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DETERMINATION OF PROTEIN PROFILE AND PRESENCE OF ANTIBODIES IN LLAMA SEMINAL PLASMA (*llama glama*)

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Dedicatory

This project is dedicated to the memory of my father, Pedro, who planted the foundation for the start of my professional career. Also my sincere thanks to my selfless mother, Tiburcia, for her support and sacrifice during my years of study.

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Summary

Internationally, Bolivia has the largest llama population followed by Peru. This makes our country a power in this renewable resource for working and generating economic development. This is mainly because of the quality of llama meat, which has nutritional properties superior to other edible animal species.

The development and research in our country of llamas is not very significant. But in other countries advanced studies have been done for years, specifically in the area of reproduction, which represents problems for intensive repopulation because of singular characteristics of semen and copulation.

The present research work was done at the UAC in Tiahuanacu (U.C.B.) and is titled: "Determination of protein profile and presence of antibodies in llama seminal plasma at three, four, and five years of age." At different dates of semen collection, protein fractions were determined, their molecular weights, presence of antibodies and concentrations of protein fractions. This determined the presence of possible components of seminal plasma caused by low percentages of successful breeding for these animals.

In the field work, a series of tests were done so the male would respond to the artificial collection of semen. It began with the mannequin technique (Peruvian) and others, which had no results. The objective was obtained with the **mannequin technique for the llama group**, which was accepted by 80% of the males.

The lab work consisted of separating the seminal plasma from the semen with a centrifuge. Electrophoresis was used to determine protein fractions. This helps to determine the number of protein fractions, their molecular weights, immunoglobulins and their concentrations.

The highest concentrations of protein fractions were found in four and five year old animals with 15 to 18 protein fractions. In weekly semen collections the last week

showed 18 fractions (four year old animals). These could possibly play an important role in nutrition, capacitation and protection of spermatozoa.

The molecular weights of the proteins in seminal plasma vary from one animal to another and dates of collection. The extreme ranges are from 2,000 to 150,000 Daltons. Five year old animals have higher molecular weights with respect to younger animals.

With respect to immunoglobulins or antibodies, proteins with molecular weights of 150,000 to 152,500 Daltons were found in animals from three to five years old. Immunoglobulins G in llama seminal plasma were found using the radial immuno diffusion technique.

Concentrations of protein fractions are variable with extreme ranges from 11.65 to 0.03 mg/ml of seminal plasma. These variations are heterogeneous in the age of the animals as well as in the collections dates. There is a slight increase in five year old animals in the third week of semen collection.

The finals results of the proteins fractions and their molecular weights show that there are antibodies in llama seminal plasma. These play an important role in spermatic viability, which is why this subject should continue to be researched to determine its specific function and look for alternatives to isolate and neutralize these antibodies and increase the fertility indexes of these animals.

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1. Introduction

South American camelid breeding dates back to ancient times. The people of the Andean regions took advantage of these animals for their meat for food, their fur for textiles, and leather for many materials. They also used them for transporting products from one region to another.

Camelids do not demand much food for feeding, which explains why they live in regions with little vegetation. In these conditions, they consume less food and nutrients. There needs to be good use of this because Bolivia is home to 2,398,572 head of camelids (UNEPCA 1997).

Currently, camelid meat and fur is in high demand at national and international levels. This situation causes farmers to commercialize and indiscriminately use the best camelids for local markets. This attitude decreases the number of reproducers and genetic resources, which if continued will cause the extinction of this natural resource.

Results from artificial insemination and natural mating show problems in males and females in their immunological systems in their reproductive systems. This is cause by the presence of immunoglobulins that act as auto-antibodies when the organism is exposed to any foreign antibody. This causes possible agglomeration of spermatozoa, which will impede motility and inhibit fertilization in post copulation or artificial insemination (Halfez 1996).

The problem lies in repopulation because of the points mentioned above and mainly in the increase in the reproduction percentage. Reports indicate fertility with semen conserved at 5°C of 40% gestations and with 33% gestations from frozen semen (Perez, 2000).

This research works hopes to identify the possible limiting factor of cryo-preservation of llama semen or autoimmune processes that are involved that are seen inside the animal

organism as a consequence of physiological and immunity factors. This would be a great scientific contribution to the fertility problem. Another important factor is to look for alternatives for processing and dilution of semen for artificial insemination. This would reduce infertility and promote advances in the livestock field of work.

1.1 General objective

Determine the protein profile and presence of antibodies in seminal plasma for three, four, and five year old llamas over a collection period of three weeks.

1.1.1 Specific objectives

-Determine the number of seminal plasma protein fractions.
-Determine the molecular weights of the seminal plasma protein fractions.
-Determine the presence of immunoglobulins in seminal plasma.
-Determine the concentration of seminal plasma protein fractions.

The hypothesis that was planted for the research is: "the number of protein fractions, the molecular weights, the immunoglobulins and the concentrations of protein fractions in llama seminal plasma vary according to age, weeks, and days in semen collection. As a consequence of these variations, the spermatozoa activity is altered, which influences reproductive activity of the llama."

2. Bibliographic revision

2.1 Camelids in Bolivia

Table 1 Plant and animal index of some animal referring to reproduction

Index	Cattle	Swine	Goats	Llama	Alpaca
Fertility (%)	70	65	64	41	47
Mortality at weaning (%)	9	-----	-----	-----	-----
Infant mortality (%)	-----	26	25	23	25
Birth rate (%)	62	-----	-----	-----	-----
Survival at weaning (%)	87	-----	-----	-----	-----
Birth weight (average kg)	29	-----	1.4	8	6
Weaning weight (average kg)	99	-----	-----	30	30
Weight at slaughter (kg)	229	-----	-----	-----	-----
Body weight (kg)	102	11	19	42	27
Extraction rate (%)	13	19	21	10	15
Intervals for births (days)	482	325	290	680	680

Soure: Statistical yearbook AGRODATA (1994).

AGRODATA (1994) has some plant animal indexes where camelids occupy the lowest indexes. These statistics are worrisome because they still do not give any emphasis on reproduction of these species even though the demand for meat is increasing mainly in international markets.

2.1.1 Geopolitics of camelids

PRODENA (1995) describes that camelid breeders at altitudes between 3,900 and 5,000 meters above sea level on the border that divides Bolivia from Chile and Peru are witnesses of the break in the native land and of the constant diplomatic controversy that the neighboring countries sustain because they enclose the rich natural resources. If not for the existence of camelids, these areas would be converted into deserts. Therefore breeders of this species, today and always, will be the unavoidable sentries of national sovereignty.

2.1.2 Dangers for germplasm

FADES (1991) says that currently the interest of non-andean countries in camelids is increasing from the point of view of Andean germplasm preservation. In recent years, foreign technicians were interested in indigenous livestock. Not to evaluate possibilities of other species in the high plane, but to learn the technology of alpaca breeding so they could import that to their respective countries.

2.1.3 Camelid production

FADES (1991) describes that fertility and the birth rate are limiting factors for the reproduction rate of the flock, on which the increase in capital livestock of the breeders depends. These elements also inhibit an effective selection of the reproducers. The physiology of the reproduction of these animals present some unique characteristics that are different than other domestic species.

2.2 Organism defenses

2.2.1 Protecting functions of the blood

2.2.1.1 General defense reactions

GURTLER (1979) said that the animal's organism receives stimulations or inhibitions from the environment against pathogenic germs. As a defense mechanism for the body there are cells that help distinguish between cellular and humoral defense mechanisms. In the immune humoral response, the specific immunoglobulins (glucoproteins) or antibodies that are formed in the reticular endothelial system are synthesized as a consequence of the intake of antigens.

2.3 Proteins

BIASOLI (1980) says that proteins, because of their organization, are macro peptides. Even though there is no definite limit in the transaction of large peptides to proteins, it is convenient to call a protein a peptide that contains more than 100 amino acids.

Some proteins have been isolated purely and crystallized. This helps to know its elemental composition. All contain carbon, hydrogen, oxygen, and nitrogen and many also contain sulfur. Other proteins contain phosphorus, iron, zinc, and cobalt. The average percentage compositions of these substances are:

- C: 51-55%
- O: 21-24%
- H: 6.1-7.3%
- N: 15-17%
- P: 0-3%

- S: 0-2.5%

2.3.1 Proteins and their functions

DREW (1989) says that chains of amino acids in live beings intervene in almost all of the biological processes.

