Embryo Production by Superovulation and Dual Siring in Alpacas

THESIS

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By

Brandon Stewart Forshey

Graduate Program in Veterinary Clinical Sciences

The Ohio State University

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Master's Examination Committee:

Jeffrey Lakritz

Carlos R.F. Pinto

Marco A. Coutinho da Silva, Advisor

Abstract

Alpacas have a gestation length of nearly one year, and therefore females can only produce one offspring per year. In order to accelerate the genetic gain of a herd, superovulation and embryo transfer techniques can be used to produce multiple embryos from genetically superior females. We hypothesized that the use of superovulation and dual siring results in the production of multiple embryos sired by both of the males. Our objectives were: 1)to determine the parentage of embryos produced from multiple ovulations on females bred by two males, and 2) to determine the effects of the time of breeding (first vs second) on the frequency of paternity.

Females received an ovulation induction agent (hCG; 1000 IU, IV or GnRH; 100ug, IM) when a preovulatory follicle >7 mm was present to induce ovulation and emergence of a new follicular wave. Starting at 60 h post-hCG/GnRH, females received twice daily intramuscular injections of FSH at decreasing doses (i.e. Day 1 = 50mg; Day 2 = 40 mg; Day 3 = 30; Days 4 to 7 = 20 mg). Administration of FSH was discontinued when half of the follicles in the growing cohort were ≥ 7 mm in diameter, or after seven days. On the last day of FSH administration, females received cloprostenol (187 μg, IM, twice) to induce luteolysis. Receptive females were bred to proven fertile males, once in the AM by one male and once in the evening by the other male and received hCG or GnRH at time of the first breeding. Embryo collections were performed 8 to 9 days after

breeding by transcervical uterine lavage without manipulating the reproductive tract per rectum. Statistical analysis was performed on data using t-test and Fisher's exact test. Significance was set at $P < 0.05$ and data are presented as mean ± SD.

Growth of multiple dominant follicles was successfully achieved in 20/21 cycles. The mean number of days of FSH administration was 5.5±1.4. Sixteen females (80%) were receptive after FSH treatment and were bred by two males (A and B). Embryo collection was performed on 15 cycles and 11 collections recovered one or more embryos.

A total of 46 embryos, 23 from breeding A-B and 23 from breeding B-A, were recovered for an average of 3.13 ±3.1 per flush. DNA amplification was performed on 23 embryos (6 from A-B, 17 from B-A). Twenty-two of the 23 embryos (96%) were determined to be sired by male B, six from breeding A-B and 16/17 from breeding B-A. A single embryo from mating B-A was sired by male A and was significantly different from the 22 embryos sired by male B. In conclusion, FSH can be used to promote growth of follicles and multiple embryos can be collected. However, differences in male fertility may have an effect on the number of embryos sired by each male.

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Vita

May 2005 .....Wauseon High School

May 2009 .....B.S. Biology, University of Findlay

Publications

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Fields of Study

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Chapter 1: Introduction

The camelid industry, specifically the alpaca industry has grown exponentially over the past couple of decades, but has recently suffered a decrease in popularity in the United States. However, in other parts of the world, particularly in South America, Australia, and New Zealand, their popularity and usage as production animals have increased. These countries have been able to use assisted reproductive techniques as a means to increasing the genetic gain of the animals and improve the quality of the fiber while still utilizing animals of low genetic merit as embryo recipients.

Assisted reproductive techniques (ART) have been used with some success in camelids with recent advancements in the field. Areas of focus have included superovulation, embryo collection and transfer, semen collection, and artificial insemination. In the United States, the main reasons for the lack of progress in ART in camelids are limited funding available for research and breed registry restrictions on offspring born from ART.

There are several reasons for progress of ART including: 1) the need to produce more offspring from desired genetic lines, and 2) to produce offspring from superior males and females that are infertile due to acquired conditions. Current success of ART in camelids is lower when compared with other domestic species such as the horse and

cow. A better understanding of the reproductive physiology of alpacas and the use of ART in these species will allow an increase in the success rate to a level comparable with those documented in the species previously mentioned.

Chapter 2: Review of Literature

2.1: Camelid Reproductive Cycle

Camelids are induced ovulators, meaning a signal must be elicited during copulation in order to cause a dominant follicle to ovulate. In the absence of mating, females display periods of receptivity separated by short intervals of non-receptivity in relation to an interval from the appearance of a dominant follicle to the next dominant follicle. In alpacas, that interval can range from 12 to 15 days (Chaves et al., 2002; Vaughan et al., 2004) and in llamas, that period can range from 20 to 22 days (Adams et al., 1990; Chaves et al., 2002). The interval is extended in the presence of a corpus luteum (CL), which can be maintained for as long as 10 days after ovulation in non-pregnant llamas (Adams et al., 1990; Bravo et al., 1991) and up to 12 days in alpacas (Brown, 2000).

2.1.1: Follicular Wave Development

Camelids develop follicular waves similar to other domestic species, but ovulation of the dominant follicle does not occur spontaneously such as in cattle and horses (Chaves et al., 2002). Follicular development can be separated in three stages: growth, maturation, and regression. At the start of a follicular wave, a cohort of eight to ten follicles measuring three millimeters in diameter are recruited and begin to develop

under the stimulation of follicle stimulating hormone (FSH). Approximately nine days after the emergence of the follicular wave, the largest follicle of the cohort will reach seven mm in diameter and become the dominant follicle (Vaughan et al., 2004). During growth and maturation, the dominant follicle will inhibit recruitment of a new wave of follicles by secreting estrogen and inhibin. The dominant follicle will continue to mature for three to five days and in the absence of a stimulus for ovulation, via natural breeding or pharmacologically, the follicle will undergo atresia (Brown, 2000). Some studies have demonstrated that a dominant follicle will develop at an equal rate on both ovaries (Fernandez-Baca et al., 1970; Vaughan, 2011; Vaughan et al., 2004) which is similar to reports in cattle (Guilbault et al., 1991), while others have shown a majority of ovulations occur on the left ovary (Picha et al., 2010). Lactation, pregnancy, and any source of progesterone, either exogenous or endogenous, can have an adverse effect on follicular size, thereby limiting the likelihood of developing a fully mature follicle (Adams et al., 1990; Bravo et al., 1991; Chaves et al., 2002). In some cases, the dominant follicle will fail to undergo atresia and grow beyond 12 to 14mm in diameter, becoming cystic (Bourke et al., 1995; Brown, 2000). In llamas, approximately 20% of these cystic follicles may become anovulatory, hemorrhagic, and undergo lutenization (Adams et al., 1991).

2.1.2: Ovulation

In contrast to other domestic livestock, camelids are induced ovulators (Adams et al., 1990; Bravo et al., 1990; Bravo et al., 1991; Fernandez-Baca et al., 1970; Vaughan et al., 2003). Ovulation has been documented to occur 30 hours after breeding or induction

via an exogenous hormone (Vaughan, 2011). There is a variation in the ovulatory response depending on the stages of follicular development (i.e. growth, maturation, regression). The most favorable ovulation rates occur when follicles ≥ seven mm in diameter are in the growing or maturation stage. During regression, there is a significant decrease in ovulation rate and the follicle may ovulate or may undergo lutenization without ovulating (Bravo et al., 1991; Ratto et al., 2003). Even though ovulation can occur in regressing follicles, the oocytes from these older follicles may be degenerating and do not develop into a viable embryo (Bravo et al., 1991; Ratto et al., 2003). There is evidence to support that fertilization of oocytes from a follicle ≥ seven mm in the growing or maturation stage of development results in decreased incidence of early embryonic death (Vaughan et al., 2003). However, there is also other data showing no significant difference in ovulation rate and pregnancy rate irrespective of where the follicle is in the developmental stages (Ratto et al., 2011a) .

