Artificial Insemination in Alpacas

Jorge Reyna

B.Sc (Hons), Msc (Sydney Uni)

Introduction

Since 1988, when the first alpaca imports from Chile took place, the Australian national herd has been increasing rapidly. Statistics show a rapid growth from 51,953 to 60,814 (17%) from 2003 to 2004 (Australian Alpacas Association, 2005), and alpacas are being kept in New South Wales, Victoria and Western Australia. The alpaca industry in this country is still an alternative activity, but attractive in economic terms, and it has the potential for long-term development. In contrast with the conditions prevailing in the Andes, Australia has excellent environmental conditions for alpaca breeding in terms of natural pastures and mild weather. There are institutions and economic resources that can be used for research and the generation of new technologies. With a strategic research plan, it will be possible to make the industry more profitable and to attract more people to invest in alpaca breeding.

Alpaca farms in Australia are situated on excellent agricultural land and there is the potential to breed animals in marginal areas, due to their capacity to tolerate harsh conditions and drastic variations of temperature, to feed on pastures that are not utilised by cattle or sheep, to tolerate high levels of dehydration and inanition, and to have a less erosive effect on the soil due to their fibro elastic pads. Alpacas also have the potential to be used for meat production in Australia as in Peru, due to their low level of fat (5-6%) and good

level of protein (21-24%) which is superior to that in beef (Hack 2001). In the long-term, when the population becomes larger, it will be viable alternative livestock.

Australia as a leader in the sheep industry has the experience, infrastructure, institutions, and assisted reproductive technologies required for genetic improvement of alpaca herds. With the development of artificial insemination, embryo transfer techniques, *in vitro* production of embryos (IVP) and the inauguration of a genetic improvement program for alpacas, it will be possible to develop a breed of extra fine Australian alpaca comparable to the alpaca kept by the Incas before the conquest in relatively few years. This will increase the production and productivity of the herd and increase our exports and earnings in the international market.

Genetic improvement of alpacas

Genetic improvement in alpacas is slow in comparison with cattle and sheep due to the atypical reproductive physiology of the species, which limits extrapolation of reproductive technologies from other domestic animals. One of the first limitations is that the males reach puberty at 1- 3 years and the prepuce adheres to the glans penis, making extrusion of the penis impossible (Pinares *et al*. 1985; Fernandez Baca 1993). This adhesion has been found to be dependent on testosterone concentrations. It has been reported that in young animals (1-2 years old) free of adhesions, that concentrations of testosterone were high relative to animals without prepuce separation (Nunez

1994). There is a potential to advance puberty in males with the application of testosterone, which needs to be studied, and also to see if a proper nutrition program can advance the release of the glans penis adhesions, and with that puberty and sperm production. This will be very useful to maximize the use of elite sires, as it will allow starting the reproductive activity of the male at an early age.

A second limitation is related to the long gestational length (11.5 months)(Hafez 2000), which limits the number of crias from a particular female to 8-10 in her whole reproductive life. In this case, the development of The Multi Ovulation and Embryo Transfer program (MOET) in alpacas could be desirable in order to maximize the use of elite females and improve the herd from the maternal side as well. An embryo transfer protocol consists in the application of hormones (FSH, hCG and/or LH) to induce follicular growth of simultaneous follicles producing multiple ovulations, and thereby giving the possibility of fertilising oocytes and producing embryos from a particularly valuable female. This animal used to superovulate is called a “donor”. Upon superovulation, the embryos obtained are transferred into females with low genetic value that will carry the pregnancy. Thus it is possible to get several offspring from one female in one attempt. Females that carry the embryos are called “receptors” and should be healthy animals with good conformation and no reproductive malformations. Research in superovulatory protocols, embryo collection and transfer has been conducted in camels (Xing-Xu *et al*. 1994; Skidmore 2004), alpacas (Palomino 1997; Gomez *et al*. 2002; Vaughan 2002) and llamas (Wilson and Chapman 1985; Correa 1992; Bourke et al. 1995;

Correa *et al*. 1997; Taylor *et al*. 2000). In the area of vitrification of embryos research has been done only on llamas (Aller *et al*. 2002; Von Baer *et al*. 2002), but a satisfactory protocol to freeze embryos still has not been described. The main problem in this research is obtaining a large number of embryos to be allocated to different freezing protocols. Probably it would be possible to collect ovaries from abattoirs in Peru and put in an *in vitro* fertilisation (IVF) system to obtain a large number of embryos to be used in several freezing protocols in order to find the optimum which will produce more viable embryos after being frozen and thawed. *In vitro* production of embryos is a novel area in camelids and has been tested in llamas only (Del Campo *et al*. 1994; Del Campo *et al.* 1994; Ratto *et al.* 2004). Some researchers take the view that there are still a lot of basic techniques to develop first before going for expensive ones which will not have a practical use in the short-term.