The functions are: Intervene in the structural support of the body, help unite skin and bones, move chromosomes from the cells and flagellum projections like the spermatozoa. It also intervenes in the storage of ions and molecules like in the case of hemoglobin, since hormones are a means of chemical communication. One of the lines of defense is proteins known as immunoglobulins.

2.3.1.1 Protein classification

BIASOLI (1980) classifies proteins according to their origin from the two following groups: native and derived.

Simple native proteins: (heloprotiens). These are formed only by amino acids. Because of their characteristics of solubility in different environments, simple proteins are subdivided into:

- Albumins, easily soluble in water
- Globulins, soluble in weak saline solutions
- Protamines, soluble in water
- Prolamines, soluble in alcohol solutions
- Histones and glutelins, soluble in acid solutions
- Scleroproteins, practically insoluble

Conjugated native proteins: (heteroproteins). This group is made up of proteins that have, apart from their protein portion, a different natured group that is called "prosthetic group." Because of the nature of this group, the conjugated proteins have different names: (Table 2).

Table 2 Conjugated proteins

Protein	Prosthetic group	Example
Phosphoproteins	Phosphate	Casein
Nucleoproteins	Nucleic acids	Virus, chromosomes
Hemoproteins	Hemo	Hemoglobin
Glucoproteins	Glucose	Gamma globulin
Lipoproteins	Lipids	Lipoproteins of the cellular membranes
Metalloproteins	Metal	Ferritin

Source: BIASOLI (1980)

Derived proteins:

BIASOLI (1980), describes this type of protein in the following way:

a) Alkali proteins

b) Acid proteins

c) Protease

d) Peptase

e) Poly peptides

f) Oligo peptides

g) Amino acids

BIASOLI (1980) also classifies proteins with the biological function that they carry out, among these important groups are:

Table 3 Other important protein groups

Class	Function
Enzymes	Catalyze all of the transformations that occur in the cells
Reserve proteins	Secure the survival of a new being, like the proteins of the clear egg, of the milk, of the seeds, etc.
Transport proteins	Transport indispensable substances for life, like hemoglobin and mioglobin, which transport oxygen.
Contractile proteins	Permit muscle, cilium, and flagellum contractions. Ex. Miocene, actin.
Protective proteins	Annul the effect of foreign proteins on the species Ej. Antibodies. Permit blood

	coagulation. E. Fibrinogen.
Hormones	Regulate functional equilibrium of the organism. Ex. Insulin, somatotrophin (growth)
Structural proteins	Form the cellular structures or serve as protection. Ex. Alfa keratin (spin, feathers, hair); collagen (tendons, bones, cartilage); elastin (ligament).

Source: BIASOLI (1980).

2.4 Enzymes and their functions

DREW (1989) explains that enzymes are proteins that catalyze virtually all biochemical reactions. Approximately 2000 enzymes have been identified that are responsible for the majority of the activities that take place in the lives of living beings.

These enzymes are large protein molecules with three dimensional shapes. Each enzyme has a special region called an active site, which unites with the molecule. The catalytic activity of an enzyme results in the union of one molecule of the substrate to the active site to form an enzyme-substrate compound.

2.5 Molecular weight

RUIZ (1997) mentions that the equivalent weight in gr/mole of a chemical substance is calculated from the atomic weights of each element. These are specified in the table of elements: Thus, S= 32, O= 16, H= 1 is used to calculate the atomic weight of H_2SO_4. Therefore, the atomic mass of one mole of atoms of a chemical element will equal the atomic weight expressed in grams. This will come specified in the periodic table of the elements.

MONRREAL (1994), says that molecular weight is the sum of the atomic weights that form the molecule.

2.5.1 Molecular weights of the proteins

BIASOLI (1980) mentions certain proteins where molecular weights can be observed. They are shown in Table 4.

Table 4 Molecular weights of certain proteins

Protein	Molecular weight (Daltons)	Number of amino acids
Insulin	4,733	51*
Ribonuclease	12,640	124
"Lisozima"	13,930	129
Myoglobin	16,890	153
Hemoglobin	64,500	574
Sero albumin	68,500	~550
Gamma globulin	149,900	~1,250
Mosaic tobacco virus	~40,000,000	~336,500

Source: BIASOLI (1980).

* Insulin, by the number of amino acids, can be considered a peptide, but in the organism it tends to form variable additions to molecular weight between 12,000 and 48,000.

RUBINSON (2001) did work on SDS gel where he found the following proteins and migrations of the same:

Table 5 Molecular weights and protein fraction distances

Name of protein	Molecular weight (KDa)	Portion in gel/cm
Ovalbumin	45.0	1.50
"Quimitripsinogeno"	27.7	3.70
Trypsin	20.0	4.90
Myoglobin	17.7	5.50
"lisozina"	14.5	6.30
Cytochrome C	12.4	7.00

Source: Rubinson (2001).

ANDIEWS (1986) shows protein fractions that are employed in the electrophoresis technique. The molecular weights are known, which standards to determine the molecular weights of other substances. They are shown on table 6.

Table 6 Certain standard proteins used normally at an international level in Gel PAGE.

Proteins	Molecular weight (Da)
"Lisozina"	14,000
Cytochrome C (horse)	12,256
Chymotrypsinogen A	23,600
Ribonuclease A	13,500
Myoglobin	17,500
Myoglobin (horse)	17,500
"Eritoaglutinina"	130,000
Insulin	11,466
B Lactoglobulin B	36,552
B Lactoglobulin A	36,724
Albumin cattle serum	67,000
Ovalbumin	45,000
Alkaline phosphatase	140,000
G- Lactoabumin	14,146

2.6 Immunology

COILA (1998) indicated that immunology is the study of immunity and cellular and molecular events that are produced after a foreign agent penetrates an organism and the biological reactions that can happen as a consequence.

2.6.1 Specific immune response

COILA (1998) says that they are made by the cells and responsible soluble factors of specific recognition and the elimination of specific antigens. These are called lymphocytes B.

TIZARD (1996) says that if the antigens are trapped and transformed by macrophages, B cells and dendrites, the development of the immune response is really a function of the cells called lymphocytes, located in the spleen, lymph glands, and the grayling. These have receptors for specific antigens and therefore can recognize whatever comes to them.

2.6.2 Globulins

DREW (1989) indicates that globulins are proteins also known as immunoglobulins, which are fundamental in the immune system. They identify, help to destroy and eliminate antigens and foreign macro molecules in the organism in general.

2.6.3 Immunoglobulins

TIZARD (1988) indicates that they are specialized proteins. The lymphocytes B, once stimulated in the presence of the antigen, synthesize and release in the middle to form the antigen-antibodies compounds. Through this mechanism, they eliminate or destroy the antigens.

2.6.4 Structure of the immunoglobulins

COILA (1988) says that they are characterized by having a high number of amino acids that vary according to order. The antibodies are made up of chains, where known areas are identified like constants and variables. (area of antigen recognition). According to their structure they are classified as:

-Immunoglobulin G
-Immunoglobulin M
-Immunoglobulin A
-Immunoglobulin D
-Immunoglobulin E

2.6.4.1 Immunoglobulins as "tetrapeptidoid" units

BARRET (1991) mentions that the chemical structure of the immunoglobulins is equal in all of its blood secretion forms because the bases have a structure of 4 peptide units per bridge of disulfide. Small variations in these peptide chains lend specificity to each immunoglobulin.

The immunoglobulin structure is based on the union of 4 peptides to form one large protein: two of these have a low molecular weight (22,000 Mr. types Kappa and Lambda)

and are known as light chains (L). The two larger peptides are Heavy chains (H) and vary in sized from one immunoglobulin to the next. The smaller of the H chains are 55,000 Mr., which are gamma and alfa. The largest are 75,000 Mr the My, 63,000 the delta and 73,000 Mr the epsilon.

2.7 Semen

HAFEZ (1996) describes it as a semi-gelatin liquid that contains masculine gamete (spermatozoa) with secretions from different accessory organs. The liquid portion of the suspension formed during ejaculation is called seminal plasma.

