.9 vs 6.0 ±

2.1). A greater proportion ($P < 0.05$) of compact COC in MII was observed after *in vitro* culture in alpacas treated with FSH. Eight expanded COC were *in vitro* fertilized with llama sperm. Embryos at two-cells and morulae stage were observed at 48 h and 7 days, respectively after *in vitro* fertilization. In summary, FSH and eCG treatments were equally effective for ovarian superstimulation and oocyte collection. COC were collected from more than 80% of follicles aspirated at laparotomy. Thirty percent of total COC collected after superstimulation were at MII stage. The number of matured oocytes per female is increased when compact COC are submitted to *in vitro* maturation.

11.2 Introduction

The application of assisted reproductive technologies in South American camelids has been limited compared to other domestic species (i.e., cattle, sheep, goat). Although superovulatory protocols and embryo transfer technology have been reported in camelids (Bourke et al., 1995; Correa et al., 1997; Ratto et al., 1997, Taylor et al., 2001, Aller et al., 2002), success has been limited due to wide variation in the ovulatory response and low embryo recovery rates. In addition, conventional cryopreservation techniques cannot be used because embryos are at a more advanced stage (hatched blastocysts), further limiting embryo production for commercial purposes. Although studies of *in vitro* maturation, fertilization and embryo culture techniques have been published in llamas (Del Campo et al., 1992, 1994), attempts to establish *in vitro* embryo production in camelids has not been reported. The development of *in vitro* embryo production techniques in camelids would circumvent some of the disadvantages of *in vivo* embryo production. Recently, by using ultrasound-guided follicular aspiration, it has been possible to collect a high number of llama oocytes from superstimulated and non-superstimulated females that can be used for *in vitro* studies (Brogliatti et al., 2000; Ratto et al., 2005). In addition, a superstimulatory treatment based on daily administration of FSH plus a unique LH dose at the end of the treatment has improved the ovarian response and oocyte collection in llamas (Ratto et al., 2005b).

Most of the superstimulatory treatments in camelids have been conducted under a natural or artificially induced luteal phase to mimic the ruminant physiology, however it is unknown whether progesterone/progestogen treatment is necessary to achieve a consistent ovarian response. In alpacas, few studies have reported a great variability on ovarian response and embryo collection when superstimulatory treatment was performed under a natural or artificially induced luteal phase (Correa et al., 1992, 1994; Ratto et al., 1994). In a recent report, eCG treatment in alpacas resulted in 3 to 7 corpora lutea and a mean of 3.9 embryos, but no details of protocol were given (Bravo et al., 2004). Follicular status at the time of superstimulatory treatment decreases the variability on ovarian response in cattle (Adams, 1999). A more consistent follicular

development has been reported in cattle superstimulated in the absence of a dominant follicle (Adams, 1994). In a recent llama study the ovarian response was consistent among females when the superstimulatory treatments were administered during the follicular wave emergence induced by follicle ablation (Ratto et al., 2005b). In addition, treatment with LH after either FSH or eCG superstimulation increased the number of expanded COC in metaphase II at the time of collection (Ratto et al., 2005b).

The objectives of the study were to compare the effects of 2 superstimulatory treatments on ovarian follicular response, COC collection rate, and the maturational stage of COC collected.

11.3 Materials and Methods

11.3.1 Animals and treatment groups

Mature nonpregnant alpacas (n = 14), ≥3 years of age and weighing an average of 75 Kg, were used during the breeding season (December-February) at the Catholic Research Station in the Province of Temuco, Chile (38° S Latitude, 72° W longitude, 100 meters altitude). The ovaries were examined daily by ultrasonography using a 7.5 MHz linear-array ultrasound transducer (Aloka SSD-500, Instruments for Science and Medicine Inc., Vancouver, Canada) to monitor follicular dynamics (Adams, 1999). When the largest follicle observed was <7 mm in diameter (i.e., ostensibly no dominant follicle), alpacas were assigned randomly to 2 groups (n = 7 per group) and treated with: 1) 200 mg FSH (Folltropin, Bioniche Animal Health Canada Inc., Belleville, Ontario, Canada) im, divided bid over 3 d, plus a single iv dose of 1000 IU of hCG (Chorulon, Intervet, Booxmer, Holland) 24 hours after the last FSH treatment, or 2) 1200 IU of eCG (Folligon, Intervet, Booxmer, Holland) as a single im dose, plus a single iv dose of 1000 IU of hCG on Day 3 of the treatment (Day 0 = start of the superstimulatory treatments).

11.3.2 Ovarian response, follicular aspiration and COC classification

The ovarian response was evaluated by ultrasonography before the scheduled time of laparotomy to determine if the surgical procedure would be done. Surgery was not done on alpacas with ≤2 follicles ≥6 mm in diameter. The number of follicles ≥6 mm in diameter were assessed during laparotomy before oocyte collection at 20 to 24 hours after hCG treatment in both groups. General anesthesia was induced by intravenous administration of 5 mg/Kg of Ketamine (Ketalar, Parke Davis, Detroit, USA) and 0.4 mg/Kg of xylazine (Rompun, Bayer Laboratory, Santiago, Chile). After endotracheal intubation, general anesthesia was maintained with isofluorane (Forene, Abbot, Buenos Aires, Argentina) for 30-35 minutes. A 10 cm incision was made through the linea alba just cranial to the mammary gland, and ovaries were exposed to count ovarian structures and collect COC. The contents of all follicles ≥6 mm were aspirated using a 21-gauge needle attached to a sterile 10 ml syringe containing phosphate buffer saline (PBS, Gibco, Grand Island, N.Y., USA) supplemented with penicillin 100 IU/ml (Gibco), streptomycin 100 μg/ml (Gibco), and 0.3% bovine serum albumin (BSA, Sigma Chemical Co., St Louis, MO, USA). Follicular fluid was transferred to a 10 ml conical tube and allowed to settle for 10 minutes.