2.1.2.1: Induction of Ovulation

Ovulation can be induced by natural breeding, pharmacologically, or occur spontaneously. One study described that approximately five to ten % of females can spontaneously ovulate (Sumar, 1999). During natural breeding, the male penetrates the cervix and deposits the semen within the uterine lumen. A substance described as ovulation-inducing factor (OIF) has been documented in seminal plasma of male camelids and has been shown to induce ovulation when administered to female camelids. (Adams and Ratto, 2011; Chen et al., 1985; Sumar, 1999; Tanco et al., 2011; Vaughan, 2007). OIF has been administered intramuscularly to female camelids and was successful

in inducing ovulation. However, induction of ovulation was not consistently achieved when OIF was placed in the uterus, suggesting that intrauterine inflammation initiated by the male during breeding allows for the OIF to enter the systemic circulation of the female (Adams and Ratto, 2013; Panez et al., 2009). The dose of ovulation inducing factor may have a positive effect on the size and function of the CL as well, with a higher dose producing a larger CL and higher concentrations of serum progesterone (Tanco et al., 2011).

Luteinizing hormone (LH) is released by the adenohypophysis in response to mating and a rise in serum concentration occurs within 15 minutes after breeding, reaching a peak concentration two hours later (Bravo et al., 1990; Bravo et al., 1991; Sumar, 1999). The amplitude of the LH surge depends on the follicle size, in which elevated levels of estrogen improve the response to ovulation induction (Bravo et al., 1991). It has also been documented that multiple breedings within 24 hours of the first do not result in another LH surge or improve ovulatory response (Sumar, 1999).

Ovulation can also be induced with the use of exogenous hormones. One example is gonadotropin releasing hormone (GnRH) or its analogues (Bourke et al., 1995; Brown, 2000; Huanca et al., 2009a; Picha et al.; Ratto et al., 2003; Sumar, 1999; Vivanco et al., 2009). When GnRH is administered, it causes a release of LH from the adenohypophysis approximately two hours after administration (Ree et al., 2009), which leads to ovulation of the dominant follicle (Peters, 2005). The efficacy of GnRH can be affected by elevated levels of progesterone, which decreases the stored amount of endogenous LH leading to a

lower LH surge that that observed in alpacas with low circulating concentrations of serum progesterone (Aslan et al., 2011; Logan et al., 2007; Pfeifer et al., 2009).

Human chorionic gonadotropin (hCG) can also be used in llamas and alpacas to induce ovulation (Brown, 2000; Correa et al., 1997; Fernandez-Baca et al., 1970; Ratto et al., 2003; Sumar, 1999). In cattle, hCG has been administered to induce ovulation and shown to have increased pregnancy rates when compared to GnRH (Aslan et al., 2011). A benefit with the use of hCG is that it by-passes the hypothalamic-adenohypophyseal axis and has direct effect on the ovary (Logan et al., 2007), and is not affected by elevated concentrations of progesterone (Giordano et al., 2012). hCG concentrations also remain elevated for an extended period of time due to its longer half-life compared to GnRH (Aslan et al., 2011; Giordano et al., 2012). One report in camelids described a 10% increase in ovulation by administering hCG to females at the time of breeding (Brown, 2000).

Another drug that can induce ovulation in llamas and alpacas is exogenous LH (Huanca et al., 2009b; Ratto et al., 2003; Sumar, 1999; Vivanco et al., 2009). LH has been used in several other species, including sheep, to induce ovulation during early anestrous (Oussaid et al., 1993), mares to induce ovulation (Jennings et al., 2009), and in cattle to induce ovulation and follicle maturation (Duffy et al., 2000). In alpacas and llamas, LH has been used with good success with only five to 14% of animals failing to ovulate a dominant follicle (Huanca et al., 2009b; Ratto et al., 2003). Of the three hormones described above, all have good efficacy to induce ovulation, however, hCG

may be the most advantageous due to its ability to have a direct effect on the ovary and availability on the market.

2.1.3: Luteolysis

The release of prostaglandin F2 alpha ($PGF_{2-\alpha}$) from the non-pregnant uterus, which causes luteolysis, has been documented in many species including horses (Ginther et al., 2009b), cattle (Stevenson and Phatak, 2010), sheep (Peterson et al., 1976), and pigs (De Rensis et al., 2012). $PGF_{2-\alpha}$ release occurs in pulses in most species, with lengths that vary between species. In cattle, these pulses occur around days 17 to 18 in the estrous cycle (Kindahl et al., 1976) compared to horses when pulses start to occur around 12 days after ovulation (Ginther and Beg, 2012). In the dromedary camel, there is evidence that $PGF_{2-\alpha}$ is consistently released during luteolysis. Skidmore et al. (1998) did not observe a pulsatile pattern of $PGF_{2-\alpha}$ secretion despite frequent sampling (i.e., every 20 minutes for eight hours). In llamas and alpacas, $PGF_{2-\alpha}$ is released in pulses from the non-pregnant uterus (Aba et al., 1997; Aba et al., 2000; Fernandez-Baca et al., 1979; Vaughan, 2011).

After $PGF_{2-\alpha}$ release from the uterus, the mechanism by which $PGF_{2-\alpha}$ reaches the ovary varies within species. In the mare, it has been shown that $PGF_{2-\alpha}$ released from the uterus reaches the ovaries via a systemic route (Ginther et al., 2009b), as opposed to ruminants in which $PGF_{2-\alpha}$ reaches the ovary through a local vascular countercurrent mechanism. (Ginther et al., 2009a). In alpacas, the mechanism of luteolysis involve both local and systemic actions of $PGF_{2-\alpha}$, and differs between the left and right uterine horns (Fernandez-Baca et al., 1979). There is a systemic and local effect of $PGF_{2-\alpha}$ release from the left uterine horn and only a local effect when released from the right uterine horn.

This observation has been demonstrated that $PGF_{2-\alpha}$ release from the right uterine horn alone will not cause complete lysis of a CL on the right ovary, but it needs the systemic effect of $PGF_{2-\alpha}$ release from the left uterine horn for complete luteolysis (Fernandez-Baca et al., 1979). Therefore, an embryo that developed from an ovulation on the right ovary must migrate to the left uterine horn to allow maternal recognition and some authors have speculated this may be the cause for the high early embryonic death rates observed in camelids (Ratto et al., 2011a). Moreover, this observation may explain why ~98% of pregnancies develop in the left uterine horn (Fernandez-Baca et al., 1979). A recent study in llamas evaluating the effect of embryo location on pregnancy rate after embryo transfer found that the highest pregnancy rate occurred when the embryo was placed in the left uterine horn with an ipsilateral CL (Trasorras et al., 2010). However in camels, there appears to be a significant difference overall in pregnancy rates based on location of embryo transfer in relationship to the CL, with the highest pregnancy rate occurring when the embryo was placed in the right uterine horn with a CL on the corresponding ovary (Tibary and Anouassi, 2010). This pregnancy rate could be due to the migration of the embryo to the left uterine horn to maintain pregnancy.