Another inconvenience which affects genetic improvement in alpacas is related to the reproductive efficiency of the herd. There are no reproductive parameters to select males apart from phenotypical characteristics like height, weight, conformation, fleece composition, testicular size, libido and limited number of descendents obtained by natural mating. It has been demonstrated in other domestic species that the greater the pressure of selection for production the less is the reproductive ability of the descendants. Animals destined to become sires could have a low sperm concentration and poor sperm motility and when they are used to improve herds could cause serious problems in their descendants by transmitting the genes for low fertility. In

conditions prevailing in the Andes, early embryo loss can affect up to 60% (Huanca et al. 2004) of the herd and due to the specific time of the year for matings, generally from December to March, it is only possible to have one “cria” per annum. This affects the number of animals available for replacement. In Australia early embryo loss has been reported, but there is no official study. On the other hand, this is not such a problem here as the female has another opportunity to mate and produce a cria.

In summary, puberty at 1-3 years, the presence of penis-prepuce adhesions, long gestational length, the limited number of offspring from a particular female and low reproductive efficiency makes a genetic improvement program in alpacas slow. That is why techniques like artificial insemination, embryo transfer and *in vitro* production of embryos (IVP) are highly recommended in order to achieve faster genetic gain in the herd.

Advantages of artificial insemination

There are several advantages of the artificial insemination (AI) program in alpacas. One of the main ones is the possibility of widespread use of elite sires to improve the performance of the national herd facilitating progeny testing. Additionally, AI will permit crossbreeding to change a production trait, will accelerate introduction of new genetics, reduce risk of spreading sexually transmitted and other diseases (lice, JD), and eliminate the need for transport of animals, reducing the need for “mobile matings”. Finally it will provide a useful tool for investigating reproductive physiology like sperm transport in the

female tract, and capacitation and time of ovulation in relation to the deposit of the sperm in the tract.

Alpaca sperm characteristics

As a preamble, it is convenient to describe briefly the ejaculatory process in alpacas. This species has a peculiar reproductive physiology in comparison to that observed in other domestic farm animals. Copula takes up to 40 minutes and urethral contractions are distributed evenly throughout the ejaculatory process (Lichtenwalner *et al*. 1995). In the past it was postulated that alpacas ejaculate in fractions like boars but it is well known these days that the ejaculation process is constant (Chipana 1997). If the male is fertile and has been used rationally, the greater the time of copulation the more probability there is of finding sperm in the ejaculate (Bravo 2001).

The seminal plasma in alpacas is gelatinous (San Martin *et al*. 1968; Garnica *et al*. 1993; Pastor 1993; Bravo *et al*. 1999; Davalos and Olazabal 2002) and it holds sperm until ovulation takes place, usually 24-30 h. post-copula (Bravo et al. 1999)(Figure 1). The function of the gelatinous seminal plasma is still unknown, but it could be a medium that protects the sperm until ovulation takes place, which is usually 24-30 h after copulation (Bravo and Sumar 1989). Another function of the gelatinous seminal plasma that has been suggested is as a medium to seal the cervix to prevent the sperm from flowing

