Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

Faculty of Veterinary Medicine
and Animal Science,
Department of Clinical Sciences

# Inter-species embryo transfer in South American Camelids

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*Uppsala*
*2015*

*Degree Project 30 credits within the Veterinary Medicine Programme*

*ISSN 1652-8697*
*Examensarbete 2015:60*

# Inter-species embryo transfer in South American Camelids
## Interspecies embryo transfer mellan små kamelider

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*Degree Project in Veterinary Medicine*

***Credits**: 30 hec*
***Level**: Second cycle, A2E*
***Course code**: EX0736*

***Place of publication**: Uppsala*
***Year of publication**: 2015*
***Number of part of series**: Examensarbete 2015:60*
***ISSN**: 1652-8697*
***Online publication**: http://stud.epsilon.slu.se*

***Key words**: Alpaca llama interspecies reproduction Peru*
***Nyckelord**: Alpacka lama interspecies reproduktion Peru*

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## SAMMANFATTNING

Embryotransfer är en metod där ett embryo i tidigt stadium flyttas från en donator till ett mottagardjur som sedan bär dräktigheten till förlossning. Det innebär att donatorhonan kan donera flera embryon under en säsong då den vanligtvis endast skulle kunna producera en avkomma. Embryo transfer är således en metod för att snabba på det genetiska urvalet i en grupp djur. Bland de mycket ekonomiskt värdefulla alpackorna i Peru, där många av herdarna saknar alternativa inkomstkällor kan embryo transfer bidra till ett mer effektivt avelsarbete och i förlängningen en bättre levnadsstandard för alpackaherdarna och deras familjer. Studien beskriver ett experiment där ett protokoll för embryo transfer från alpacka till lama utförs under fältmässiga omständigheter på en forskningsstation i de peruanska Anderna. Forskningsstationen saknade rinnande vatten och fast elektricitet men var utrustad med fångstfållor och möjlighet att immobilisera djuren under proceduren. Experimentet pågick under två perioder under 2014, en under april med 48 försök och en under september med 20 försök. För att göra resultaten jämförbara eftersträvades att omständigheterna kring experimenten var så lika som möjligt, djur ur samma flock användes, hölls i samma område och proceduren utfördes av samma tekniker. Resultatet visar att embryo transfer går att utföra under de fältmässiga omständigheterna men att det finns en signifikant skillnad i hur lyckat försöket är beroende på om det utförs under djurens normala parningsperiod (januari till april) eller ej, främst beroende på alpackahonornas parningsvilllighet. En intressant observation är att lamorna i samma flock som deltog i ett parallelt experiment inte visade någon signifikant skillnad i parningsvillighet vid de olika tillfällena.

## SUMMARY

Embryo transfer is a method where an early stage embryo is transferred from a genetically valuable donor female to a surrogate mother that carries the pregnancy to term. This enables the donor female to donate several embryos during a period when she could normally only produce one offspring. The method has the capacity to speed up the genetic improvement in a group of animals. In rural Peru where many inhabitants lack an alternative source of income other than that which the alpacas generate, embryo transfer can potentially aid in the breeding of the economically important animals. The paper describes an experiment in which a method for embryo transfer is tested under simple conditions at a field station in the Peruvian Andes. The field laboratory lacked running water and electricity but was equipped with basic restraining pens and immobilization equipment. The method used is based on one which was previously found to be successful, but was simplified to fit the basic conditions. The experiment took place during two periods in 2014, the first one in April with forty-eight attempts and the other in September with twenty attempts at embryo transfer. Other than season, no factors were altered; the same procedure, the same animals, and the same operators were utilized. The result showed that it is indeed possible to perform embryo transfer under spartan conditions but that there is a significant difference in overall success rate depending on whether the experiment is performed within or out of the normal mating season of the alpacas (January to April), mainly due to lack of libido among the females. An interesting observation is that the llamas in the same herd in a parallel experiment did not show season variability in their willingness to mate.

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## INTRODUCTION

There are four types of South American camelids, two of which are domesticated, the alpaca (*Vicugna paco*) and the llama (*Lama glama*); the other two being wild, the vicuna (*Vicugna vicugna*) and the guanaco (*Lama guanicoe*). All four are likely to have a shared common ancestor with the Asian and African camelids (*Camelus spp.*); a now extinct animal that lived in North America about 3 million years ago. The alpaca and the llama are believed have been domesticated 6000 – 7000 years ago in the highlands of Peru (Wheeler, 1995). The alpaca is kept for its fine quality fiber which is used in textile production whereas the llama is mainly used as a beast of burden. In addition, both species are slaughtered for their meat. Highly valued and cherished by the Incan and Pre- Incan societies, the two domesticated species faced near-extinction at the time of the European invasion and subsequent colonialism. The population was decimated by 80-90% in less than 100 years (Wheeler, et al., 1995). This population crisis most likely necessitated hybridization between the two species which has led to an overall reduced quality of the alpaca fiber. Since then, Peruvians and others alike have faced the task of regaining the exceptional fiber quality that the alpaca has the potential to produce, as found among the Incan mummies. This calls for extensive breeding with a well-defined goal.

### *Importance of South American Camelids in Peru*

Around 3 million alpacas live in Peru today, constituting the majority of the world alpaca population (Kershaw-Young, et al., 2012) (DAP, 2015). Although Peru is developing quickly as a nation, 23% of the population is still living in poverty as of 2013 and many of those are living in rural areas (WB, 2015). A specialized agency of the United Nations, The International Fund for Agricultural Development (IFAD) has identified a number of important factors which contribute to rural poverty in Peru (IFAD, 2015) among these being:

- Inadequate agricultural research, training and financial services
- Ineffective animal and plant health services

The alpacas' high quality fiber is a very important source of income for the local farming community of the Andes, where few other domestic species can graze and reproduce successfully.