Cumulus oocyte complexes were examined using a stereomicroscope at a magnification of 15x and categorized according to the number of cumulus cell layers and the appearance of the oocyte cytoplasm. The COC were categorized as expanded, compact (3 or more layers of granulosa cells tightly surrounded the oocyte), denuded, or degenerated (pyknotic granulosa cells and vacuolated ooplasm). Expanded COC were denuded immediately by repeated aspiration and expulsion through a Pasteur pipette, and fixed with 1:3 acetic acid/ethanol for 24 h. After the fixation period, oocytes were stained with 1% orcein (w/v) in 45% acetic acid (v/v) and evaluated using phase-contrast microscopy (400x). Nuclear morphology was assessed according criteria established previously (Ratto et al., 2005b): oocytes with an intact nuclear membrane were in the germinal vesicle stage (GV), oocytes with no distinct nuclear membrane or nucleolus were in the germinal vesicle break down stage (GVBD), oocytes with a

metaphase plate and no polar body were in metaphase I stage (MI), oocytes with a metaphase plate and a polar body were in metaphase II stage (MII), oocytes with fragmented ooplasm, no distinct nuclear membrane or nucleolus, with irregular clumps of chromosomes within the ooplasm, or with chromatin that was not recognizable at any stage of meiosis were considered degenerate (D).

11.3.3 *In vitro* maturation

Compact COC with 3 or more layers of granulosa cells were matured *in vitro* using the technique previously described (Del Campo et al., 1994). Briefly, COC were cultured in groups of 12 in 50 μl drops of maturation medium consisting of TCM-199 (Gibco), supplemented with 10% heat-treated fetal calf serum (Gibco), 0.2 mM sodium pyruvate (Sigma), 0.5 μg/ml FSH (Sigma), 1 μg/ml estradiol-17β (Sigma), and 25 μg/ml gentamycin (Sigma). Drops were covered with mineral oil and cultured for 26 hours with 5% CO_2 and high humidity. After the *in vitro* maturation period, COC were denuded, fixed, stained, and evaluated as described above.

11.3.4 In vitro fertilization and embryo culture

Eight expanded COC from one female in the FSH group were fertilized *in vitro* using the technique previously described (Del Campo et al., 1994). In brief, llama epididymides were collected from mature males at a slaughterhouse in Arica, Chile (15° S latitude, 70° W longitude, at sea level) and transported on ice in a thermos to the Catholic University in Temuco by air within 5 h of slaughter. The tissue temperature upon arrival was between 5-7°C. Epididymal tails were dissected and placed in small Petri dish containing Sperm-TALP media supplemented with 3mg/ml of BSA (Bavister and Yanagimachi, 1977). Sperm were recovered under stereomicroscopy by puncturing and squeezing the tissue, and aspirating with a 30-gauge needle attached to a 1 ml syringe. Sperm from 5-6 epididymal tails were pooled and evaluated for motility. Samples with ≥75% progressive motility were centrifuged in a discontinuous percoll gradient (1 ml of 45% over 1 ml of 90% percoll) for 15 minutes at 700 g. The

supernatant was removed and the pellet was suspended with Sperm-TALP and centrifuged at 350 g for 6 minutes. The supernatant was removed and the final pellet was suspended with Fert-TALP (Bavister and Yanagimachi, 1977) supplemented with 6 mg/ml of fatty acid-free BSA (Sigma) and 10 μg/ml heparin (Sigma) to a final concentration of 1 x 10^6 spermatozoa/ml.

Expanded COC were washed with PBS supplemented with BSA, and then transferred into a small Petri dish with a 50 μl drop of spermatozoa suspension and covered with paraffin oil. Gametes were co-incubated at 38.5°C in air with 5% CO_2 and high humidity for 18 hours (Day 0 = *in vitro* fertilization). After *in vitro* fertilization, 3 presumptive zygotes were fixed and stained to evaluate sperm penetration or pronuclear formation, whereas the remaining zygotes (n = 5) were washed in TCM-199 supplemented with 10% FCS and 25 μg/ml of gentamicyn and cultured in a 50 μl drop of TCM-199 containing bovine oviductal epithelial cells at 38.5°C in air with 5% CO_2 and high humidity for 8 days (Eyestone et al., 1986). Embryo development was evaluated on Day 2 and Day 7 of *in vitro* culture (Day 0 = *in vitro* fertilization).

11.3.5 Statistical Analyses

Parametric data were compared between groups using Student's t-test and proportional data were compared by Fisher's exact test.

11.4 Results

The ovarian follicular response (number of follicles ≥6 mm) on Day 4 (schedule day of laparotomy) did not differ between FSH and eCG groups (20.0 ± 7.5 vs 27.0 ± 3.3; P = 0.5). However, 3 alpacas from FSH group had only 2 follicles ≥6 mm on Day 4 and were, therefore, not sumitted to the follicular aspiration procedure. There was no difference (P = 0.7) between FSH- and eCG- treated groups in the number of follicles aspirated or the number of COC collected (Table 11.1; Figure 11.1a). There was no difference (P = 0.5) between groups in the number of expanded COC; however, a

greater proportion ($P < 0.05$) of COC were compact in the eCG-treated group (Table 11.2; Figure 11.1bc). A higher mean number and proportion of denuded COC were observed in the FSH-treated group (Table 11.2).