Exogenous $PGF_{2-\alpha}$ or its analogues have been used to induce luteolysis in camelids. As documented in other domestic species, the CL in female llamas has been shown to be responsive to one dose of exogenous prostaglandin on or after day 5 post induction of ovulation (Bianchi et al., 2012). Severe side effects and deaths have been documented with the use of the natural $PGF_{2-\alpha}$ analogue, dinoprost, in these species. In

camelids, the synthetic analogue$PGF_{2-\alpha}$, cloprostenol, has been safely used to induce abortion after mismatings (Smith et al., 2000).

2.2: Ovarian Steroids

The endocrine events during reproductive cycles in alpacas are very similar to other species, with a few differences that will be discussed next. Estrogen will increase and decrease in serum concentration in association with follicular growth and regression, respectively (Bravo et al., 1991; Chaves et al., 2002; Sumar, 1999; Vaughan, 2011). Female alpacas may show prolonged periods of receptivity due to the rise in estrogen concentration along with a short period of non-receptivity caused by the low levels of estrogen during the regression of a dominant follicle and very early development of the next follicular wave (Sumar, 1999; Vaughan, 2011; Vaughan et al., 2003). Elevated serum concentrations of estrogen do not induce a pre-ovulatory LH surge as seen in spontaneous ovulating species (Brown, 2000). However, high concentrations of estrogen are required to sensitize the hypothalamic-pituitary axis to release LH in response to breeding or other form of ovulation induction in alpacas (Bravo et al., 1991). Estrogen concentrations decrease approximately 18 hours after mating or exogenous induction of ovulation (Bravo et al., 1990).

In camelids, serum concentrations of progesterone begin to rise three to four days after induction of ovulation or mating and reach peak levels around eight to ten days (Aba et al., 1995; Bravo et al., 1990). A positive correlation has been shown between the serum concentration of progesterone and the size of the CL (Bravo et al., 1991; Sumar, 1999). Similar to other domestic species, female camelids will not be receptive to the

male when elevated concentrations of progesterone (i.e. > 1ng/ml) are present (Cotton, 2008).

2.3: Oocyte and Embryo Transport

Oocyte and embryo transport has been well documented in other species and is facilitated by ciliary beating of the epithelial cells that line the uterine tubes and contractions of the tubal smooth muscle (Brussow et al., 2008; Kolle et al., 2009; Kolle et al., 2010). Currently in camelids, there are limited data on tuball transport and the exact timing of entry of the embryo into the uterus is unknown. It has been documented that embryos can be collected from the uterus in females 5 days after breeding (Brown, 2000; Sumar, 1999; Tibary and Anouassi, 2007). A study evaluating the time (day five, six, or seven after breeding) and location of embryo collection (uterine tube vs. uterus) reported that, on day five, most embryos were collected from the uterine tubes while on days six and seven, most embryos were collected from the uterus (Cervantes et al., 2011). These observations suggest that alpaca embryos may arrive in the uterus between days five and six after mating.

2.4: Superovulation (SO) and Embryo Transfer (ET)

Both eCG, FSH, or a combination of both hormones have been used to induce growth of multiple dominant follicles in camelids (Correa et al., 1997; Nowshari and Ali, 2005; Ratto et al., 1997; Vaughan, 2007; Vyas et al., 2004; Vyas et al., 2002), and treatments for alpacas are summarized in Table 1.

2.4.1: Equine Chorionic Gonadotropin (eCG)

n	Treatment Started	Drug	Dose	Frequency	Length	PGF2-α admin.	Time of Breeding	hCG/ GnRH	Response	Reference
13	P4/E2, PG – day 0 Remove P4/E2 – day 7	pFSH	Dec doses 420 mg	BID	days 8-16		Between days 5-8 of pFSH q12h	LH at breeding	0.54 emb/ female	(Vivanco et al., 2009)
		eCG	300 IU	Once day 16						
13	P4, PG – day 0 Remove P4 – day 7	pFSH	Dec doses 230 mg	BID	days 5-9		Between days 5-8 of pFSH q12h	GnRH at breeding Buserelin	1.23 emb/ female	(Vivanco et al., 2009)
		eCG	500IU	Once day 7						
13	P4 GnRH – day 0 Remove P4 – day 7	pFSH	Dec doses total 440mg	BID	days 5-9		Between days 5-8 of pFSH q12h	GnRH at breeding Buserelin	0.15 emb/ female	(Vivanco et al., 2009)
9	GnRH with >8mm follicle – day 0	eCG	1000 IU	once day 2		day 7 cloprostenol	1 day post PG administration	GnRH at breeding Buserelin	2 emb/ female	(Vivanco et al., 2009)
21	>7mm follicle, given 4ug buserelin day 0	pFSH	Dec doses total 200 mg	BID	5 days	day 6 PGF	1 day post PG administration		2.7 ±0.4 emb/flush	(Huanca et al., 2009a)
20	>7mm follicle, Buserelin Day 0	eCG	750 IU	once day 2		day 6 PGF	1 day post PG administration		2.7 ±0.7 emb/flush	(Huanca et al., 2009a)
7	Largest follicle <7mm without a corpus luteum	FSH	200 mg total	BID	3 days			1000 hCG 24h post FSH	Follicles >6mm = 20 ±7.5	(Ratto et al., 2007)
7	Largest follicle <7mm without a corpus luteum	eCG	1200	Once day 0				1000 hCG day 3	Follicles >6mm = 27 ±3.3	(Ratto et al., 2007)

Table 1: Comparison of different superovulation protocols in alpacas

In camels, the use of eCG for a superovulation protocol has shown some good evidence, however, it appears to be inconsistent in the number of follicles produced, ranging from one to six per animal, and number of embryos collected, ranging from zero to four per animal (Khatir et al., 2007; Vyas et al., 2004; Vyas et al., 2002). A study evaluated the use of FSH compared to eCG with and without exogenous progesterone prior to SO and found FSH administration during luteal phase produced the greatest number of embryos (Vyas et al., 2002). Anovulatory follicles have also been observed after superovulation in camels lasting greater than 6 days after mating (Nikjou et al., 2008). In cattle, large anovulatory follicles can be due to the hypothalamus becoming less sensitive to the positive feedback effects of elevated estrogen levels, thereby altering the release of endogenous GnRH/LH pulses causing maturation of the follicle and subsequent ovulation (Wiltbank et al., 2002).

eCG has also been used in a variety of amounts from 750 to 1200 IU in alpacas (Huanca et al., 2009a; Ratto et al., 2007; Vivanco et al., 2009). One study determined that the best response in terms of number of corpus lutea was administering one injection of eCG on day 7 (Vivanco et al., 2009). There was also an increase in the number of anovulatory follicles in alpacas with the use of eCG and an overall increase in percentage in the number of females that had anovulatory follicles (34%) compared to FSH (Huanca et al., 2009a).