2.7.1 Seminal plasma

ILLERA (1994) says that it is the fraction of semen after separating the spermatozoa from the accessory gland secretions for centrifugation. They are characterized by having substances that are not found in any other part of the organism, and are a source of nutrients and energy for the spermatozoa after being ejaculated by the reproducing apparatus of the male.

2.8 Immunology of reproduction

HAFEZ (1996) mentions that autoimmunization, the iso-immunization of males and females with spermatozoa counterparts, produce equivocal effects on the reproduction of antibodies. In the distance of the feminine tract, the antibodies unite to the spermatozoa, inhibiting or neutralizing their activity and thereby destroying them.

The main reproduction antigens are spermatozoa. And in the female immune response system they are recognized in the uterus, neck of the uterus, and the vagina; where immunoglobulins known as A are found. These possibly confront with spermatozoa and form an antigen-antibody compound.

2.8.1 Autoimmunity

MORENO (1996) says that autoimmunity is not necessarily pathologic or occasional, since all normal individuals, especially during the course of some infectious diseases, have autoimmune phenomenon.

During ontogeny, the cells of the immune system randomly develop the capacity to respond against antigens, foreign or domestic. The majority of T and B linfocitos that recognize domestic antigens are eliminated immediately after the generation or inactivity in the periphery. Thus, it precedes the development of an autoimmune disease in the majority of the cases.

2.8.2 Immunological aspects of spermatozoa

HAFEZ (1996) says that one of the functions of the hematotesticular barrier is the immunological isolation of the gametes in development. It is important because spermatocytes, spermatides, and spermatozoa, because they are their own antigens, are sequestered, later to be freed, are easily recognized as foreign cells by the immune system of an adult male.

2.8.3 Immunological studies

FECUNDITAS (1998), says that the presence of immunoglobulins A, G and M on the surface of spermatozoa can cause infertility. The IgA antibodies are produced locally in the genital tract of the male as in the case of the woman, while IgC and IgM are circulating.

When the antibodies appear, they unite to the surface of the spermatozoa and hamper mobility, penetration of cumulus from the "pelucida" of the egg, and the ovular-spermatozoa interaction. As a consequence, this influences fertilization as well as the destruction of spermatozoa by the immune system.

2.9 Determination of antibodies

MASCARO (1979) mentions that blood serum proteins (albumin and globulins) can be separated many different ways, which are: precipitation, electroforesis, ultracentrifugation, centrifugation, chromatography, etc.

Through electroforesis and other methods, they separate and are soaked in a stabilizer. The globulins in the serum are then connected with an electric current of 110-220 volts for 14-18 hours. They then migrate towards the anode and different color fractions make it possible to distinguish albumins and globulins (alfa, beta, gama).

2.10 Concept of the electroforesis technique

VALLS AND Del CASTILLO (1998) mention that electroforesis is to define migration of certain coloides charged in the bosom of a continual electrical field that separates small ions, minerals or from the ionization of amino acids, and peptides. The fundamental requirements of electroforesis are that the molecules, in one phase, have a positive or negative net charge.

Under the effect of an electric field applied to the positively charge molecules, they will move towards the cathode (-), while the negatively charged will move towards the anode (+). The movement takes place in a liquid, generally supported in an inert solid substance (paper, semisolid gel) constituting the conductor from the electric current generated by the application of en external voltage to the system.

2.10.1 Parameters that influence electric mobility

a) External parameters

- **Electric field:** This is the most essential and is the electric field produced by the potential difference in the terminals of the electrophoretic tray.

- **Temperature:** Temperature control is important because it can help in the denaturalization of certain substances to appear from convection currents.

b) Parameters related with the solvent and with the electric system:

- **Electrical constant:** The electrophoretic mobility is directly related with the same.

- **Viscosity:** It is inversely related to electrophoretic mobility. It increases viscosity of the absorption solution, and the resistance for friction is increased, which opposes movement of the charged particles through the electric field, reducing the electric mobility.

- **Ionic force:** Sets the charge density around the central ion and therefore the intensity of the relaxation effects and the electromagnetic reduction upon increasing the ionic force increases the electric mobility.

- **The nature of ions:** Components of the absorption solution that is very important in the separation of proteins.

- **pH:** Plays an important role in the preparation of "anfoteros."

c) Parameters related to solute

- **Charge:** It is an intrinsic property, even though it is bound to the solvent properties.

- **Shape and size of the ions:** This can play an important role when electrophoresis is done on an inert support where a filtration process is produce though pores.

2.10.2 Migration of molecules

For molecules with the same net charge, the smallest will migrate faster than the larger ones. However, one large molecule with a high net charge can move at the same velocity than a smaller one with a low net charge, thus they will appear in the same area at the end of electrophoretic separation. Therefore, if the samples are separated in one band of gel, it does not necessarily mean that there is one component in that band.

2.10.3 Types of electrophoresis

The ionic species with distinct effective electrophoretic mobility show differences in migration velocity, being found at the end of the electrophoretic experiment separated in different areas. They are then treated then as electrophoretic areas that can be divided into three different groups:

a) front mobile electrophoresis

b) Electrophoresis on an inert support

c) Isotacophoresis

In terms of use, route, and regime of separation, the distinct electrophoreses can be classified as indicated in the following table:

Table 7 Types of electrophoresis according to use and regimen of separation

Electrophoresis	Use	Means of separation	Regime
Area electrophoresis	Analytical	Solid support or gel	Discontinual
Rightly said	Preparative	Solid support gel, or liquid phase	Continual or Discontinual
Front mobility electrophoresis	Analytical	Liquid phase	Discontinual
Esotacophoresis	Analytical	liquid phase	Discontinual
	preparative	liquid phase	Continual or Discontinual
Electrofocus	Analytical	Solid support, or gel	Discontinual
	Preparative	Liquid phase	Continual or Discontinual

2.10.4 Electrophoresis on gels

The use of gels as a means of supporting electrophoresis is an alternative for solving some problems associated with the use of cellulose acetate paper like absorption and low resolution of repaired compounds.

A gel is a three dimensional polymeric net. Those used in electrophoresis are polymers with crossed tissues that should be inert and not interact with the molecules that are being studied. It is particularly important that they do not contain ionizable groups. The electrophoresis gels are not granular, but reticular from polymeric molecules surrounded and embedded in absorption solution. The spaces between the gel molecules are pores.

When voltage is applied, the charged molecules migrate to the appropriate electrode. The molecule that moves with the gel shows a frictional resistance in its movement. This depends on the size relations of the gel pores and the radius of the molecule. A molecule

smaller than a gel pore will move faster than another molecule whose size is similar or larger than the pore. The gels acts as sieves when molecules pass through.

2.10.5 Types of gels

a) Starch gels

The first electrophoretic tests of protein separation on gels were done with starch. It is prepared by heating a suspended amount of potato starch hydrolyzed in a absorption solution. When the suspended matter is transparent, a 1 mm deep mold is poured into mold and extended on a solid support like glass or plastic plates.

Contact between the absorption solution and the gel is done using paper filtering strips or suitable cushions. The sample problems are absorbed on small pieces of filter paper that are situated in grooves cut in the gel. After electrophoresis, the filter papers are separated from the samples and the gel can be cut in various caps that can be examined in different ways.

b) Polyacrylamide gels

Polyacrylamide gel is without a doubt the most widely used support in electrophoresis and fundamental research. It is obtained through acrylamide polymerization. This leads to large chains with a good variable proportion with crossed tissues dependant on the amount of added bisacrylamide

2.10.5.1 Advantages of polyacrylamide gel

1. Good mechanical resistance

2. Reproducible porosity
3. No absorption or electro-osmosis phenomenon
4. Minimal diffusion
5. Excellent hydrodynamic stabilization
6. Possibility of being used with a large variety of absorption solutions
7. Possibility to analyze small amounts of samples (0.1 μg)
8. Naturally transparent

The polymerization of the "acrilamida" monomer and the divalent monomer NN'-metilenbisacrilamida can induce photo-chemically in the presence of riboflavin through TEMED (N,N,N,N',N', tetrametilendiamina) and addition of oxidant (ammonic persulfate in the presence of free radicals). The gel is prepared directly in the desired absorption solution or in water, penetrating in this last case in the absorption solution through gel diffusion.

