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Interspecies embryo transfer in South American Camelids

A field study

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Inter-species embryo transfer in South American Camelids

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Embryo transfer mellan alpacka och lama
En fältstudie

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SUMMARY

The aim of this study was to investigate the possibility of performing inter-species embryo transfer under actual field conditions in the natural habitat of South American camelids. In this study embryos from llamas were transferred to alpaca recipients. In a parallel study alpaca embryos were transferred to llama recipients. All animals used were free of uterine and ovarian abnormalities detectable by rectal palpation and /or ultrasound examination. Embryos were harvested twice after single ovulation in 10 mated female llamas and non-surgically transferred to synchronized alpaca recipients. The field laboratory was located in the Peruvian highlands and was equipped with basic restraining pens but lacked electricity and running water. The use of alpacas as recipients for embryos of the slightly larger llama may be relevant for future preservation of the wild South American camelids. Furthermore we wanted to determine if it is possible to perform successful inter species embryo transfer under basic field conditions. The result of this study is promising with a 44% pregnancy rate after inter-species transfer.

SAMMANFATTNING

I detta examensarbete utreddes möjligheterna för lyckad embryoöverföring mellan två arter av små kamelider i deras naturliga habitat under fältmässiga förhållanden. I detta arbete beskrivs hur embryon från lamor flyttas till alpacka-surrogatmödrar. Parallellt med denna studie utfördes en annan studie med omvänd embryoöverföring. Alla djur som deltog i försöket var, enligt gynekologisk undersökning, fria från sjukdom. Tio stycken lamahonor parades naturligt varefter man samlade in embryon icke kirurgiskt och överförde dessa till hormonellt synkroniserade alpacka honor. Fältlaboratoriet som användes var beläget i det peruanska höglandet och saknade rinnande vatten samt elektricitet. Att använda alpackor som mottagare av embryon från den något större laman är intressant ur ett bevarandeperspektiv då de vilda små kameldjursarterna; vikunja och guanaco är hotade. Vidare undersöktes om det är möjligt att utföra mellanartlig embryoöverföring under basala fältförhållanden. Resultatet av studien är lovande med 44 % dräktighet efter embryoöverföring mellan olika arter.

Innehåll

INTRODUCTION

Compared to other domestic livestock the South American camelids, SAC, have long been neglected by the world of research. For 5000-6000 years alpacas and llamas have been crucial for the survival and culture of the people living in the high altitudes of the Andes (Wheeler, 1995). Approximately 300,000 families in South America still depend on breeding South American camelids for their main source of income (Aba, 1998). There are two types of South American camelid production, the traditional South American way where the animals are extensively kept in harsh but natural conditions on the high altitudes of the Andean highlands and, increasingly, the production of SACs under more advantageous conditions at sea-level where they are bred not only for fiber production but also as companion animals. In the last few decades, interest in research and the improvement of reproduction technology in these animals has grown, reflecting a growing interest among breeders in the U.S, Europe and Australia to breed these animals for shows, fiber production and as pets. The alpaca industry in the U.S has grown so rapidly that valuable animals are being smuggled out of Peru to be sold for enormous amounts on the black market. This has led to genetic erosion of the Peruvian alpaca herds. A study states that poor farming families in the Andes are seventeen times more likely to get out of poverty if the genetic quality of the livestock herd were improved by targeted breeding. It is the poorest of local Peruvian breeders that would benefit most from more efficient genetic gain. (Kristjanson, et al., 2006) (Sexton & Saitone, 2005). Assisted reproductive techniques such as artificial insemination (AI) and, to a lesser extent, embryo transfer (ET), are routinely used in other livestock production. The potential to increase the rate of genetic improvement in SACs with the help pf these techniques is great. The alpaca farmers of Peru would benefit from an efficient technique to increase the number of offspring from genetically valuable animals. Animal welfare would be improved as a result of the decrease in live animal transport and disease transmission that are associated with international animal trade. Since all SACs can interbreed it is possible to exploit the use of reproductive biotechnologies in reproduction management and in research (Gray, 1954, p. 289 in Wheeler 1995). Reproductive biotechnologies such as embryo transfer, could thus also enable the preservation of endangered species such as guanaco and vicuna.

Many studies on reproduction technologies in the llama have been conducted far from the high altitudes of their natural habits. Studies on alpacas are more common in South America but currently alpaca research is growing across the globe. Very few studies on the wild SACs have been done. The transfer of alpaca embryos to llamas has been done a few times before and was first reported in 2001 (Taylor, et al., 2001) . The use of the llama as recipient has many advantages. The llama is bigger, has a wider birth canal and gives birth to crias (alpaca or llama offspring) with higher bodyweight than the alpaca. For preservation reasons inter species embryo transfer with alpaca recipients is interesting. In a situation of acute need for reproduction biotechnologies to help an endangered group of animals it is not always possible to choose which animals one can work with, nor is it possible to choose the ideal location and facilities. It is essential to know which animals are possible recipients and under what conditions a positive result can be expected. The purpose of this study is to investigate the

possibility to use alpacas as surrogate mothers to llama crias under basic field conditions and thus gain knowledge to the field of inter-species embryotransfer. The usage of alpaca recipients to llama embryos has to my knowledge only been done twice before, with the result of two live llama crias (Sumar, 2013) and six live crias (Huanca, et al., 2012), respectively

LITERATURE REVIEW

Inter-species embryo transfer has been proved to be a possible as a tool to protect important genetic material from endangered species (Andrabi & Maxwell., 2006). It has been used in several species (Comizzoli, et al., 2000). A problem that often occurs is the sparse knowledge of the reproductive features and behavior of wild species since background data from research is often limited; a way to solve this problem could be to use a closely related but not endangered species as a model. This was done in 1981 in the first attempt at inter-species embryo transfer, when a gaur (Bos gaurus) embryo was successfully transferred to a Holstein cow (Stover & Westrom, 1984). In the camelid family a recent attempt to save the rare wild Bactrian camel (*Camelus bactrianus ferus*) resulted in the birth of four healthy calves. This was also the first attempt of inter-species embryo transfer in Old world camelids (Niasari-Naslaji, et al., 2009). In South American camelids two reports have been published. Taylor (Taylor, et al., 2001) reported the first two alpaca crias born from a llama surrogate mother. The study took place in Montana U.S.A; the crias were healthy and well accepted by their surrogate mothers and the rest of the llama herd. In 2008 Sumar published an article where transfer of alpaca embryos to llamas resulted in four pregnancies with three live born crias (Sumar, 2008, pp. In Sumar 2013, pp 175). In the same study 3 alpacas were diagnosed as being pregnant with llama embryos and the pregnancies resulted in 2 healthy llama crias. In both cases non-surgical methods of embryo collection and embryo transfer were used. In an article about the birth weight and growth curve of llama crias arising from a inter-species embryo transfer, Huanca et al. showed that both birth weight and weight at one year of age was higher in llama crias born to llama mothers than the ones born to alpaca surrogate mothers (Huanca, et al., 2012). However, this article does not mention technique, background or the result of the embryo transfer program. In Sumar 2013 a successful attempt with a guanaco embryo transferred to a llama female by Von Baer is mentioned; ”The first two reports of interspecies transfer in South American Camelids where published by Taylor et al. (2001) and von Baer (personal communication)” (Sumar, 2013, p. 175).

