

Reproductive Biotechnologies in Swedish Male Alpacas

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Cover: Happy boys at “Norrängens Alpacka”, Sweden
(photo: MC. Abraham)

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Abstract

Alpacas have become more popular during the last decades. The herds have been built up by importing live animals since reproductive biotechnologies, for example artificial insemination and semen preservation, are not well-developed in this species. A major problem is the viscosity of the seminal plasma which hinders processing or evaluation of the semen. Enzymes have been used to deal with the viscous seminal plasma but they may damage spermatozoa or render them incapable of fertilization. The use of reproductive biotechnologies would permit the introduction of new genetics without the need to import live animals, thus improving animal welfare and reducing the risk of spreading diseases. Therefore, our aim was to improve reproductive biotechnologies to help develop the Swedish alpaca breeding industry.

Laboratory techniques were performed to select the best spermatozoa with Single Layer Centrifugation (SLC), in order to improve cryopreservation. These techniques were developed first using bull semen. There was an improvement in sperm quality in the SLC-selected samples, particularly from poor quality semen. In addition, the SLC technique could be modified to process small volumes.

Alpaca epididymides were obtained after routine castration for husbandry purposes, with the intention of comparing semen extenders using extracted epididymal spermatozoa. Most of the organs came from pre-pubertal animals and therefore did not contain spermatozoa. Nevertheless, a decision-making tool for alpaca husbandry under Swedish conditions was developed. We suggest a combination of testicular size and body condition score as a tool for decision-making in the selection of potential sires for animal husbandry under Swedish conditions.

A phantom was designed and built to collect semen samples in Sweden, and semen collection trials were also performed in Perú. The advantages and disadvantages of different semen collection techniques were evaluated. However, the problem with semen viscosity still has to be solved. Therefore a semen collection method should be established so that semen handling methods can be developed. We conclude that a phantom could be the best method to use for semen collection in Sweden, since it is a fairly simple technique and, as far as we are aware, there are no animal welfare concerns.

Keywords: animal welfare; artificial insemination; decision-making tool; epididymal spermatozoa; semen preservation; Swedish alpaca industry; *Vicugna pacos*

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J. Puhakka

Dedication

To Ale, Clara and Tobias...mis amores

It always seems impossible until it is done
Nelson Mandela

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List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

- I Abraham, M.C., Johannisson, A. & Morrell J.M. (2016). Effect of bull sperm preparation on development of blastocysts *in vitro*. (Submitted).
- II Abraham, M.C., Puhakka, J., Ruete, A., Al-Essawe, E., de Verdier, K., Morrell, J.M. & Båge, R. (2016). Testicular length as an indicator of the onset of sperm production in alpacas under Swedish conditions. *Acta Veterinaria Scandinavica*, 58 (10), pp. 1-8.

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Abbreviations

AI	artificial insemination
ALH	amplitude of lateral head deviation
ART	assisted reproduction techniques
AV	artificial vagina
BCF	beat cross frequency
BCS	body condition score
CASA	computer-assisted semen analysis
CITES	convention on international trade in endangered species of wild fauna and flora
COCs	cumulus-oocyte complexes
EE	electroejaculation
FITC-PNA	fluorescent isothiocyanate-conjugated lectin
GAGs	glycosaminoglycans
HOST	hyposmotic swelling test
IVF	<i>in vitro</i> fertilisation
IVITA	instituto veterinario de investigaciones tropicales y de altura (veterinary institute for tropical and high altitude research)
LIN	linearity
mL	millilitres
OIF	ovulation-inducing factor
PBS	phosphate-buffered saline solution
PI	propidium iodide
SACs	South American camelids
SCSA	sperm chromatin structure assay
SLC	single layer centrifugation
SLU	Swedish university of agricultural sciences
Spz	spermatozoa
STR	straightness

SVA	statens veterinärmedicinska anstalt (Swedish veterinary institute)
TB	toluidine blue
VAP	velocity of the average path
VCL	curvilinear velocity
VSL	straight line velocity
WOB	wobble
β-NGF	β -nerve growth factor
μL	microliters
%DFI	DNA fragmentation index

1 Introduction

1.1 South American Camelids: Alpaca, Llama, Vicuna and Guanaco

South American camelids, also called New World camelids, are members of the Camelidae family together with the Old World Camels: Dromedary, Bactrian, and Wild Bactrian (Table 1). Alpacas and llamas were domesticated around 6000 years ago, and camels 5000 years ago. Guanacos and vicunas have never been domesticated; they are protected by the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES, Appendix I and II).

Table 1. *Classification of camelids and other artiodactylids (Fowler, 2010).*

Class--Mammalia
Order--Artiodactyla
Suborder--Suiformes--Hippos, swine, peccaries
Suborder--Tylopoda--Camelids
Old World genus and species
Camelus dromedarius--Dromedary camel
C. bactrianus--Bactrian camel
C. bactrianus ferris--Wild bactrian camel
New World genera and species
Lama glama--Llama
L. guanicoe--Guanaco
Vicugna pacos--Alpaca
V. vicugna--Vicuna
V. vicugna mensalis (Peruvian)
V. vicugna vicugna (Argentine)
Suborder--Ruminantia--Cattle, sheep, goats, water buffalo, giraffes, deer, antelopes, bison

Alpacas are reared for fibre production and, in some places such as Perú and Australia, they are considered a healthy source of meat. They are also kept as companion animals and for exhibitions. Recently people have started to use them for animal-assisted therapy in human medicine, since it has been shown that people feel less stressed when they are in contact with these animals.

There are several differences between ruminants and camelids (Table 2).

Table 2. *Differences between South American camelids and ruminants (adapted from Fowler, 2010).*

	South American camelids	Ruminants
Evolution	Evolutionary pathways diverged 40 million years ago	Evolutionary pathways diverged 40 million years ago
Blood	Red blood cells elliptical and small (6.5µ); predominant white blood cell is neutrophil; leukocytes up to 22.000	Red blood cells round and larger (10µ); predominant white blood cell is lymphocyte; leukocytes up to 12.000
Foot	Foot has toenails and soft pad. Second and third phalanges are horizontal	Foot has hooves and sole. Second and third phalanges are nearly vertical.
Digestive System	Foregut fermenter, with regurgitation, rechewing and reswallowing Stomach - 3 compartments, resistant to bloat Dental formula - I 1/3, C 1/1, PM 1-2/1-2, M 3/3 x 2= 28-32	Same (parallel evolution) Stomach - 4 compartments, susceptible to bloat Dental formula - I 0/3, C 0/1, PM 3/3, M 3/3 x 2= 32
Reproduction	Induced ovulator No estrous cycle Follicular wave cycle Copulation in the prone position Placenta diffuse Epidermal membrane surrounding fetus Cartilaginous projection on tip of penis Ejaculation prolonged	Spontaneous ovulation Estrous cycle No follicular waves Copulation in standing position Placenta cotyledonary No epidermal membrane on fetus No cartilaginous projection on tip of penis Ejaculation short and intense
Respiratory System	Soft palate elongated; primarily a nasal breather	Soft palate short; nasal or oral breather
Urinary System	Kidney smooth and elliptical Suburethral diverticulum in female at external urethral orifice Dorsal urethral recess in male at junction of pelvic and penile urethra	Kidney smooth and lobed No suburethral diverticulum Dorsal urethral recess in some species

There are two breeds of alpacas: “Huacaya” and “Suri”. Huacaya fiber is short and crimped, giving the appearance of Corriedale sheep wool (Fowler, 2010). Suri fiber is longer, not crimped and hangs down alongside the body.

1.2 South American Camelids in Sweden

In recent years, the international interest in breeding South American Camelids (SACs) has increased together with the demand for more accurate information about their health care and animal husbandry. In Sweden, alpacas have become progressively more popular during the last decades; however, knowledge about optimal management practices and disease panorama under Swedish conditions is still limited (de Verdier & Bornstein 2010; Björklund 2014; Abraham *et al.*, 2016).

1.2.1 A new rurality in Sweden?

A new rurality is defined as “a concept that describes the most recent changes in agriculture and rural areas where people reinvent themselves in order to supply not only products to fulfil nutritional demands, but also recreation and leisure opportunities, secure future environmental sustainability, promote growth, counteract depopulation, promote gender equality and offer regions and nations a sense of history and tradition” (Rytkönen, 2014). Although it is an emerging concept, its importance is growing in different regions in Europe, including Sweden (SKL, 2014). Alpaca production could be a potential new rurality in Sweden (*Figure 1*) and a good example of multifunctional agriculture such as the use of marginal land. Moreover, there is potential to integrate this animal production with other livestock production, agrotourism or recreation, for example “alpaca trekking”.