In recent decades international interest in a developed alpaca industry has grown rapidly (Espinoza, 2010). The export of Peruvian alpacas is regulated by the Peruvian government; however, the high demand for these animals on the international market has led to unauthorized export over the last few years. In the latter half of the 20th century, interest in refining alpaca fiber blossomed and developed into an international industry with an impassioned following worldwide. It is not uncommon for high quality machos (male alpacas) to be sold for hundreds of thousands of dollars (Saitone & Sexton, 2007). The Peruvian government limits the number of exported alpacas, which in theory should protect the domestic gene pool; however, the resulting high demand has made illegal export on the black market extremely lucrative. The illegal export consists mainly of those animals with a high genetic value. This fact alone threatens to further impoverish the Peruvian alpaca gene pool and endanger the important fiber industry which stands for a large share of the local export market value (Espinoza, 2010). This export does not only impact the domestic gene pool negatively; it is likely to have a multitude of negative effects on animal welfare and disease control (Saitone & Sexton, 2007).

Peru's population has a wide variety of ethnic origins. The Altiplano region, where most alpacas are bred, is one of the poorest areas of Peru, mainly populated by indigenous Quechua and Aymara-people. A study about the poverty of alpaca herders in the town of Puno off the Titicaca shore states that the likelihood of escaping poverty is 17 times greater if the household has taken action to improve the genetic quality of the livestock herd (Kristjanson, et al., 2006). Such actions could, for example, include using one male to mate with each female and thus retain information of the paternal lineage of the offspring, and to ensure the selected parent animals are of compatible color and fiber type.

The two different phenotypes of the alpaca, the huacya (Figure 1) and the suri (Figure 2) are not considered to be breeds since two suri bred together can produce a huacaya offspring - it is merely a trait. The huacaya alpaca has a dense coat similar to that of the sheep, while the suri alpaca has longer, straighter fiber. At first sight the suri fiber appears as dread locks, they are however on closer inspection found to be unfelted.

It is not desirable to crossbreed suri with huacaya as different qualities are appreciated in the different fiber types.


**Figure1 Huacaya**

Figure 2 Suri

Before the European invasion of South America only three mammals were domesticated on the continent, the guinea pig, the alpaca and the llama (Dimond, 1999). Since then a multitude of animals have been introduced to Peru, but none have proven to adjust satisfactorily to the very specific and demanding climate. Some species harm pastures too much and others suffer and die from altitude sickness.

## LITERATURE REVIEW

In general, the subject of camelid reproduction is a relatively uninvestigated field of research. Embryo transfer in general and the narrower field of interspecies embryo transfer in particular would seem to require further development before many conclusions can be drawn. Few of the reports published on the subject give conclusive results and even fewer are applicable to the conditions of the Altoplano regions of Peru. Despite recent interest in this field of study, much of the available literature is outdated. Alpacas have a relatively long mean generation span, with females reaching sexual maturity at age 2-3. The gestation period of the alpaca is 11 months, and the birth of more than one cria (baby-alpaca) is atypical, leading to slow genetic advancement. Traditional agrarian methods still hold sway in the Andean regions where veterinary service and contemporary animal husbandry techniques are not readily available. As such, of the alpacas born in the Peruvian Andes, roughly half survive to weaning. Most fatalities occur due to gastrointestinal overgrowth of *clostridia spp.* followed by secondary septicemia (Fernandez-Baca, et al., 1970).

### *Reproductive Physiology of South American Camelids*

Camelid reproduction is unique. Unlike all other ruminants, camelids do not display estrus behavior but are instead induced ovulators, with waves of follicular growth that sometimes overlap (Vaughan, et al., 2003) (Adams & Sumar, 1990). Most female alpacas have a follicle large enough (above 7 mm) to ovulate if given enough stimulation (Bravo & Sumar, 1989). These stimuli include the sound and smell of a fertile male, the copulation act as well as components in the semen. Under natural conditions, they must receive enough stimulation in order to ovulate; the copulation is a lengthy procedure averaging 25 minutes (Vaughan, et al., 2003). Given enough stimulation the alpacas will ovulate 30 hours later.

Semen is deposited in the uterus following an excessive physical stimulation of the uterine wall carried out by the cartilage formation on the glans penis of the adult male. The males produce semen of extremely high viscosity and very low sperm count. The viscosity of the semen is attributed to excessive amounts of mucopolysaccharides (Garcia, et al., 1993) and it is believed that the viscosity of the seminal fluid keeps the sperm protected in the harsh environment of the uterus for the first few days after copulation. The post-copulation uterus contains bacteria, blood and possibly inflammatory substances post coitus (Bravo, et al., 1996). Despite the seminal fluids' protective nature, the uterine climate presents a difficult environment for the spermatozoa. The seminal fluid does also contain ovulation-inducing substances, (Kershaw-Young, 2012) and it is possible to induce ovulation solely by injecting seminal fluids intramuscularly (Adams, et al., 2005). The excessive and distinctive vocalization produced by the male during copulation known as "orgeling" is also a contributing factor to the induction of ovulation. It has been shown that a percentage of the alpacas will ovulate solely after being exposed to the sound of orgeling (Bravo & Sumar, 1989). Mating alpacas repeatedly during one reproductive period used to be commonplace but has proven to be unnecessary (Knight, et al., 1992), having little or no effect on the proportion of females ovulating or conceiving. It is also possible to artificially induce ovulation in female alpacas; hCG and GnRH have been shown to be effective if administrated in sufficient amounts (Sumar, 2013).