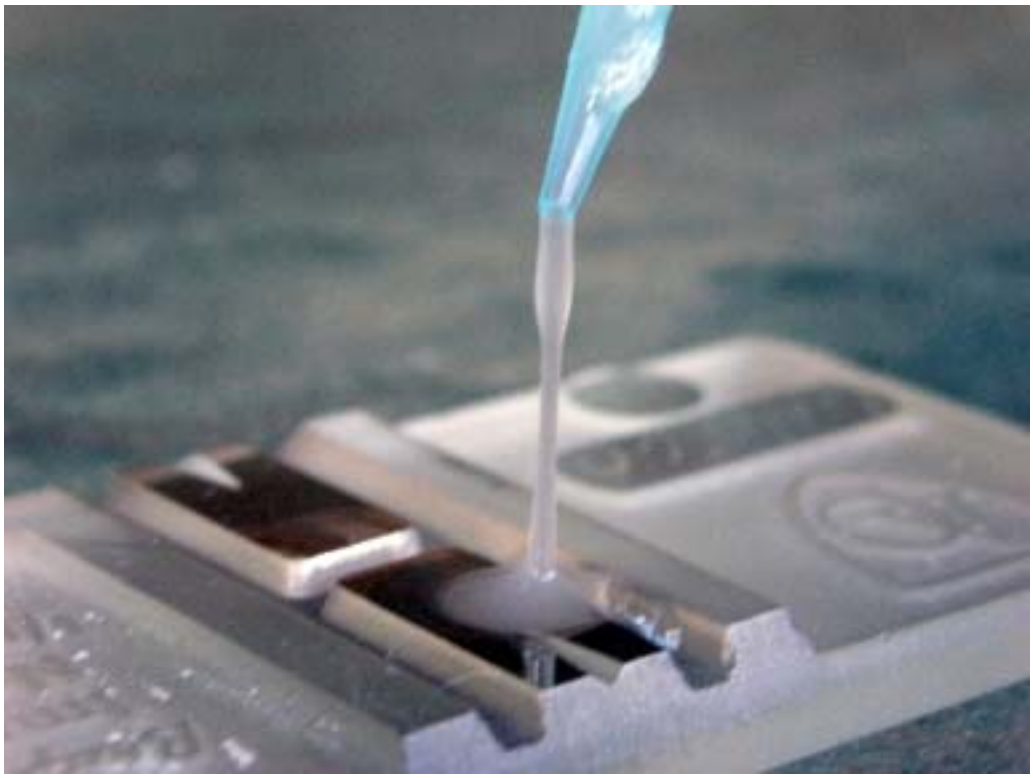

Figure 1: Alpaca sperm is highly viscous, which makes it difficult to obtain a homogenous sample when it is mixed with extender for artificial insemination and/or freezing procedures.

back from the uterus to the vagina. The most interesting function of the seminal plasma in alpacas recently reported is as an ovulation inductor. Sixty

percent of the females treated with an i.m. dose of 0.8 – 1.0 ml of seminal plasma ovulated. The mechanism involved is unknown, but may involve a protein that has a GnRH or LH action that stimulates the ovulatory follicle to ovulate (Sapana *et al*. 2002).

The volume of the ejaculate reported was 0.8 to 3.1 ml (Bravo *et al*. 1997; Bravo *et al*. 2000; Flores *et al*. 2002). In our preliminary field trials the average was 2.57 ml (range 1 to 6 ml). Ejaculates collected by mannequin and artificial vagina (AV) contain foam on top due to the constant penetration of the penis allowing air onto the sperm (Figure 2). A large amount of foam on the top of the ejaculate could yield an extra ml of sperm in an hour or two.

The predominant colour of the sperm varies from slightly milky white to creamy and depends on the concentration of the sperm contained (Fernandez-Baca 1970; Garnica *et al*. 1993; Flores *et al*. 2002; Vaughan 2003). In our field trials we collected sperm that looked crystal white (2 x 10^6 sperm/ ml), slightly milky (74 – 80 x 10^6 sperm/ ml) and milky creamy (170 - 220 x 10^6 sperm/ ml). The more concentrated the sperm is, the more milky the sample is. Our ranges were variable in agreement with the ranges reported by other researchers *(*Garnica *et al.* 1993*;* Garnica *et al.* 1995*;* Bravo *et al.* 1997; Chipana 1997; Flores *et al*. 2002; Vaughan 2003).

Figure 2: Collection tube showing an unusually high volume of ejaculate (6 ml), slightly milky with foam on top (contained 17 x 10^6 sperm/ml) collected at the first attempt from Wiston, a male from La Hacienda, Marulan – NSW.

Regarding the morphometric characterization of alpaca sperm-heads, in alpacas a high degree of sperm polymorphism within and between animals

has been found (Buendia *et al*. 2001). It is possible to classify alpaca sperm according to the size of the head in the following categories: pyriform, short, normal, round or long (Figure 3). In other species this has been identified as a characteristic which can be useful in the prediction of fertility. Further research needs to be conducted to elucidate if sperm morphology is important for fertility in alpacas.

Current limitations of artificial insemination in alpacas

There are several limitations to the use of AI in alpacas; one of the most important is that a reliable technique to collect semen needs to be developed. It seems that the use of a mannequin and artificial vagina (AV) is the best technique so far, but the ejaculate varies within the same animal and between animals as well. Another limitation is related to the length of copulation, which goes from 17 to 43 minutes in the field trials conducted at our laboratory. This extended time of copulation means you must maintain a constant temperature of the AV in order to give the conditions the animal needs to produce a good ejaculate. Temperature of the collection tube is important as well as the sperm will remain there until the end of the copula. This must be 37 ° C at all the times, as 1°C up or down may damage or even kill the sperm.