1.3 Reproductive anatomy and physiology of the alpacas

South American camelids have unique anatomical, behavioural and physiological reproductive characteristics. They differ from other domestic animals (Table 2) and, as knowledge advances, it is becoming clear that extrapolations from other domestic livestock are not relevant to camelids (Argañaraz *et al.*, 2015). They are usually classified as non-seasonal breeders but this is still under debate. Seasonality seems to be related to the environmental constraints and management systems; in the harsh conditions of the high Andes they typically display sexual activity during the warm months, whereas in milder habitats alpacas can be sexually active throughout the year

(Aba, 2014). This is also related to their nutritional status and the possibility of fulfilling their requirements under different conditions.

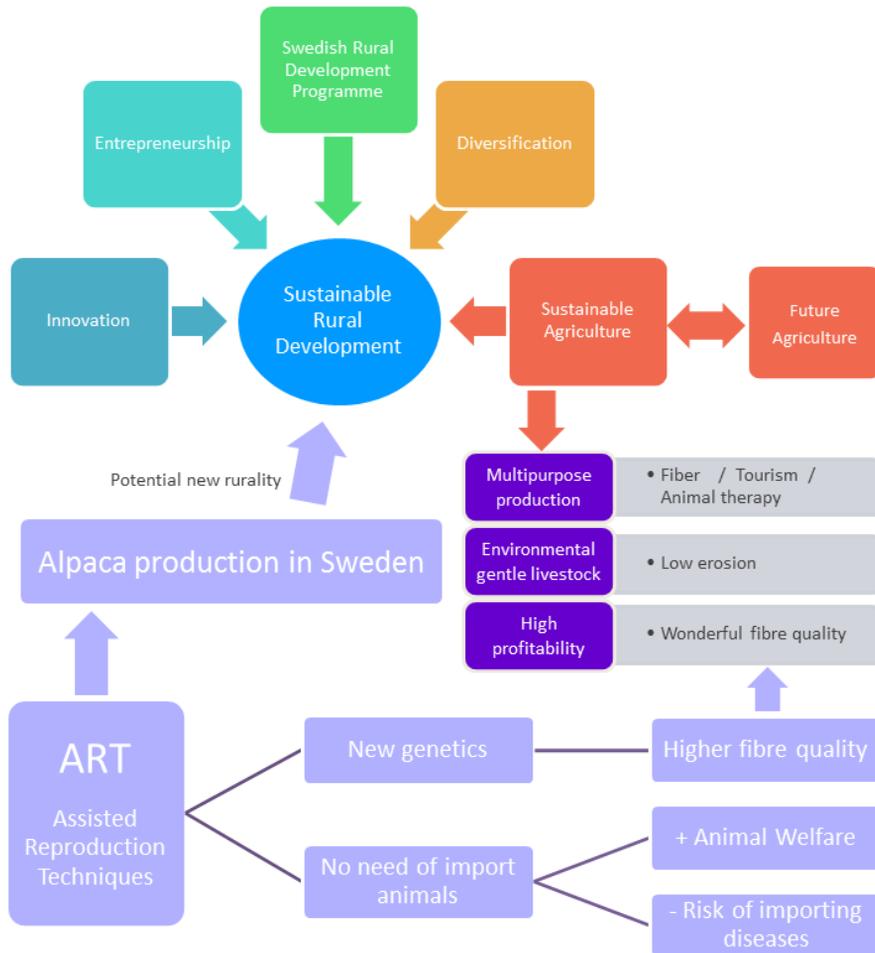


Figure 1. Flow diagram presenting alpaca production in Sweden as a potential new rurality, where all of the components end in sustainable rural development.

Females are induced ovulators, which means they require copulation to trigger the luteinizing hormone responsible for ovulation. In addition, it is known that a potent factor named “Ovulation-inducing factor” (OIF) is present in the seminal plasma and has been identified as 27kDa protein β -nerve growth factor (β -NGF) by Kershaw-Young *et al.* (2012).



Figure 2. The tip of the penis ends in a curved, fibro-cartilaginous projection.

The gestation period is on average 345 days and pregnancies are mainly established in the left uterine horn irrespective of whether ovulation occurs on the right or left side. Nutrition is an important factor that influences the ovarian activity. Females need to reach approximately 65% of their mature body weight to have ovarian activity, to be able to become pregnant and not to have stunting or parturition problems (Vaughan, 2015a).

Male alpacas have a fibro-elastic penis, 1-2 cm in diameter and 35-40 cm long, which has a prescrotal sigmoid flexure to retract it into the prepuce in the non-erect state (*Figure 2*). The tip of the penis ends in a curved, fibro-cartilagenous projection, which is probably an adaptation to facilitate the passage through the cervix (Fowler, 2010). Semen is deposited in the uterine horns. The prepuce of immature males adheres to the glans penis; therefore they cannot exteriorise their penis for mating until they secrete high levels of testosterone as they approach puberty. Puberty is influenced by genetics and body weight, among other factors.

The only accessory sex glands present in alpacas are a pair of bulbourethral glands and a small prostate; ampullas and seminal vesicles are lacking. The testes are relatively small and non-pendulous; testicular size can be measured as mean testicular length (Galloway, 2000; Abraham *et al.*, 2016).

Mating is in sternal recumbency lasting on average about 15- 20 minutes (range 15 to 60 minutes). The male vocalizes during mating with a characteristic guttural sound called “orgling”. Ejaculation occurs throughout

the entire mating period, called “dribble ejaculation”, producing an ejaculate of low volume, low sperm concentration and high viscosity (Tibary & Vaughan 2006; Morton *et al.*, 2008).

1.4 Characteristics of alpaca semen

Evaluating the characteristics of the semen in SACs is problematic for several reasons: the difficulty of obtaining consecutive ejaculates from the same individuals, the peculiarities of copulation and ejaculation in these species, and the high viscosity of the semen, which makes evaluation difficult. Alpaca semen has a low sperm concentration (0.3 – 150 million/mL) and low volume (1-2 mL in average) (Stuart & Bathgate, 2015). The seminal plasma is very viscous and distributed throughout the ejaculate, rather than being a distinct gel fraction or gel plug. This viscosity impedes semen assessment as it entraps the spermatozoa causing them to move in an oscillatory manner with limited progressive motility (Garnica *et al.*, 1993; Deen *et al.*, 2003). Furthermore, it hinders the use of stains to evaluate morphology and homogenous mixing with extender, limiting contact with the cryoprotective agents during cryopreservation (Kershaw-Young & Maxwell, 2012a). Until a few years ago it was thought that glycosaminoglycans (GAGs) were responsible for the high viscosity (Ernst *et al.*, 1995). However, Kershaw-Young & Maxwell (2012a) identified a protein: mucin 5B as the most likely viscosity-causing factor.

There are several factors affecting semen characteristics: duration of copulation, effect of successive ejaculations, individual male variation, age, season, semen collection technique among others (Tibary & Vaughan, 2006; Morton *et al.*, 2010a; Bravo, 2014).

1.5 Semen collection techniques

Collection of semen from camelids presents many difficulties due to the nature of their copulatory behaviour and the slow process of ejaculation, as described above. The main techniques used are the artificial vagina (AV), electroejaculation or post-mating aspiration from a female (Tibary & Vaughan, 2006).

1.5.1 Artificial vagina

Semen can be collected using an AV by allowing the male to mount a phantom or a receptive female. The penis is diverted into a hand-held AV or the AV may be fixed inside the phantom. Using a receptive female as a stimulus, males can be trained by encouraging them to mount the phantom. The phantom is

placed behind a receptive female, allowing the male to see and smell her during the entire collection. The temperature and pressure inside the AV are very important. Successful collection of semen by AV has been reported in alpacas (Sumar & Leyva, 1981; Vaughan *et al.*, 2003; Huanca & Adams, 2007; Morton *et al.*, 2010a) and llamas (Lichtenwalner *et al.*, 1996; Von Baer & Hellemann, 1999, Huanca & Gauly, 2001).

1.5.2 Electroejaculation

Electroejaculation should always be performed under general anaesthesia. An electroejaculator, commonly used in sheep and goats, could be used for camelids but it is important to consider that the response to the electrical stimulus varies amongst males. The probe is inserted into the rectum to stimulate sex glands and induce ejaculation. This technique has been used successfully in llamas (Director *et al.*, 2007; Giuliano *et al.*, 2008), alpacas (Fernandez-Baca & Calderon, 1966) and vicunas (Giuliano *et al.*, 2013).

1.5.3 Post-mating aspiration

This technique can be used as a routine to perform semen evaluation on a sample aspirated from the female genital tract after mating (Tibary *et al.*, 2014; Dascanio, 2014).

1.5.4 Epididymal spermatozoa

Spermatozoa (spz) collected from the epididymis can be used. Epididymal spz do not have the same restrictions as ejaculated spz because they have not been exposed to seminal plasma; therefore, they provide a good model for research and can be obtained readily when animals are castrated for husbandry purposes. Previous studies in other species, including goats, red deer, dogs, and humans, reported successful epididymal sperm collections with pregnancies after artificial insemination (Cary *et al.*, 2004).

1.5.5 Other techniques

Other techniques have been described with variable results: intravaginal condom (Mogrovejo, 1952; McEvoy *et al.*, 1992); vaginal sponge (San Martin *et al.*, 1968); urethral fistulation (Von Kubicek, 1974) and deviation of deferent ducts (Quintano, 2002; Perez *et al.*, 2014).