There were no differences between groups in the number of expanded COC in the respective nuclear stages at the time of collection (Table 11.3). Of the total expanded COC recovered from the FSH and eCG treatment groups, the proportion that were at the MII stage was 74% (34/46) and 67% (42/63), respectively (Figure 11.1d).

Differences between groups in the mean number of compact COC that reached the the GV, GVBD, and MI stages after 26 h of *in vitro* culture were not significant ($P = 0.2$; Table 11.4), but a greater proportion ($P < 0.05$) of COC reached the MII stage in the FSH group and a higher proportion ($P < 0.05$) of degenerate COC was observed in the eCG group. The total number and proportion of COC that reached the MII stage (expanded COC at the time of collection plus compact COC after *in vitro* culture) did not differ between groups ($P = 0.3$; Table 11.5).

Two of 3 expanded COC that were fixed and stained after 18 h of *in vitro* fertilization were found in pronuclear stage, the third one was unidentified (Figure 11.1e). Five of 5 presumptive zygotes that were co-cultured with oviductal cells developed into 2-cells and morulae stage on Day 2 and 7 after in vitro fertilization, respectively (Figure 11.1f). None of these embryos developed into blastocyts stage at day 8 of *in vitro* culture.

Table 11.1 Collection of cumulus-oocyte complexes (mean ± SEM per alpaca) from alpacas after ovarian superstimulatory treatment with FSH or eCG followed by hCG.

End Point	FSH (n = 4)	eCG (n = 7)
Number of follicles aspirated*	29.5 ± 8.3	26.7 ± 3.4
Number of COC collected*	26.2 ± 8.4	23.3 ± 3.7
COC recovery rate*	105/118 (89%)	163/187 (87%)

* No significant difference between groups (P = 0.7).

Table 11.2. Morphologic characteristics (mean number of COC ± SEM per alpaca) of COC collected from alpacas after superstimulatory treatment with FSH or eCG followed by hCG.

	FSH (n = 4)	eCG (n = 7)
Expanded COC	11.5 ± 2.9 46/105 (44%)	8.8 ± 2.8 62/163 (38.0%)
Compact COC (≥3 cumulus cell layers)	12.5 ± 4.3 50/105[a] (48%)	14.3 ± 2.6 100/163[b] (61%)
Denuded	1.8 ± 0.8 [a] 9/105[a] (8%)	0.1 ± 0.1 [b] 1/163[b] (1%)

[ab] Values within rows with different superscript are different (P < 0.05)

Table 11.3 Nuclear status (mean number of COC ± SEM per alpaca) of expanded COC at the time of collection from alpacas after superstimulatory treatment with FSH or eCG followed by hCG.

Treatment group	Expanded COC Collected	Morphology of oocyte nucleus*				
		GV	GVBD	MI	MII	Degenerated
FSH	11.5 ± 2.9	0	0	1.2 ± 1.2	8.5 ± 1.9	1.7 ± 0.8
(n = 4)	(100%)	(0%)	(0%)	(11%)	(74%)	(15%)
eCG	8.8 ± 2.8	0.1 ± 0.1	0.6 ± 0.4	1.7 ± 0.6	6.0 ± 2.1	0.4 ± 0.2
(n = 7)	(100%)	(2%)	(6%)	(19%)	(68%)	(5%)

GV: germinal vesicle, GVBD: germinal vesicle break down, MI: metaphase I, MII: metaphase II
* No significant differences between treatment groups for any end point.

Table 11.4 Nuclear status (mean number of COC ± SEM per alpaca) of compact COC (≥3 cumulus cell layers) after in vitro culture for 26 hours in alpacas given a superstimulatory treatment of FSH or eCG followed by hCG.

Treatment group	Compact COC Collected	Morphology of oocyte nucleus				
		GV	GVBD	MI	MII	Degenerated
FSH	12.5 ± 4.3	0.3 ± 0.3	0.3 ± 0.3	2.3 ± 1.3	13.7 ± 2.1	0
(n = 4)	(100%)	(2%)	(2%)	(14%)	(82%)[a]	(0%)[a]
eCG	14.3 ± 2.6	0	0.7 ± 0.3	3.8 ± 2.4	9.1 ± 1.9	1.1 ± 0.4
(n = 7)	(100%)	(0%)	(5%)	(23%)	(64%)[b]	(8%)[b]

GV: germinal vesicle, GVBD: germinal vesicle break down, MI: metaphase I, MII: metaphase II
[ab] Proportions within columns are different ($P < 0.05$)

Table 11.5. Total number and proportion (%) of alpaca COC at second metaphase collected immediately (*in vivo* maturation) or after 26 h of *in vitro* culture (*in vitro* maturation) after FSH or eCG superstimulatory treatment followed by hCG.

	COC collected*	*In vivo* matured*	*In vitro* matured*	Overall*
FSH	26.2 ± 8.4	8.5 ± 1.9	10.2 ± 3.7	18.7 ± 2.1
(n = 4)	(100%)	(32.0%)	(39.0%)	(71.0%)
eCG	23.3 ± 3.7	6.0 ± 2.1	9.1 ± 1.9	15.1 ± 1.9
(n = 7)	(100%)	(26.0%)	(39.0%)	(65%)

*No significant difference between treatment groups (P = 0.3).