The device used in alpacas to collect semen is not just an artificial vagina (AV), as used by all alpaca researchers in the past. The concept needed to be

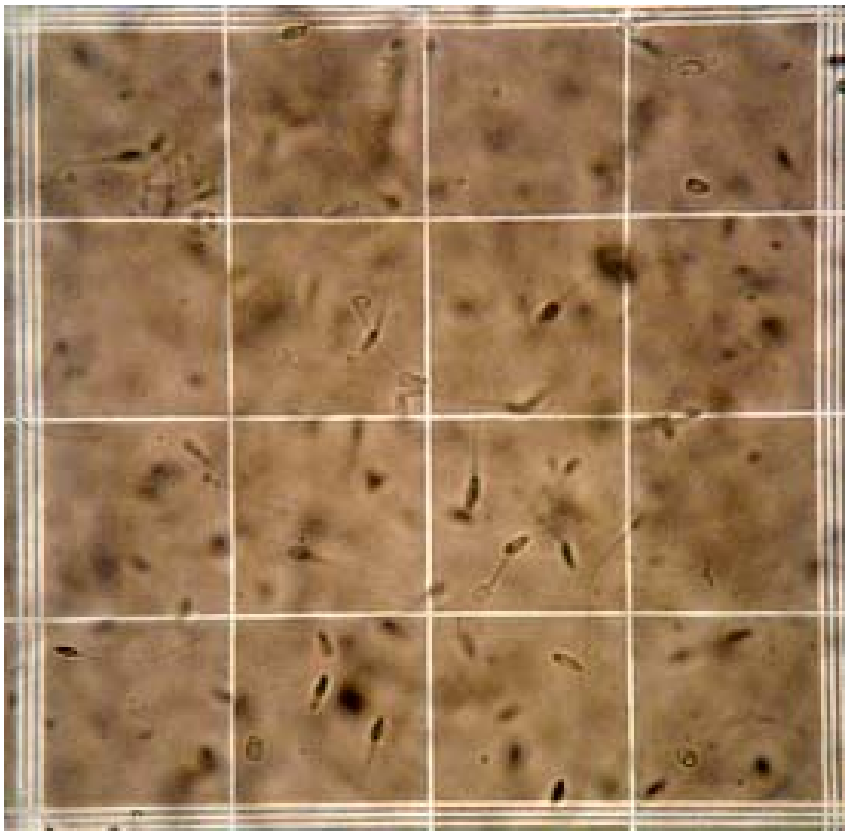

Figure 3: Alpaca sperm under the microscope. It is possible to visualise normal, round, pyriform and long heads. Dilution 1/20

changed to give better results. The device has 3 parts: artificial vagina, cervix or uterine neck, and finally uterine body and horns. In other words, it is an artificial reproductive tract that we will from now on name AART (Alpaca

Artificial Reproductive Tract). Future research needs to come up with a design that better simulates the real female reproductive tract in order to stimulate the male to produce a good ejaculate.

Problems related to alpaca semen are its unique mucoid character, and low spermatozoa concentration and low motility in the ejaculate (McEvoy *et al*. 1992; Bravo and Johnson 1994; Vaughan 2002). Other problems that should be mentioned are the lack of techniques to store semen in chilled or frozen form which we will discuss later on, the frequency of use of the male and the characteristic of induced ovulation in the female. Regarding about the frequency of use of the males, a few papers have been published in the past using a small group of animals in Peru, but the recommendations may not be useful for our purposes. Generally speaking, animals in the Andes have low body condition, due to exclusive feeding on natural pastures of predominantly low quality. There almost certainly are genetic and nutritional components which will define the frequency of use of the males, and there is a lot of research to be conducted in this area under Australian conditions.

The induced ovulation observed in alpacas, as in the other camelids, may be an issue when artificial insemination is being used. The time that ovulation takes place in alpacas has been studied before (San Martin *et al*. 1968) and has been reported to occur 24 h after natural mating under conditions prevailing in the Andes. There are other reports on llamas and camels but these can be taken as references only. It has been described before in other species that are considered spontaneous ovulators (cows and ewes), that

time of ovulation is highly influenced by several factors like season and photoperiod, breed, age, presence of the ram (in the case of the ewe), differences between flocks, body condition and the synchronisation protocols used. It will be necessary to perform a study under Australian conditions to determine the time of ovulation in alpacas taking into account natural mating, GnRH and hCG treatments, and time of the year as well, in order to time insemination with ovulation and thereby get better pregnancy rates. Transrectal ultrasound can be used for this purpose as a non-invasive technique that allows visualisation of the ovaries and other structures, determining the time of ovulation by disappearance of the dominant follicle, as described in the ewe (Reyna 2005).