1.6 Semen evaluation

When performing semen analysis, different factors should be taken into consideration. Examination in summer may result in a suboptimal semen

sample from a male that otherwise would have normal fertility (Dascanio, 2014). Too frequent mating may also negatively affect semen quality. The collection method, nutritional status and age need to be critically evaluated. According to Tibary & Vaughan (2006), the major problem in semen analyses in this species is the lack of standard methods for collection and examination.

Volume: the volume ranges from 0.5 to 2 mL.

Colour: semen should appear opaque white. If aspirated after mating from the vagina of the female, it may have a slight reddish colour due to blood contamination.

Viscosity: to evaluate this parameter a “thread test” is used. The test is performed by pulling semen up from a surface, the length of the threading is measured (*Figure 3*).

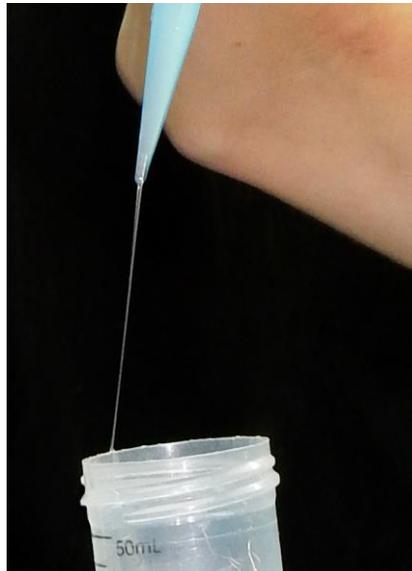


Figure 3. Thread test to evaluate camelid semen viscosity.

Sperm concentration: concentration is generally estimated using a hemocytometer. Sperm concentration is highly variable, affected by age, method of collection and frequency of ejaculation.

pH: The pH of the alpaca semen is between 7.5 and 8 (Morton *et al.*, 2008).

Sperm motility: motility analysis is difficult because of the viscous nature of the ejaculate. Normally oscillatory motility is seen rather than progressive

motility. Motility should be 30% or greater for a mature breeding male (Dascanio, 2014).

Sperm morphology: mature camelid spz present the same anatomical features as other domestic mammals. Ideally, more than 50% normal morphology should be present in a sample from an adult male in active breeding (Dascanio, 2014). Morphology can be assessed with different stains.

Biochemical components: the biochemical composition of camelid semen is similar to that reported for other livestock species.

Viability: the viability is measured by assessing the membrane integrity as follows:

- The ratio of live/dead spz may be assessed using an eosin-nigrosin stain (Dascanio, 2014).
- Flow cytometry analysis after staining the spz with mixtures of fluorophores, such as Syto-16 and Propidium iodide (Kershaw-Young & Maxwell, 2011)
- Hypoosmotic swelling test (HOST) is used to obtain the percentage of spz with functional membranes (See Materials & Methods Section)

Acrosome integrity: Kershaw-Young & Maxwell (2011) validated a method to assess acrosome integrity in alpaca spz using fluorescent isothiocyanate-conjugated lectin (FITC-PNA).

Chromatin integrity: the DNA in the spz is highly condensed and known as “chromatin”. There are different techniques to evaluate the chromatin integrity:

- Toluidine blue: Carretero *et al.* (2011, 2012) validated this technique in alpaca and llama spz (See Materials & Methods Section).
- Sperm chromatin structure assay (SCSA) assessed by means of the metachromatic stain acridine orange using flow cytometry. Intact double stranded DNA fluoresces green whilst damaged, single stranded DNA fluoresces red. The ratio of the red fluorescence to the red+green fluorescence indicates the proportion of spz with damaged chromatin, measured as the DNA fragmentation index (%DFI).
- Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL): Kershaw-Young & Maxwell (2011) validated this method for alpaca spz.

1.7 Reproductive biotechnologies in SACs. Possibilities and limitations

In the latter part of the 20th century, SACs were exported from South America to other countries, where populations are continuously expanding. The herds

have been built up by importing live animals which, apart from the implications of animal welfare during the long transport, carries the risk of importing parasites and pathogens, including some important zoonoses. An alternative to importing live animals could be to use reproductive biotechnologies (also known as assisted reproduction technologies: ARTs).

Moreover, reproductive biotechnologies such as semen cryopreservation, artificial insemination and embryo transfer can have a significant impact on livestock industries. In SACs, improving cryopreservation techniques would contribute to the development of programs of genetic improvement. None of these reproductive biotechnologies are well-developed in males in this species due to the problems with semen collection and the viscous nature of the seminal plasma (Morton *et al.*, 2008; Kershaw-Young & Maxwell, 2011; Stuart *et al.*, 2015). The application of some technologies in female SACs have increased in recent years, for instance synchronization of ovarian follicular development, ovarian superstimulation, embryo transfer (Trasorras *et al.*, 2013), inter-species embryos transfer (Pacheco *et al.*, 2015) and oocyte cryopreservation by vitrification (Ruiz *et al.*, 2013).

1.7.1 Artificial insemination

Artificial insemination (AI) is an important technique used to ensure fast genetic progress in domestic species. This technique has many advantages as it allows more efficient use of genetically superior males by inseminating more than one female with a single ejaculate and thus increasing the number of offspring. Moreover AI can prevent the spread of venereal infections that can cause a decrease in fertility. Although the first report about AI in camelids was in 1968 by Fernández-Baca & Novoa, the use of this technique remains limited and insemination trials are still rare (Bravo, 2014; Skidmore *et al.*, 2013). This could be due to the difficulties in collecting and handling the semen (Skidmore *et al.*, 2013).

1.7.2 Semen cryopreservation

Semen cryopreservation in other species not only increases the efficiency of breeding, but also allows easy transport and storage of semen from superior sires (beyond the lifespan of the donor), as well as the prevention and control of disease. In comparison with other domestic species, there have been few investigations of freezing methods for alpaca spz (Morton *et al.*, 2007). The development of an efficient method of cryopreserving alpaca spz could be used as the basis for developing similar methods for preserving spz from vicuna and guanaco; both of these species are protected by the CITES (Appendix I and II).

2 Aims of the thesis

- To develop methods of handling and assessing alpaca sperm quality in both epididymal and ejaculated sperm samples.
- To develop improved methods for sperm selection and preservation.

3 Materials and Methods

In the present study, there was a collaboration between two universities (Swedish University of Agricultural Sciences and San Marcos National University from Perú). In addition, SVA (National Veterinary Institute), Swedish veterinarians and Swedish alpaca owners formed part of this project, sending alpaca testicles from castrations or *post mortem* analysis.

A summary of the methods used in the thesis is provided in *Figure 4*, and more detailed descriptions are presented in Papers I and II. The methods are organized as follow:

	<p>Sweden</p> <ul style="list-style-type: none">• Laboratory techniques (semen evaluation and selection techniques)• Epididymal spz (testicles from routine castrations and cadavers)• Semen collection trials (design of phantom with AV)
	<p>Perú</p> <ul style="list-style-type: none">• Semen collection trials (phantom with AV and post-mating aspiration)

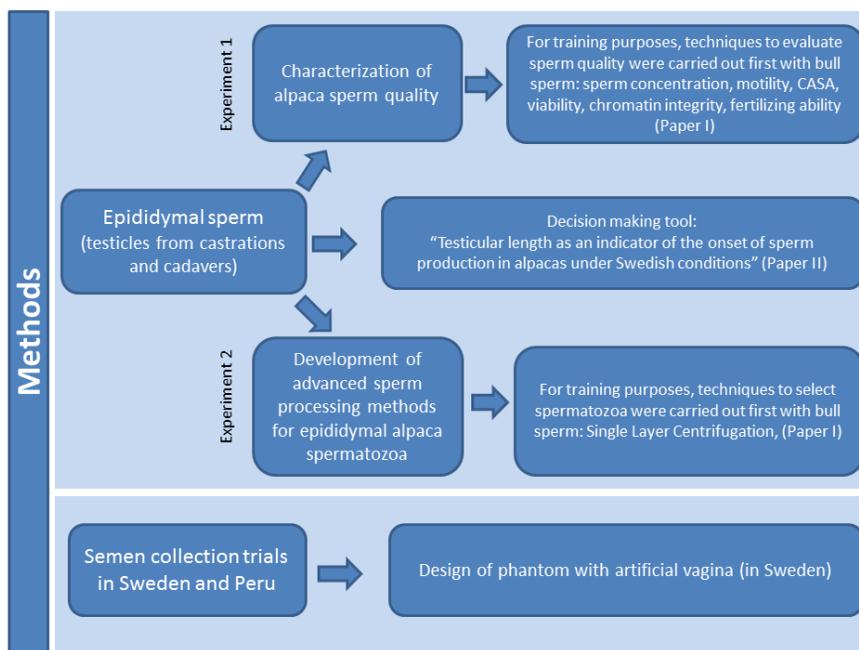


Figure 4. Summary of the methods.