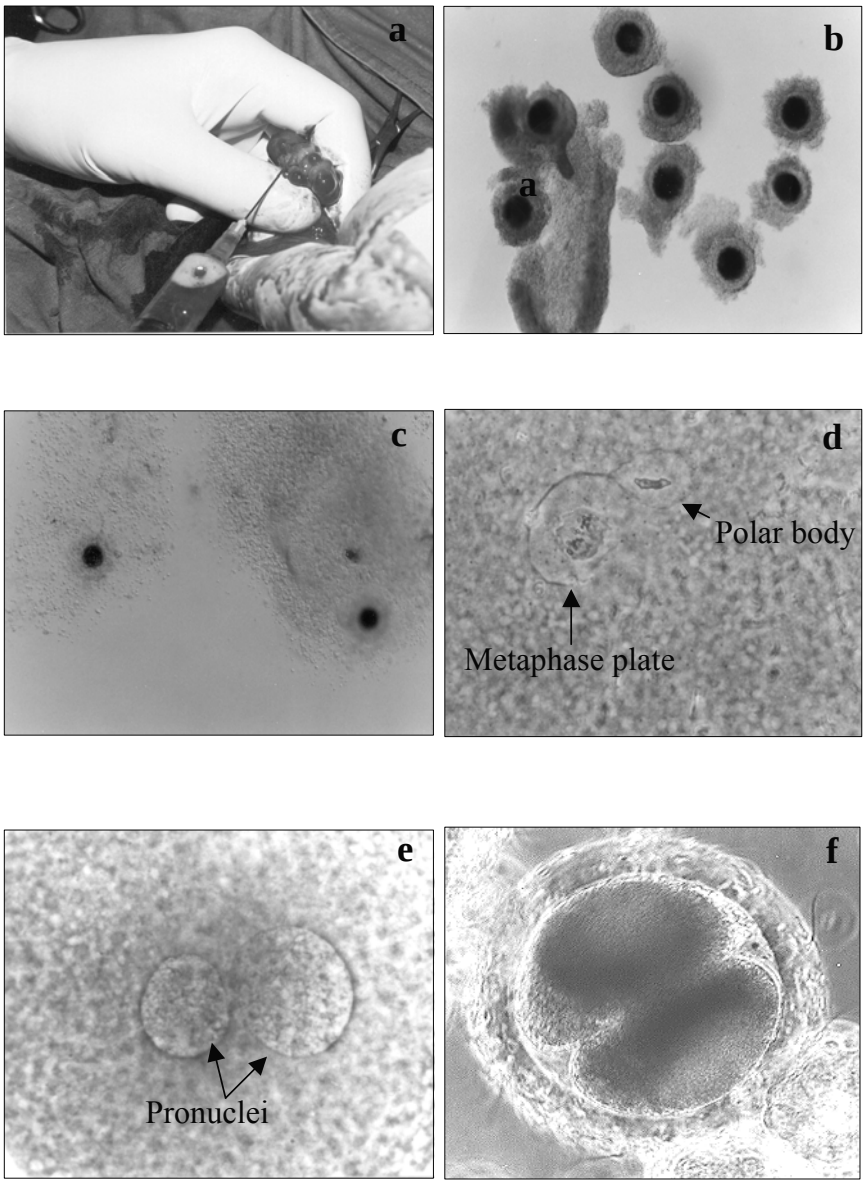

Figure 11.1. a) Collection of cumulus-oocyte complexes (COC) by follicular aspiration in alpacas after ovarian superstimulatory treatment. b) Compact COC with ≥3 layers of cumulus cells, and c) expanded COC collected 20-24 h after ovarian superstimulatory treatment. d) Expanded COC in meiosis II after staining with orcein-acetic acid. e) Alpaca zygote with the presence of two well-defined pronuclei after 18 h of *in vitro* co-incubation with llama sperm. f) Two-cell alpaca x llama embryo on Day 2 after *in vitro* fertilization and co-culture with bovine oviductal epithelial cells.

11.4 Discussion

The present study demonstrated that superstimulatory treatment with either FSH or eCG induced the development of a high number of follicles available for follicular aspiration. Approximately 30% of the total COC collected per alpaca were mature at the time of follicular aspiration and suitable for immediately *in vitro* fertilization. This proportion increased to 70% when compact COC were matured *in vitro* for 26 h. Thus a considerable number of COC per alpaca can be subjected to *in vitro* fertilization and embryo production.

The number of follicles observed after superstimulatory treatment in this study was higher than those reported in previous llama studies in which the superstimulatory treatment was initiated at random stages of follicular development (Brogliatti et al., 2000; Miragaya et al., 2002). However, the number of follicles in this study, although still higher, was close to that observed in llamas in which gonadotropin treatments were initiated after follicular wave synchronization by follicular ablation technique (Ratto et al., 2005b). Of 14 alpacas, only 3 (20%) failed to develop follicles >6 mm in diameter, and of those that responded, all had more than 8 follicles >6 mm.

Despite the poor response obtained in 3 females from the FSH treatment group, the number of follicles available for collection was high in both groups. These females were treated with the absence of <7 mm diameter follicle (i.e., ostensibly no dominant follicle), a stage in which was expected the lack of the inhibitory effect of the dominant follicle over the rest of the follicles. Perhaps the criteria to start the superstimulatory treatment used in this study, i.e, absence of follicles in <7 mm diameter, was not sufficiently rigorous to define dominance. Although a superstimulatory treatment combined with a natural or artificially induced luteal phase was not included in this study, apparently the presence of progesterone does not appear to be requisite for a superstimulatory response.