The early development of embryos consists of several stages, the first stages from conception to morula consists of cell division without growth and occurs within the zona pellucida. The zona pellucida is a membrane that consists of glycoproteins, originates from the ovary and serves the important purposes of inhibiting polyspermy and later of protecting the egg and early embryo. Depending on the species the zona pellucida breaks down 6-11 days post conception, the embryo can expand in volume and develops from morula to a blastocyst.

As in other species the embryo is fertilized in the fallopian tubes but, unlike in other animals, the embryo hatches out of the zona pellucida before it descends to the uterine body (Del Campo, et al., 1995). It is therefore not possible to directly extrapolate the technologies used in assisted fertilization of other ruminants such as artificial insemination or cryopreservation of semen, eggs or embryos.

Although the left and the right ovary are equally likely to ovulate and are similar in physiology, the uterus of the camelid is not physiologically symmetrical. Almost all pregnancies are carried in the left horn and a number of physiological traits are significantly different between the two horns. This means that the embryo, if conceived in the right oviduct, has a relatively greater distance to travel before it is possible to implant in the uterine wall of the left horn. This is debatably the reason why the camelids have a relatively high early embryo death (Fernandez-Baca, et al., 1970) (Daniel Diaz, et al., 2010).

It is safe to assume that the greater theme of the reproduction is identical between the small camelids even if some details may vary.

*Embryo Transfer*

Embryo transfer is a valuable method to increase the rate of genetic progress, and exchange important breeding material internationally, without the need to transport live animals. Embryo transfer has the potential to benefit all alpaca breeders: the rural alpaca farmers as well as the international alpaca-breeding community. Furthermore, embryo transfer would serve the same purpose while avoiding the spread of disease and minimizing risks to animal health and transportations costs usually associated with the international trade of livestock. Embryo transfer is the technique in which a fertilized embryo is moved to a uterus not belonging to the genetic mother. In other species and in particular humans; it is often used in combination with in vitro fertilization (IVF) where the egg and the spermatozoa are placed in a petri-dish and an embryo is conceived outside of a living organism.

In animal husbandry and breeding, embryo transfer is a method to more fully utilize the reproductive capacities of the most valuable females. Embryo transfer has the potential to revolutionize the selection of dams for breeding in much the same way as artificial insemination has revolutionized the selection of sires. It has the advantages that the selected female donor animal does not have to become pregnant but is still available to participate in breeding. For example, mares can continue be used in competitive training and still produce offspring. As mentioned previously, embryo transfer also enables the possibility of importing valuable animals without the negative effects concerning animal welfare and disease control. It also enables the same valuable female to produce many zygotes for transfer during the period in which she would normally be able to produce only one offspring. This is particularly valuable following multi-ovulation protocols in species that normally only carry a single fetus.

Embryo transfer is a widely used technique in cow breeding. Between 2010 and 2012, between 500000 and 800000 embryos were transferred yearly (Stroud, 2012). The International Embryo Transfer society has developed a grading scale to evaluate the quality of bovine embryos obtained from flushing (Bo & Mapletoft, 2013). This scale is not entirely applicable to the camelid embryos as almost all embryos found from flushing the uterus of camelids have developed to stage 8 and the evaluation is in part based on the quality of the cells in the zona pellucida which is not present at this stage.

Embryo transfer can also be utilized as a method to help preservation of endangered species (Niasari-Naslaji, et al., 2009)(Amstislavsky, 2006). The first interspecies embryo transfer from the critically endangered Bactrian camel to the more common dromedary was successfully carried out in 2009 and can be a valuable tool in their preservation efforts. The method could also be applied to assist the preservation of the vicuña, the genetic ancestor to the modern alpaca, which is in risk of becoming an endangered species according to the IUC (Lichtenstein, 2012)

Since the development of non-surgical embryo transfer, the procedure does not involve general anesthesia. The method used in this experiment is non-invasive and involves negligible impact on animal welfare. To avoid complications related to rectal palpation, epidural lidocaine was administered, care was taken to select operators with sufficiently small hands to not traumatize the rectum, and excessive lubricant was used to avoid injuring the animals.

*Interspecies Embryo Transfer in Camelids*

Embryo transfer in South American camelids was first reported in 1968 (Novoa & Sumar, 1968). The experiment was performed on alpacas; the technique used was based on the methods used for embryo transfer in ewes and is a surgical method where the embryos are obtained through laparoscopy. The first non-surgical embryo transfer was performed on llamas some years later in USA (Wilson Wiepz & Chapman, 1985). Since then, the technique has developed rapidly and almost every year since, a new article has been published. Embryo transfer is now so widely developed in South American camelids (Vaughan, et al., 2013) as well as in camels (Anouassi & Tibary, 2013) that it is considered a routine procedure in many countries. Interspecies embryo transfer on the other hand is still at an experimental stage. The first successful attempt at embryo transfer between alpaca and llama was reported in 2001 (Taylor, et al., 2001) followed by von Baer in 2003 (oral communication). There are many potential benefits of utilizing llamas to carry alpaca crias, llamas give birth to larger crias, possibly leading to healthier crias with a greater chance of survival. Llamas have a lower market value and are more easily reproduced (Sumar, 2008) as cited in (Vaughan, et al., 2013) and (Sumar, 2013).

*Season Dependency in South American Camelids*

Conflicting conclusions have been drawn on the matter of whether alpacas have a distinct reproductive season or not. Even though some experts consider the alpacas to be non-seasonal breeders (Vaughan & Tibary, 2006) (Fernández-Baca, 1993) it is customary to breed alpacas exclusively in the warmer and rainier season in the Andes.