3.1 Sweden

3.1.1 Laboratory techniques

Different techniques to evaluate sperm quality and select the best spz were performed at the Clinical Sciences laboratory at the Swedish University of Agricultural Sciences, mainly using bull spz as a model.

Sperm selection: Single Layer Centrifugation

Single Layer Centrifugation (SLC) through a species-specific colloid is a sperm selection technique that has been shown to improve sperm quality in different species. For training purposes two experiments of SLC were performed with bull spz using fresh semen (Yulnawati *et al.*, 2014) and frozen straws (Paper I). For SLC, an aliquot of semen was layered on top of a column of colloid Bovicoll (previously known as Androcoll-B) and the tubes were centrifuged at 300 x g for 20 minutes. Each sperm pellet was transferred to a clean tube and resuspended in semen extender, and the effect of SLC on bull sperm quality was evaluated. In the first experiment, a large volume of extended semen (15 mL) was prepared on 15 mL Bovicoll; in paper I, three variants of the SLC were compared: "Small" (4 mL of colloid in a conical

centrifuge tube), “Mini” (1 mL of colloid in a conical centrifuge tube) and “Mini-EP” (1 mL of colloid in a 1.5 mL Eppendorf® tube), each with 200µL thawed bull semen.

Sperm concentration

To measure the sperm concentration of the semen samples a Nucleocounter SP 100 (Chemometec, Allerød, Denmark) was used (Paper I).

Sperm motility

Sperm motility was assessed by computer-assisted semen analysis, CASA, using the SpermVision™ (Minitüb, Tiefenbach, Germany) connected to an Olympus BX 51 microscope (Yulnawati *et al.*, 2014 and Paper I). Eight fields were analysed, evaluating at least 1000 spz, and several kinematics were recorded, that is total and progressive motility (%), velocity of the average path (VAP; µm/s), curvilinear velocity (VCL; µm/s), straight line velocity (VSL; µm/s), straightness (STR; VSL/VAP), linearity (LIN; VSL/VCL), wobble (WOB; VAP/VCL), amplitude of lateral head deviation (ALH; µm), beat cross frequency (BCF; Hz) and hypermotility (%). The yield of motile spz after SLC was calculated as follows:

$$\text{Yield (\%)} = \frac{(\text{total number of motile spermatozoa after SLC})}{(\text{total number of motile spermatozoa before SLC})} * 100$$

Viability: Hypoosmotic swelling test

The hypoosmotic swelling test was used to obtain the percentage of spz with functional membranes. The sample was incubated at 37°C for 1 h, in a 100 mOsm fructose-Sodium Citrate solution. After incubation, a drop was placed on a slide with a cover glass and a total of 200 spz was evaluated by phase-contrast microscopy (Paper I).

Chromatin integrity: Toluidine blue

Toluidine blue (TB) stain allows the differentiation of spz according to the degree of chromatin condensation. Smears were prepared, air-dried and fixed with ethanol. Afterwards the smears were stained with working solution, rinsed and air-dried, protected from light. A total of 200 spz was evaluated by phase-contrast microscopy (Olympus BH 2). According to Carretero *et al.*, (2010), spz with normal chromatin condensation stain light blue; spz with some degree of decondensation stain light violet: intermediate; those with a high degree of decondensation stain dark violet.

Sperm chromatin structure assay

Chromatin integrity was evaluated using the SCSA via flow cytometry of acridine orange-stained spz (Evenson & Jost, 2000; Goodla *et al.*, 2014). Aliquots of semen samples were mixed 1:1 v/v with TNE buffer, snap-frozen in liquid nitrogen and transferred to a -80°C freezer until analysed. The DNA fragmentation index (%DFI) is calculated as the proportion of spz fluorescing red out of the total population: spz with red and spz with green fluorescence (Paper I).

Fertilizing ability of the selected spermatozoa

In vitro fertilization (IVF) trials were performed in order to evaluate the fertilizing ability of the bovine spz selected with Bovicoll (Paper I).

The methods used have been described previously by Abraham *et al.*, 2012 and Laskowski *et al.*, 2016 with slight modifications. Bovine ovaries were collected at a slaughterhouse and transported to the laboratory. Cumulus-oocyte complexes (COCs) were aspirated and collected in search medium; only good quality COCs were selected for maturation. After maturation, COCs were washed and pipetted and then transferred to wells with fertilization media. The spz were thawed and two sperm selection techniques were performed: Mini-SLC and swim-up, as control. After evaluation, spz were added to the oocytes and incubated. After 22h, fertilized oocytes were denuded and cultured in a specific medium; cleavage was checked and the number of blastocysts developed by day 7 and day 8 were recorded. The cleavage rates and blastocyst rates were calculated from the number of fertilised oocytes. On day 8, all blastocysts were graded, fixed and stained. The number of nuclei was recorded.

3.1.2 Epididymal spermatozoa

Testicles and epididymis from male alpacas were obtained from routine castrations and from *post mortem* evaluations (Paper II).

Testicles from routine castrations

Testicles were obtained from on-farm routine castrations of male alpacas on several Swedish farms. The pairs of testicles were placed in plastic bags with phosphate-buffered saline solution (PBS) and were transported to the laboratory in styrofoam boxes containing a cold pack at 4°C, where they arrived within 24-48 hours of surgery.

Testicles from cadavers

Testicles were obtained at necropsy from male alpacas from different Swedish farms. The *post mortem* evaluations were conducted at Eurofins in Skara,

Sweden. The pairs of testicles were placed in plastic bags and were transported to the laboratory, where they arrived within a week of death.

Procedure

After the tunica albuginea, connective tissues and blood vessels were removed, the length of the testicles was measured with a ruler (*Figure 5*). The epididymis was separated from the testes, the cauda epididymis was isolated, cut into 4-5 pieces and placed in a petri dish, in pre-warmed semen extender AndroMed (Minitüb, Tiefenbach, Germany) or INRA 96 (IMV Technologies, L'Aigle, France) for samples from castrations and in PBS for samples from cadavers. After incubation (10 minutes, at 37°C in 5% CO₂), the presence of spz was verified. If there were motile spz, staining with FITC-PNA was performed (Cheng *et al.*, 1996).

The relationship between mean testicle length and the individual's age was analysed with a non-linear regression model (Paper II). We tested for the effect of age and mean testicle length on the presence of spz using logistic generalized linear models (Paper II).

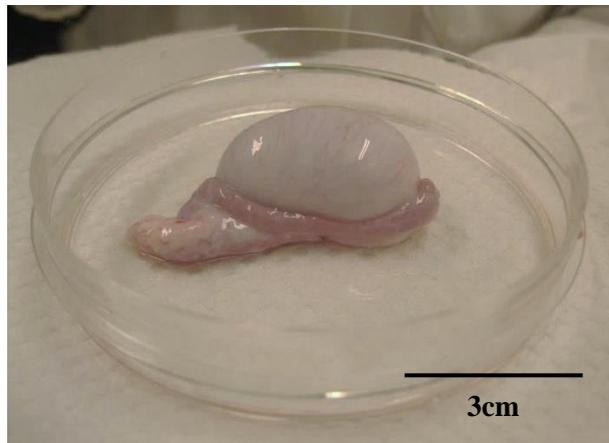


Figure 5. Alpaca testicle and epididymis after removal of tunica albuginea, connective tissues and blood vessels.

3.1.3 Semen collection trials

Animals and facilities

The semen collection trials were performed on a private farm situated near Sala (61 m above sea level), 70 km from the Swedish University of Agricultural

Sciences in Uppsala. It has pasture-based husbandry where there are approximately 100 animals divided in single sex groups (*Figure 6*).



Figure 6. Group of males at a private alpaca farm in Sweden.

Design of phantom and Artificial Vagina

The phantom was constructed following the specifications provided by a research group in Australia (Morton *et al.* 2008; Kershaw-Young, 2012b). The base of the phantom consisted of a plastic plant-pot covered with a synthetic hide, which was attached with Velcro to facilitate removal and cleaning, whereas the neck consisted of a foam pipe covered with the same hide (*Figure 7*). The AV was prepared following a description made by Morton 2008. A special rubber tube was used with a silicone liner inside. The ends of the silicone liner were then folded back over the AV and secured with rubber bands. The chamber between the hard rubber tube and the liner was filled with hot water (45°C) via the valve. The AV was inflated by blowing in the air valve and a camel semen collecting glass was placed in one end. The whole unit was wrapped in an electric blanket.

3.2 Perú

3.2.1 Semen collection trials

The aim was to compare two semen collection methods in alpacas: a phantom with AV and post-mating aspiration. After the evaluation of the sperm sample (motility, concentration and membrane integrity), SLC was performed to extract spz from the viscous seminal plasma. Smears were

prepared and 50 μ L of sample was fixed in a tube with formol saline for morphology evaluations.



Figure 7. Phantom and artificial vagina (a-c: MC Abraham; d: P Nicholls).