Sperm transport in the female reproductive tract has been studied before. Reports available confirm that the spermatozoa reach the site of fertilisation 6 h after copulation in alpacas and remain there for up to 30 h until ovulation takes place and the sperm reaches the oocyte and fecundation take place. This means that the sperm is well adapted to survive up to 30 h in the female tract. It may be a capacitation effect in the uterus that makes the sperm remain potentially fertile. This could be related to the gelatinous consistency of the semen. It would be interesting to study this phenomenon because it could be a key to improving fertility in artificial insemination.

In summary, lack of a reliable technique to collect consistently good quality samples, the long period of copulation, the unique characteristics of the alpaca sperm regarding viscosity, low motility and low concentration, and the

nature of induced ovulation in alpacas, make difficult the development of biotechnological tools like artificial insemination to improve herds. Regrettably due to the differences from other domestic species it is not possible to extrapolate current technologies to alpacas, which makes research in this field very challenging.

Semen collection techniques in alpacas:

Several techniques have been used in the past to collect semen from alpacas. One of the first techniques used was intravaginal condoms (Mogrovejo 1952). The problem with this technique was that it did not consider that the deposit of sperm in alpacas is intrauterine. Copulation of a male with a female that has a condom did not last more than 5 minutes and the ejaculate was incomplete. A second technique used was electroejaculation (Fernandez-Baca 1970), but the problem was that the semen usually was contaminated with urine, which affects motility and can kill sperm, and it was stressful for the animals and could compromise future reproductive life if not conducted carefully. Another technique described was the use of post copula sperm aspiration from the vagina (PCSAV)(Pastor 1993) . This technique was described as simple and economic to use in order to obtain ejaculates from alpacas to study semen physiology, but the problem was that the sample was mixed with vaginal secretions that may alter semen characteristics. Regrettably there are no further reports on the technique. Finally, the latest technique described is the use of a mannequin (dummy female) and an artificial reproductive tract (AART), which has been reported to be effective to collect quality samples of

semen (Aller *et al*. 1997; Chipana 1997; Perez 1997; Bravo 2001; Davalos and Olazabal 2002; Vaughan 2002; Huanca *et al*. 2004). The problem with the sperm quality obtained by this latest technique is that it varies within and between males, making it hard to obtain a consistently good quality sample needed to test different freezing protocols. There is no explanation for this problem, but it could be related to the design of the AART and the need to improve it to better simulate the anatomy of the alpaca's reproductive tract. On the other hand, it is not only important to select the animals for acceptance of the mannequin, libido and testicular size, it is also necessary to evaluate consistency in semen production. Studies in the area of freezing of sperm need a reliable technique of collection that guarantees the provision of reasonable ejaculates from the experimental process.

Field trials demonstrated that the training of the male to mate with the mannequin is a simple procedure. Animals that had not been used for mating service for a while were keen on mannequin mating and produced an ejaculate at the first attempt. In contrast, animals which had been used regularly for natural matings were reluctant, and they needed audiovisual stimuli in the form of a real female being mated by a male near by. It was frequently observed that some males had difficulties finding the entrance of the vagina. Others were uncomfortable when mating the mannequin, manifested by a constant movement and sometimes withdrawal of the penis and standing up for a few seconds. It was noticed that some animals were scared of the mannequin and moved away. Additionally, it was found that the back height of the mannequin was a bit low for some animals, and a piece of

wood was used to increase it and give the animal easy access to the AART (Figure 4).