Background

South American camelids

Camelids are ruminants but have only three compartments of the stomach compared to the four compartments of other ruminants. Their digestion is much like other ruminants but they

have evolved independently of, and parallel to, that of the four compartment ruminants (Fowler, 2010). The camelids of South America are well adapted to the challenging conditions of the Andes just as the Old world camelids are adapted to the deserts of their habitats. The SACs live mainly but not exclusively in the treeless pastoral high altitude zone called altiplano in the Andes (Brown, 2000). Ninety percent of all llamas and alpacas in South America are found in Peru and Bolivia. SACs and Old World camelids belong to the *Camelidae* family. The Old World camelids belong to the genus *Camelus* and consists of the one-humped camel (*Camelus dromedaries*) and the two-humped camel *(Camelus bactrianus*). Recently the wild two-humped camel in Mongolia has been recognized as a third specie (*Camelus bactrianus ferus*). (Fowler, 2010) The South American camelids include four species of the llama genus; the domesticized Llama (*Lama glama)* and Alpaca, *(Vicugna pacos)* and the wild Vicuna (*Vicugna vicugna)* and Guanaco *(Lama guanicoe*). Both groups can interbreed within their genus (Kadwell, et al., 2001). It is commonly believed that the alpaca descended from the vicuna and the llama from the guanaco, but researchers have long argued this matter and some claim that both domestic SACs descended from domesticated guanacos (Wheeler, 1995). A more recent study using mitochondrial and microsatellite DNA showed that the alpaca did, in fact, descend from the vicuna and the llama from the guanaco (Kadwell, et al., 2001). The same paper also reveals proof of massive hybridization between alpacas and llamas, explaining the taxonomic confusion regarding the domestic forms of SACs in the resent past (Kadwell, et al., 2001).

When the Spaniards conquered the Inca society in 1532 a genetic bottleneck in the camelid population occurred. It is estimated that 90% of all llamas and alpacas were eliminated within a short period of time. The Inca society had strict breeding strategies: the State maintained vast herds to supply the army with pack llamas and the community with alpaca fiber. Findings of camelid mummies suggest that two phenotypes of llama were bred, one with a harsh fleece which was used as a pack animal, and another breed that produced fleece for textiles. Also the alpaca is believed to have been divided into two breeds, one with extra fine fiber and another with harsher fiber. Today the use of llama fiber for textiles is marginal and no breeds of llamas or alpacas producing fine fiber exist. The most probable cause of the coarsening of the fiber and lack of uniformity in llamas and alpacas today is hybridization of both species in the chaos that followed the fall of the Incan empire. (Wheeler, 1995) (Wheeler, et al., 1995). The hybridization has to some extent continued until today since some breeders mate alpacas and llamas together to increase bodyweight and amount of fiber of the “alpaca”. The fiber is then sold as “alpacafiber” to buyers that do not discriminate against the lower quality (Kadwell, et al., 2001). Serious alpaca breeders across the world are struggling to reconstruct the fine lines of alpacas that existed before the conquest of South America. Reproduction biotechnologies are potentially one way to reach that goal.

The Llama

The llama is the largest of the four llamoids. They weigh 110-250 kg and stand 100-120 cm at the withers. The llama has traditionally mainly been used as a beast of burden; the fiber of the llamas is harsher than the fiber of alpaca but has been used for blankets and rugs. Llamas are also a common source of protein in the Andean kitchen. The domestic llama features two

phenotypes, the Kara has less fleece on the neck and head whereas the Chacko is woolly on the neck, ears and head (Fig.1). Llamas can be multicolored, with coloration ranging from white to black and brown. Fleece quality is uneven and there is a wide variation of fiber diameter. The llama is considered to derive from the larger of the two wild SACs, the guanaco (*Lama guanicoe*). It is possible that two types of llama existed before the Spanish conquest, one kind with uniform color that was used for textiles and another breed that was a beast of burden and meat producer.

Fig. 1, *A group of lamas of kara phenotype a snowy day on the Peruvian Altiplano.*

The Alpaca

Alpacas *(Vicuna pacos*) weigh between 55 and 90kg and reach 75-95cm at the withers. They are bred and kept for their unique fiber quality. They have colors in all shades from white to brown and black. They are preferably uniform in color but spotted and multicolored animals do occur. There are two phenotypes of alpaca; the huacaya (Fig. 2) that has a dense, spongy fleece consisting of crimped fibers that are shorter than those of the less common suri phenotype. The suri has longer, straighter fibers that fall in waves on each side of the body. Alpaca fibre is and always has been highly valued by the textile industry.

Fig. 2.At the *Right; A group of female huacaya alpacas with llamas and Andean peaks in the background. At the Left; a suri alpaca*

Vicuna and Guanaco

Of all the South American camelids the vicuna is the one that possess the finest fiber, and its fiber is also the most valuable from the animal kingdom (Wheeler, 1995). It is also the smallest of the four SACs and lives wild in areas of extreme elevation in the Andes. It has been a severely endangered species but has recently reached a more stable population due to rigorous protection programs in the area. According to IUCN (International Union for Conservation of Nature) it is still at risk of being endangered. The Guanaco (*lama guanicoe*) is the largest of the South American camelids. It is widely distributed both in the highlands and at sea level from north of Peru to the south of Chile but the majority of the population lives in Argentina. IUCN labels both the Guanaco and the Vicuna as Conservation dependent species.