The collection rate (87%) in this study was similar to that of our previous study in llamas (75%; Ratto et al., 2005b) in which transvaginal ultrasound-guided follicle aspiration was used to collect oocytes. The collection rate in these camelid studies was higher than that reported for cattle (52%; Looney et al., 1994; 38%; Goodhand et al., 1999). It is unknown whether or not the high recovery rate was influenced by the effect of LH or hCG treatment at the end of superstimulatory treatment. The use of LH or hCG at the end of superstimulatory treatment has been reported to improve the oocyte collection rate in cattle and horses (Brogliatti et al., 1997; Cook et al., 1993). Another plausible explanation of this feature is that follicles protrude more distinctly from the surface of the ovary in llamas than in cattle (Adams et al, 1989) and in a study involving follicular trans-illumination (Del Campo et al., 1994), COC were attached to the wall of the exposed follicular hemisphere (i.e., above the adjacent ovarian surface) in 91% of llama follicles compared to 65% in cattle. Perhaps the more superficial location of the COC facilitates collection by aspiration.

Although the proportion of expanded COC in this study were lower than that reported for our previous llama study (Ratto et al., 2005b), 67% and 74% of the expanded COC collected in the respective treatments groups were at second metaphase similar to that reported in superstimulated llamas (80%; Ratto et al., 2005b). A higher proportion of *in vitro* matured oocytes were observed in the FSH-treated group. Similar to that observed for llama oocytes collected after gonadotropin treatment and subjected to in vitro maturation for 27 to 30 h (Miragaya et al., 2002).

Although, a low number of llama COC were submitted to *in vitro* fertilization and *in vitro* embryo culture, apparently COC were competent to be penetrated after co-incubation with llama sperm (pronuclear formation), and to develop until morulae stage after co-culture with bovine epithelial cells.

In conclusion, FSH and eCG treatments were equally effective for ovarian superstimulation and oocyte collection. Cumulus-oocyte complexes were collected from more than 80% of follicles aspirated at laparotomy. Thirty percent of all COC collected

after superstimulation was at the MII stage. *In vitro* maturation resulted in an increase in the total number of mature oocytes available for *in vitro* fertilization to 70% of collected oocytes. To our knowledge this is the first report of successful alpaca x llama embryos production after heterologous *in vitro* fertilization.

12.0 GENERAL DISCUSSION

12.1 Ovarian follicular wave synchronization and fixed-time natural mating in llamas

The breeding management of llamas and alpacas in the high Andes communities is based on sexual behavior pattern, i.e., a sexual receptive female is bred with the male without any knowledge regarding the ovarian physiological status. On small farms, the system consisting of repetitive mating of the receptive female until signs of rejection of the male by the female is manifested, results in several matings over a long period of time. However, multiple mating does not guarantee high conception rates. In addition, it is detrimental for males, i.e., decreases the sperm quality, and increases labor and time. The situation on large farms is even worse. A group of females with unknown ovarian follicular status are introduced into a small pen and bred with a group of males on just one day, and a second mating is allowed 2 weeks later. A female is considered pregnant when it rejects the male at the second mating. These breeding practices require a great deal of time and human labor, but pregnancy rates are only 40 to 50%.

To our knowledge, at this time, only one study has reported the use of estradiol alone or in combination with progesterone to synchronize follicular wave emergence in alpacas (D'Occhio et al., 1997). No systematic attempts have been reported to synchronize ovarian follicular wave emergence in llamas followed by ovulation induction and to determine the effect on subsequent fertility.

A new wave of follicular development can be induced in cattle approximately 2 days after GnRH or LH treatment, but the efficacy of the ovarian follicular synchronization will depend on the presence of a large viable dominant follicle capable

of ovulation (Pursley et al., 1995; Martinez et al., 1999, 2000). Unlike cattle in which ovulation has been reported to be quite variable among studies (50% to 78%); a large proportion of llamas ovulated (80%) after LH treatment given at random stages of follicular development, resulting in a large proportion of llamas with new follicular wave emergence (Chapter 5, Experiment 1). The effect of LH on ovulation induction was consistent in another llama study (Chapter 6); therefore we adopted LH treatment as our basic protocol for inducing ovulation and follicular wave synchronization in the llama and alpaca OIF studies in the present thesis (Chapter 7, 8, and 9).

The rationale for the use of estradiol combined with progesterone was based on evidence that llamas exhibit a follicular wave pattern similar to that described in cattle and share similar features such as recruitment, selection and dominance during follicular development (Adams, 1990). Based on these features, we assumed that both FSH and LH might play important roles in recruitment and dominance, respectively. The combination of estradiol plus progesterone treatment has been reported to be efficacious for synchronizing wave emergence and ovulation in cattle regardless of the stage of ovarian follicular development at the time of the treatment (Bo et al., 1994,a,b). However, estradiol plus progesterone treatment did not have a clear effect on synchronizing follicular wave emergence in the same fashion as that observed after LH or follicle ablation treatment (Chapter 5, Experiment 1). The lack of a clear effect may have been related to the dose or duration of treatment with estradiol and progesterone.

Higher doses of estradiol and progesterone, including a more prolonged progesterone treatment for 6 to 9 days, has been used in cattle to effectively synchronize follicular wave emergence (Bo et al., 2002). However, the dose of estradiol in our llama study was similar to that of a previous study in alpacas (D'Occhio et al., 1997) in which a single administration of 0.5 or 2 mg of estradiol without progesterone induced follicular regression and new wave emergence regardless of the stage of follicular development.