The following parameters should be taken into account to prepare the gel:

1. Gel concentration: that comes from the amount of acrylamide and bisacrylamide present in a 100 ml solution (P/V) of the gel without polymerizing. It represented by C.

$$C = \frac{a + b}{V} \times 100$$

V is the volume of gel that it contains. "a" is grams of acrylamide and "b" grams of bisacrylamide.

2. The grade of reticulation that represents the percentage in weight (P/W) of bisacrylamide in gel:

$$R = \frac{b}{a + b} \times 100$$

Concentration C can vary between 3 and 30 percent. It influences the mechanical properties and porosity, being that those with low concentrations are more fragile and difficult to manage. Their firmness can be increased adding an inert solid like sucrose.

2.10.5.2 Obtaining gel

To obtain gel, it is essential that you remove the oxygen from the middle by using nitrogen current or a pump. The jellification can accelerate, increasing the temperature to 40°C. The gel is prepared with the absorption solution more adequate for the separation that will be done. But a pH higher than 6.5 will always have to be present since the absorbers with low pH inhibits jellification.

2.10.5.3 Gel preparation

Polyacrylamide gels can be prepared in columns or in fine caps. In the first case, the gel is polymerized in a glass or plastic cylinder tube with an internal diameter of 5 mm and a length of 70-100 mm. With the longer tubes the components can be separated with

similar mobility but it requires a few hours of separation. This leads to an important diffusion in the separated areas.

Once the gel is polymerized on the inside of the tube, the sample is placed in the top part of the tube and is filled with absorption solution. It is placed vertically in the electrophoretic apparatus.

2.10.5.4 Refrigeration

It is necessary to refrigerate the system and it is good that the electrophoresis absorber circulates to avoid changes in pH. Once the colorant has moved to the bottom of the tube, the food source is disconnected and the gels are extracted from the tube.

2.10.6 Electrophoresis characteristics in gel

The proteins can be set with precipitating agents and analyzed by "tincion" or through radioactivity. The gels of poliacrilamida in fine caps are useful for analysis of various samples under the same electrophoretic conditions and have some advantages compared to those in columns.

1. It dissipates heat better
2. It can be used horizontally or vertically
3. There is better resolution power

A groove former or comb is inserted in the apparatus before the gel polymerizes and is taken out afterwards. This permits separate small bowls to be formed for each sample. The samples are introduced into the bowls, generally with "glicocola" or with sucrose to increase density.

2.10.7 Electrophoresis in "poliacrilamida" gel - (PAGE-SDS)

In protein molecules with different net charges, the differences in charges can be eliminated through the formation of compounds with anionic detergents. SDS: CH_3-$(CH_2)_{10}$-CH_2OSO_3-NA^+.

At a neutral pH, in the presence of SDS at 1% and 2-mercaptoetanol 0.1 M. the majority of the proteins disassociate in individual untwisted polypeptide chains since the 2 M.E. destroys the disulphate bridges and the SDS unites to such chains through hydrophobic interactions.

The amount of SDS tied per unit of protein masses is constant, 1.4 gr. SDS/1gr. The proteins acquire an identical charge/size relation and in these conditions, the separation only depends on the P.M. The gel and absorber used in this electrophoresis both contain SDS 0.1 % p/V.

2.10.8 Determination of molecular weights

Various polypeptides are used for unknown molecular weights and their relative emigration velocities are determined. The molecular weight of other polypeptides can be determined whose P.M. is unknown. A linear relation is found that graphically represents the mobilities vs. the molecular weights.

Table 8 Relative concentrations and percentages of serum immunoglobulins and mammary secretions of cattle and hogs

Animal	Immunoglobulins	Concentration			Immunoglobulins		
		Serum	Colostrum	Milk	Serum	Colostrum	Milk
Cow	IgG	11.0	47.6	0.59	50	81	73.0
	IgG_2	7.9	2.9	0.02	36	5	2.5
	IgM	2.6	4.2	0.05	12	7	6.5
	IgM	0.5	3.9	0.14	2	7	18.0
Hog	IgG	21.5	58.7	3.00	89	80	29.0
	IgM	1.1	3.2	0.30	4	6	1.0
	IgA	1.8	10.7	7.70	7	14	70.0

Source: Blood (1983)

2.11 Characterization of molecular proteins

Lehninger (1994) says that after a protein has been isolated in a pure form, its homogeneity should be established. This effect was common for free electrophoresis and for the sedimentation in the ultracentrifuge. These were substituted by more simple methods like chromatography of molecular exclusion, electrophoresis in "poliocrilamida" gels, and isoelectric focus.

Once the homogeneity is established for the protein, it can characterized using a succession of methods to establish:

a) Molecular weight

b) Content of simple or multiple chain polypeptides

c) Molecular weight of polypeptide chains

d) Amino acid composition

e) Amino acid sequence

2.12 Determination of molecular weight by chromatography of molecular exclusion

Lehninger (1994) explains that molecular exclusion does not measure the true weight of an unknown protein but rather the radius of a perfect non-hydrated sphere that has the same passing velocity for the column than the unknown protein. If the protein markers and the unknown are spherical, the method directly proportions the molecular weight.

This chromatography can even give the approximated molecular weight of one determined protein in very complex mixtures, supposing that the protein possesses a characteristic biological activity, or a property that can be measured. Usually it is possible to determine the approximate molecular weight of one type of enzymes.

Having the extract pass through the shephadex column and determining the portion of peak catalytic activity of enzymes, the presence of other enzymes does not interfere since each one goes passes through the column independent from the others at a velocity that is determined by its radius. The molecular exclusion columns are a great way to measure the association and disassociation of protein molecules.

2.13 Measurement of immunoglobulins

BLOOD (1983) indicated that the methods available for measuring immunoglobulin serum include the following:

1. Indirect:

a) A refract meter to measure total proteins in the serum.

b) A zinc sulfate turbidity test to calculate the levels of immunoglobulins.

c) Precipitation test with sodium sulfate.

2. Direct:

a) Electrophoresis on paper to calculate the profile of protein serum

b) Radial diffusion test to identify or quantify the subclasses of immunoglobulins.

2.14 Antigenic correspondence between field stock and reference of B. canis by SDS-PAGE

Larsen (1998), did research with the following objectives:

- Compare by electrophoresis on SDS-PAGE two field stock with reference stock RM 666 and M(-).

- Determine the correlation between protein profiles

- Observe the behavior of the infected animal serums opposite the antigenic fractions of the different stocks.

Stocks were used as antigens that were analyzed by electrophoresis in the gel SDS-PAGE. The protein profile showed six main bands in the stocks RM 666 and the C field. There were differences between the patterns of the stocks M (-) and the B3 fields in the 31 KDa band.

The rows were transferred to nitrocellulose membranes and were faced with positive animal serums with isolation and negative serums with the Western Blotting technique. The 31 KDa protein fractions were recognized by positive serums in the reference stocks as well as the field stocks.

2.15 Electrophoresis in 10% polyacrylamide gel

VINO (1996) did research on electrophoresis with poison from *Lachesis muta* and *Bothrops neuweid.* They had 10% polymerization in SDS PAGE. The pattern lane in the electrophoretic chamber described the approximate molecular weight of the total crude poison fractions and those obtained through chromatography of molecular exclusion.

2.16 Proteins and antibodies in human semen

2.16.1 Accessory sex tissues and their secretions

SANZ (2002) mentioned that the prostate, seminal vesicle, and the bulbourethral or Cowper gland provide the ejaculated material its chemical composition and more than 90% of the total volume of seminal plasma. These tissues secrete prostaglandin (200 μg/ml), sperm (3 mg/ml), fructose (2 mg/ml), citric acid (4 mg/ml), and high concentrations of zinc (150 μg/ml), proteins (40 mg/ml) and specific enzymes like immunoglobulins, protease, esterases, and phosphatase.

Currently, there is limited knowledge of the physiological functions of these secretions, with the exception of some roles in the coagulation processes and lyses that occur in seminal plasma.