Previous experiments conducted in our laboratory point to the need to modify the mannequin, and especially the AART, to make the animal more comfortable with the mating process and to obtain a better quality of semen. The first field trials showed that the mannequin needs to be rebuilt, using a softer material and probably modifying the shape as well. For some animals it takes time to find the entrance of the vagina and an operator is required to lead the penis. Other animals find the vagina, but may feel uncomfortable, withdraw the penis and stretch their legs, and then come back again. It seems to be a matter of training as well, but it is important to have a design that simulates the female in the copulation position (Figure 5). Also, the external insulation seems to be insufficient when collection is performed outdoors, as the AART goes from 45°C at the start of the collection to 37°C at the first 5 minutes, 36.5°C at 10 minutes, 36°C at 15 minutes and 35°C at 20 minutes. The electric blanket that covers the collection tube seems to be effective at maintaining the temperature indoors, keeping the initial temperature of 40°C after 45 minutes, but when it was used outdoors the collecting tube lost 3°C. The new design has been built and is double insulated (internal/external) to avoid the temperature drop (Figure 6). It would be desirable to keep the collection tube at 37°C, as temperatures over or under this will affect the

Figure 4: Monty mating Consuelo, a mannequin developed by Dr. Jane Vaughan at Belbourie Alpaca Stud, Wilton Park, NSW.

Figure 5: A natural mating, inspiration for the new mannequin that is being testing at our laboratory.

Figure 6: PC the new mannequin in the posture adopted by the female in real mating. PC has been made with a wooden frame and wire. It has internal insulation (fibre glass) and external insulation (high density foam) to avoid loss of temperature during the collection procedure. The skin is made with wool carpet and is easy to remove for convenient cleaning.

sperm quality. Another source of loss of temperature from the AART could be the metal valve. It would be interesting to replace that part with a plastic part or cover it with insulation as well.

The AART needs to be modified. Reviewing the anatomy of the female reproductive tract it was found that the total length of the tract is 28-30 cm between the labia and the uterine horns. The length of the penis is between 35-40 cm (Hafez 2000). This means that the deposit of semen may take place at the end of the uterine horn. The current length of the AART is only 25 cm which means that the penis is reaching the glass collection tube (Figure 7). This could be a cause of variability of ejaculates in the AART and could be more obvious when the libido is not high, causing poor semen quality at collection. On the other hand, the simulation of the cervix that is being used is 4 cm long, when the literature describes it as 2-3 cm long. Anatomical reviews of the female alpaca reproductive tract describe the cervix as having 2 irregular annular or spiral folds, and it is very important to stimulate the penis properly to try to imitate this anatomical characteristic. The idea is to simulate as closely as possible the reproductive tract of the female in order to get a sample that is the same as when natural mating take place. Probably it is a matter of improving the AART design, or it could be the case that it is a normal characteristic of the species to ejaculate good and bad quality sperm independently of the AART design used. Probably there are animals which exhibit more consistency in semen production than others. This needs to be clarified.

Figure 7: Current Alpaca Artificial Reproductive Tract (AART). The liner is showing in white, with the collecting tube at the end. The whole apparatus is wrapped with an electric blanket to keep it warm during the time of collection, and fitted inside the mannequin.

Currently our laboratory is working on the liner design. We have 3 types to be tested: the first is straight all around with a diameter of 4 cm, the second has variable diameter (4 to 1.5 cm) with an imitation of the cervix rings, and finally the last one is straight all around at 1.5 cm in diameter with an imitation of the cervix rings. Latex is being used for liners for AV in bulls and rams for decades but the problem is that it can be toxic for the sperm. In the case of these species, due to the short ejaculatory pattern the sperm hardly ever come in contact with the latex, so it is not a problem. In alpacas, due to the long ejaculatory process, the sperm will be in contact with the latex for more than 20 minutes, and this will cause death of the sperm or reduced quality. The alternative product that seems to be safe for the sperm is liquid silicone medical grade (Prosil 8). This product comes in two parts, A and B, which are necessary to mix by weight 100:50 respectively. The consistency is very liquid and it is necessary to paint the moulds with the mix, but it takes too long to dry and we found that is necessary to put in an oven at 120° C to accelerate the curing process. Ten to twelve layers to obtain the desirable thickness is required. The texture of the liner made by silicone is very smooth, extremely elastic and sticky that feels like real skin.

In summary, several techniques to collect sperm from alpacas have been tested in the past and the use of a mannequin fitted with the AART seems to be the best technique so far. There are still a few modifications to work on to find the best design that will be as close as possible to the real female. Given these conditions and proper training, we are expecting to have consistency in the ejaculates obtained from our experimental animals.