The use of a progesterone device (CIDR) containing 0.33 g of progesterone has been reported in llamas and vicuñas (Chaves et al., 2002; Aba et al., 2005). In the first study, the progesterone device induced follicle regression in those females which had a dominant follicle in the growing or early static phase, while in the second study, although the stage of follicular development was not stated, no follicular activity was observed during the entire period of progesterone implant. However, results from both studies did not show whether the suppressive effect of progesterone is able to induce a new follicular wave. Recently, it was reported that intramuscular administration of 200 mg of progesterone given 3 times, once every 2 days, induced follicular wave emergence with a new follicle capable of ovulating 12 days after progesterone administration (Vaughan, 2001). Although the ovarian effects of dose and duration of estradiol/progesterone treatment in llamas remain to be investigated, we need to consider the possibility that a different intra- and/or extra-ovarian mechanism to control follicular growth may be inherent to induced-ovulator species when compared to mechanisms in other ruminants where ovulation is a spontaneous process.

Although synchrony of wave emergence was affected by the treatments in Experiment 1 (Chapter 5), treatment-induced wave synchrony did not influence ovulation rate subsequent to natural mating in Experiment 2 (Chapter 5). The ovulation rate observed in the control group in Experiment 2 (Chapter 5) was unexpectedly high (93%), suggesting that follicles in growing, static, and regressing phases are capable of ovulating, contrary to the results of a previous study (Bravo et al., 1991). Although ovulation rate was not affected by treatment, the pregnancy rate was higher in synchronized than non-synchronized females (Chapter 5, Experiment 2). The disconnection between ovulation rate and pregnancy rate suggests that ovulatory capability is not directly related to the capability of the oocyte to become fertilized and develop into an embryo.

12.2 Different stimuli affecting ovulation induction in llamas and alpacas

Although information has been reported for the use of hormonal treatments to induce ovulation in llamas and alpacas, knowledge of ovulation intervals and corpus luteum formation after treatment is very limited. Results among studies are difficult to compare because data have been collected using different hormonal protocols (including hormonal preparations and doses); furthermore, methods (slaughterhouse or laparoscopy techniques) and observational intervals to evaluate ovarian structures have been varied. In addition, some studies have attempted to correlate the use of only hormonal profiles with ovarian function without any evidence of ovarian structures.

Direct comparison between the effects of mating and hormonal treatment on ovulation and luteal kinetics has not been reported previously in llamas. Presently, there is a major concern whether or not the use of GnRH agonists has an adverse effect on the luteal phase when such treatments are used for ovulation induction in both superstimulated and non-superstimulated women (Romeu et al., 1997). Proper luteal function is critical to establish successful development of fixed-time artificial insemination protocols and for recipient synchronization in embryo transfer programs for new world camelids. In the light of these concerns, the study of the effect of hormonal treatments on ovulation induction and luteal function in llamas was necessary. Results of the study described in Chapter 6 show that the effects of mating and hormonal treatment on interval to ovulation, ovulation rate and luteal function are similar and these hormonal preparations are suitable for control of ovarian function for breeding management.

The interval from mating or LH/GnRH treatment to ovulation did not differ among groups (29-30 h), and was similar to the interval previously reported for llamas after hCG or GnRH administration (27-29 h; Adam et al., 1992). However, the interval from mating to ovulation was nearly 1 day shorter than previously reported (2 days; Adams et al., 1991a; Adam et al., 1992). This difference was attributed to examination frequency. The developmental kinetics of the CL induced by hormonal treatment in the

present study were similar to those induced by natural mating and consistent with those observed in previous studies (England et al., 1969; Adams et al., 1990, 1991a). Unlike in women, in which serum progesterone concentration has been reported to be lower in GnRH than LH-treated patients (Humaidan et al., 2005), plasma progesterone concentration for the entire sampling period in this study (Chapter 6, Figure 6.2) did not differ between naturally mated and hormonally treated females. Similar to observations in women (Humaidan et al., 2005), no significant difference was observed on ovulation rate in llamas after GnRH and LH treatment. However, a peculiar feature has been reported in women after the use of these hormonal treatments. For instance, ovulation induction with GnRH has a beneficial effect on oocyte maturation compared with that observed in LH-induced patients, but lower implantation and pregnancy rates have been observed in GnRH than in LH-induced patients (Itskovitz et al., 2000; Fauser et al., 2002). The defective luteal phase observed in GnRH-treated woman has been attributed to a shorter duration of endogenous LH surge than that observed under physiological conditions compromising the luteogenic process and corpus luteum formation (Hoff et al., 1983; Chandrasekher et al., 2004). An interesting feature is that GnRH-induced women treated with exogenous progesterone to compensate for the luteal defects did not improve embryo implantation and pregnancy rates (Humaidan et al., 2005). Unfortunately, our study did not evaluate plasma LH concentration, oocyte morphology, fertilization or implantation; however, due to the results of the studies involving women, it would be interesting to determine the oocyte competence after these hormonal treatments. In addition, the llama is an exciting biological model to evaluate a direct effect of ovulation induction treatments without the interference of a long exposure of GnRH-antagonist treatment commonly used in combinations with these hormones to control the endogenous LH surge in women. In fact, llamas and alpacas have become a valuable model for the studies of ovulation-inducing factor present in mammals. As stated earlier, in most induced ovulator species, stimulation of the female genital tract by penile intromission is the most effective stimulus to induce ovulation. However, this notion was challenged when ovulation was induced by intra-vaginal or intramuscular administration of semen in Bactrian camels (Chen et al., 1985; Xu et al., 1985; Pan et al., 1992).