2.16.2 Antibodies associated with seminal plasma and spermatozoa

YEPE (2000) studied the presence of anti spermatozoa antibodies in the seminal plasma cervical fluid or fluid attached to the spermatozoa of 75 infertile pairs. Using the immunobeads test, YEPE determined the inhibiting capacity of seminal plasma and the lymphocyte proliferation to evaluate the immuno-modulating capacity of semen as a

participant in the primary pathogenic mechanism of the spermatozoa antibodies, for which YEPE found the following:

Associated with spermatozoa	**Antibodies in the seminal plasma**	**Antibodies in the cervical fluid**
%	%	%
IgG- 100	IgG-50	IgG-90
IgA-70	IgA-16	IgA-90
IgG-35	IgG-25	IgG-80
IgA-10	IgA-7	IgA-45

2.16.3 Immunological studies

GONZALES (2000) mentions that the immunoglobulins A, G, and M can cause infertility. The IgA antibodies are produced locally in the genital tract of the male and female, while the IgG and IgM are circulating. These attach to the surface of the spermatozoa and inhibit mobility, cumulus penetration in the pellucid area of the egg, in egg-spermatozoa interaction, and in fertilization as well as the destruction of spermatozoa.

2.16.4 Proteins secreted by the seminal vesicle

According to mediconet.com (2002) [Semenogueilina?] is a protein that produces semen coagulation as well as the proteolytic enzymes: pepsinogen, lysozyme, alpha amylase hyaluronidase. Proteinase inhibitors: Alpha 1-anti-tripsine and Alpha antiquimotrypsin.

The bacterial and viral infections in the male genital tract are important etiological factors in male fertility, which leads to deterioration of spermatogenesis, and alters the spermatic function. More than 60% of the patients with acute epididymus show drastic alterations in the spermatogenesis that is later reversible with antibiotic treatment.

Because of the barrier infection, hemato-testicular barrier can form antisperm antibodies that can be detected in the serum and seminal plasma and have been observed to have an adverse effect in the spermatic function.

The male reproductive tract is an immunologically privileged site, where the hemato-testicular barrier formed by straight unions between the Sertilo cells, protects the germinal testicular cell phases, which express unique antigens that can stimulate the autoimmune response.

2.16.5 Anti spermatozoa antibodies

FECUNDITAS (2002) says that the antibodies are produced when the immune system responds to the spermatozoa as if they were foreign elements. Its presence immobilizes and agglutinates the spermatozoa, impeding them from reaching the ovule. They are normally detected in serum or seminal plasma. It is estimated that 50% of spermatozoa united with antibodies cause 5-10% male infertility, and 10-15% female infertility.

3. Methods and materials

3.1 Location

The present research work has been developed in the Biochemical and Animal Nutrition Lab at the Rural Academic Unit in Tiahuanaco at the Catholic University of Bolivia located in the community of Achaca, Ingavi province. It is located 57 km from the city of La Paz, La Paz international freeway. It is located at 68°42'28" South and 16°35'41" West at an altitude of 3,856 meters above sea level.

3.2 Sample size

The research work was done with 6 male llamas selected at their fertile age, of which semen was collected to obtain seminal plasma.

The semen collection dates were distributed to imitate alternate breeding practiced at centers for South American camelid experimentation and reproduction. In this type of breeding, the male reproducers are 10% of the total female population and are separated into two groups for a period of one week. During this week they breed and rest for seven days to be replaced by the second group. After the seven days they replace the second group.

The minimum age for breeding is three years, considering that 100% of the males at this age have foreskin liberation. Five years old is considered the best age for reproduction. The animals involved had the following chronological characteristics.

Table 9 Number and age of animals

Males	Number	Age (approx. years)
	2	3
	2	4
	2	5

3.3 Materials for semen collection

- Llama hindquarters mannequin
- Artificial vagina
- Latex covers
- Nylon covers
- Collection tubes
- Water thermos
- Pasteur pipettes
- Clinical thermometers
- Equipment for boiling water
- Livestock: One female llama
  One male cow

3.4 Equipment and materials for separation and conservation of seminal plasma

- Small stepladder
- Centrifuge
- Centrifuge tubes
- Hypodermic syringes (cap. 1 ml)
- Eppendorf conservation vials
- Refrigerator

3.5 Equipment for separation and determination of molecular weight of proteins and immunoglobulins

- Camera
- Milipore filters (Holders)
- Analytical balance (brand: A.N.D.)
- Fraction collector
- Potentiometer
- Collecting tubes
- Electrophoretic camera (brand: BIO-RAD)
- Multiple transformer (power source 400 mA) (brand: BIO-RAD)
- Radial immuno diffusion plates (brand: DIFFU PLATE)

3.6 Reactants and solutions for separation and determination of molecular weight of proteins and immunoglobulins

- PAGE gel system ("poliacrilamida") (brand: SIGMA)
- "Comaisse" blue (brand: SIGMA)
- PAGE tampon: Glycine, SDS, TRIS, "Acrylamida-bisacrilamida (brand: SIGMA)
- Ammonium Persulfate (brand: SIGMA)
- TEMED, Glycerol (brand: SIGMA)
- Standard of high and low molecular weights (brand: SIGMA)
- B-mercaptoethane (brand: SIGMA)

3.7 Methodology

3.7.1 Animal acquisition

The animals used in this research were acquired from the Department of Oruro, Carangas province, Community of Belen de Choquecota. A total of 12 males at their reproductive age were selected between the ages of 3 and 5.

To purchase the livestock they were selected for their desired phenotypic characteristics, taking into account the following aspects: good conformation, good self possession, good

testicular development, foreskin liberation; discarding animals with any kind of defect, especially those related to the reproductive system. To determine their ages, chronological and dental information was taken.

3.7.2 Animal transport to research location

The animals were moved to installations at the Rural Academic Unit at Tiahuanaco, where they adapted to the conditions of the area (little variation from that of their origin) and the food (native pastures).

3.8 Field work

3.8.1 Sanitation

A parasite analysis was done for each of the animals involved in the research. Gastrointestinal parasite eggs were found in the animals for which an internal parasite removal was conducted. Vitamins were immediately administered to assure better responses and efficiency to semen collection.

3.9 Animal training and semen collection technique

Once the animals are accustomed to their surroundings, they were trained for mounting and ejaculation, testing different techniques and obtaining the best responses and acceptance with the "mannequin llama group." This technique was developed by a group of thesis writers conformed of R. Flores, P. Delgado, F. Flores and R. Fernandez at the installations of the UAC-T. With this technique 0.5 to 6.0 ml of semen was collected.

This technique consists of holding a female llama in the ventral position (copulation position) and coupling the group mannequin at the rear of the llama. The artificial vagina was put into the mannequin with all the accessories enabling the male to copulate.

3.9.1 Animal selection

The animals that responded to the mentioned mannequin with the corresponding mount and ejaculation were taken to do the present research. The positive response to this technique was 80% of the total males, resulting in 6 selected animals, two from each age.

3.9.2 Semen collection

For this activity, the female was prepared (as explained in 3.9) held on the platform (in copulation position) and the llama group mannequin was placed at the posterior of the llama, to which the artificial vagina was inserted. (see photo No. 1)

3.9.2.1 Arming the artificial vagina and its characteristics

a) Arming the vagina

- The collecting tube was inserted and held with the nylon cover.
- The latex covers were inserted in the internal face of the artificial vagina (plastic tube). The latex cover was firmly held to both tube extremes to avoid entry of water or air.
- It was then filled with tempered water (average of 37°C) in the intermediate chamber, which is the space between the plastic tube and the latex cover on the artificial vagina.

b) Characteristics of the artificial vagina

- Size: 25 cm long and 5 cm wide
- Final and constant temperature of 37°C (animal body temperature), caused by using warm water and with a clinical thermometer.
- Pressure: This was measured by the touch, obtaining similar pressure as the natural vagina, filling the space where the concentrated water or intermediate space is located with air.

3.9.2.2 Mount and ejaculation

The artificial vagina is found in the female prepared with the mannequin with a semen collecting tube. After the male initiated copulation, the ejaculated semen slowly collected in the collecting tube. The copulation lasted for 20-30 minutes for the male. In this type of copulation, the male had various movements of rest until reaching the end of its libido. The male then stepped way from the female, which indicated to continue work with the next male (photograph No. 2).