There is credible evidence that the female reproductive physiology is not affected by the time of the year. An extensive study involving laparoscopic inspection of female internal genitalia showed no statistically significant seasonal variability. The ovaries are found just as likely to have one or more follicles above 7 mm year round, independently of rainfall, photoperiod or temperature (Bravo & Sumar, 1989). Another study confirmed these results, finding no difference between sexual receptivity, ovulation or fertilization rate year round (Fernandez-Baca, et al., 1972). Yet a third study comparing fertilization rate of females mated prior to culling showed no difference in rate between on season mating and off season mating (Sumar, et al., 1993). The latter study is of special interest to this work since the experiment was carried out at the same research station, IVITA at Abra la Raya. On the other hand, this study does not properly investigate the subject of seasonal dependency of the sexual *behavior* since all females were selected on the condition that they were receptive to the male. The sexual receptivity of female alpacas is a poor indicator of whether or not the female has a follicle large enough to ovulate (Vaughan, 2010).

The herd of alpacas and llamas are held in the treeless zone in the Altiplano; the average rainfall is between 250 and 500 mm annually and is almost exclusively limited to the months between November and April. The nutritional value of the pastures is heavily dependent on the amount of rain. Approximately 80% of the fetal growth occurs in the third trimester, and milk yield as well as growth of the newborn is strongly associated with the nutritional status of the dam (Burton, et al., 2003). Thus it would only be natural for these animals adapt to the season variability so that the births of the young coincide with the vegetation growth spike between January and March. It has been recorded that alpacas are less likely to copulate during dry season than rainy season in New Zeeland (Pollard, et al., 1994). There are no studies to be found involving survival rate of the crias based on the time of year they were born, though fertile pastures should increase the likelihood of survival.

### SPECIFIC OBJECTIVES

The objectives of this experiment are twofold; to evaluate the viability of embryo transfer from alpacas to llamas in an Andean field laboratory as a potential model for other camelids, and to investigate the role that seasonal variation might play in the successful reproduction of South American camelids in general.

The hypothesis of this work states that embryo transfer can be performed in the field with similar success rates as in larger facilities.

## MATERIALS AND METHODS

The protocol used for embryo transfer is based on the previously successful transfer protocol as described by Sumar (2013). The protocol was a single ovulation protocol where the embryos are obtained and transferred in a non-surgical manner.

### The Animals

The studied group of animals consisted of 10 female and 10 male donor alpacas and 20 female surrogate llamas though only two were utilized in the experiment due to limited numbers of obtained embryos. The animals live in Abra la Raya on the border between the two districts Cuzco and Puno, in Peru. They were kept in two separate herds, one consisting of males and one of females along with their suckling young. After weaning, the young alpacas and llamas are moved to a different pasture where they remain until two years of age when the females are reunited with their mothers and the males are taken to join the herd of male adults. The animals are owned and cared for by the field station IVITA, a filial to San Marcos University in Lima, Peru. The majority of the alpacas are of the huacaya phenotype but a few present the suri coat.

The llamas used in the experiment were characterized as either Khara or Chaco, two different phenotypes.

All common coat color are represented in the alpacas in the experiment, from black, dark chocolate, light brown to white, but the white ones are by far the most common.

The alpacas and llamas at the field station are kept in a traditional manner with extensive grazing on pastures at an altitude of 4300 m above sea level. The pastures are situated downstream from a glacier, giving the pastures continuous water supply, even during the dry season. The total herd size is around 320 animals, consisting of llamas, alpacas and sheep.

The animals are regularly treated with anthelmintic medication twice a year and the young ones are treated at the time of weaning. The animals do not receive any supplement food or minerals.

For this report, the males were matched with the selected females based on phenotype, huacaya with huacaya, suri with suri as well as color of the fiber, white with white and light brown with light brown etc. No other criteria were taken into consideration.

Two of the donors were suris and the rest were huacaya, five were currently nursing a cria, the others had already weaned their cria or lost it.

The body condition score of the ten donor animals ranged between two and five, distributed as shown in Table 1.

**Table 1 Body condition score of donor alpacas**

| | |
|---|---|
| Mean | 3.2 |
| Median | 3 |
| Sample standard deviation | 0.9189 |

The body weight of the same animals ranged between 57 and 68 kg, and were distributed as shown in Table 2

**Table 2 Weight of donor alpacas in kilograms**

| | |
|---|---|
| Mean | 62,2 |
| Median | 62 |
| Sample standard deviation | 4.517 |

The two recipient llamas weighed 99 kg and 95 kg and had a body condition score of three and two respectively. The larger one was of the Khara phenotype and the slightly smaller of the Chaco.

*Facilities and Equipment*

The laboratory was placed in close proximity to the alpacas' pastures and was suitable for holding animals. The most important equipment is listed below:

*For selection*

A pen to separate and sort the animals, a battery driven ultrasound and a probe suitable for trans rectal examination of the genital tract, paint to mark the animals fur, scale and a tripod for weighing animals.

*For embryo transfer*

Gasoline power generator, electric heating plate, electric lamps, tables, one for the laboratory equipment and one for the animals to rest during the procedure, electric heated basin and thermometer, thermos. Stereoscope, gloves, alcohol infused cotton swabs, syringes and needles for injections, petri dishes, Foley catheter size 14 with stylet and an agtech Zona filter (Orlando, USA), Kelly forceps, embryo transfer set, including straws and a transfer gun. Ropes and blindfold to restrain the animals.