Animals and facilities

Animals were kept in a traditional manner (extensive grazing on pastures at 4300 m above sea level), in a group of approximately 300 alpacas and llamas, at La Raya-Pata which is situated between Cuzco and Puno Districts in Perú (Figure 8). These animals belong to the field station IVITA-Maranganí (San Marcos National University, Lima, Perú).

Semen collection with a phantom

Males were exposed to the phantom and those which demonstrated interest were selected for training and semen collection. Males were allowed to mate until they stood up voluntarily, indicating no further interest.

Post-mating aspiration

This technique, described by Alarcon *et al.* (2012), sometimes is used in Perú to collect semen samples. Samples were aspirated from the female genital tract immediately after mating using a uterine pipette fitted with a syringe.



Figure 8. A group of alpacas and llamas at La Raya-Pata (4300 m.a.s.l.), situated between Cuzco and Puno, Perú.

4 Results

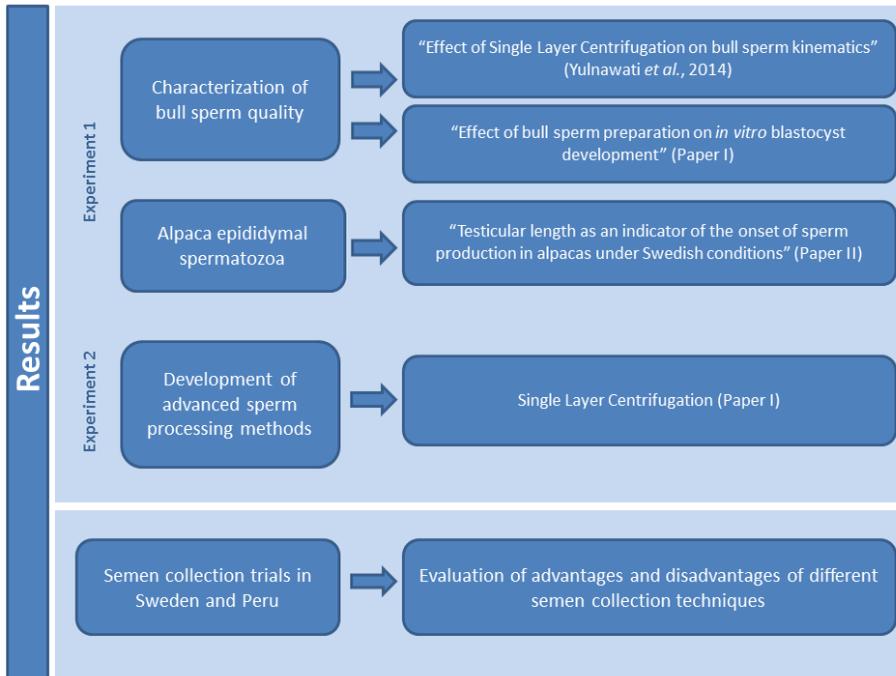


Figure 9. Summary of the results.

4.1 Effects of Single Layer Centrifugation on bull sperm kinematics

In Table 3 the sperm kinematics before and after SLC are presented (Yulnawati *et al.*, 2014). While total motility, progressive motility, and VSL were similar, VAP, VCL, ALH and hypermotility were lower in SLC samples. STR, LIN and BCF were significantly higher in SLC samples compared with unselected samples. Table 4 shows the improvement after SLC of the samples with the

poorest motility; STR, LIN, WOB, ALH, BCF and hypermotility were improved after SLC. The effect of SLC on hemospermic samples is shown in Table 5. In the hemospermic sample, total motility, progressive motility, ALH and hypermotility were reduced compared to non-hemospermic samples while VSL and STR were increased. After SLC, total motility, progressive motility and ALH increased, while VSL, VAP, VCL, STR and hypermotility decreased.

Table 3. Mean (\pm SD) sperm kinematics before and after single layer centrifugation ($n = 80$).

Parameters	Uncentrifuged	SLC	Significance (p)
Total motility (%)	86.0 \pm 3.2	88.2 \pm 3.3	NS
Progressive motility (%)	83.6 \pm 3.4	84.4 \pm 3.8	NS
VSL ($\mu\text{m/s}$)	62.4 \pm 4.3	62.1 \pm 4.1	NS
VAP ($\mu\text{m/s}$)	94.7 \pm 5.3	89.8 \pm 5.6	0.0037
VCL ($\mu\text{m/s}$)	191.6 \pm 13.6	180.0 \pm 14.1	0.0303
STR (VSL/VAP)	0.65 \pm 0.04	0.69 \pm 0.05	0.0046
LIN (VSL/VCL)	0.32 \pm 0.03	0.34 \pm 0.03	0.0021
WOB (VAP/VCL)	0.49 \pm 0.02	0.49 \pm 0.02	NS
ALH (μm)	7.0 \pm 0.5	6.5 \pm 0.6	0.0146
BCF (Hz)	22.3 \pm 1.2	23.8 \pm 1.3	0.0015
Hypermotility (%)	48.2 \pm 9.7	36.5 \pm 13.8	0.0023

NS = non-significant difference ($p > 0.05$)

Table 4. Changes in sperm kinematics after single layer centrifugation for the bulls with the poorest sperm quality in uncentrifuged samples.

Parameters	Uncentrifuged	SLC	Significance (p)
Total motility (%)	84.5 \pm 3.9	87.8 \pm 3.4	NS
Progressive motility (%)	81.5 \pm 3.9	83.5 \pm 3.7	NS
VSL ($\mu\text{m/s}$)	90.0 \pm 3.4	89.5 \pm 3.9	NS
VAP ($\mu\text{m/s}$)	184.5 \pm 12.0	178.0 \pm 11.1	NS
VCL ($\mu\text{m/s}$)	61.5 \pm 1.4	65.0 \pm 3.4	NS
STR (VSL/VAP)	0.68 \pm 0.01	0.73 \pm 0.04	0.063
LIN (VSL/VCL)	0.33 \pm 0.02	0.37 \pm 0.03	0.012
WOB (VAP/VCL)	0.49 \pm 0.02	0.50 \pm 0.01	NS
ALH (μm)	6.6 \pm 0.49	6.1 \pm 0.56	0.068
BCF (Hz)	22.6 \pm 1.1	24.6 \pm 1.7	0.07
Hypermotility (%)	41.0 \pm 11.9	31.4 \pm 10.1	0.076

NS = non-significant difference ($p > 0.05$)

Table 5. Effect of hemospermia on sperm kinematics and effect of single layer centrifugation.

Parameters	Normal (n=3)		Hemospermia	
	Uncentrifuged	SLC	Uncentrifuged	SLC
Total motility (%)	87 ± 6	90 ± 3	61	94
Progressive motility (%)	85 ± 7	86 ± 3	60	91
VSL (µm/s)	98 ± 13	97 ± 6	97	86
VAP (µm/s)	200 ± 30	200 ± 11	203	169
VCL (µm/s)	57 ± 5	59 ± 4	72	57
STR (VSL/VAP)	0.58 ± 0.04	0.60 ± 0.01	0.73	0.66
LIN (VSL/VCL)	0.28 ± 0.03	0.29 ± 0.01	0.35	0.33
WOB (VAP/VCL)	0.49 ± 0.01	0.48 ± 0.01	0.47	0.50
ALH (µm)	7.6 ± 0.5	7.4 ± 0.5	5.9	7.1
BCF (Hz)	22 ± 1	23 ± 0.4	23	22
Hypermotility (%)	59 ± 9	57 ± 6	13	5

4.2 Effect of sperm preparation on *in vitro* blastocyst development

4.2.1 Comparison of three variants of a sperm selection technique

Total number of spz, yield of motile spz after SLC, progressive motility and chromatin damage are shown in *Figure 10*. Of the three treatments, Mini-SLC produced the highest yield of motile spz. For progressive motility, there was no significant difference between Mini and Mini-EP SLC; however, Small-SLC resulted in significantly lower motility than the other two treatments. There was no significant difference between Mini-SLC and Mini-EP for membrane integrity, although Small-SLC contained significantly fewer membrane-intact spz than the unselected sample. Sperm chromatin damage, %DFI, was significantly lower in the selected samples than the unselected control (1.84 and 2.99 respectively, $p = 0.036$), with no significant differences between the SLC treatments ($p > 0.85$).

4.2.2 *In vitro* fertilization trials

The results of the IVF experiment are presented in Table 6. Cleavage rate and blastocyst rate did not differ significantly between groups. There was no statistically significant difference in the total number of cells present in the blastocysts between Mini-SLC and Control (96.05 ± 36.86 and 91.57 ± 39.07 , respectively).