Reproduction in South American camelids

The South American camelids have unique features in their reproduction that lead to poorer conventional breeding results for alpacas and llamas compared to other domestic species. They have a comparatively long gestation period (341 and 344 days respectively) and both sexes are older when breeding can start (Sumar, 1996). In traditional Peruvian breeding systems mating of females is done at two years of age in alpacas and three years in llamas. It is not recommended to use males under 3 years of age for breeding even though both sexes can be fertile earlier. Puberty and sexual maturation is closely linked to individual nutrition state and body weight (Brown, 2000) (Sumar, 1996).

All camelids are induced ovulators with ovulation 1 to 3 days post coitus. Ovarian activity occurs as waves of follicular growth and regression which tend to overlap. The duration of each wave varies with an average time of 10-18 days. SACs have no estrus period and can be sexually active during the course of the whole year. In their natural habitat of the Peruvian

highlands both domestic and wild SACs breed during the warmest months of the year: December to March. (Aba, 1998) (Sumar, 1996) (Fowler, 2010). Llamas and alpacas have a bicornual uterus, the cervix is two to five cm long and has two to three ring like structures which can complicate insertion of uterine catheters. The ovaries are covered with a bursa and the oviduct is embedded in the mesosalphinx. The oviducts open into the uterine horns with a tight sphincter that makes retrograde flushing of oviducts difficult. The male has a fibrous penis with a harder cartilage tip that helps the penetration of the cervix. During intercourse the penis penetrate the uterine horn and the semen is deposited inside the uterus. Copulation lasts for approximately 15 to 40 minutes with the female in sternal recumbence. Ejaculation is continuous.

Camelid semen is a gelatinous, highly viscous mass in which the spermatozoa are retained. Unlike the spermatozoa in other domestic species the SACs´ sperm motility is oscillatory and not progressive when contained in the ejaculate mass (Bravo, et al., 1997). The sperm concentration and ejaculate volume are also lower than in bull or ram semen. The seminal plasma includes an identified protein that induces ovulation. (Kershaw-Young & Maxwell, 2012) (Bravo, et al., 1997) (Sumar, 1996). Fertilization takes place in the ampulla of the uterine tube. In alpacas and llamas the embryo reaches the uterus as a hatched blastocyst. The time at which the embryo enters the uterus varies; in alpaca it appears to happen 6.5 days to 7.5 days post mating, in llamas slightly later. (Fowler, 2010) (Taylor, et al., 2000) .

All camelids have a placenta of the diffuse epitheliochorial type. Placental capillaries are deeply indented in the chorionic and uterine epithelia during the latter part of gestation making the distance across the diffusion pathway smaller than in other species with epitheliochorial placenta. This is believed to be an adaptation to pregnancy at high altitudes. In llamas and alpacas ovulation occurs with equal frequency in both ovaries but although multiple ovulations do occur, twin pregnancies are very rare. It is likewise rare for the fetus to occupy the right uterine horn, the embryos that originate from the right ovary most often migrate to the left horn and attach there. It is thought that 98% of the pregnancies in alpacas are carried in the left uterine horn (Vaughan & Tibary, 2006) (Sumar, 1996).Maintenance of pregnancy seems to be totally dependent on the corpus luteum (Sumar, 1988). Maternal recognition of pregnancy happens by day 10 in pregnant llamas but the mechanism is not yet fully understood. (Del Campo, et al., 1995). Under natural conditions embryo mortality and conception rates are highly variable; the reasons for this is not fully understood but environmental and nutritional constraints may be important (Saun, 2008). It is estimated that 10-15 % of the pregnancies are lost within 60 days due to early embryotic death. Extreme cases of a 60-80% loss of embryo in the first 90 days have also been reported (Vaughan & Tibary, 2006)

Reproduction biotechnologies

Reproduction technologies are being used effectively in almost all domestic animals. As an example AI has revolutionized the dairy production worldwide. The reproduction

biotechnologies that are currently being used in camelids are generally similar to those used in small ruminants like sheep. (Miragaya, et al., 2006).The sheep has been a good model for the development of these technologies and was also the first species to be cloned (Tibary, et al., 2005). Camelids are not as well studied as other domestic species and offer new and different challenges for researchers in the field. Breeders and animals both have much to gain from the further development of these technologies in camelids, for instance export of frozen embryos and frozen semen could radically diminish costs and eliminate health problems linked with quarantine and transport of live animals. (Tibary, et al., 2005).

One challenge that has been identified is the understanding of the follicular dynamics of camelids. This has allowed development of synchronization and superovulation protocols from which embryo transfer technologies benefit. The ovarian reaction to the different superovulation protocols that have been tried is still variable but the technique is improving. The collection and preservation of camelid semen is another crucial field that is the focus of research. Data on pregnancies obtained by preserved semen are limited and low compared to other species. The high viscosity of camelid seminal plasma complicates progress to develop cryopreservation of sperm for AI since it hinders the contact between the sperm membranes and the cryoprotective compounds. It is also more difficult to collect semen from SACs because of the mating position as well as the semen composition. The males show less willingness to mount a dummy and ejaculate into an artificial vagina, which is the most common method used, than for example the ram and the bull. However, progress in this field has been seen lately (Bravo, et al., 1997). The use of cryopreserved semen could possibly lead to a drastic genetic improvement comparable to that seen in the cattle industry (Miragaya, et al., 2006). Research has also been done on in vitro oocyte maturation, in vitro fertilization, intracytoplasmic sperm injection and even nuclear transfer. (Miragaya, et al., 2006) (Tibary, et al., 2005).

Embryo transfer

Embryo transfer in alpacas is a well-developed technique that currently plays only a minor role in the breeding of SACs compared to other livestock. The technique enables a naturally fertilized embryo to be moved into the uterus of a female that may not be the biological mother of the offspring that she will produce. Even though the use of embryo transfer in SACs is far behind that of more conventional domestic animals such as cattle and sheep it is considered a standard procedure in many large breeding herds in Australia and North America. (Vaughan, et al., 2013) In Peru, where alpacas and llamas are being kept traditionally, such methods are still far beyond reach. The first recovery of an alpaca embryo was made surgically as early as 1968 by Novoa and Sumar, who flushed the uterus of a super ovulated alpaca in a retrograde fashion from the uterus towards the oviduct. (C.Novoa & Sumar, 1968, p. in Sumar 2013). In 1974 Sumar and Franco reported the first birth of an embryo transferred alpaca; surgical procedures for both the retrieval and transfer of the embryos were used here as well. The first time a non-surgical procedure without super ovulation of the female resulted in a live born llama was in 1985 (Wiepz & Chapman, 1985). A report on commercial embryo transfer programs from 2000 has given a 66% pregnancy rate in llamas (Taylor, et al., 2000). In 2008 a pregnancy rate of 40 % in embryo transfer

programs in alpacas was reported (Sumar, 2008, p. in Sumar 2013 p. 170) and Vaughan reports similar results of 43.2 % from Australia in a large retrospective study (Vaughan, et al., 2013). If early embryo development and the trigger of maternal recognition in SACs could be better understood it is possible that the pregnancy rates could increase. Still the use of embryo transfer can allow a donor to produce up to 10 offspring every year instead of only one, and even more offspring can be produced if the donor is superovulated. (Sumar, 2013). Further research in long term preservation of camelid embryos, either by cryopreservation and/or vitrification is needed. This technique could have a great impact on multiplication of camelids of high genetic value and could increase wild, threatened or genetically unique camelid populations (Miragaya, et al., 2006).