## The Procedure

*The selection*

The animals used in this experiment were selected two days prior to the first mating. All females in the herd were examined rectally, alpacas by ultrasound and llamas manually. All pregnant females were excluded. Any animals too nervous for handling and all animals with apparent diseases were excluded. The majority of the individuals in the selected group was of proven fertility and was between 2 and 13 years old. Of the susceptible 23 alpacas, the ones with the widest pelvic size were selected as donors. There were 20 possible llamas available as recipients. The males were palpated and external genitalia were visually examined; all of them were adult and had reached sexual maturity. All males that showed any abnormality or sign of disease were excluded. No consideration was taken of the genetic value of the animals selected, fiber quality or other aspects that did not affect reproduction. The animals were simply elected for systemic and reproductive health and status (e.g. they had reached sexual maturity and were not currently pregnant).

*Mating 1*

The 10 female alpacas selected for embryo donation were introduced to one male each. Only five were receptive of the male. The recipient llamas were also exposed to a male in order to identify those that were receptive, but none were allowed to mate. Of the first nine females introduced to a male, only four showed interest in the male; and those four were treated with GnRH 1.5 ml (Conceptace buslorelin acetate 84ug/ml, Agrovet Market, Peru) intramuscularly. More receptors were not likely to be needed since so few of the donors mated. The other recipients were left untreated and were kept for the second part of the experiment that was taking place the next week.

*Embryo transfer 1*

On day six after mating 1

The experiment took place in a field laboratory in close proximity to the pasture where the animals are usually held. The facilities do not have running water or a fixed electrical supply. The electricity needed for heating, light and the microscope was provided by a portable generator run with gasoline. Water for basic hygiene was found in a nearby stream.

Flushing medium consisting of physiological saline solution combined with serum from crias of the same herd, at 1 ml per 1000 ml. Antibiotics (Pen-duo-strep; Penicillin procaine 200 000IU+ dihydrostreptocillin sulfate 250000 IU, Agrovet Market) were added to the flushing medium.

The five donor alpacas that were previously mated were restrained with a rope around the abdomen and hind legs, and their face was covered with a towel. They were placed on a table and given an epidural injection of 1.5 ml lidocaine (Lidocaina, hydrochloric lidocaine 2%, Laboratorios Unidos, S.A). The tail was wrapped in a cloth and tied dorsally to the rope encircling the abdomen. The perineal area was washed with soap and water after the rectal ampulla was manually emptied of fecal matter. The left hand of the operator was liberally lubricated and carefully introduced into the rectum of the donor. The vulva lips of the donor were held open by an assistant and the catheter was introduced in the vagina. The catheter used was size14 FR with a metal stylet fixed into position by a pair of Kelly forceps, thus making sure that the stylet's end was always secure in the very tip of the catheter with no risk of escaping through the opening at the side, which would lead to significant risk of harming the animal. With the left hand guiding transrectally, the Foley catheter was introduced through the cervix into the uterus. This procedure was performed with varying degrees of difficulty but the procedure never exceeded 15 minutes. Once the catheter was introduced into the uterus the balloon of the catheter was inflated with air and the pressure was manually controlled by the left hand of the operator. The correct position was confirmed by gently pulling the catheter caudally; if resistance from the cervix was apparent, the balloon was properly placed in the uterus and the stylet could be removed. The uterus was flushed two or three times with temperate flushing medium, approximately 100 ml each time. The operator massaged the uterus to confirm sufficient exposure to flushing medium. The collected media was filtered through an Agrotech filter. Once the lavage was complete, the balloon was deflated and the catheter removed.

The media was passed through a filter and the filtrate discarded, the last five to ten ml of the feed being collected and transferred to a petri dish. The filter was rinsed with 5 ml of medium sprayed through a needle gauge 16 and the rinsing fluid, as well as the feed fluid, was carefully examined by microscope for the presence of an embryo. The procedure was performed as rapidly as possible and the petri dish placed on a heated surface to protect any possible embryos from the cold.

After the procedure the donor females were treated with prostaglandins (Lutaprost, Sodium Cloprostenol 0.263 mg/ml Agrovet market) to induce luteolysis and thus ensure a new wave of follicles for the next mating.

Due to the lack of embryos no recipient llamas were needed at this stage of the procedure. For more information on how the transfer was executed see "embryo transfer 2"

*Mating 2*

Two weeks after the first copulation, the same donor females were once again introduced to one male each; this time only three alpacas accepted the male. Three recipient llamas were prepared to receive embryos and were thus treated with GnRH.

*Embryo transfer 2*

Following the same procedure described above, two embryos were obtained, both were hatched blastocyst and of good quality.

The embryos were washed with commercial embryo washing fluid Embryo wash solution (Syngro Holding, Bionisce, Pullman, WA. USA).

The embryos were isolated and placed in a transfer straw in the following manner; cotton soaked in polyvinyl alcohol, holding medium, air, holding medium with the embryo, air and holding medium. The procedure was done making sure the embryo was never shocked by fluctuating temperatures.

The recipient llamas were treated with 1.5 ml azopromazine (Promazil 1% azopromazine, Montana) intramuscularly. The llamas were restrained with a rope around their abdomen, placed on a table and given an epidural injection of 1.5 ml lidocaine as described above. The tail was wrapped in a cloth and tied dorsally to the rope encircling the abdomen. The perineal area of the females was washed with soap and water after the rectal ampulla was manually emptied of fecal matter. The left hand of the operator was liberally lubricated and introduced in the rectum of the llama. The straw with the embryo was placed in a transfer gun just prior to the transfer. The vulvar lips of the recipient were held open by an assistant and the straw was introduced in the vagina. The transfer pipette was guided through the cervix by the left hand of the operator and introduced in the left horn of the uterus; the contents of the straw were deposited.