The results obtained in the alpaca (Chapter 7, Experiment 1) and llama (Chapter 7, Experiment 2 and 3) studies support earlier observations made in Bactrian camels (Chen et al., 1985; Xu et al., 1985; Pan et al., 1992) for the presence of an OIF in the seminal plasma. Ovulations ranged from 93% to 100% of the females when given by intramuscular administration (Chapter 7, Experiments 1, 2, 3; Chapter 8, Chapter 9). In addition, the presence of an OIF may not be restricted to the seminal plasma of induced ovulator species, since 26% (5/19) of the llamas ovulated after intramuscular administration of bovine seminal plasma (Chapter 8). Results of this study (Chapter 8) clearly indicate that OIF is a conserved molecule among species because cattle and alpaca OIF was able to induce interspecies ovulations. That ovulation rate with bull seminal plasma was lower than alpaca or llama seminal plasma suggesting that bull OIF may be a partially conserved molecule or present in a lower concentration. It is not known whether or not bull OIF chemical structure is related to that of camelids, but different kinds of selection pressures could have exerted an influence on the degree of conservation and bioactivity among species.

Ovulations did not differ between GnRH and seminal plasma treated females. But when we analyzed LH plasma concentrations, the duration of the surge in LH was significantly greater after treatment with seminal plasma than with GnRH. Progesterone secretion from subsequent CL was double that of the GnRH group (Chapter 7, Experiment 3). These observations provide a rationale for the hypothesis that OIF and GnRH effect pituitary LH release differently and are different molecules. The prolonged LH concentration observed for the whole period of sampling after seminal plasma treatment could have an effect on the corpus luteum formation and function, as evidenced by the higher secretion of progesterone. This observation provides a rationale for the hypothesis that the degree of luteogenesis is directly proportional to the duration of the preovulatory LH surge, as reported in other species (Chandrasekher et al., 1994; Bomsel-Helmreich et al., 1989; Peluso, 1990; Ishikawa, 1992).

Contradictory results have been obtained in ovulation induction when seminal plasma was given by intrauterine deposition. In the first study (Chapter 7, Experiment 1) none of the alpacas ovulated when seminal plasma was infused in the uterine horn, while the opposite result was observed in second study (Chapter 9). In addition, ovulation did not occur in alpacas after intrauterine deposition of LH (Chapter 7, Experiment 1). The results of intrauterine deposition in Experiment 1 (Chapter 7) raised the question whether or not a physical stimulus was necessary to induce mucosal laceration in order to improve OIF absorption. Copulation in alpacas and llamas is prolonged (30 to 50 minutes), and ejaculation is intrauterine. A transient inflammation of the endometrium is observed as a result of repeated abrasion by the penis (Bravo et al., 1996). Therefore, we hypothesized that absorption of OIF in seminal plasma subsequent to natural mating could be facilitated by the hyperemia of the excoriated endometrium. To test this hypothesis, we conducted the second study to determine whether intrauterine curettage can influence the ovulation mechanism after intrauterine deposition of alpaca seminal plasma (Chapter 9). Although we were unable to test the hypothesis that OIF absorption depends exclusively on a disrupted uterine mucosa (Chapter 9), we suggest that intrauterine curettage facilitated the absorption of OIF as evidenced by a higher ovulation rate than that observed in the non-curettage intrauterine group. A plausible explanation behind the different outcome regarding the intrauterine deposition of seminal plasma seems to be attributed to deposition site and dose. A total of 2 ml of seminal plasma was infused in the uterine horns (1 ml in each horn) (Chapter 9), while only 1 ml of seminal plasma was infused in the uterine body (Chapter 7, Experiment 1). Deposition at 2 different sites may further increase the efficacy of absorption and ovulation induction.

12.3 Superstimulatory treatment and oocyte morphology in llamas and alpacas

One of the most important limitations to the establishment of the embryo transfer technique in llamas and alpacas has been the lack of a consistent ovarian superstimulatory protocol. This limitation has been associated with low embryo recovery rates and a more advanced embryonic stage (hatched blastocysts) at the time of collection. The development of an *in vitro* embryo production technique for camelids could partly circumvent the disadvantage observed in the in vivo embryo production process, but as a first step it is necessary to study the morphological and physiological characteristics of camelids oocytes. The studies conducted in Chapter 10 and 11 were done to develop a consistent superstimulatory protocol and to evaluate its effects on oocyte morphology in llamas and alpacas.

The most important finding of the *in vitro* study described in Experiment 1 (Chapter 10) was that a great proportion of COC were mature at a shorter time interval of *in vitro* culture (78% at 28 h) than previously reported in which 36 h of *in vitro* culture was necessary to achieve second metaphase in a large proportion of llama oocytes (Del Campo et al., 1992). The difference may be attributed to the oocyte quality classification used before the in vitro culture in the earlier study. In addition, a longer period of time (18-20 h) from the ovary collection and oocyte aspiration in the earlier study may have affected COC quality. Interestingly, the 28 h interval is similar to *in vivo* results (Chapter 6) in which ovulation was detected by ultrasonography 29.8 ± h after an ovulatory stimulus.