3.10 Identification of collected semen

While the semen was being collected it was identified carefully with names and dates to maintain organization. This work phase was done in the following table:

Table 10 Collection weeks and days

Age (years)	Animals	Collection weeks					
		First		Second		Third	
		Tues	Fri	Tues	Fri	Tues	Fri
3	A B						
4	A B						
5	A B						

3.11 Laboratory work

3.11.1 Separation of seminal plasma from semen

The obtained semen was centrifuged at 4,000 r.p.m. for 40 minutes (see photograph No. 3), which allowed for complete separation of the spermatozoa from the seminal plasma. The floating substance was separated with a 1 ml syringe, suctioning the seminal plasma that was left on top. It was later to be stored in eppendorf vials.

3.11.2 Seminal plasma conservation

The seminal plasma was conserved in eppendorf vials at a temperature of -15°C (freezing) to avoid denaturalization of the components.

3.12 Determination of protein profile of seminal plasma

3.12.1 Validation

- Validation of the electrophoretic technique

In the current research work, the protein patterns of high and low molecular weight were previously taken into account from SIGMA BRAND No. M3788 and M3913, which

contains proteins from 36,000 to 205,000 and 6,500 to 66,000 mol/Wt respectively. Concentration is from 2 to 3.5 mg/ml. The "liofililizado" content was dissolved with 100 µl of distilled water and separated into 20 µl volumes, which were stored frozen.

To understand the behavior of these proteins in laboratory working conditions, the patterns were separated in PAGE – SDS from 8-15% concentration, obtaining good results at 10%.

After doing this, the distances were then measured, as well as the numeration of the fractions and band area with the "universal electrophoresis gauge" from BIO-RAD, with which concentration was determined.

-Validation of the radial immunodiffusion technique

DIFFU-PLATE plates were used from Bioscientific S.A. for Immunoglobulins A, G and M. Five µl of seminal plasma and blood serum was planted in plates and incubated for 42-72 hours for observation of the reaction.

3.12.2 Protein electrophoresis

BIO RAD (1990) indicates that through the electrophoresis method on "poliacrylamida" gel (PAGE) the number of protein fractions and their molecular weights were determined for each one of the samples. In our case we worked with llama seminal plasma.

For electrophoresis (PAGE), an analytical balance was used (brand: A.N.D. 12 gr. Capacity) for the solid reactants and eppendorf micropipettes were used for liquids (capacities: 100 to 1000 µl, 10 to 100 µl and 5 to 50 µl) according to the needs and requirements (annex No°2). The solutions prepared were:

a) Acril solution – bis acrilamida
b) Buffer tris 0.5 M. pH 6.8 (Buffer stacking)
c) Buffer tris 1.5 M. pH 8.8
d) Buffer line
e) Sample buffer (without B-Mercaptoethenol)

f) Blue dye solution with Coomassie R250

g) Fading solution

***Gel preparation**

Table 11 Gel preparation protocol

Solution or reactant	Equilibrium gel	Separation gel			
		8%	10%	12%	15%
Acril-bis acrilamida	0.5 ml	7.9 ml	990 µl	1.2 ml	1.6 ml
Buffer tris 0.5 M pH 6.8	380 µl	---	---	---	---
Buffer tris 1.5 M pH 8.8	---	750 µl	750 µl	750 µl	750 µl
SDS 10%	30 µl	30 µl	30 µl	30 µl	50 µl
TEMED	3 µl	6 µl	3 µl	3 µl	3 µl
Distilled water	2.1 ml	1.3 ml	1.2 ml	990 µl	0.6 ml
10% Ammonium Persulfate	30 µl	100 µl	30 µl	30 µl	50 µl
Total volume	**3 ml**	**3 ml**	**3 ml**	**3 ml**	**3 ml**

Source: SIGMA (2000)

The reactants dissolved according to the protocol were mixed and reacted forming a polymer in the run plates. The gels were prepared at 10%. This concentration was previously selected doing runs in the polymers whose concentrations are detailed in Table 11. The equilibriums were marked in the gels with a comb, designed to deposit the samples in the seminal plasma. (see photograph No. 4)

***Sample runs**

- The plates (glass) were adjusted with pressure hooks on a vertical support so that the run tampon would not move. A comb was put on the top of the intermediate of the plate so that together with the run tampon they form a space or lane to deposit the samples.
- It was deposited in gel between the glass plates in the four draft parts on the bottom of the plate and in the resting space above the plate, in equilibrium gel (liquid state) before polymerization.
- The samples were put onto the plates with a volume of 10 µl with a Hamilton syringe and in the central slots high and low molecular weight patterns were filled. (brand: SIGMA).

- The prepared plates were introduced to the tray or electrophoretic chamber, in which was installed a multiple transformer to regulate the desired amount of energy. (see photograph No. 5).
- The run tampon was loaded for each of the electrophoretic chamber behaviors, both positive and negative (SDS).
- At first the run lasted 30 minutes with 50 volts in the equilibrium gel (staking), with the goal that all of the samples were uniformly located to start to run in the gel. To conclude with a voltage of 150 and 8 mA for approximately the final 3 hours.
- When the run was done, the polymer was retired and deposited in the dye solution for 60 minutes. The tinting gel was revealed in the acetic acid, methanol, and water solutions for 20 minutes each.
- The colored gel dried between two cellophane pieces and was placed on a glass plate. It was left to dry at room temperature for 24 hours.

***Determination of the molecular weights**

- The molecular weights were determined by measuring the run distance of the pattern proteins, whole molecular weights are known.
- The data was graphed on millimeter axis paper. The axis was used for the known molecular weights and the abscissa axis was expressed for the distances traveled by the known proteins (in cm) in the polymer. The found points formed a line, over which the distances were inter-polarized from the proteins present in the seminal plasma.

***Radial Immunodiffusion**

- The Immunodiffusion radial plates were used to determine the presence of immunoglobulins: Ig A, Ig M, and Ig G, for which 5 µl of seminal plasma is deposited in the agar of the plate. (see photograph No. 6)
- It was incubated in the inverted position by first humidifying the plate with water vapor for 48 and 72 hours according to each immunoglobulins.

- After letting the above time pass, a halo was formed around the deposited sample, which indicated the presence of antibodies.

***Quantification of proteins**

- The "electroforgram" measured the area for each protein fraction and the height of the gel was considered. The volume of the protein fractions of the patterns could then be determined. With this data, the protein fractions of the seminal plasma inter-polarized and the concentrations were calculated according to the information matrix from 2 to 3.5 (mg/ml).

3.13 Study factors

- Collection week
- Age of the animal
- Collection days

3.14 Response variables

- Number of protein fractions
- Molecular weight of the proteins
- Presence of immunoglobulins
- Protein concentration

3.15 Experimental design

The design to find statistical differences was the Jerárquico factorial Design, (Calzada Benza, 1990), whose model is:

$$Y_{ijk} = \mu + \alpha i + \beta(i)j + \gamma k + \alpha * \gamma(ik) + \varepsilon(ijk)m$$

Where:

Y_{ijk} = Experimental observation

μ= General measurement of the samples

αI = Effect of the I-esima week of collection

$\beta(i)j$ = Effect of the j-esima day of collection added to the I esima week

γk = Effect of the k-esima age of the animals

$\alpha*\gamma(ik)$ = Effect of the interaction of the I-esima week by the k-esima age

$\varepsilon(ijk)m$ = Experimental error

i = 1; 2 and 3 collection weeks

j = 1 and 2 days of collection

k = 3, 4 and 5 years, age of the animals

m = 2 repetitions

The DUNCAN test helps us compare the significance with the results found in this research. The S.A.S. statistical analysis was used to obtain more reliable information.

3.16 Spatial arrangement

The semen collections to obtain seminal plasma were done according to the distribution that is detailed in table 12.