*Follow-up*

The outcome of the transfer was investigated by transrectally guided ultrasound on day 21, 90 and 180 after transfer, and the resulting births.

*Analysis*

The data obtained regarding how many copulations per trail, how many obtained embryos per flush and how many pregnancies per transfer was analyzed and compared with the data obtained in April using wolframalfa.com's Wilson score confidence interval for binomial parameters with continuity correction. Assumed confidence interval was set at 95%.

## RESULTS

Of the twenty attempts at embryo transfer in September, twelve failed due to female lack of libido among the alpacas, two embryos of high quality were obtained and transferred to recipient llamas, and both llamas were pregnant 15 days after transfer.

Comparing this to data obtained in the same facilities in a larger study using the same method in April the same year (48 alpaca matings, 22 embryos were attained, and only four alpacas refused to mate) there is a statistical difference between the sexual receptivity in the two groups ($p<0.05$), leading to a significant difference in the overall success rate ($p<0.05$). (See figures 3, 4 and 5) Although there were no indications that the alpacas that did mate were less likely to ovulate and conceive when mated in September compared to April, or that the quality of the embryos was lower, the data are too limited to draw any conclusions.

*Statistical Analysis*

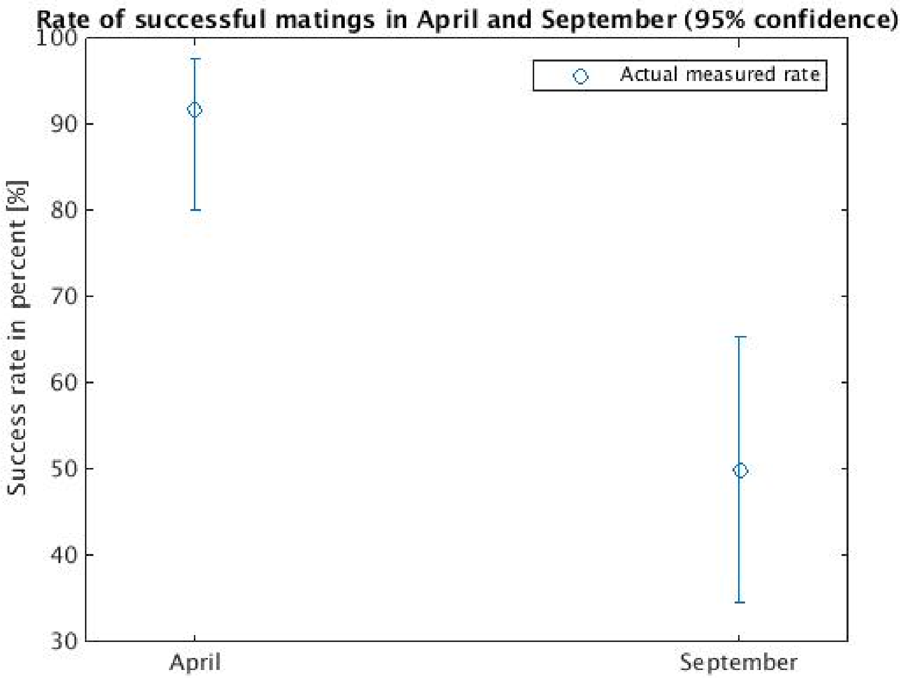



**Figure 3 Rate of successful matings in April and September**

One of the eight alpacas that did mate was diagnosed as being pregnant and was subsequently not flushed.