Table 6. Summary of results obtained by IVF of bovine oocyte (n=320) from abattoir-derived ovaries. Comparison between Mini-SLC and Control (swim up). Cleavage and blastocyst rates calculated from the number of fertilized oocytes

Variable	Mini-SLC	Control
Cleavage rate %	84.25 ± 5.29	80.5 ± 5.25
Cleavage rate above 2-cell stage %	90.5 ± 1.0	89.5 ± 5.06
Blastocysts developed by day 7 % (n)	15.62 ± 7.18 (25)	11.25 ± 8.5 (18)
Blastocysts developed by day 8 % (n)	27.50 ± 3.53 (44)	22.50 ± 5.40 (36)

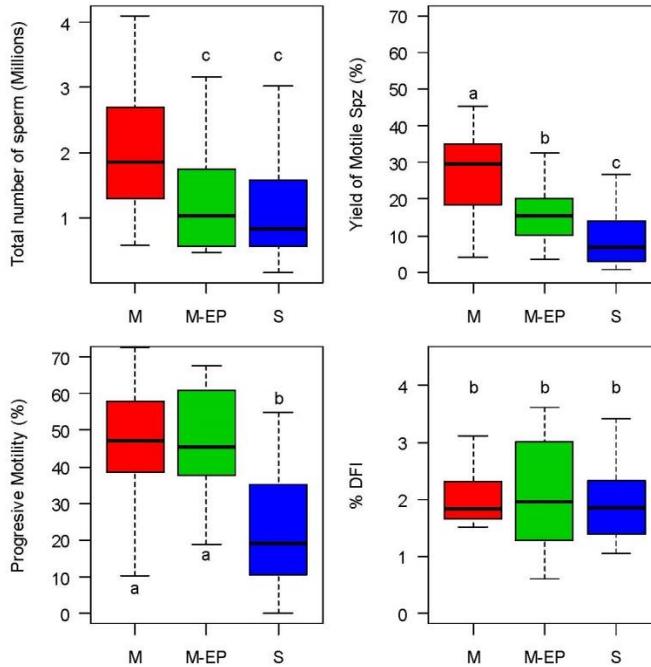


Figure 10. Results of total number of spermatozoa (spz), yield of motile spz after Single Layer Centrifugation (SLC), progressive motility, and chromatin integrity (%DFI=DNA fragmentation index). Selected samples: “M”= Mini SLC (1 mL of colloid in a conical centrifuge tube); “M-EP”= Mini SLC (1 mL of colloid in an Eppendorf® tube) and “S”= Small SLC (4 mL of colloid in a conical centrifuge tube). Unselected sample: “C” = Control. Labels “a”, “b” and “c” indicate significant differences between variables.

4.3 Recovery of epididymal spermatozoa

The presence of spz in testicles from castrations (Figure 11) and cadavers was determined. This study is presented in detail as Experiment 2 in Paper II. The results of the distribution of mean testicular length by age groups and the

presence of spz in the testicles from castrations and cadavers are presented in Table 7 and Figure 12 and Figure 13. If the spz were motile, FITC-PNA stain was performed to evaluate the acrosome integrity (Figure 14).



Figure 11. Alpaca testicles from routine castration, animals of different ages, “a”= 9 years old male, “b”= 2 years old male, and “c”= 18 months old male.

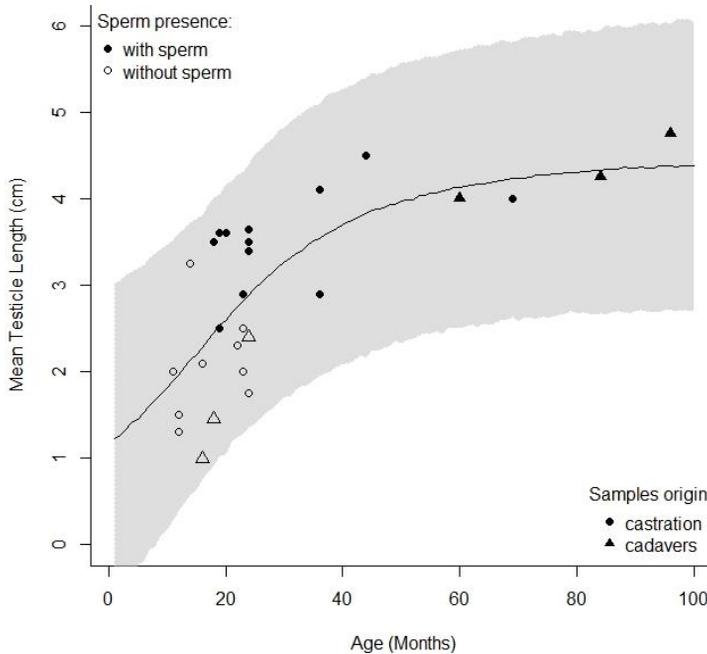


Figure 12. Length increment model for alpaca testicle length. Testicles from castrations (n = 22) and cadavers (n = 6).

Table 7. Mean testicular length, number of alpacas by age group and sperm presence (Testicles from castration n=22; testicles from cadavers n= 6). ^a: Age at castration/ necropsy

Mean length (cm)	Age ^a (months)	Sperm presence (%)	
		Castration	Cadavers
<3	12-23	1/6 (17)	0/3 (0)
	24-35	1/4 (25)	-
	>36	1/1 (100)	-
3 - 3.9	12-23	3/4 (75)	-
	24-35	3/3 (100)	-
	>36	-	-
>4	12-23	-	-
	24-35	-	-
	>36	4/4 (100)	3/3 (100)

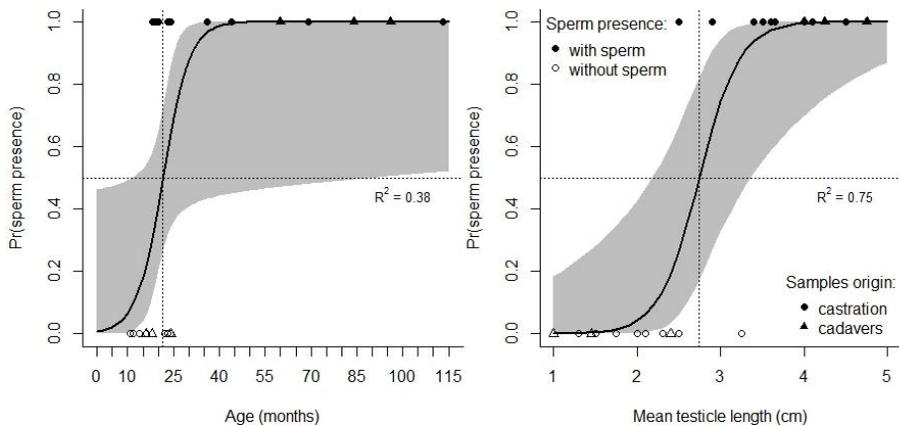


Figure 13. Probability of sperm presence in alpaca testicles. Samples from castrations and cadavers (n = 28) explained by: age and mean testicle length. Black circles represent organs from castrations, black triangles represent organs from cadavers; filled circles represent organs containing spermatozoa (spz), open circles represent organs without spz. Animals with testicular length ≥ 3.8 cm would be producing spz (median probability of sperm presence is ≥ 0.99); and animals with testicular length ≤ 1.6 cm will not be producing spz (median probability of sperm presence is ≥ 0.01).

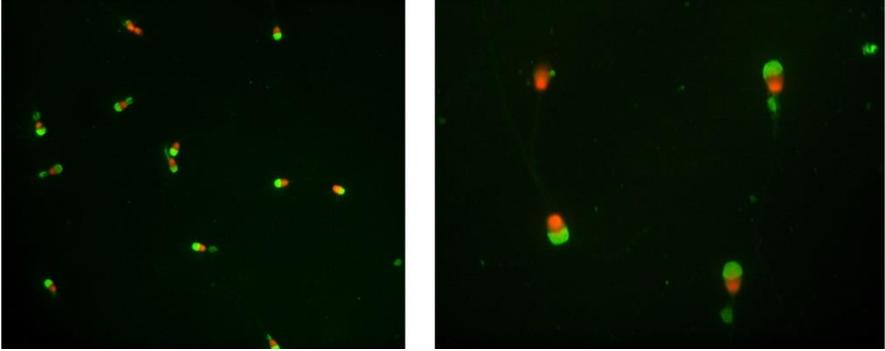


Figure 14. Alpaca spermatozoa stained with FITC/PNA (Cheng *et al.*, 1996). Green: intact acrosome; red: non-intact acrosome. Photo: E Al-Essawe.

4.4 Semen collection trials in Sweden

Attempts were made to collect semen samples with the phantom on three occasions. The first time, two males approached the phantom and mounted it (*Figure 15*), but no semen samples were obtained. Afterwards, in order to improve the design of the phantom, some modifications were made to facilitate the access of the penis to the AV. On the second occasion, a few millilitres of very viscous semen were collected from one of the three males. On the third occasion, a small volume of semen was obtained from a different male. The spz were immotile on reaching the laboratory.



Figure 15. Male mating the phantom in Sweden. Photo: JM Morrell.

4.5 Semen collection trials in Perú

Eight animals were selected to be trained with the phantom, although only six were kept for the experiment because two lost interest during training. Three of the six males responded to the AV, resulting in the collection of between 0.5 to 1 mL of samples, consisting mainly of foam (*Figure 16*). The mating duration was between 15 and 50 minutes. Smears were prepared for morphology evaluation (*Figure 17*).