Table 12 Distribution of collections by age, weeks, and days

Treatments							
Age (years)	Animals	Weeks of collection					
		First		Second		Third	
		Tue	Fri	Tue	Fri	Tue	Fri
3	A B						
4	A B						
5	A B						

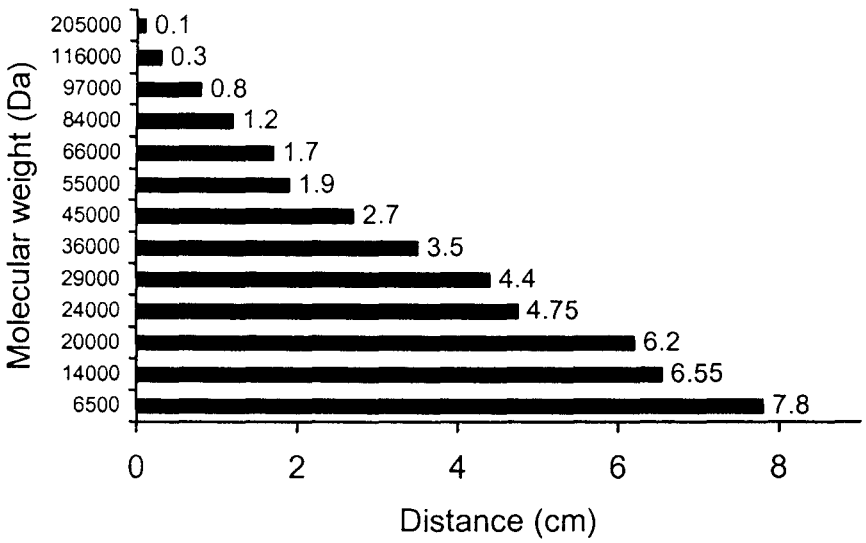
4. Results and discussion

4.1 Electrophoresis of proteins and immunoglobulins

4.1.1 The pattern

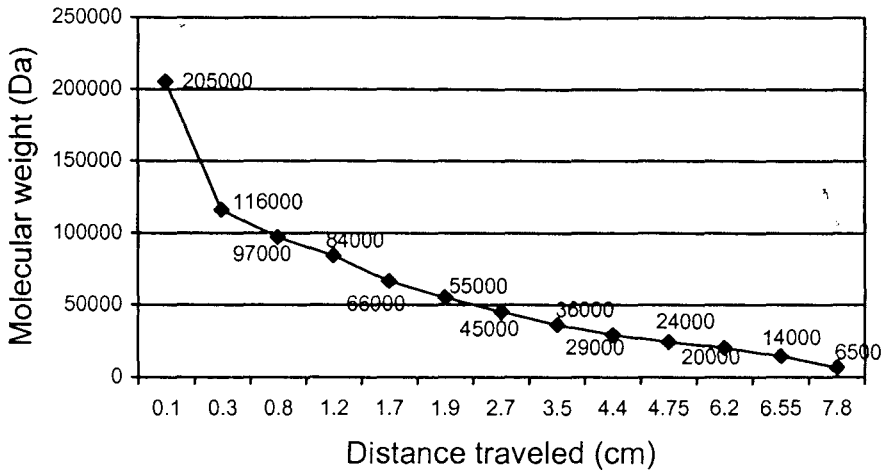
Figure 1 shows the differences in distance traveled by the protein fraction patterns (SIGMA 2000), according to the molecular weights in the gel (SDS-PAGE). These reference statistics were used for comparison and determination of the number of protein fractions and the molecular weights of llama seminal plasma with respect to the fraction variations by age, weeks and days of collection.

Figure 1 Protein pattern and distance traveled on the electrophoretic plate.



The high molecular weights expressed in Daltons (Da) are those that have traveled less distance and those that have low molecular weights travel longer distances. To obtain the molecular weights of the protein fractions that make up llama seminal plasma, the travel distances have been measured on the electrophoretic plate and were inter-polarized on the curve obtained with the protein patterns (graph 1).

Graph 1 Curve of molecular weights of protein patters



4.2 Protein fractions in llama seminal plasma

Table 13 Number of protein fractions

Collection weeks							
Age (years)	Animal	First		Second		Third	
		April 18	April 21	May 1	May 4	May 15	May 18
3	A	6	6	12	16	12	11
	B	6	9	12	9	11	11
4	A	11	8	11	15	11	11
	B	9	9	8	9	18	18
5	A	16	11	14	9	18	10
	B	15	9	8	8	18	17

Table 13 shows the distances of protein fractions obtained according to age, collections weeks and days of the week. It shows that there were more protein fractions in the last two weeks of collections in four and five year old animals and in the first day of the week.

Table 14 General averages and their standard deviation

Age	**Average**
3 years	10.08 ± 2.89
4 years	11.5 ± 3.42
5 years	12.75 ± 3.81
Week	**Average**
First	9.58 ± 3.12
Second	10.91 ± 2.75
Third	13.83 ± 3.38
Day	**Average**
First	12.0 ± 3.75
Second	10.8 ± 3.28

The general averages of protein fractions and their standard deviations according to the study factors are shown in table 14.

Table 15 Variance analysis for protein fractions

Source of variation	**G.L.**	**S.C.**	**Energy Source**	**Pr> 0.05**
Age	2	42.7222	2.58	0.0964 n.s.
Weeks	2	113.3880	6.85	0.0004 **
Weeks * Age	4	85.7777	2.59	0.0621 n.s.
Days (weeks)	3	18.5000	0.75	0.5355 n.s.
Error	24	198.5000		
Total	35	458.8888		

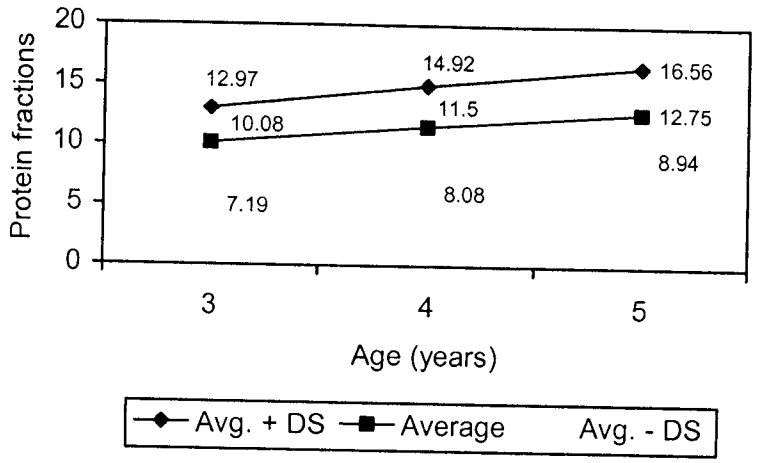
C.V. 25.12%

The variance analysis (Table 15) shows significant differences ($p<0.05$) between weeks, but not between ages ($p>0.05$) or in the interaction of weeks by age, or in added days in the week.

The variation coefficient is 25.12%, which expresses the reliability of the research results. The planted hypothesis is accepter for the factor weeks. That is to say that the number of protein fractions in llama seminal plasma vary depending on the week of collection. Meanwhile, there is no variation in factors of age and days of semen collection, which rejects the hypothesis for these factors.

4.2.1 Protein fractions between llama ages

Graph 2 Average protein fractions between ages



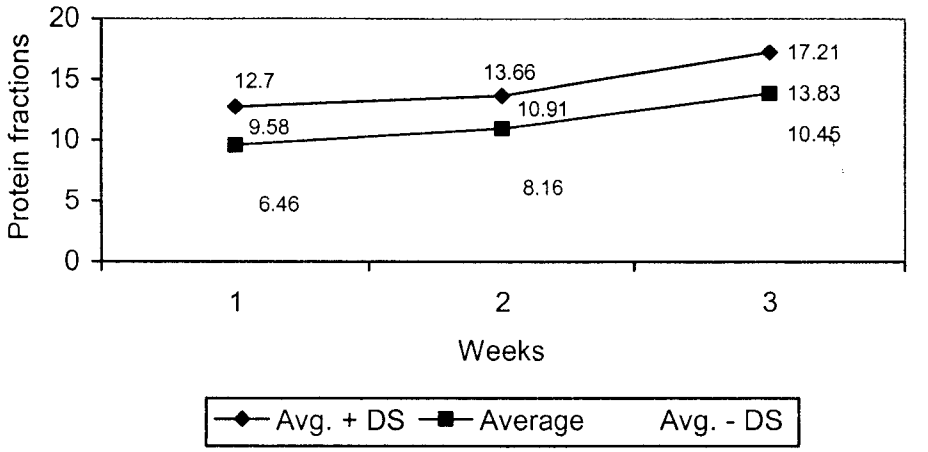
Graph 2 shows protein fraction variations according to age. Three year old llamas have an average of 10.08 ± 2.89 protein fractions. Five year old animals had an average of 12.75 ± 3.42 protein fractions, which was higher than younger ages. These results show an increase in the number of protein fractions with the age of the animals. The variance analysis (Table 15) shows that the protein fraction levels between the animals are similar.