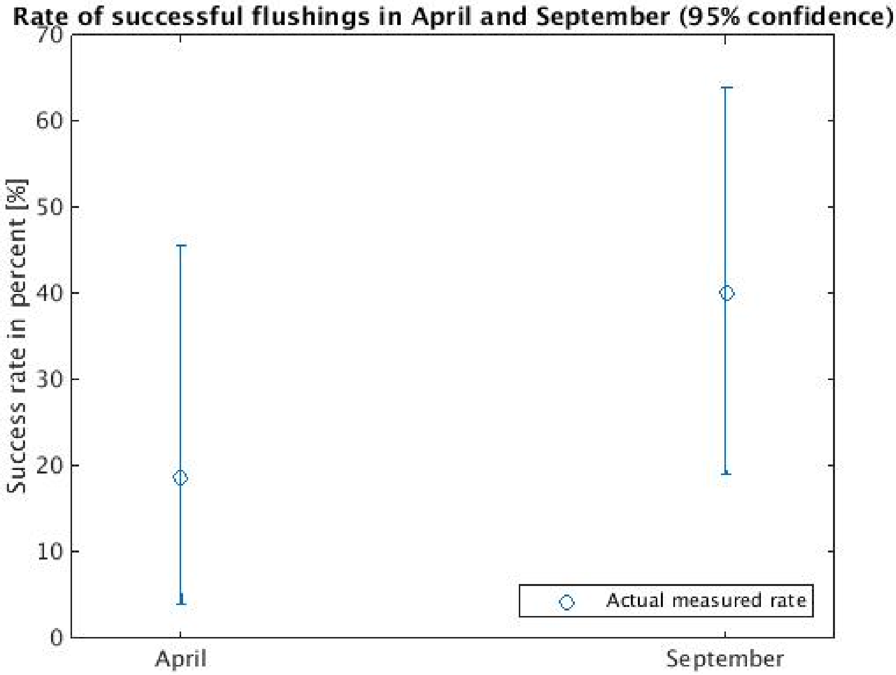
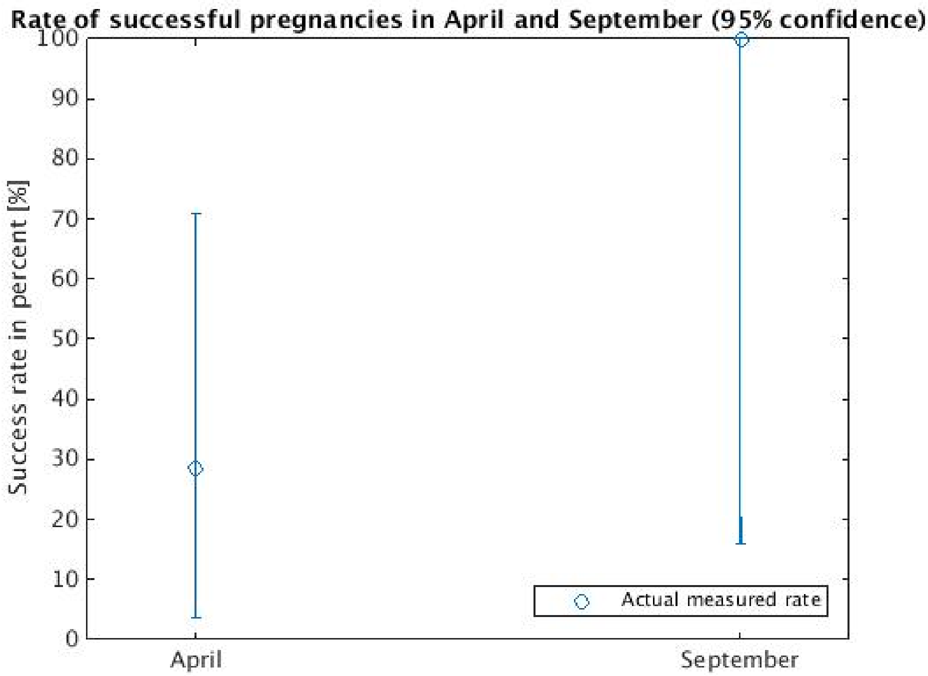



**Figure 4 Rate of successful flushings in April and September**

Of the embryos obtained in April, five were of quality grade 3, and three unfortunately dried out in the petri dish due to technical error. None of these embryos were




transferred.

**Figure 5 Rate of pregnancies per embryo transfer in April and September**

## DISCUSSION

Of the twenty attempts at mating in September, twelve failed due to lack of libido among the female alpacas, one of these females was excluded resulting in 7 females flushed. Two embryos of high quality were obtained and transferred to recipient llamas; both llamas were pregnant 15 days after transfer. The greatest loss of potential pregnancies was subsequently found in the conception and collection of embryos and not in the interspecies transfer and implantation.

Compared to the receptiveness of the females in the same flock earlier the same year within the traditional mating season, when 44 of 48 females mated, there is a strong statistical difference, seemingly dependent on season.

Information on the number of embryos found per flushing varies from study to study. Vaughan et al (2013) found that of 822 donors flushed in a single ovulation protocol, an embryo was encountered in 660 cases (80.3%). Those results are far better than found in the present study where 2 out of 7 flushings (28.6 %) were successful. This could partially be explained by the lack of ultrasonographic diagnostics of ovulation in this current study. It is unknown how many of the 7 females in this study actually ovulated. Sexual receptivity is not a sufficient indicator of presence of a follicle mature enough to ovulate (Vaughan, et al., 2003). Partially this failure remains unexplained, but factors that may result in the outcome of failed flushing include, but is not limited to, infertility of the paternal animal, delayed ovulation which would result in the embryo still in the uterine tube on the day of flushing, uterine infection resulting in an early destruction of the embryos and inexperienced embryo transfer technicians unintentionally losing embryos.

Of the 553 embryos transferred in Vaughan et al's 2012 retrospective study 235 resulted in delivery of a live cria, indicating a success rate of 42.5% in the single ovulation program. The much larger multi ovulation program showed similar results 1657 of 3962 transferred embryos resulted in a live cria born representing a success rate of 41.8%. Of the two transferred embryos in this study, both llamas were diagnosed as pregnant 15 days after transfer. It is not possible to compare this data to the results Vaughan reported since none of the crias in this current study are due to be born until after this paper is published.

The conclusion that can be drawn from these results is simply that transfer of embryos between different species of camelids is possible. This has been proven before (Niasari-Naslaji, et al., 2009) (Taylor, et al., 2001).

The present study in Peru is interesting since it is likely, or at least possible, that the physiology of the animals differs when exposed to different environmental factors such as food supply, altered disease spectrum, air pressure, and temperature, as well as daily and annual light variability. Moreover, it is unique in that it describes a working protocol that is carried out under very basic conditions in a field laboratory lacking both electricity and running water in the environment where most alpacas live. The more extensive research on alpaca reproduction has focused on animals living in a drastically different environment than their natural habitat, often on other continents for example (Pollard, et al., 1994) and

(Vaughan, et al., 2013). The animals used in this experiment are held in the same traditional manner that alpacas have been for centuries. Even though the results showed lower success rate when compared to the results reported from other countries, it is a great success to show that it possible to carry out the procedure in field conditions. This opens the possibility to bring the embryo transfer laboratory to the alpacas, rather than the other way around, leading to less stress for the animals and less risk of spreading diseases.

One might argue that the need for reproductive techniques is the greatest in these spartan conditions, not only because most alpacas live under these circumstances, but also because the shepherds living there are so very dependent on the income generated by the alpacas for their survival and to preserve the cultural heritage of the Andean highlands (Espinoza, 2010).