MATERIALS AND METHODS

Animals

All animals were kept free range in a group consisting of 300 female alpacas and llamas including crias. The males are herded on a different pasture in a group of 70 animals. The pastures are located in the Andean highlands, Abra de Raya, Marangani, Peru at an altitude of 4300 m above sea level. The experiment was done in September which is the end of the dry season in Peru. The pastures that the animals live on is meager at this season and consist mainly of the harsh Peruvian feather grass called "Ichu" which is endemic to the area. No extra feed was supplied during the experiment but the pasture where the animals were kept is located downstream of a glacier and therefor it is lush even during the dry season.

Ten non-pregnant, lactating llamas with a history of good fertility were to be used as embryo donors and thus were mated with ten llama males known to have sired crias previously. The males were examined visually and by palpation before mating. Twelve female alpacas, ten with a history of good fertility and two maidens, were chosen as recipients. Since September is not generally the breeding season most animals in the herd had been mated earlier and were now pregnant. The recipient alpacas were chosen after clinical examination including ultrasonografic evaluation of the uterus; only not pregnant, reproductively healthy animals were included in the study. The pelvic size of the recipient animals was also taking into consideration, animals with a narrow pelvis being excluded. The females were kept separated from the males during the experiment.

The experimental design described in this work was compressed to 41 days (Fig. 3).

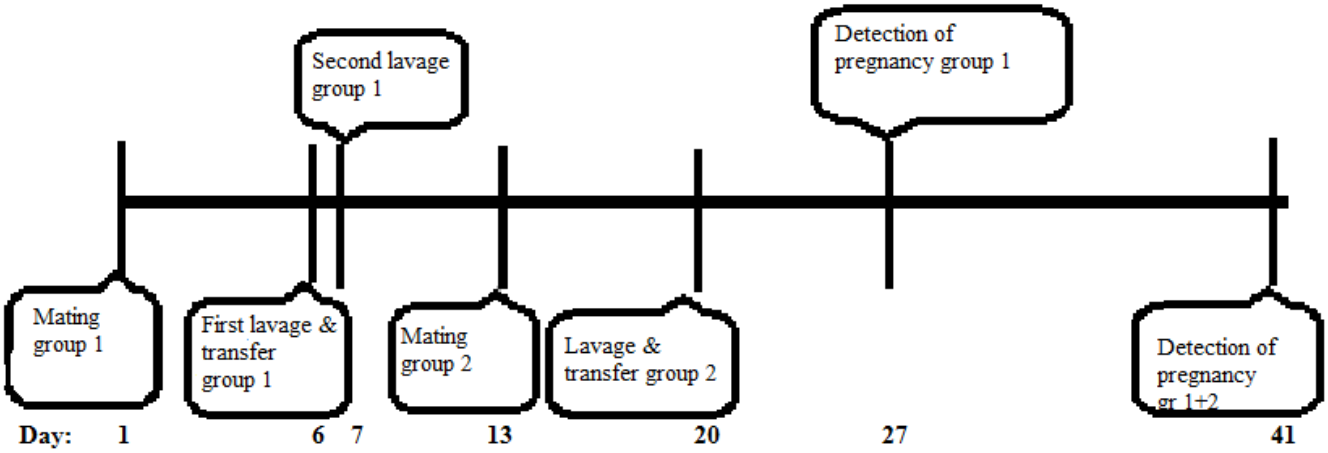

Figure 3. *Experimental design for the study*

The embryo donors´ age ranged between 3-11 years and their body condition was scored at a scale from 1 to 5 (Body condition score BCS) with 5 meaning obese. The BCS of the donating animals was 3-4. They weighed 72-121kg. The recipients were aged between 1. 5-8. 5 years, with BCS 2-5 (1 individual with BCS 2 and 1 with BCS 5) and they weighed 46-76 kg (Table 1 and 2) Two days prior to the first mating the donor llamas were chosen after a clinical examination including rectal palpation of the uterus and ovaries. Six of the donors where of chacko phenotype and four of kara phenotype. The recipients were mainly huacaya but the group included two suri. (see Table 2). All animals with any signs of disease were excluded.

Table 1. *Animals: Age, condition and phenotype of donor llamas*

Name	Date of birth	BCS	Weight	Phenotype	Lactating
L040	210310	3	97	K	YES
L027	170111	3	98	CH	YES
L074	50308	3	95	CH	YES
L039	60208	3	105	CH	YES
L038	280111	3	85	CH	YES
L042	110201	3	115	CH	YES
L059	70208	3	72	CH	YES
L014	30111	2	85	K	YES
L005	140103	3	121	CH	YES
L075	290311	4	110	K	YES

Table 2. *Animals: Age, condition, species and phenotype of recipient females*

Name	Date of birth	BCS	Weight	Phenotype	Lactating
	Alpacas				
H069	29 01 08	3	70	H	YES
H026	13 01 08	2	60	H	YES
H013	01 01 11	4	61	H	YES
H038	03 02 13	3	58	H	MAIDEN
S040	21 01 06	4	76	S	YES
H047	16 01 11	3	62	H	YES
H047	23 11 12	4	59	H	YES
S001	01 01 09	5	71	S	NO
H052	15 02 13	3	46	H	MAIDEN
	Llamas				
LO61	03 05 09	3	99	K	YES
L068	24 02 08	3	95	CH	YES
L017	19 01 06	3	125	K	YES

Conditions

The small field laboratory used is located at a high altitude and the work was performed in field conditions. The building used was approx. 3x3m and lacked running water and electricity. All equipment had to be brought by car. The equipment used was;

- Gasoline engine as power source
- Three electric lamps.
- Three tables, two for equipment and one for the animals.
- Scales and a tripod
- Electric heat plate
- Electric heated basin + thermometer
- Thermos
- Microscope
- Gloves, syringes and injection needles
- Petri dishes
- Foley catheter size 16 with mandrine
- Agrotech filter
- Forceps
- Embryo transfer set, including straws and pistolette.