Liquid storage of sperm:

Liquid storage of sperm is a technique that reduces sperm death and degeneration, can be considered as a reversible inactivation of the sperm, and has been successfully developed in other domestic species like bulls, rams and boars. This could be achieved using low temperatures in conjunction with extenders that reduce or arrest the metabolism of the sperm and thereby prolong their fertile life. In comparison with frozen-thawed sperm, liquid storage sperm requires lower sperm numbers per AI dose (Vaughan 2003). Irrespective of diluent, dilution rate, temperature or other conditions of storage the sperm deteriorate as the duration of the storage increases (Maxwell and Salamon 1993). Changes occurred during the storage of sperm, including reduction of motility, reduction of DNA integrity, and morphological and biochemical changes which affect fertility and may cause embryo mortality. Several extenders used in camelids and other domestic species were examined in an attempt to prolong the storage-life of alpaca semen at 4°C, but the best results indicate that motility was 50% at 24 h and then declined to 45% at 48 h (Bravo 2001; Vaughan 2002). There is still a lot of work to be done in this area, taking into account dilution, temperature of storage and length of storage.

Liquid storage of alpaca sperm will be an interesting practical approach, reducing mobile matings and stress for the animals, and giving the possibility of maximizing the use of one ejaculate according to the sperm concentration and motility of the sample.

Freezing of sperm:

Freezing sperm offers the many advantages of long term storage and flexibility of use (Vaughan 2003). Genetic evaluation and selection programmes make possible progeny testing and is thus a powerful tool to achieve better production and productivity performance of the national herd. However, one of the main problems of the use of frozen semen is that during the freezing and thawing procedures a high percentage of cells die as a result of damage to the membrane structure (Quinn and White, 1966). The rest of the sperm, which survived the freezing process, lost viability and showed impairment of function (Watson 2000). The injury during freezing procedures is due to the formation of intracellular ice crystals, which disrupt intracellular organelles (Watson 2000). As a consequence, fertility after AI with frozen-thawed sperm is poorer than with fresh sperm, and this can be compensated for by using a greater number of sperm in the insemination dose (Watson 1995). In alpacas, as we mentioned before, low sperm concentration and low motility in their ejaculate make it harder to achieve commercial fertility rates upon insemination with frozen-thawed sperm. Unfortunately, we may find in the future that there are excellent males which will not be suitable for sperm freezing due to low concentration and low motility of the ejaculate. These animals can be potentially used for *in vitro* production of embryos as a reduced amount of live sperm is required to fertilise a large group of oocytes.

Upon sperm collection in alpacas, due to the viscous nature of the samples it is necessary to use hydrolytic enzymes like Trypsin or Collagenase *(Bravo et*

al. 2000) or to use mechanical stirring (Vaughan 2003) in order to liquefy the sperm and to facilitate mixing with the extender. The extender is required to protect sperm against cold shock during freezing procedures. A good extender has the following functions:

- provides nutrients as a source of energy.
- protects against the harmful effect of rapid cooling.
- ensures the pH will be maintained (buffer).
- maintains osmotic pressure and electrolyte balance.
- inhibits bacterial growth.
- increases the volume of the ejaculate.
- protects sperm cells during freezing.

There are different types of cryoprotectant compounds like glycerol, ethylene glycol and methanol. These compounds are able to penetrate the cytoplasm of the cell. The mechanism of action of these compounds is described by a depression of the freezing point. Most of the sperm preservation protocols used glycerol, which was used for the first time in 1949 by Polge *et al*. To protect the sperm against cold shock, egg yolk and a surfactant is used, and it has been demonstrated that the addition of surfactant improves post-thaw fertility in other domestic species.

At the moment, a successful protocol for freezing and thawing alpaca sperm has not been yet described and motility after thawing goes down to 20-40% (Bravo *et al*. 2002; Valdivia *et al*. 2003; Vaughan 2003). Probably one of the

main problems will be the viscous nature of alpaca sperm, making it hard to achieve a homogeneous mix. Biochemical studies of the sperm would be desirable, in order to develop an extender that will give a better motility after thawing and thereby better fertility rates for AI. It would be desirable to freeze epididymal sperm from alpacas, as it has contains no seminal plasma to study if survival rates are better. If survival rates are better using epididymal sperm it could indicate that the problem is the seminal plasma which makes it hard to obtain a homogeneous mix with the extender and exposes the sperm to cold shock.