Seasonal dependency is apparent in this study and is a factor to take into consideration when breeding in areas resembling the natural environment of the alpacas. This statement is in agreement with other publications such as (Pollard, et al., 1994) and (Vaughan & Tibary, 2006). An interesting curiosity is that llamas do not seem to show the same season dependency. An experiment carried out at the same time using animals from the same herd where the similar procedure was performed, the only difference being that llamas served as donors and alpacas as recipients, showed a completely different result. Even though similar in body condition score, the llamas showed no unwillingness to mate (Tollig, 2015). In fact, the experiment showed good results; of the 20 llamas introduced to a male, 13 produced an embryo. It is difficult to speculate why this may be so, perhaps the alpacas have a stronger seasonal dependency than llamas, or perhaps other factors yet unaccounted for play a role. Further research is needed before definite conclusions can be drawn

*Limitations of embryo transfer*

The technique of embryo transfer still needs refining before it can properly substitute export of live animals. One of the major remaining problems is the need for a method of preserving the embryos, which would be required for the transportation of embryos over great distances. There is a well-described method for preserving bovine, equine and ovine embryos (Arav, 2014), including commercially available medium and equipment needed for the procedure. Since the camelid embryos are not harvested at the same developmental stage as bovine embryos, (hatched blastocysts versus morula), camelid embryos are not protected by the zona pellucid (Bravo & Sumar, 1989) (Bravo, et al., 1996). This gelatinous capsule shields the morula from the environment outside of the uterus while being transferred or stored.

Based on the results found in this study it is possible to transfer embryos between females living comparatively near to each other. It is still unknown how long an embryo can survive outside the uterus and, more importantly, what conditions are ideal for embryo survival. The method used in this study attempted to mimic the conditions in the uterus, trying not to shock the embryo with different temperatures, osmolarity etc., none of these assumptions have been scientifically tested for alpacas. The medium used was a commercial medium for bovine embryos, and the evaluation of the quality of the embryos before transfer was roughly based on that used for bovine embryos (Bo & Mapletoft, 2013). It is not unlikely that the evaluation scale is somewhat inadequate since excessive consideration taken of the shape of the embryo and the appearance of the zona pellucida which, as previously mentioned, is per definition not present in the hatched blastocyst.

In my opinion, it is not possible to completely rely on interspecies embryo transfer from alpaca to llama for the proliferation of the alpaca on a population basis since that would lead to a greatly reduced number of llamas born and a lack of receptor animals in the next generation. Neither should it be viewed as a substitute for a goal-oriented breeding program, but merely one of many tools to improve the breeding of alpacas in the Andean highlands.

I see a possible difficulty with the technique in that it will potentially impair the possibility of selecting dams based on some of their reproductive capabilities. Qualities such as the capability to carry a fetus to full term, low incidence of maternally-derived birth difficulties and twin pregnancies, high milk yield and good quality colostrum, and appropriate maternal behavior, are most likely heritable, but cannot easily be selected for in an embryo transfer program. If used incorrectly, embryo transfer can be used as a method to breed females that do not have the ability to mother offspring naturally, which is not only illegal in countries like Sweden but can possibly lead to reproductive problems in the next generation. A possible way to lessen this problem is to only use animals with a known reproductive history as donors.

I would like to stress that this method is by no means a way to avoid the very important work of genetic evaluation of the herd. Embryo transfer is not a short cut to a successful breeding program. Quite the contrary, I think it calls for extensive and unbiased evaluation of breeding females and careful matching with the right male. The technique is still a cumbersome and expensive procedure and most likely only pays off economically and genetically if enough time is spent on selecting only the best donor animals to participate. This job requires extensive knowledge of genetics, the herd, the breed, and the species as well as the market in which the product will be sold. The donors should be well above average for the genetic group. It is also important to take in consideration that the recipients will not contribute genetically to the next generation and valuable traits may be lost.

## Method Errors

*Animal selection*

The animals chosen as donors and recipients were selected based on their lack of pregnancy. The selection took place in a period of the year when generally all fertile female alpacas were pregnant. It is not possible to identify the reasons why they were not pregnant this September. Some of them may have failed to conceive or may have conceived but failed to carry a pregnancy barren females have significantly lower pregnancy rate than females that have had one or more cria (Daniel Diaz, et al., 2010). Others were used in the experiment in the spring and were therefore not pregnant at this time of the year. It is not hard to imagine that one lost pregnancy is correlated to future reproductive failures. For example Knight et al. in their report on fetal loss in 1995 describe two alpacas that lost their fetuses between day 60 and day 120 of pregnancy for three consecutive matings (Knight, et al., 1995).

It is unlikely that the experiment in April would have impaired the likelihood of conception in September as other scientists have been able to recuperate a multitude of embryos from the same female during a breeding season (Del Campo, et al., 1995).

The females and the males are all part of the same genetic group and thus related to each other. Although the group is supposedly large enough to reduce the possibility of inbreeding to an insignificant factor, the matching of parent animals based on fiber type and color undoubtedly leads to a much smaller genetic pool. The most common type of alpaca was the white huacaya, whereas suri coat as well as all other color than white most likely is a risk factor of unintentionally being coupled with a close relative such as son, brother or father, disputably leading to a smaller chance of conception as well as to reduced embryo survival.

## Conclusion

It is possible to carry out embryo transfer between alpacas and llamas in a rudimentary field laboratory. Any trials to repeat this experiment should be executed within the time period of the natural breeding season for best results. Alpacas in their natural environment are to be considered seasonal breeders.

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