- Ropes and blindfold to restrain the animals.
- GnRH 84ug/ml (Conceptase, Agrovetmarket s.a., Lima, Peru)
- Acepromazin, 1% (Promazil, Montana)
- Lidocain 2% (Lidocaina, Laboratorius unidos)
- Physiological saline solution mixed with serum from crias in the same herd (10ml/1000ml)
- Pencillin prokain 200 000IU+ dihydrostreptocillinsulfat 250 000 IU (Pen-duo-strep).
- Prostaglandin 250ug/ml (Lutaprost, Agrovetmarket s.a ; Lima, Peru)
- Embryowash solution (Syngro Holding, Bionisce, Pullman, WA. USA)

Day 0, Mating.
The 10 female llamas that were chosen for donation were weighed and mated with a male each. All of them were receptive to the male. The recipient alpacas were also weighed and exposed to a male in order to identify receptive females, although they were not allowed to mate. Six receptive female alpacas were treated with 1.5ml Gonadotropin releasing hormone (GnRH) intra-muscularly in order to synchronize ovulation with the donors. The other six recipients were left untreated and saved for the second part of the experiment.

Day 6, Embryo transfer
The embryo donors where tranquilized with Acepromazin, 1 ml 1% intramuscularly. One by one they were moved to the small field laboratory, restrained and placed on a small table in sternal recumbancy. Caudal epidural anesthesia was induce with 1.5ml Lidocaine 2%. The tail was tied up to the dorsum, feces where removed from the rectum and the perianal area was washed with soap and water. A silicon Foley catheter size 16 (5.3mm) was used. The catheter was guided transrectally through the cervix and into the uterine body. When the catheter was in the correct position the balloon of the catheter was inflated with 10-15ml air and the stylette was withdrawn. The flushing medium consisting of physiological saline solution mixed with serum from crias in the same herd (10ml/1000ml) and antibiotics (see brand above).An assistant used a 110ml syringe to flush the media at 38°C through the Foley catheter and into the uterine body. The uterus was flushed twice with ca 70-100 ml media/flush. Each lavage included rectal guidance and gentle massage of the uterine horns in order to fully evacuate the fluid. The fluid was filtered through an Agtech Zona Filter with a capacity of 120ml. After the lavage the catheter was rinsed with flushing media into the filter.

The contents of the filter were emptied into a petri dish and the filter was also rinsed with a small amount of medium. After the procedure the donors were injected with 1ml prostaglandin 250ug/ml (Lutaprost) to avoid pregnancy .The embryos were washed once with a commercial embryo wash. They were all hatched blastocysts and were classified according to their visual appearance (Grade 1- excellent, Grade 2- moderate to good, Grade 3- poor). Only Grade 1 to 2 were transferred (Fig 5). A pipette attached to a Uno-pet transfer-straw was used to load the embryo into the transfer straw. The transfer straw was loaded with; cotton, holding medium, air bubble, holding medium containing the embryo, air bubble, holding medium, polyvinyl alcohol (Fig.6). The loaded straw was stored on a 38°C heat plate until transfer which was executed within two hours.

When all the donors had been flushed, one recipient at a time was taken to the laboratory. The recipients were restrained but remained standing during the procedure. After washing of the perineal area with soap and water the pistolette with the straw containing the embryo was introduced into the vagina and its tip was transrectally guided into the uterine body where the embryo was deposited, preferably in the left uterine horn. The pistolette was covered with a plastic sanitary cover while passing through the vestibulum and vagina to protect the uterus from contamination. On the first day of uterine flushing only one embryo was found and transferred.

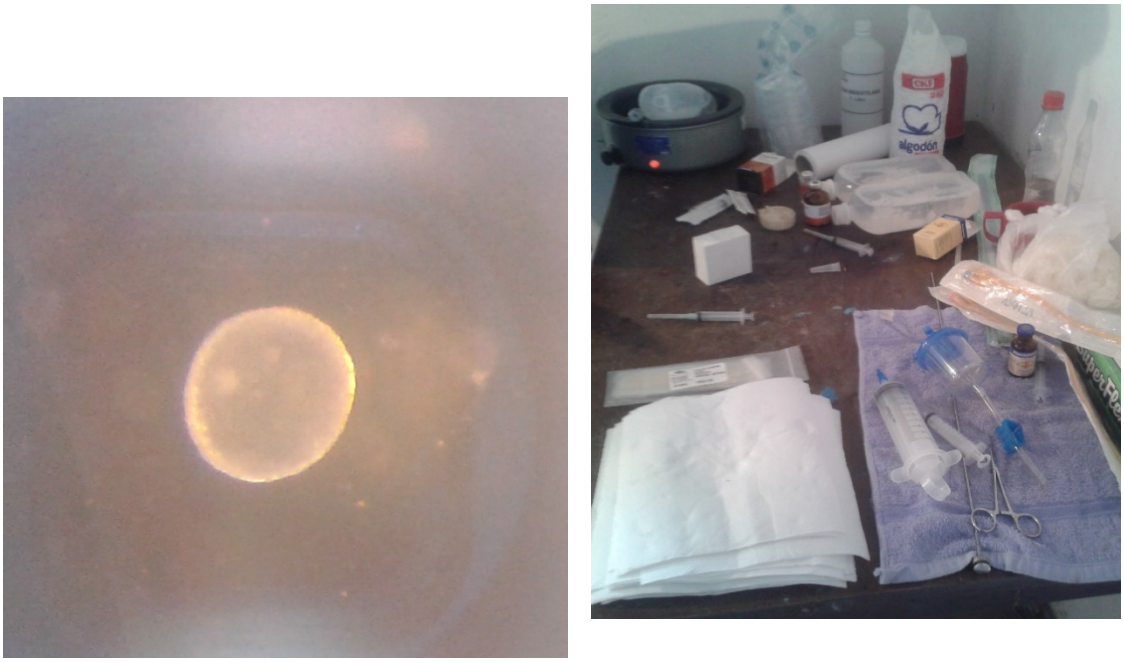

Fig 4. *Left: Llama embryo grade 1. Right: Some of the equipment used.*

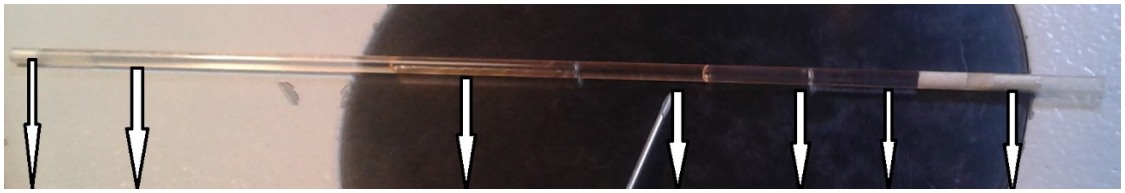

Fig 5. *Embryo transfer straw containing cotton, holding medium, air bubble, holding medium with the embryo, air bubble, holding medium, polyvinyl alcohol.*