Artificial insemination:

Before artificial insemination (AI) it is necessary to synchronise ovulation in the females. It is important to mention that alpacas are induced ovulators which means that the copula stimulus is necessary to produce the luteinising hormone (LH) responsible for ovulation. Female alpacas and llamas present periods of receptivity of up to 36 days with short periods of non-receptivity that may last 2 days (Hafez 2000). The variability of sexual receptivity may be attributable to the degree of follicular maturity and the production of oestradiol, as observed in other domestic species. When the female is receptive, the copula will cause the release of LH and ovulation will take place 30 h later (Bourke *et al.* 1995; Bravo *et al*. 1996). Sexual receptivity in the female is not always indicative of the presence of an ovulatory follicle containing an oocyte with high fertilisation potential and normal embryo developmental competency (Bravo et al. 1991). This is very important to take into account in insemination

programs where females are induced to ovulate with GnRH (Bourke *et al*. 1995), hCG (Adam *et al*. 1989), LH (Taylor *et al*. 2000) or seminal plasma (Sapana *et al*. 2002). It is possible that we are inducing ovulation in a follicle that has not yet completed its development or has possibly started to regress, and the results can be low viability of the oocyte and thereby low fertility rates. There was no significant relationship between follicle diameter and time of ovulation post mating (Adams *et al*. 1990), which indicates that even immature follicles can ovulate at the same time as mature follicles in natural mating and probably upon hormonal application. It seems that there is a need to determine the optimal time for mating alpacas according to follicle diameter. Probably a treatment with progesterone or progestogens will be necessary to inhibit follicular growth and then at the time of withdrawal of progestogens all the animals will have a synchronised follicular wave and it will be possible to predict the time when a mature follicle will be present. This means that with the induction of ovulation by exogenous hormones the oocytes released will be more competitive and this may increase fertility rates upon natural mating or artificial insemination.

Artificial insemination in South American camelids has been described before via rectum or laparoscopy (Calderon *et al*. 1968; Fernandez-Baca 1993; Bravo *et al*. 1996; Perez 1997; Aller 1998; Huanca *et al*. 2004). Artificial insemination via rectum is performed introducing the left hand side and, upon finding and stabilising the cervix, a pipette is introduced into the vagina and through the cervix. Then, when the uterus is reached, a syringe containing the sperm is connected into the end tip of the pipette and the sperm is deposit ed

into both uterine horns (Bravo *et al*. 1996). This is a simple technique, but it requires practice and patience to be developed. The second technique uses laparoscopy and the animals need to be sedated. Then the animals are placed on a cradle with the head down at 60° angle. Then two skin incisions are made cranial to the udder at 2 cm from the *linea alba*. A pyramidal trocar cannula is inserted on the left which will be replaced by the telescope and a second trocar cannula is inserted through the right incision which is replaced by a grasping forceps. Upon finding the dominant follicle (ovulatory follicle), the grasping forceps is replaced with an insemination pipette that contains the sperm in a syringe at the other end. Finally, sperm is deposited in the uterine horn ipsilateral to the ovary that contains the ovulatory follicle (Bravo *et al*. 1996).

Fertility rates in alpacas upon transcervical artificial insemination using fresh sperm vary from 40 to 67% (Bravo *et al*. 1996; Bravo *et al*. 1999; Huanca *et al*. 2004), and in the case of frozen-thawed sperm fertility rates vary from 26 to 37% (Apaza *et al*. 2000; Bravo *et al*. 2000). More research needs to be conducted to find the optimal dose and time of insemination to yield better pregnancy rates, but it is also important to consider the synchronisation protocol and hormones used to induce ovulation as these could affect sperm transport and oocyte competence.

The future

There is still a lot of research to be conducted in order to develop artificial insemination in alpacas. First it will be necessary to test the new mannequin that has been built to make sure that it maintains the temperature of the AART and gives the male the opportunity to express in full his mating behaviour and thereby to obtain a good quality ejaculate. To this end the design of the AART is crucial, as it is the way the penis will be stimulated to produce a good quality ejaculate. Ensuring the collection of a good ejaculate in terms of sperm concentration and motility is the starting point to develop a protocol to chill/freeze alpaca sperm. It is important to test extenders, times and temperatures of sperm processing, as well as packaging and the addition of novel supplements like enzymes and antioxidants which will improve viability of the sperm upon thawing. Protocols for synchronisation of the follicular wave and induction of ovulation need to be revised with the objective of finding the best ovarian response, as represented by the number of viable oocytes to be fertilised upon artificial insemination. Finally, techniques to deposit the sperm into the uterine horn and dose of sperm need to be studied further to get higher fertility rates.

Contact details:

Jorge Luis Reyna Zeballos

7/520 New Canterbury Rd

Dulwich Hill, NSW 2203

Sydney – Australia

Home +61 2 956 813 70

Mobile: +61 4 28 ALPACA

E-mail: Jorge_Llama_Guy@yahoo.com.au

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