Superstimulatory treatments (FSH or eCG) were equally efficacious in inducing multiple follicle growth in llamas (Chapter 10, Experiment 2) and alpacas (Chapter 11). The superstimulatory response was relatively consistent among animals in both studies and was attributed to the emergent stage of follicular wave development at the time treatment was initiated. Based on the results of Chapter 5 (Experiment 1), follicular wave emergence was expected 2.3±0.3 d after follicular ablation; hence, treatment was initiated 2 d after ablation in the llama study (Experiment 2, Chapter 10). The number of

COC collected after superstimulatory gonadotropin treatment in the llama study (~11 per llama) was approximately half that in the alpaca study (~24 per alpaca), a difference that may be attributed to a greater ovarian response and to the laparotomy technique used to collect COC in the alpaca study. However, in the llama study the recovery rate ranged from 71% to 74% using ultrasound-guided follicular aspiration, and it is still greater than that described for cattle (~50%). Further investigation is required to determine whether LH or hCG-induced maturational changes might improve the COC collection rate by weakening its attachment to the follicular wall in both llamas and alpacas. In a study of the attachment and location of COC in llamas (Del Campo et al., 1994), llama follicles were found to protrude more prominently from the ovarian surface than those of cattle, and 90 % of llama COC were located in the hemisphere adjacent to ovarian surface. This facilitated the location of the COC by transillumination without follicular dissection, and it probably allowed the high collection rate observed in these species.

Although, a higher proportion of expanded and matured COC were collected from llamas after eCG treatment (Chapter 10, Experiment 2), over 80% of the expanded COC in both treatment groups were in metaphase II. The reason that the number of expanded COC after FSH or eCG treatment in the llama study (Experiment 2, Chapter 10) was higher than observed in superstimulated alpacas (Chapter 11) may be attributed to individual variation in response. The proportion of matured oocytes in the alpaca study for the expanded COC category, although lower, seems to be closely related between studies. The *in vitro* maturation culture of those immature COC in the alpaca study (Chapter 11) increased the proportion of COC in second metaphase over the total number of collected COC. In the llama study, superstimulation with eCG was associated with a slightly higher proportion of expanded COC and COC in MII compared to superstimulation with FSH. Although this feature did not differ between groups in the alpaca study, a higher proportion of matured COC were observed in FSH-treated females after 26 h of *in vitro* culture. Similar results were described in a recent llama study (Miragaya et al., 2002) in which a higher proportion of COC collected after FSH treatment were in the second metaphase than in eCG-treated females after in vitro

maturation for 27-30 h. Finally, either of the superstimulatory treatment are useful to obtain oocytes suitable for immediate *in vitro* fertilization, i.e., *in vitro* maturation is not required.

13.0 GENERAL CONCLUSIONS

13.1 Ovarian follicular wave synchronization study in llamas

1. LH and FA treatments were most effective for inducing follicular wave synchronization, while estradiol-progesterone treatment was intermediate.
2. Ovulatory capability extends through a greater proportion of the growing, static and regressing phases of dominant follicles than previously thought.
3. Follicular wave synchronization did not influence ovulation rate subsequent to natural mating, but synchronization was associated with a higher pregnancy rate.
4. Follicular wave synchronization followed by a single pre-scheduled mating resulted in acceptable pregnancy rates and may form the basis of new breeding management schemes that obviate the labor-intensive need for testing behavioral receptivity in llamas.

13.2 Ovulation induction studies in llamas and alpacas

1. Ovulation rate, interval to ovulation, and luteal development were similar among llamas that were mated naturally or treated with LH or GnRH.2. Hormonal treatments (LH and GnRH) are equally reliable for inducing ovulation and normal luteal function, and both are suitable for use in synchronization for artificial insemination protocols or embryo transfer programs.
3. Our experiments documented unequivocally the existence of an ovulation-inducing factor in the seminal plasma of alpacas and llamas.
4. Ovulation-inducing factor in the seminal plasma of alpacas and llamas induces ovulation in more that 90% of females when given intramuscularly.

5. Although, OIF and GnRH were effective to induce ovulation in llamas, OIF induced a higher and more prolonged LH secretion from the pituitary gland than GnRH affecting the development and function of the corpus luteum as evidenced by a higher progesterone production.
6. Presence of an OIF in the seminal plasma of spontaneous ovulator species (bull) suggests that OIF is a conserved molecule among species.
7. The delivery route for seminal plasma treatment influences the effect of OIF in ovulation induction in alpacas. Ovulation rate was detected to be higher in intramuscular than intrauterine-treated females, however, ovulation rate increased when seminal plasma was infused after intrauterine curettage.
8. Disruption of the lining endometrium mucosa facilitates OIF absorption and increases ovulation rate.

13.3 Superstimulatory treatment and oocyte morphology studies

1. Llama oocytes collected from follicles about 3 to 6 mm in diamaeter from slaughterhouse ovaries reached the MII stage as early as 28 h after *in vitro* culture.
2. FSH and eCG treatments were equally effective in inducing ovarian superstimulation when administered during follicular wave emergence in llamas.
3. LH treatment after ovarian superstimulation in llamas permitted the recovery of a preponderance of expanded COC (80 to 90% of total collected), most of which were in MII (64 to 79% of total collected).
4. Superstimulation of llamas with eCG was associated with a slightly higher proportion of expanded COC and COC in MII compared to superstimulation with FSH.

5. FSH and eCG treatments were equally effective for ovarian superstimulation and oocyte collection in alpacas. COC were collected from more than 80% of follicles aspirated at laparotomy.
6. Thirty percent of COC collected from alpacas after FSH and eCG superstimulation treatment were at MII stage. The number of matured oocytes per female increased when compact COC were subjected to *in vitro* maturation.
7. Both FSH and eCG superstimulation of llamas and alpacas are useful for the collection of oocytes, that are suitable for immediate *in vitro* fertilization (i.e., *in vitro* maturation not required).

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