In llamas, eCG has been used in doses ranging from 500 to 1500 IU. An early study documented that 1000 IU eCG administered 7 days after induced ovulation produced 1.3 embryos per flush. However, there were multiple females that underwent

premature ovulation and therefore were not receptive to the male. This study also reported that the number of CLs on the ovary had a negative effect on recovery rate. This may be attributed to excessive ovarian response that limits the function of the fimbriae, decreasing the ability to collect the oocytes being released (Bourke et al., 1995).

Another study using 1200 IU eCG documented a recovery rate of 1.8 embryos per flush with two matings (Aller et al., 2002). Trasorras et al. (2010) obtained a recovery rate of 3 embryos per flush with eCG administration beginning in the absence of a large follicle. In another study by the same author, dose of eCG was also compared (500 vs. 1000 vs. 1500 IU) and found 1500 IU to produce the greatest number of follicles (Trasorras et al., 2009). Researchers administering eCG (500 IU once daily for three days) to llamas recovered 0.75 embryos per flush (Correa et al., 1997). Similar to what has been documented in alpacas and camels, llamas seem to have an increase in the number of anovulatory follicles after superovulation using eCG (Correa et al., 1997). Since eCG has an extended half-life, therefore one injection is need to induce a superovulatory response, this may lead to an increase in anovulatory follicles. It appears that the use of eCG produces multiple dominant follicles, however, the high incidence of anovulatory follicles after treatment results in lower efficiency of the superovulatory protocol.

2.4.2: Follicle Stimulating Hormone (FSH)

FSH has been utilized with a variety of doses ranging from 200 to 400 mg in equal or decreasing doses over three to five days in camels. A study evaluated the use of FSH in static doses as a superovulatory hormone following an hCG induced luteal phase.

The report documented that approximately 90% (17/19) of females responded (> three follicles ovulated) but less than 60% of flushes performed recovered embryos for a total of 30 (1.51 embryos per donor) (Vyas et al., 2004).

In alpacas, FSH has been used in a variety of protocols including administration in decreasing doses over three to five days ranging from 130 to 440mg of FSH total or as equal dosing totaling 200mg in alpacas (Huanca et al., 2009a; Picha et al.; Ratto et al., 2007; Vivanco et al., 2009). There was a wide range of responses in these studies, from all alpacas responding to stimulation (Picha et al.) to 80% response with a 53% recovery (Huanca et al., 2009a), which is comparable to results in camels (Vyas et al., 2004). There was also a large range of recovery rates from 0.15 to 2.7 embryos / flush (Huanca et al., 2009a; Vivanco et al., 2009). A report in llamas documented the development of 12.5 follicles per female after dominant follicle removal (DFR) and the use of oFSH (ovine FSH) twice a day over 3 days (Sansinena et al., 2007). Another study in llamas documented a recovery rate of 2.5 embryos per flush using FSH in decreasing doses (220mg in twice daily for 5 days) with (Correa et al., 1997). FSH has also been used to improve oocyte yield in alpacas, producing ~ 20 dominant follicles per female (Ratto et al., 2007).

The timing of breeding after superovulation treatment using FSH in llamas has also been evaluated. Females were administered 20 mg of FSH twice-daily for 5 days. One group was bred at the last FSH administration and the other group was bred 36h after FSH with a second mating 12 hours after the first mating in both groups. The second group had increased superovulatory response and ovulation rate, potentially as a result of

more time for the follicles to mature, thereby allowing a better response to breeding and ovulation induction. There was a decrease in percentage of embryos recovered from the uterus based on CL formation in the 36 hour group however; there was no difference in the total number of embryos recovered. The authors suggest this may be due to the fimbriae being unable to collect all the oocytes that are released at the time of ovulation (Ratto et al., 1997). Response rate using FSH varied within all reports ranging from 0% to 37% of llamas failing to develop more than 2 dominant follicles (Bourke et al., 1995; Trasorras et al., 2009), which in agreement to early results in cattle, where up to 30% of females fail to respond to treatment (Guilbault et al., 1991). Overall, the use of FSH as a superovulatory hormone produces an adequate and fairly consistent result when compared to eCG.

2.4.3: Combination of eCG & FSH

Several studies assessed the use of a combination of FSH and eCG in camelids. Nowshari and Ali (2005) discovered using 400mg FSH alone was less effective than administering 2000IU PMSG (eCG) prior to 400mg FSH in recovery rate and quantity of transferable embryos (Nikjou et al., 2008). Llamas administered a combination of the two (500 IU eCG day 1 and 156 mg FSH in deceasing doses for 4 days) recovered 0.5 embryos per flush. (Correa et al., 1997). In alpacas, one report using varying doses of FSH and eCG in combination documented a range of 0.54 embryos per female collected to 1.23 embryos per female (Vivanco et al., 2009). Overall, it appears that the combination of the two hormones may not improve the efficiency of the procedure when comparing each hormone individually.

2.4.4: Embryo Collection and Associated Factors

Currently, there is no standard treatment protocol to induce growth of multiple follicles and ovulation, and a large variability in the response and recovery rate. However, studies have shown that better results are achieved when superovulation treatment is initiated in the absence of a dominant follicle and around the time of emergence of a new follicular wave (Tibary et al., 2005). This large variability could be due to the limitations of the fimbria to capture all of the oocytes and the limited knowledge about superovulation in camelids (Brown, 2000). Embryo recovery rates may also be low due to failure of ovulation, *in vivo* fertilization, or gamete transport (Ratto et al., 2011b). One recent study analyzed the success of embryo transfer and identified factors that affect the success of SO-ET procedures such as, ease of rectal palpation, skills of technician, placement of the embryo in left uterine horn during transfer, and avoid stressing the animals (Sumar, 2011).

With most of the studies in camels, ovarian dynamics and uterine condition were monitored via ultrasonography per rectum. Embryo collection was performed in a standing, or sternal position with sedation through a catheter that was placed into the uterus through the cervix. The placement of the catheter and monitoring of uterine filling was done per rectum, with collection of the fluid by gravity (Nikjou et al., 2008; Nowshari and Ali, 2005; Vyas et al., 2004; Vyas et al., 2002). In llamas, it has been documented that breeding a female twice within a 24 hour period increased embryo recovery rate (Bourke et al., 1995).

Research conducted in cattle has identified two key factors that can affect the outcome of a superovulation protocol: age of the female and regimen of FSH administration (i.e. dose and frequency). There was a peak number of embryos collected from cows at 5.5 years of age, then decrease in number collected as the cow ages (Lerner et al., 1986). Also, failure to sustain elevated serum concentrations of FSH and the natural variation among animals may play a role in the inconsistent response rate of superovulation in cattle (Hackett and McAllister, 1992). These factors may also have an effect in camelids however, our knowledge on the factors that affect the success of SO-ET in alpacas is very limited, and most procedures have been adapted from other species.

2.5: Heterospermic Insemination

Heterospermic insemination is defined as the insemination of females with semen from more than one male at the same time (Nelson et al., 1975; Stewart et al., 1974). This procedure has been used in many species including cattle, pigs, rats, chickens, rabbits, and sheep (Berger and Dally, 2001; Martin and Dziuk, 1977; Parrish and Foote, 1985; Sharma and Hays, 1975; Stewart et al., 1974). Heterospermic insemination has been traditionally used as a method to determine differences in fertility between males by inseminating females with equal numbers of sperm and evaluating the paternity of the progeny (Berger, 1995; Berger and Dally, 2001; Martin and Dziuk, 1977). In rats, it has been documented that males with increased fertility will sire more offspring in a litter (Sharma and Hays, 1975). Heterospermic insemination has also been shown to increase the fertility of the mixed semen and is commonly used by the swine industry (Stewart et al., 1974). An increase in pregnancy rate has also been documented in cattle with the use

of heterospermic insemination (Nelson et al., 1975). Also, insemination with two different sires has recently been used in canine reproduction to produce puppies from multiple sires in the same litter and increase the fertility of the semen.