Post-mating aspiration was performed in 14 females, in two groups. Samples were collected and then transported to the lab in a thermos with warm water (approximately 10 minutes' drive). There was a problem with the temperature during the transportation of the first group of samples; no further evaluation could be done because the samples were overheated (47°C). The second set of samples was difficult to evaluate due to the viscosity and the presence of blood. SLC was performed in two samples but only erythrocytes were found in the pellet.



Figure 16. Samples collected with an artificial vagina in Maranganí, Perú.



Figure 17. Alpaca spermatozoa stained with Williams' staining (1920). The semen was collected with an artificial vagina. Photo: A. Jansson

5 Discussion

5.1 Semen characteristics and semen handling

One of the aims of this thesis was to develop methods of handling and assessing alpaca sperm quality in both epididymal and ejaculated sperm samples. It was intended that epididymal extracted from post-castration organs would be used to test different semen extenders, to develop SLC for alpaca spz and to assess sperm fertilizing ability using a heterologous zona binding assay, before progressing to using these techniques with ejaculated spz. Since most of the castrations were carried out in pre-pubertal animals, it was not possible to obtain enough spz for these studies. In the absence of alpaca spz, techniques to evaluate sperm quality were carried out with bull spz for training purposes, resulting in the co-authorship of an article (Yulnawati *et al.*, 2014). In addition, testing variants of SLC with small volumes of semen was done with bull semen instead of alpaca semen (Paper I).

Camelid semen is characterized by a highly viscous, low volume ejaculate with a low sperm concentration; spermatozoa exhibit low progressive motility (Kershaw-Young & Maxwell, 2012b). The viscosity of the semen is one of the major impediments to the development of reproductive biotechnologies in these animals. In order to reduce viscosity, many techniques have been tested: pipetting, needling, vortexing, centrifugation, and use of non-specific enzymes. Morton *et al.* (2008) reported that centrifugation, needling and vortexing were ineffective or sometimes detrimental to spz. The use of enzymes is still controversial. According to Giuliano (2010), collagenase successfully reduced llama seminal plasma viscosity while sperm function was maintained. Conversely, in the alpaca, collagenase impaired sperm function (Morton *et al.*, 2008). In other studies, camelid semen was diluted with cryoprotectants (Deen *et al.*, 2003: dromedary camels; Santiani *et al.*, 2005: alpacas), reasonable post-thaw motility was achieved, but fertility was low and not commercially viable after insemination of frozen-thawed spz (Kershaw-Young & Maxwell, 2011).

The SLC technique has been shown to improve sperm motility in enzyme-treated samples from llama (Trasorras *et al.*, 2012) and in non-enzyme-treated samples from camel (Morrell, *personal communication*). According to Trasorras *et al.* (2012) colloid centrifugation would be the best method for separation of llama spz due to the possibility of working with ejaculates with a low sperm concentration. In addition, the result with the hemospermic bull sample showed that sperm kinematics are improved if the semen is processed by SLC (Yulnawati *et al.*, 2014) suggesting that epididymal sperm samples or samples contaminated with uterine fluids could benefit from processing by SLC.

Since alpaca spz are only poorly motile and do not readily pass through a colloid, we wanted to develop a protocol that used a smaller volume of colloid than the standard protocol. Previous studies in red deer used only 1 mL of colloid in an Eppendorf tube (Anel-Lopez *et al.*, 2015) instead of the more usual protocol of 4 mL colloid in a 10-15 mL centrifuge tube (Morrell *et al.*, 2009). However, previous studies have shown that the use of small diameter tubes reduces the surface area between the colloid and the sample, thus restricting the number of spz that can pass through the colloid (Morrell, *personal communication*). For this reason, we decided to compare the three variants of SLC described in Paper I.

In accordance with the previous studies on bull semen samples (Yulnawati *et al.*, 2014) our results showed a positive influence of SLC on several sperm kinematic parameters that may be important for fertilization (Paper I). A lower prevalence of chromatin damage was found in the selected samples compared with the unselected control, which is consistent with the findings of Goodla *et al.* (2014) for bull spz. However, other authors (Jiménez-Rabadán *et al.*, 2012; Anel-López *et al.*, 2015) reported no differences in chromatin damage between controls and SLC-selected samples for frozen goat and red deer spz respectively, perhaps reflecting a species difference.

In summary, good results were obtained using a Mini version of SLC, indicating that it is possible to use 1 mL of colloid instead of 4 mL to prepare frozen-thawed bull sperm samples. For maximum yield it is important to use a tube with a large diameter to optimise the contact between the colloid and the sperm sample. Such a modification would enhance its usefulness and acceptability in a variety of laboratory settings and could be applied to other species, such as SACs.

5.2 Recovery of epididymal spermatozoa

Although this technique has been mainly oriented to recover germplasm from individuals of endangered species that have died (Anel *et al.*, 2002; Santiani, 2012), it could also be used in other species as a source of spz to develop handling techniques for ejaculates.

However, these sperm samples can be contaminated with blood and cellular debris, which adversely affect sperm survival and may produce reactive oxygen species that damage spz during freezing (Muñoz-Fuentes *et al.*, 2014). Therefore, sperm selection techniques are recommended to separate the spz from cellular debris.

Previous studies in other species (goats, red deer, dogs, and humans) reported successful epididymal sperm collections and pregnancies (Cary *et al.*, 2004). This technique has been used in SACs by other researchers (Santiani, 2012; Morton *et al.*, 2007 and 2010b). Epididymal spz provide a good model for research because they have not been exposed to seminal plasma. This is particularly relevant for SACs because epididymal spz are free from viscous seminal plasma. Epididymal spz can be obtained when animals are castrated for husbandry purposes, or from the slaughterhouse in countries where these animals are kept for meat production. A disadvantage of the technique could be the age at which animals are castrated. In our study, the majority of the organs came from young animals, which appeared to be pre-pubertal; therefore only a few organs contained spz, which did not provide enough material to test different semen handling procedures.

Even though a low number of samples were obtained in this study, it could theoretically be a useful source of spz from organs that would otherwise be discarded, for example in countries where large numbers of alpacas are slaughtered for meat production. Furthermore, there are no welfare issues associated with the collection of spz from this discarded material. The disadvantage, as discovered here, is that males may be castrated before they start to produce spz, which negates the usefulness of this technique as a source of material (see later discussion on testicular length as an indicator of sperm production) and it is not possible to obtain multiple samples from the same animal, as would be the case for ejaculated spz.

5.3 Semen collection techniques

Artificial insemination is still under development in this species due to problems with semen collection, in other words, the inability to collect consecutive ejaculates from the same individuals and the viscous nature of the semen, which make evaluation, dilution and preservation difficult (Morton *et*

al., 2008). Although we were not able to collect useable samples in our attempts in Sweden, it was important to establish that the males were interested in the phantom and could probably be persuaded to ejaculate into the AV if trained. With proper training it should be possible to establish a semen collection routine and assessment. Thus we have established the basis for future projects in Sweden.

The mating duration was variable, between 15 to 50 minutes, which could have affected the survival of the spz. In previous studies by other groups, the mating time was limited to 20 minutes (Szymkowicz, 2012) to prevent a possible decrease in semen viability and cold shock to the spz. Another option could be to keep alpacas at the University's animal facilities, which would give the possibility to work in a more controlled environment, avoiding long distances and waiting time between semen collection and semen evaluation.

Electroejaculation is another semen collection technique used in numerous species. There are a number of reports regarding its use in camelids (e.g. Director *et al.*, 2007; Giuliano *et al.*, 2010) that consider it to be a safe and reliable method, which gives complete samples routinely. The disadvantages are that it has to be performed under general anaesthesia and the samples could be contaminated with urine during collection. The latter can be avoided if the male is encouraged to urinate before anaesthesia (Director *et al.*, 2007).

Post-mating aspiration is still a controversial technique (Pacheco Curie, 2008) and could have some implications for animal welfare. One of the disadvantages is the semen sample could be incomplete and diluted with other secretions from the female genital tract (Bravo *et al.*, 2000), and there is the possibility of transmitting infection between animals if the sample is subsequently used for artificial insemination. Neither post-mating aspiration nor electroejaculation would be the first option to use in Sweden.

5.4 Testicular length as an indicator of sperm production in alpacas under Swedish conditions

The wide range when the onset of puberty in alpacas can occur, between 1 to 3 years of age, makes management decisions difficult and may be influenced by the conditions under which animals are kept. By using a model of testicular length that accounts for variability in age and body condition score, we tried to predict when alpacas can be expected to start producing spz in Sweden. We found that if only one parameter is considered, testicular length is better than age alone to estimate sperm production but that the inclusion of body condition score and age together with testicular length gives better predictability than a single parameter. Therefore, we suggest using a combination of these

parameters as a tool for decision making in the selection of potential sires. In addition, it is important to know what testicular size should be considered as normal for the identification of pathological changes, e.g., hypoplasia or degeneration.