Day 7, Embryo transfer

The procedure was repeated on day seven when five more embryos where obtained and transferred. The donors where under the influence of prostaglandin when flushed the second time. This made no notable difference to the procedure.

Day 13, Mating

The 10 donating females were mated once again with 10 new males. Since the evaluation of the animals and previous transfer three of the recipient alpacas had been discharged for various reasons. Thus only three possible recipients remained. All of these were injected with 1.5ml GnRH intramuscularly. Three receptive llamas were also prepared with GnRH.

Day 20, Lavage and transfer

The procedure of uterine lavage and transfer of the obtained embryos were repeated following the same protocol as on day six and seven. In total six embryos were collected. Three of these were transferred into alpaca recipients and three intra-species transfers were made into llama recipients.

Day 27 Diagnosis of pregnancy group one

The six alpacas that received embryos on the first day of transfer where examined by ultra-sound to determine pregnancy.

Day 41, Diagnosis of pregnancy group two
All the recipients in the second group were examined by ultrasonography to diagnose pregnancy. The first group of recipients was examined again to confirm pregnancy.

RESULTS

Two animals were found pregnant on day 27 but only one of them was still pregnant on day 41. In the second group five out of six animals were pregnant on day 41. The lavage and collection of embryos in the first group were done six days post mating due to an error in identifying the day of mating as Day 1, and were thus repeated on day seven. According to previous studies the hatched embryo of the llama enters the uterus on day 6.5-8. It has also been suggested that harvesting embryos at a longer post-mating interval might give even better results (Taylor, et al., 2000; Cervantes, et al., 2008; Sumar 2013) Therefore it was no surprise that only one embryo was obtained on day 6. When the procedure was repeated on day 7, five more embryos were obtained. On day 7 small amounts of blood were found in the lavage fluid from two animals, probably due to inflammation because of damage done to the cervix and uterus membranes during the prior lavage. No embryos were obtained from these individuals. The embryos were graded. All but one embryo were considered to be grade 1 quality and one embryo was considered to be a grade 2. All embryos were thus fit for transfer.

The same llamas were mated and used as donors for a second group of recipients. This time they were only flushed once, on day 7 post mating. No signs of inflammation were seen this time and again a total of six embryos were obtained. Five of these were grade 1 and one grade 2. In the first group of recipient alpacas the transfer resulted in two pregnancies on day 27 but only one animal was still pregnant at 41 days post mating, giving a success rate of 16. 7% in the first group. In the second group embryos where obtained from six out of ten mated animals. Unfortunately three llamas had to be used as recipients in the second group since some alpacas were excluded from the recipient pool. All three alpacas were found to be pregnant on day 28 post mating as were two out of three llamas, giving a transfer success rate of 83%. The transfer results of group one may have been lowered by the fact that all the donors were treated with prostaglandins on the first day of flushing. The repeated flushing could also have had a negative influence on embryo viability due to inflammation caused by the lavage on day six.

The result in total was that embryos were recovered from 60% of the donors and the pregnancy rate after transfer was 50%, not including the one animal that suffered early embryonic death and was barren on the second ultrasound examination.

Table 3. *Embryo collection and transfer group 1, donor llamas*

Nr.	Name	Day of collection	Obtained embryo	Embryo grading
1	L042	7	NO	
2	L074	7	NO	
3	L039	6	YES	2
4	L005	7	YES	1
5	L059	7	YES	1
6	L027	7	YES	1
7	L040	7	YES	1
8	L014	7	NO	
9	L038	7	YES	1
10	L075	7	NO	

Embryos obtained from 60% of the mated llamas

Table 4. Recipient *alpacas group 1*

Nr	Name and phenotype	Pregnant day 27	Pregnant day 41
1	H069	NO	NO
2	H047(230102)	YES	NO
3	S001	NO	NO
4	H052	NO	NO
5	H013	NO	NO
6	H038	YES	YES

Transfer resulted in 16, 7% pregnancy on day 41 post mating (day 34 post transfer)

Table 5. *Embryo collection and transfer group 2, donor llamas*

Nr	Name	Obtained Embryo	Embryo Grading
1	L042	NO	
2	L074	YES	1
3	L039	NO	
4	L005	YES	1
5	L059	YES	1
6	L027	NO	
7	L040	YES	2
8	L014	YES	1
9	L038	YES	1
10	L075	NO	

Embryos obtained from 60% of the mated llamas

Table 6. *Recipient alpacas and llamas group 2*

Nr	Name, species and phenotype	Pregnant day 28
1	S040	YES
2	H026	YES
3	H047	YES
4	L017	NO
5	L061	YES
6	L068	YES

Transfer resulted in 83% pregnancy rate on day 28 post mating (day 21 post transfer)

DISCUSSION

This study can confirm that it is possible to obtain pregnancy through interspecies embryo transfer under basic field conditions, however is it impossible to value the efficiency of this method if it were to be repeated. Several factors may have contributed to the outcome of this study. It was conducted in September which is not a regular breeding time for SACs in Peru. All the females were primarily selected 7 months earlier as they were part of a similar study. That study is not likely to have affected their fertility and was the main reason that they were not pregnant at this time of year. The details of the original collection are unknown. All but two of the recipients had a history of giving birth to live crias according to the herd manager. Two of the recipients were maiden (Fig. 4), providing a factor of uncertainty to the reproductive function of these animals. Ideally animals with a well-documented and flawless reproductive history should have been used. The animals used were all part of the same herd and therefore inbreeding is possible. The herd is large enough (approximately 370 animals) to make this risk small but it is not impossible that this might lead to reduced embryo survival. All the donors were lactating at the time of the embryo collection (Fig. 3); it has previously been seen that this does not affect the ovulation and embryo recovery in llamas (Aller, et al., 2002).We were able to obtain embryos after 60% of the matings, this is a higher percentage than mentioned in small scale llama studies reviewed by Vaughan et al. (2013)