Many factors contribute to fertilization rate, however, these factors can be categorized into two groups; the number of sperm in the ejaculate and the quality of the ejaculate (motility, morphology, survival rate, etc.) (Gage and Morrow, 2003; Gage et al., 2004; Pizzari et al., 2008). Insemination with high numbers of spermatozoa has been shown to increase the fertility of semen in chickens (Martin et al., 1974). In sheep, it has been documented that there are no male-female interactions when using heterospermic insemination (Berger and Dally, 2001).

Another factor that may affect fertility and production of offspring from multiple sires is the time of breeding in relation to ovulation. Recently, the effect of the time of breeding in relation to ovulation using semen from two different stallions and oocyte transfer in mares was evaluated. Mares were inseminated with semen from one sire approximately 12 hours before oocyte transfer, which corresponded to ovulation, and again two hours after transfer with semen from a different male. Authors used parentage testing of the embryos and documented that >90% of the oocytes were fertilized by the sire from the first insemination (Carnevale et al., 2004). For this reason, mating with two proven males 12 hours prior to ovulation would allow sperm ample time to reach the oviduct for fertilization. However, in rats, it has been documented that males with the advantage of time or order of breeding will fertilize more eggs compared to another male when mating occurs prior to ovulation (Sharma and Hays, 1975). A study in boars

reported that difference in the viability of pooled sperm in the reproductive tract may contribute to difference in fertilization rates in males of similar fertility based on homospermic insemination (Stahlberg et al., 2000). Currently, there is limited information about in vivo insemination with two different males at different times prior to ovulation.

Chapter 3: Experiment

3.1: Introduction

Camelids have a gestation length of almost one year (Adams, 2007), and therefore females can only produce one offspring per year. In order to accelerate the genetic gain of a herd, assisted reproductive techniques such as embryo transfer (ET), can be used to produce embryos from genetically valuable females that are harvested and transferred into surrogate females that will then carry the pregnancy to term. ART has been used with limited success in New World camelids. In other species, such as bovine and equine, ART has been mainly used for two reasons: 1) for rapid multiplication of certain genetics lines, and 2) to produce offspring from animals that otherwise would be infertile. In camelids, the advance of ART has been very slow due to the scarcity of financial support for research and breeding registry regulations. Currently, very few veterinary practices around the world perform embryo transfer in alpacas, and mostly are located in South America and South West Pacific (Australia and New Zealand).

ET has been successfully performed in alpacas by applying similar techniques used in other species. Despite low success rates obtained with ET and the lack of a standardized procedure, very few prospective studies have been performed on ET in alpacas. Moreover, the few veterinary practices performing ET commercially do not

publish their procedures and/or results frequently, resulting in a scarcity of information in the literature.

Superovulation (SO) is one strategy that can be used in association with ET to increase the number of embryo/offspring produced from valuable females per cycle. The goal of combining SO-ET is to further increase the genetic selection of the herd by reproducing in large scale the genetics of the most valuable females and utilizing the less valuable females as surrogate dams. SO-ET has been extensively studied in bovine species and efficient methods to induce multiple ovulations and the production of multiple embryos have been established. As a result, SO-ET has been widely used in cattle operations across the globe.

In camelids, there have been limited studies on camelid SO-ET and to this date, there is no consensus on an effective treatment protocol. Induction of multiple ovulations has been achieved using equine chorionic gonadotropin (eCG) and follicle-stimulating hormone (FSH). Variations on the drug, dose, and frequency of administration between studies prevent a direct comparison of results and establishment of a standard protocol treatment. Recently, decreasing doses of porcine FSH were used to induce SO in alpacas resulting in production of multiple embryos (Huanca et al., 2009b)

In an attempt to increase the genetic diversity of embryos produced from a single cycle, females can be mated to different males during estrus, a procedure termed dual siring. Dual siring or heterospermic insemination has been used in many species including swine, rabbits, chickens, cattle, and most recently in canines. In bulls, rabbits, and chickens, it has been used experimentally to assess differences in fertility between

two males (Flint et al., 2003; Parrish and Foote, 1985; Tajima et al., 1989). In dogs however, the use of dual siring has been aimed at the production of puppies from different males in the same litter.

Currently, there is no published information on the use of dual siring in combination with SO-ET in alpacas. The use of dual siring can be very advantageous to the alpaca industry, by allowing production of multiple genetically diverse embryos during a single cycle. Therefore, our hypothesis was that the use of SO-ET and dual siring would result in the production of multiple embryos from different sires in the same cycle. Our objectives were to determine the effects of: 1) the use of dual siring on the paternity rates of embryos produced by SO-ET and 2) time of breeding (first vs second) on the frequency of paternity.

3.2: Materials and Methods

3.2.1: Superovulation Protocol

A total of 17 female alpacas (50 to 80 kg), ranging in age from 3-15 years old, and two males (male A and male B) of good fertility were used during this study. Females were group-housed in stalls while the males were housed individually and all procedures were previously approved by the IACUC at The Ohio State University.

Each morning, a male with known high libido was brought to the stall of females and allowed to mount the females for approximately 5 minutes per pen. Receptivity was determined when the female assumed sternal recumbency (cushed position) and allowed the male to mount; however breeding was not allowed at this time. Transrectal ultrasonography (US) utilizing a linear 7.5MHz prostate transducer (UST-660-7.5, Aloka,

Japan) was performed on receptive females to determine ovarian status. For ultrasonography, animals were manually restrained in the stall, either standing or in a cushed position. Prior to insertion of the transducer, approximately 20cc of lube was inserted into the rectum and a small amount of lube was also applied to the end of the transducer. During the US exam, digital still images and videos were recorded using a digital recorder and the size and number of all follicles as well as the time and number of ovulations were recorded. Receptive females displaying a dominant follicle $\geq$ 7 mm in diameter were enrolled in the study and received 1,000 IU hCG IV (Chorulon, 1,000 IU/mL, Intervet, Merck, USA) or 100 ug GnRH IM at that time (day 0) to induce ovulation and emergence of a new follicular wave. Females were monitored twice daily by US to confirm ovulation of the dominant follicle. Figure 1 outlines the protocol used in this study.

Approximately 60 hours after ovulation induction, treatment with porcine Follicle-Stimulating Hormone (pFSH) (Folltropin, 20 mg/mL, Bioniche, Canada) to induce growth of multiple pre-ovulatory follicles was initiated. pFSH was administered intramuscularly in the semimembranosus or semitendinosus muscle twice daily at decreasing doses (Table 2). Treatment was discontinued when half of the growing follicles in the cohort reached $\geq$ 7mm in diameter or continued for a maximum of 7 days.