In line with Galloway (2000), our results showed a large variation in mean testicular length and the presence of spz at different ages. It would be beneficial to include more data from older animal or with larger testicles. Such animals were limited in our study (Paper II) due to the relatively new trend of castrating animals when they are around 1 year old. If there is an opportunity of collecting larger testicles, the probability of finding spz is higher and thus the possibility exists to test sperm preparation techniques such as SLC, new semen extenders and cryoprotectants to improve freezing ability.

A significant positive effect of body condition on the testicle length increment rate was observed in our study, in accordance with Galloway's findings in Australian herds (Galloway, 2000). Alpaca husbandry in Sweden is likely to be more similar to Australian conditions than Peruvian conditions, especially in terms of nutrition and thus body weight. Fowler indicates that overweight is probably a more common problem than emaciation in alpacas in the Western world. Although body condition score was generally good in the animals examined in the study in September, a previous study carried out in Sweden by Björklund (2014) found that emaciation can be a problem in weanling alpacas. It is worth to mention that there are two different systems of production of SACs in the world: the traditional Andean herding strategies, in a pastoral economy in the dry highlands, with a high altitude grassland between 3000 and 4800m above sea level, and the other system under very different and more favourable conditions, at a low altitude of no more than 800m above sea level (Miragaya *et al.*, 2006). These husbandry conditions are likely to result in differing availability of nutrients and thus possibly the onset of puberty.

5.4.1 Breeding management

By detecting puberty in males as early as possible, owners are able to separate females and males to avoid undesirable matings, to use elite males strategically in breeding programs, and to castrate the ones that are not intended to be used as stud males.

According to Vaughan (2005, 2015a, b) and Pearson (2013), when selecting males for breeding, owners should choose early-maturing males with large testicular size. Selecting for large testicular size, together with an evaluation of the sexual behavior, BCS and the detachment of preputial adherences, is likely to improve male reproductive efficiency. To assist with selection of potential male sires, it is recommended to perform a breeding soundness examination in

young males starting at 6 months of age and at the beginning of each breeding season.

5.5 Reproductive biotechnologies in Sweden

To be able to utilise reproductive biotechnologies in Swedish alpacas, the problem with semen viscosity still has to be solved, and therefore a semen collection method should be established so that semen handling methods can be developed. We conclude that the use of the phantom could be the best method to use for semen collection in Sweden. Although a training period is needed so that the males can become accustomed to the phantom and AV, it is a fairly simple technique with high potential and, as far as we are aware, there are no animal welfare concerns.

6 Concluding remarks

Semen handling and sperm evaluation techniques:

- Good results were obtained using a Mini version of SLC with bull spermatozoa. Such a modification would enhance its usefulness and acceptability in a variety of laboratory settings and could be applied to alpacas, as well as other SACs or other species in general, especially when handling small volume samples.

Semen collection techniques:

- Epididymal spermatozoa provide a good model for research because they have not been exposed to seminal plasma. This is particularly relevant for SACs because epididymal spermatozoa are free from viscous seminal plasma. Furthermore, there are no welfare issues associated with the collection of spermatozoa from this discarded material.
- In Sweden, semen collection with a phantom is more likely to be used for alpacas than other techniques such as post-mating aspiration or electroejaculation.
- Alpaca males were interested in the phantom we designed and, if they are trained, semen samples can be collected routinely. Thus we have established the basis for future projects in Sweden.

Breeding management:

- We suggest a combination of testicular size and body condition score as a tool for decision making in the selection of potential sires for animal husbandry under Swedish conditions.

7 Future challenges

- It would be interesting to compare the colloid centrifugation and enzyme extraction of spermatozoa and to determine the ability of the spermatozoa to survive freezing while retaining functionality.
- The phantom we designed seems to work, however it still need to be refined and males need to be trained in order to establish a semen collection routine.
- The development of reproductive biotechnologies in alpacas could be a model for conservation biology of wild camelids, such as vicunas, which are protected in several countries (CITES, Appendix I and II).
- It would be important to evaluate the effect of using reproductive biotechnologies on genetic improvement, e.g. for fleece quality. This can be very useful not only for Sweden but also for Perú and other countries where alpacas are indigenous.
- Regarding the measurement of testicular size, it would be interesting to do additional measurements in Sweden on the same males at different seasons of the year, to investigate whether there is seasonality of sperm production.
- Furthermore, there is a need for more research on early embryonic death in these animals, which seems to be a problem in several countries. If we are able to select the spermatozoa using single layer centrifugation before doing artificial insemination, we could reduce early embryonic death, or at least prevent any that is due to sperm chromatin damage.

8 Populärvetenskaplig sammanfattning

Alpackan tillhör kameldjuren, liksom laman, guanakon och vikunjan, Alpackor är starka och tåliga djur och dessutom intelligenta, vänliga och nyfikna. De hålls framför allt för produktion av fiber/ull eller som hobbydjur. Människor blir mindre stressade när de får vara tillsammans med dessa underbara djur. Nyligen har alpackor börjat användas inom så kallad djurterapi eftersom deras speciella karaktär lyser upp dagen för äldre, sjuka, barn eller för människor som behöver varva ner. Alpackor anses vara miljövänliga eftersom de inte trampar sönder ömtåliga marker. Verksamhet baserad på alpackor kan bli en ny nisch inom det svenska lantbruket och ett bra exempel på multifunktionalitet. Dessutom finns potentialen att integrera alpackaverksamhet med annan djurproduktion, agroturism eller rekreation, till exempel organiserade vandringar med alpackor.

I Sverige har alpackor blivit allt mer populära under de senaste decennierna. Flockarna har byggts upp genom livdjursimporter eftersom moderna reproduktionstekniker som till exempel spermasamling, spermakonsivering och artificiell insemination inte är välutvecklade för alpackor. Ett stort problem är att den trögflytande sperman är ett hinder för att processa eller bedöma spermerna. En möjlighet att frigöra spermier är att lösa upp den trögflytande sperman med enzymer, men dessa kan orsaka skador på spermerna eller göra dem odugliga för befruktning. Genom att använda moderna biotekniker i reproduktion skulle nytt avelsmaterial kunna introduceras utan att behöva importera livdjur, och därigenom förbättra djurvelfärd och minska risken för smittspridning. Vårt mål var därför att förbättra biotekniker inom reproduktion, som spermakonsivering och artificiell insemination, för att bidra till att utveckla den svenska alpackaaveln.

Vi utvecklade laborietekniker för att kunna utvärdera kvaliteten hos sperma och välja ut de bästa spermerna i syfte att förbättra frysförvaring. Teknikerna utvecklades först genom att använda tjursperma som modell. Det

var möjligt att förbättra spermakvaliteten genom att selektera de bästa spermerna med en speciell centrifugeringsteknik, särskilt från prover med dålig spermakvalitet, och det var också möjligt att modifiera tekniken för att behandla små volymer av fryst sperma.

Tanken var att jämföra olika spädningvätskor för sperma som samlats från bitestiklar från kastrerade alpackor. Tyvärr kom de flesta organen från alpackor som inte uppnått könsmognad och därför inte innehöll några spermier.

En utvärdering gjordes av för- och nackdelar med olika sätt att samla sperma från levande djur. I Sverige finns lagstiftning som säkrar djurvälståndet, och därför är spermasamling med en så kallad fantom, ett konstgjort hondjur, den metod som är mest lämplig jämfört med andra mer invasiva tekniker som att samla sperma ur de honliga könsvägarna efter betäckning eller att använda elektroejakulation, spermasamling med hjälp av elektroder. En fantom för spermasamling designades och byggdes i Sverige, och försök med spermasamling gjordes också i Peru. Vi lyckades inte fullt ut att samla sperma vid de kortvariga försöken i Sverige, men responsen från handjuren var positiv. De var intresserade av att betäcka fantomen och med ytterligare träning skulle det vara möjligt att rutinmässigt samla sperma. Metoden har sålunda etablerats i Sverige och kan användas här i framtida projekt.

Dessutom har ett hjälpmedel utvecklats för att kunna ta rätt skötselbeslut för alpackor i Sverige. Det är en statistisk modell som förutsäger när en alpackahane förmodas börja producera sperma, något som är viktigt att känna till för att undvika oönskade dräktigheter eller bestämma lämplig tidpunkt för kastration.

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“Every person that passes through our lives is unique. Always leaves a bit of themselves and takes a bit of ourselves. There will be those who will take a lot, but there won't be those who will not leave anything. This is a clear proof that two souls don't meet by chance.” (Jorge Luis Borges)

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“Cada persona que pasa por nuestra vida es única. Siempre deja un poco de sí y se lleva un poco de nosotros. Habrá los que se llevarán mucho, pero no habrá de los que no nos dejarán nada. Esta es la prueba evidente de que dos almas no se encuentran por casualidad.” (Jorge Luis Borges)

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Maria Celina Abraham, Uppsala, March 2016