The number of embryos obtained might have been higher if the donors would have been examined by ultrasound to verify the presence of a mature dominant follicle on the day of mating. It would likewise have been useful to control the sperm quality and quantity of the males before mating to exclude the possibility of infertile males. The animals in this study are part of a large group and kept in a free range manner. Even though care was taken not to

stress the animals they were unaccustomed to handling as well as being separated from the group. The whole experience was likely to be stressful to all animals. The role of stress in camelids is not fully known but have been associated with early embryotic death and abortions (Vaughan & Tibary, 2006); for a better result socialized animals should be chosen. To use alpacas as surrogate mothers for llama crias might not be the ideal in production since the alpaca is usually the species that becomes more valuable with faster genetic improvement. Alpacas also have a higher rate of early embryonic death than llamas and have narrower birth passages. Production on a bigger scale was not the aim of this study and therefor these concerns were not taken into consideration.

Furthermore the season must be considered, although SACs are generally thought to be non-seasonal breeders (Vaughan & Tibary, 2006). However some researchers claim that this is not entirely true (Brown, 2000) (Pollard, et al., 1995). Pollard, et al. saw that alpacas bred during the rainy season in New Zealand showed less libido than at other times of the year. In a large retrospective study in 2013 there was a significantly higher embryo transfer success rate in summer compared to spring and winter in Australian, single ovulation alpacas (Vaughan, et al., 2013). In a study that was conducted parallel to the current one the alpacas, both male and female, showed lack of libido when they were put together (Winblad von Walter, 2015). In traditional small scale Peruvian farms where males and females are kept together all year round, the animals mate and give birth in the warm months of December to March. In September, when this present study took place, the pastures contains less energy and protein and even though all animals had an acceptable to high BCS (Fig 3 and 4) it is not impossible that the low energy forage lowered their fertility. Ideal BCS in recipient alpacas seems to be 2.5-3.5 according to Vaughan et al. (2013) who showed a significantly lower success rate in thin animals (BCS $\leq$ 2). All but two recipients in the current study were lactating (Fig. 4), but it has previously been shown that this does not affect the pregnancy rates in alpacas (Vaughan, et al., 2013). That study was based on results from Australia; it is likely that those animals were held under more advantage conditions with a diet adjusted to lactation. The animals used in this study did not receive extra fodder and although it is natural that alpaca females are lactating when mated, the usual breeding season provides higher energy pastures. Lack of good feed during early pregnancy might cause a higher rate of early embryonic loss (Brown, 2000). It would have been interesting to weigh and score BCS repeatedly during the study and register any sign of weight loss.

It has been seen that pregnancy rates after embryo transfer in alpacas are higher when the embryo is placed in the uterine horn ipsilateral to the corpus luteum (Picha, et al., 2013). In this study no ultrasound was done to confirm ovulation or location of corpus luteum. The embryos were placed randomly inside the uterus. The mechanism of maternal recognition of pregnancy of SACs is multifactorial and not fully understood; it seems to be associated with the site of embryo deposition and embryo migration seems to play an important role (Picha, et al., 2013).

Taylor did the first inter-species embryo transfer in SACs and had a 100% pregnancy rate since both transferred embryos resulted in pregnancy (Taylor, et al., 2001). The only attempt before this one to use alpacas as recipients for llama embryos resulted in a pregnancy rate of 50% post transfer (Sumar, 2013). The result in this study is similar but the group of animals is

larger (12 transferable embryos compared to 6 in Sumar 2008 as mentioned in Sumar 2013). Unfortunately we did not have enough alpaca recipients available and we were thus forced to use llamas as recipients for three of the embryos. Counting only true inter-species transfers, 4/9 transferred embryos resulted in pregnancies giving a pregnancy rate of 44%. This is comparable to similar results in embryo transfer program within the specie in alpacas (Sumar, 2013) (Vaughan, et al., 2013). According to Vaughan 2013 an embryo transfer success rate over 40% in alpacas is considered to be satisfactory. The results in the second group were better (3/3 compared to 1/6 pregnancies in the first group), indicating that the first batch of embryos might have been negatively influenced by the repeated flushing and the injection with progesterone.

At the time of writing fresh results from Peru arrived. At a follow up ultrasound examination conducted approximately 2.5 months post transfer, a high rate of embryonic loss was seen. Only one alpaca and the two llamas were still pregnant at this time. This year (2014) the spring was late in the Peruvian highlands. The late rains caused a lack of good pastures. The area where the animals in this study are kept has a good supply of water from a nearby glacier but after a prolonged dry season this might not been enough to keep the animals in a positive energy balance. Llamas are generally better adapted to meagre pastures than alpacas and in a mixed herd the alpacas are disadvantaged. In early studies on alpaca reproduction conducted in the harsh environment of their natural habitat, early embryo mortality as high as 83% was seen (Sumar, 1996).

It cannot be excluded that the basic field conditions that were part of this study did not provide good enough hygiene and precise enough methods to get a better result than 1/9 pregnancies, although this is unlikely since similar rate of loss of pregnancies in the llamas were not seen. It would be valuable to repeat this study in the normal breeding season for Peruvian llamas and alpacas and to use a larger group of animals. Ideally a new study would be located at high altitude but under more controlled conditions with animals used to handling. Extra feed and care should be provided to eliminate the risk of lowered fertility due to nutritional constraints.

CONCLUSIONS

The early pregnancies established in the alpaca recipients confirm that it is possible to transfer llama embryos to alpaca recipients under basic field conditions. However the high proportion of early embryonic death in this study suggests that this is not ideal. There are many benefits of using reproduction biotechnologies to allow more rapid propagation of individuals with high genetic merit and desired qualities such as color or fine fibre. If lines of llamas or alpacas of relict population closer to the pre-conquest breeds could be identified, reproduction biotechnologies such as embryo transfer, intra or/and inter species, would be crucial to insure their preservation and cultivation. In the case of wild camelids it is also highly desired to protect all genetic variations of each species; inter species embryo transfer could be one of the tools to do so. To really know how well fitted alpacas are to surrogate as mothers to llama embryos more studies with larger groups of animals are needed.

References

Aba, M. A., 1998. Hormonal Interrelationships in reproduction of female Llamas and Alpacas. *Acta Universitatis Agriculturae Sueciae,* Volume 35, pp. 11-17.