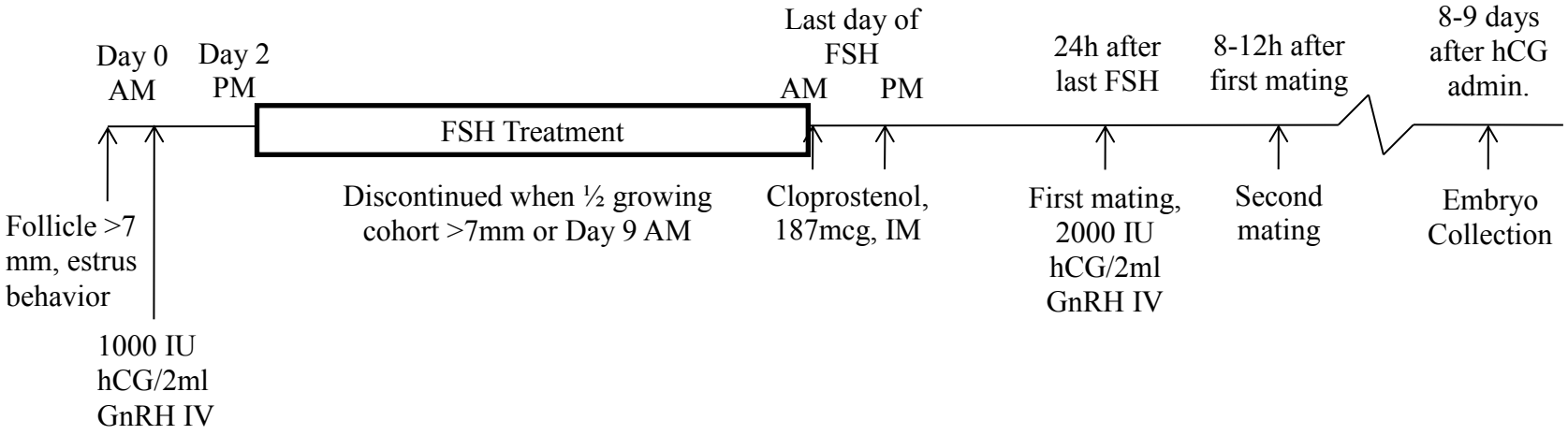

Figure 1: Superovulation schedule

Day	Dose (mg)
2 – PM	50
3 – AM/PM	50/40
4 – AM/PM	40/30
5 – AM/PM	30/20
6 – AM/PM	20/20
7 – AM/PM	20/20
8 – AM/PM	20/20
9 – AM	20

Table 2: pFSH treatment protocol

On the last day of pFSH treatment, females received two doses of 187mcg of Cloprostenol (Estrumate, 250mcg/mL, New Jersey, USA) IM approximately eight hours apart to induce luteolysis. Females were then exposed to males starting 24 hours after the last pFSH administration. If the animal was not receptive to the male, additional doses of cloprostenol were administered twice daily until breeding or for a maximum of 4 days.

Blood samples (3 mL) were collected using a 20g 1" needle on a 3cc syringe from the jugular vein for later progesterone analysis. Blood was obtained at the time of the first hCG/GnRH administration, on the last day of pFSH treatment, daily until breeding, on the day of breeding, and the day of collection. Blood samples were centrifuged (800G for ten minutes) and serum was transferred into a 1.5mL cryovial and stored in a -80^0 freezer for later analysis.

3.2.2: Breeding

Two males of good fertility (male A and male B) were used in this study. Fertility of males was determined in a previous experiment (Coutinho da Silva et al., 2012). Each female was mated by the two males approximately eight to twelve hours apart and the

order of breeding (first or second) was randomly assigned at the beginning of the study, then alternated for subsequent females. All females were hand-mated and successful breeding was determined by visual and manual inspection. The length of each breeding and the interval between the first and second matings were recorded. Mating proceeded until the male ceased voluntarily. Immediately after the first breeding, females received 2,000 IU hCG IV or 100 ug GnRH IM to assist in ovulation induction.

3.2.3: Embryo Collection

Embryo collections were performed approximately 8-9 days after the first breeding. Females were sedated (Xylazine, 0.25 mg/kg, TranquiVed, 20mg/mL, Vedco, MO, USA) and the animal was secured in a cushed position on a hydraulic table. Additional sedation was giving as needed. The perineum was washed with warm water and iodine scrub. After drying with a clean towel, a sterile 25 cm sigmoidoscope (Welch Allyn, KleenSpec®, New York, USA) connected to a light source was inserted through the vulva into the vagina and the external os of the cervix was visualize.

A 12 Fr catheter (Cook®, Partner Animal Health, Michigan, USA) with a stylet was advanced through the cervix, and placed into the uterine body. If the cervix was not easily catheterized, 10 mg of Buscopan (20mg/ml, Boehringer Ingelheim, CT, USA) was administered IV to promote relaxation and dilation of cervix. Once through the cervix, the cuff on the catheter was inflated with five to seven mL of air and the stylet was removed (Figure 2).

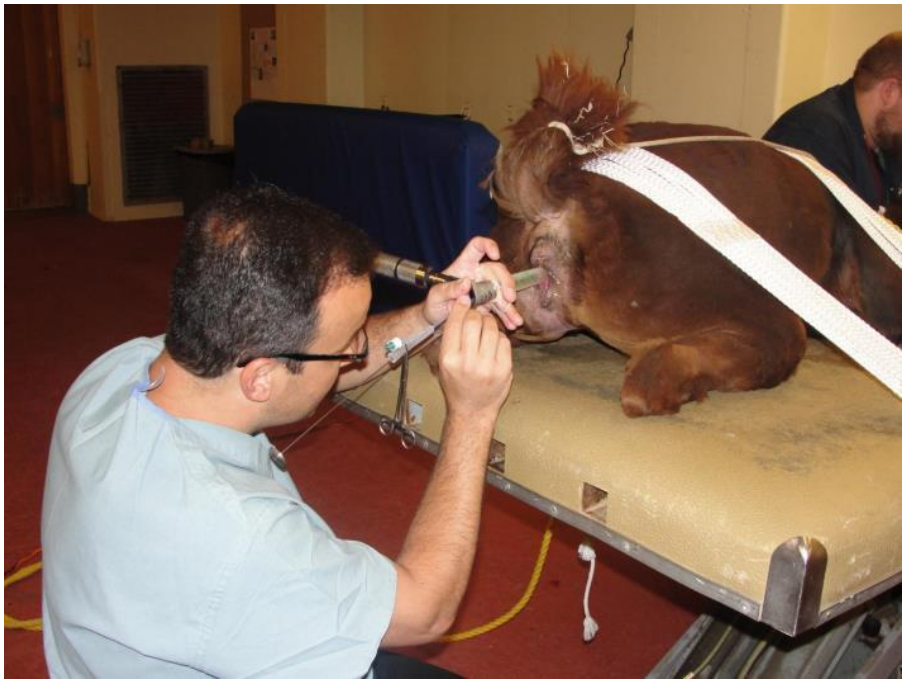

Figure 2: Cervical catheterization

The catheter was connected to a 1L bag of flush media (Emcare complete ultra-flush media, Agtech, Kansas, USA) using Y tubing (Agtech Rapid Flow, Kansas, USA) (Figure 3). Both uterine horns were flushed simultaneously using gravity flow. The recovered fluid was drained into a filter (Millipore® 75um embryo filter, Labstock, Dublin, Ireland) and this process was repeated several times. The line was flushed with remaining fluid in the bag. If fluid was retained within the uterus, 10 IU oxytocin (20 U/mL, Vedco, MO, USA) was administered IV to aid in uterine clearance of remaining fluid. Ultrasonography per rectum was performed throughout the procedure to monitor uterine filling and evacuation.

Figure 3: Collection performed using Y tubing

The contents in the filter were transferred into a search dish (90mm round polystyrene search dish, Agtech, Kansas, USA) and examined under a stereoscope (Nikon SMZ 1500, Nikon, New York, USA) for the presence of embryos. The number of embryos collected and stage of development were determined. Embryos were rinsed through multiple drops of phosphate buffered saline and individually transferred into cryovials with minimal volume of medium (< 10 ul). Embryos were stored in a -80^0

freezer until DNA analysis for parentage determination. Embryos were submitted for parentage determination via microsatellites to UC-Davis Veterinary Genetics Lab.

3.3: Statistical Analysis

T-test was used to test for significant differences in mating times, day of collection, progesterone concentrations, and ovulations. Fisher's exact test was used to test for significant differences in embryo parentage. Data presented as mean ± SD.

3.4: Results

Administration of FSH was successful in inducing growth of multiple pre-ovulatory follicles in 20/21 (95%) of cycles (Table 3). Females were treated with FSH for an average of 5.5 ± 1.4 days. After treatment, four females failed to become receptive to the male and were eliminated from the study. Therefore, embryo collection was attempted in 16 cycles and successful catheterization of the cervix was achieved in 94% (15/16). The female that was not catheterized was confirmed to have a cervical deviation on postmortem exam. Forty-six embryos and eight unfertilized oocytes (UFOs) were recovered from 15 uterine flushes. The average number of embryos collected per flush was 3.13 ±3.1 (range 0-10), with 11/15 (73%) flushes yielding at least one embryo.

# of cycles	# cycles w/> 2 follicles	# cycles bred	# uterine flushes	# of embryos	# of UFOs	Embryos per flush	# of flushes with ≥1 embryo
21	20 (95%)	16 (80%)	15 (94%)	46	8	3.13 ±3.1	11 (73%)

Table 3: Superovulatory response, breedings, and collection rates

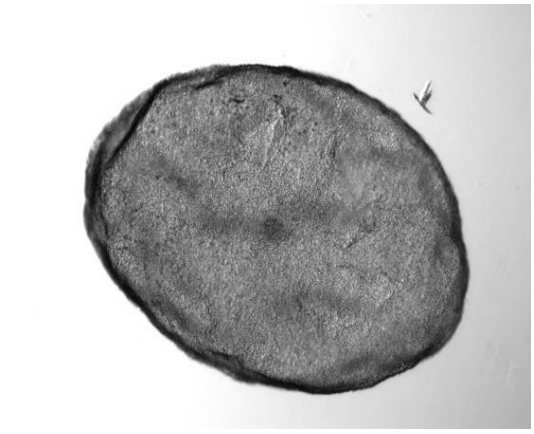

Figure 4: Day 8 embryo

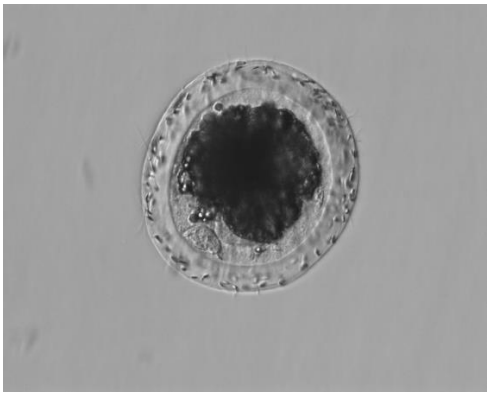

Figure 5: Unfertilized oocyte with accessory sperm

The mean number of cloprostenol injections females received at the end of FSH treatment was 3.4±1.7. There was no difference ($p>0.05$) in the length of mating between male A (20.3±4.2 mins) and male B (21.1±5.2 mins). The interval between first and second matings and number of ovulations were also similar between breeding order (i.e. A-B vs B-A) (Table 5). Nine females were flushed when male A bred first and six

females were flushed when male B bred first with an equal number of embryos obtained from each group (A-B = 23 embryos; B-A = 23 embryos).

Ten flushes were performed eight days after breeding, with seven being successful, and five flushes were performed nine days later, with four being successful. There was a tendency for number of ovulations between day eight and day nine collections to be different (P=0.075), 10±4.4 and 6±1.6 ovulations respectively. On day eight, a mean of 4.2±3.3 embryos were collected per female and was significantly different compared to the collection rate on day nine at 0.8±0.45. There was no significant difference in the number of successful flushes between groups. The average serum progesterone concentration at the time of flush was 27.1±16.7. Concentrations of serum progesterone at the time of flush were not significantly different between flushes performed on day eight compared to day nine, 24.4±17 and 32.6±16.6 respectively (Table 4).

Day of Flush	# of flushes (n)	# of ovulations	# of embryos	# of successful flushes	Serum progesterone concentration (ng/ml)
8	10	10±4.4[a]*	4.2±3.3[a]	7 (70%)[a]	24.4±17[a]
9	5	6±1.6[a]*	0.8±0.45[b]	4 (80%)[a]	32.6±16.6[a]

Table 4: Day of flush collection rate and serum progesterone concentrations
Values with different letters within each column are different P<0.05, *tendency (P=0.075)

Male order	Length of breeding (min)	Interval between 1st and 2nd breeding (h)	# of ovulations	# bred	# of Ovulations per cycle	# flushed	# of embryos
Overall	A = 20.3±4.2 B = 21.1±5.2	10.37±1.1	137	16	8.6±4.0	15	46
A-B	A = 20.6±4.7 B= 21.6±4.4	10.42±0.94	70	9	7.8±4.0	9	23
B-A	A = 20±3.8 B = 20.4±6.4	10.32±1.4	67	7	9.6±4.0	6	23

Table 5: Breeding data

Both male were proven fertile by a previous experiment based on embryo collection rates where male A had a 49% collection rate and male B had a 43% collection rate. Forty-six embryos were submitted for parentage testing; however, DNA amplification and parentage determination was only successful in 50% (23/43) of the embryos. The order of breeding did not affect the paternity of embryos. Overall, more embryos were sired by male B (22/23; 96%) than male A (1/23; 4%) (Table 6).

Breeding	n	Male A (%)	Male B (%)
A-B	6	0^{a}	6^{b} (100)
B-A	17	1^{a} (6)	16^{b} (94)

Table 6: Embryo data based on mating order and parentage
Values with different letters within each row are different ($P<0.05$)

3.5: Discussion

Assisted reproductive techniques have been used in many other species, including cattle, mares, and camelids in order to improve the overall genetics of the herd. In our study, superovulation was achieved in 95% of the cycles. This result was in agreement with other studies evaluating SO protocols involving the use of FSH in llamas (90%) and camels (89%); however it was higher compared with results obtained in alpacas (57%) (Ratto et al., 2005; Ratto et al., 2007; Vyas et al., 2004).