Aller, J., Rebuffi, G. & Alberio, R., 2002. Successful transfer of vitrified llama (Llama glama) embryos. *Animal reproduction science,* Volume 73, pp. 121-127.

Andrabi, S. & Maxwell., W., 2006. A review on reproductive biotechnologies for conservation of endangered mammalian species. *Animal reproduction science,* pp. 2213-243.

Bravo, P., Flores, U., Garnica, J. & Ordenez, C., 1997. Collection of semen and artificial insemination of alpacas. *Theriology,* Volume 47, pp. 619-626.

Brown, B. B., 2000. A review on reproduction in South American camelids. *Animal reproduction science,* Volume 58, pp. 169-196.

C.Novoa & Sumar, J., 1968. Coleccćon de huevos in vivo y ensayos de transferencia en alpacas.. *Tercer Bolentín Extraordinario IVITA. Universidad Nacional Mayor de San Marcos, Lima, Peru,* pp. 31-34.

Cervantes, M. F. et al., 2008. Relacion entra el dia de collecion y la recuperation de embriones en alpacas superovuladas. *Rev Inv Vet Perú,* Volume 22, pp. 125-128.

Comizzoli, P., Mermillod, P. & Mauget, R., 2000. Reproductive biotechnologies for endangered mammalian species. *Reproduction, nutrition development,* Volume 40, pp. 493-504.

Del Campo, M., Del Campo, C., Mapletoft, G. & Adams, R., 1995. The application of new reproductive technologies in south american camelids. *Theriology,* Volume 43, pp. 21-30.

Fowler, M. E., 2010. *Medicine and Surgery of Camelids.* 3 ed. s.l.:Blackwell Publishing, Ltd.

Gray, A., 1954. Mammalian Hybrids. *Technical Communication, Commonwealth Bureau of Animal Breeding and Genetics, Edinburgh, Bucks, England.),* Volume 10, p. In Wheeler 1995 p.289.

Huanca, T. et al., 2012. Evaluacíon del peso al nacimiento, destete, al año de edad y curva de crecimiento de alpacas y llamas cría nacidas por transferencia de embriones interespecies. *Spermova,* pp. 44-46.

Kadwell, M. et al., 2001. Genetic analyses reveals the wild ancestors of the llama and the alpaca. *South American Camelid domestication ,* pp. 2575-2584.

Kershaw-Young, C. & Maxwell, W., 2012. Seminal plasma components in camelids in comparisons with other species. *Reproduction in domestic animals,* Volume 47, pp. 369-375.

Kristjanson, P., Krishna, Anirudh & Radeny, I. L. R. I. M., 2006. Pathways into and out of poverty and the role of livestock in the Peruvian Andes.

Miragaya, M., Chaves, M. & Aguero, A., 2006. Reproductive biotechnology in South American camelids. *Small Ruminant Research,* Volume 61, pp. 299-310.

Niasari-Naslaji, A. et al., 2009. Interspecies embryo transfer in camelids: the birth of the first Bactrian camel calves (Camelus bactrianus) from dromedary camels (Camelus Dromedarius). *Reproduction, Fertility and Development,* Volume 21, pp. 333-337.

Picha, Y. et al., 2013. Chronology of early embryonic development and embryo uterine migration in alpacas. *Theriogenology,* Volume 79, pp. 702-708.

Pollard, J., Littlejohn, R. & Moore, G., 1995. Seasonal and other factors affecting the sexual behavior of alpacas. *Animal reproduction science,* Volume 37, pp. 349-356.

Saun, R. V., 2008. Effect of nutrition on reproduction in llamas and alpacas. *Theriology,* Volume 70, pp. 508-514.

Sexton, R. J. & Saitone, T. L., 2005. Alpaca lies? Do Alpacas Represent the Latest Speculative Bubble in Agriculture?. *Department of Agricultural and Resource Economics.*

Stover, J. & Westrom, W., 1984. Reproductive studies unit of the New York Zoological Society. *Zoo Biology,* Volume 3, pp. 335-341.

Sumar, J., 1988. Removal of the ovaries or ablation of the corpus luteum and its effect on mantenance of gestation in the alpaca and llama. *Acta Vet. Scand. Suppl.,* Volume 83, pp. 133-141.

Sumar, J., 2008. Alpacas finas obtenidas en vientres de llama por transferencia de embriones.. *Agro Noticias,* Volume 338, pp. 132-134.

Sumar, J. B., 1996. Reproduction in llamas and alpacas. *Animal Reproduction Science,* Volume 42, pp. 405-415.

Sumar, J. B., 2013. Embryo transfer in domestic South American camelids. *Animal Reproduction Science*, Volume 136, pp. 170-177.

Taylor, S. et al., 2001. Alpaca offspring born after cross species embryo transfer to llama recipients. *Theriogenology,* Volume 55, p. 401 (Abstract).

Taylor, S., Taylor, P., James, A. & Godke, R., 2000. Successful commercial embryo transfer in the Llama (Llama glama). *Theriogenology*, Volume 53.

Tibary, A., Anouassi, A., Sghiri, A. & Khatir, H., 2007. Current knowledge an future challenges in camelid reproduction. *Society of Reproduction and Fertility supplement,,* Volume 64, pp. 297-313.

Tibary, A., Anoussi, A. & Khatir, H., 2005. Update on reproductive biotechnologies in small ruminants and camelids. *Theriogenology,* Volume 64, pp. 618-638.

Vaughan, J., Mihm, M. & Wittek, T., 2013. Factors influencing embryo transfer success in Alpacas- A retrospective study. *Animal reproduction science,* Volume 136, pp. 194-204.

Vaughan, J. & Tibary, A., 2006. Reproduction in female South American camelids: A review and clinical observations. *Small ruminant research,* Volume 61, pp. 259-281.

Wheeler, J., 1995. Evolution and present situation of the South American camelidae. *Biological Journal of the Linnean Society,* Volume 54, pp. 271-295.

Wheeler, J., Russel, A. & Redden, H., 1995. Llamas and Alpacas: Pre-conquest breeds and post-conquest hybrids. *Journal of archaeological science,* Volume 22, pp. 833-840.

Wiepz, D. W. & Chapman, R. J., 1985. Non-surgical embryo transfer and live birth in llama. *Theriology,* Volume 24, pp. 251-257.

Winblad von Walter, A., 2015. *Inter species embryotransfer in small camelids*. Uppsala: s.n.