In our study, we administered decreasing doses of FSH for a variable length of time, based on the follicular growth of each animal. The strategy for superovulation used

in our study was derived from studies in cattle and horses (Lerner et al., 1986; Logan et al., 2007; Scoggin et al., 2002). In these species, decreasing doses of FSH were used in order to promote the growth of more follicles yet allowing the follicle to mature during treatment. The use of decreasing doses of FSH (52, 48, 40, 32, 28 mg divided in twice daily injections) has been previously reported in alpacas (Huanca et al., 2009a). In our study, the daily dose of FSH was decreased by approximately 20% and limited to a maximum dose of 400 mg of pFSH.

During four treatment cycles, females successfully developed multiple dominant follicles but failed to become receptive to males. The lack of response to $PGF_{2-\alpha}$ in these females was confirmed by elevated serum progesterone concentrations (i.e. > 1ng/ml) even after > 6 doses of $PGF_{2-\alpha}$. $PGF_{2-\alpha}$ was administered starting on day 6 post-mating, at a time that the CL is expected to be responsive to $PGF_{2-\alpha}$. A recent study evaluated the effects of a single $PGF_{2-\alpha}$ administration at different stages during the luteal phase in llamas and documented that complete luteolysis is achieved starting six days after induction of ovulation following a single administration of $PGF_{2-\alpha}$, with variable results on day five, and no effect prior to day five (Bianchi et al., 2012). A potential explanation for the lack of response observed in our study is that these females may have ovulated prematurely during FSH treatment and the newly formed CL was not susceptible to $PGF_{2-\alpha}$.

The mean number of days of pFSH treatment was 5.5±1.4 with 63% (10/16) cycles treated for five or six days. In cattle, most superovulation protocols utilize decreasing doses of FSH for four days (Mapletoft et al., 2002). Based on our results, it

appears that female alpacas may require additional days of treatment. However, our study did not objectively evaluate the effects of the length of FSH treatment on the superovulatory response.

The use of exogenous FSH to induce superovulation has been linked with an increase in incidence of ovarian follicular cysts in ruminants (Todoroki et al., 2004). In our study, development of cystic follicles (i.e. anovulatory follicles > 14 mm) after FSH treatment was observed in 33% (7/21) of cycles. Our results are in agreement with previous reports in llamas (44%) and alpacas (34%) (Huanca et al., 2009a; Huanca et al., 2009b). Potentially, maturation of some dominant follicles is delayed or incomplete by exogenous FSH administration, resulting in the inability to respond to ovulation inducing agents.

We successfully catheterized the cervix and flushed the uterus in 94% (15/16) of the attempts. The unsuccessful catheterization of the cervix was performed in a female that had been previously catheterized successfully in a different cycle. At the completion of the study, a slight deviation in the cervix was discovered during the post mortem exam that was believed to be the reason for the difficulty of catheterizing the cervix.

In our study, embryos were collected either eight or nine days after breeding, which is one or two days later than conventional procedures for ET in camelids (Vaughan et al., 2013). The reason for delaying embryo collection in our study was to allow for collection of larger embryos, yielding more material for DNA analysis. In a previous study, our lab documented a significant increase in the diameter of the embryos collected

on day eight as compared to day six or seven after breeding (Coutinho da Silva et al., 2012).

Embryos were recovered in 73% (11/15) of the flushes with an average of 3.13±3.1 embryos per flush. Our embryo collection rate is higher than previously reported data in alpacas, 53% (Huanca et al., 2009a) and 60% (Ratto et al., 1997). Our average number of embryos was also higher than reports in camels (1.51 embryos), llamas (1.3 embryos), and alpacas (0.5-2.7 embryos) (Bourke et al., 1995; Huanca et al., 2009a; Vivanco et al., 2009; Vyas et al., 2004). There was however a significant difference in embryo recovery rate when compared to the day of collection. The day eight collection rate was 4.2±3.3 compared to day nine, which was 0.8±0.45 embryos/flush. This dramatic decrease in collection rate could be partially explained by the relative lower number of detected ovulations in alpacas flushed on day 9 than on day 8. The embryo recovery rate (i.e. number of embryos recovered per ovulation) in our study (33%) was slightly below previously reported rates for superovulated alpacas,41% (Huanca et al., 2009a) ,and llamas, 48% (Correa et al., 1997). A previous study by our lab documented a recovery rate of 47% from non-superovulated females (Coutinho da Silva et al., 2012) . In our study, we did not perform rectal manipulation of the uterus during the embryo collection procedure, which is in contrast to other studies in llamas and alpacas (Aller et al., 2002; Sumar, 2011). Potentially, manipulation of the uterus during the procedure may improve embryo collection rates by moving embryos into suspension and facilitating drainage of the fluid from the uterus.

Induction of ovulation at the start of the study was initially performed by administration of hCG. However, some females were unresponsive to hCG and thus, GnRH was administered in subsequent cycles. Potentially the lack of ovulation in response to hCG may have been caused by the development of antibodies, which are known to reduce the efficacy of hCG on other species such as cats, horses, and cattle (Giordano et al., 2012; Siddiqui et al., 2009; Sullivan et al., 1973; Swanson et al., 1995; Swanson et al., 1996). The females that did not respond to hCG were also used in a previous study that involved induction of ovulation using hCG. To our knowledge, this is the first report of alpacas failing to respond to hCG administration after multiple doses.

Of the 46 embryos collected, parentage identification was not possible for 23 embryos due to the lack of DNA amplification. The exact cause for lack of amplification remains unclear. The batch containing these 23 embryos was shipped separately from the second batch. Potentially, samples were thawed during transport and the embryos moved to the lid of the tube, before dehydrating. This would prevent DNA extraction since the lid of the tubes was not rinsed during processing. The lack of parentage information in 50% of the embryos collected did not affect our ability to determine the absence of an effect of the order of breeding of the paternity of embryos, as 96% of the embryos were sired by one male, regardless of the order of breeding.

The overall goal of our study was to develop a method to produce multiple embryos from different sires within the same cycle. In order to assure fair sperm competition and allow equal chances of fertilization to both males, our study utilized two highly fertile males. Moreover, breedings were performed > 12 hours before ovulation

allowing sufficient time for sperm from both sires to travel into the uterine tubes and complete capacitation.

Despite our attempt to control some of the factors that are known to affect fertility rates (i.e. male fertility, time of breeding in relation to ovulation), most of the embryos collected were sired by one male. We speculate that small differences in fertility between the two males may have been magnified during sperm competition within the female reproductive tract. Lastly, differences in semen quality between males could have been responsible for the disproportional paternity results. In other species, heterospermic insemination has been used to determine difference in fertility, with the more fertile male fertilizing a greater proportion of oocytes (Berger, 1995; Berger and Dally, 2001; Martin and Dziuk, 1977; Sharma and Hays, 1975). Therefore, it would have been advantageous to perform semen analysis on the males used in this study. However, semen collection and evaluation in male alpacas is challenging due to the lack of repeatable methods of semen collection and the gelatinous nature of the ejaculate, and was beyond the scope of this project.

3.6: Conclusion and Future Considerations

In conclusion, the superovulation protocol used in our experiment produced an adequate response in the majority of females. The embryo collection procedure was successful in recovering multiple embryos from females without the requirement for manipulation of the reproductive tract per rectum. The use of two different males may not be an efficient method to produce embryos from multiple sires in the same cycle. Further

studies should focus on the factors that affect fertilization rates during sperm competition using a similar model of dual siring.

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