According to the numerical results obtained, age influences the amount of protein fractions. This is due to higher body development by older animals and consequently the accessory organs (prostate and bulbourethral glands) of the fully developed reproductive system.

As a consequence the older animals, because they have more protein fractions in the seminal plasma, probably provide a higher amount of nutrients to the spermatozoa (Biasoli, 1980), with fertility indexes higher than those in younger animals. This asserts that seminal plasma proteins play an important role in nutrition, protection and capacitation of the spermatozoa.

4.2.2 Protein fractions between collection weeks

Graph 3 Protein fraction averages between collection weeks



According to graph 3, the third week of collection shows an average of 13.83 ± 3.38 protein fractions. This is higher than the second and first collection weeks, which have average of 10.91 ± 2.75 and 9.58 ± 3.12 protein fractions respectively.

Table 16 Comparison of protein fraction averages between weeks (Duncan)

Weeks	Average (protein fraction)	Group
3	13.83 ± 3.12	A
2	10.81 ± 2.75	B
1	9.58 ± 3.12	B

The Duncan test (Table 16) shows differences between the averages in collection weeks with 13.83 ± 3.38 protein fractions for the third week. This is statistically higher with respect to the second and first weeks of collection. The first and second weeks of collection were similar with averages of 10.91 ± 2.75 and 9.58 ± 3.12 protein fractions respectively.

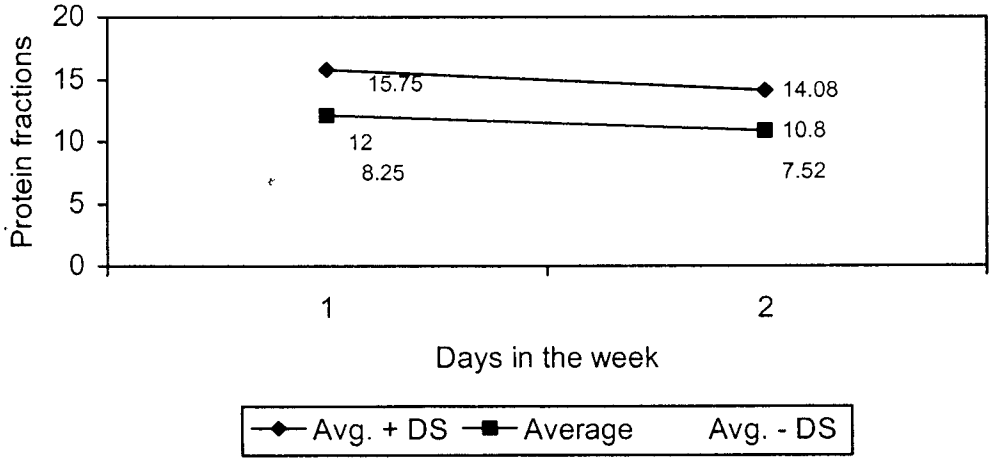
In the first week of collection, the animals showed lower amounts of protein fractions in seminal plasma, possibly because of low pregnancy percentages. An increase of protein fractions in the following weeks would indicate that the pregnancy percentages are higher.

The artificial collection of llama semen during the first weeks may cause stress, which is caused by a low protein secretion from the male accessory glands. With a higher frequency and semen collection time, the animals respond better to the artificial vagina, which permits the obtaining and evidencing a higher number of protein fractions (graph 3).

4.2.3 Protein fractions between collections days in the week

The variance analysis (Table 15) shows no significant differences ($p>0.05$) between collections days during the week according to ANVA, rejecting the hypothesis. The accessory glands of the male reproductive system, on collection days during the same week secrete similar quantities of protein fractions (graph 4).

Graph 4 Protein fraction averages between collection days in the week



The first collection day shows an average of 12 ± 3.75 protein fractions, which is higher than the second with an average of 10.8 ± 2.28 protein fractions. The animals secrete the most protein fractions in the first collection day of the week.

Proteins play an important role of the physiology of the male reproduction apparatus. They provide great advantages to the spermatozoa and also act negatively (Fecunditas

2002) because of the presence of enzymes that produce semen coagulation and inhibit spermatic motility.

Llama semen's high viscosity is attributed to the presence of certain conjugated proteins that semen dilution difficult. The presence of pepsinogens, "alfa amilasa", hyaluronidase, lysozymes, and protease inhibitor (mediconet 2002) inhibit the development and nutrition of spermatozoa.

4.3 Protein fractions molecular weights of llama seminal plasma

Because of particularities that these results show about molecular weights and because of the specificity of each one (specific proteins, proteins that have other unique biological functions, etc.) they can't work with compound averages, or do statistical comparisons until they do not have to count on complementary information of earlier studies. For this reason, only the results are described.

The length and molecular weight of the proteins is variable. They can be made up of hundred to thousands of amino acids with different sequences. Therefore, their molecular weights can also vary from thousands to millions. (Coila, 2001).

In llama seminal plasma, results have been obtained of proteins with variable molecular weights. This indicates the existence of proteins that are structurally different and according to size, they can fulfill catalytic functions (according to specificity) of transport, tampon effect, etc.

Proteins with high and low molecular weight and the number of protein fractions in seminal plasma influence the fertilizing capacity of spermatozoa. This affirmation is based on the existence of diverse proteins with distinct functions. This way a protein with a high molecular weight (150,000 Da) denotes the presence of globulins, whose function is to protect, generated by an auto immune process of proteins with a positive or negative effect.

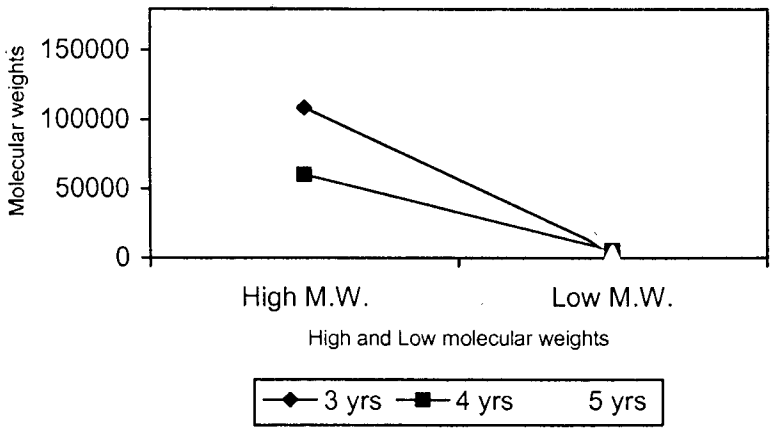
Other protein fractions (66,000 Da to 70,000 Da) implicate the presence of albumin, whose function is to transport and have a protein tampon effect in biological liquid.

Analyzing protein fraction after protein fraction, we find proteins with enzymatic activity, which can be from hydrolysis, lyases, transferase, etc. Each one has its own different action mechanism and the need of a specific substrate present in the *in vitro* seminal plasma (liquefaction of seminal plasma) or in different deposit places for seminal plasma in the female genital tract where it is in contact with corporal liquids of the same animal receptor.

4.3.1 Molecular weights of protein fractions between ages

Protein fraction molecular weights in the seminal plasma of different animals in terms of age, show variable protein molecular weights, of which are described to be the best representatives.

Graph 5 Molecular weight ranges between ages



Graph 5 shows the molecular weight ranges with proteins between 108000 Da and 4500 Da in three year old animals. Four year old animals have proteins molecular weights that range from 60000 Da and 5000 Da. Five year old animals have protein molecular weights that range from 152500 to 3500 Da.

Five year old animals have proteins with high molecular weights compared to those of other ages. This could be because of better corporal and sexual organ development that five year old animals have.

Proteins with high molecular weights have positive effects on spermatozoa metabolism. During the research work, it has been observed that five year old males had better sexual behavior and a better copulation response. This would indicate that five years old is the ideal age for breeding to have the highest number of protein fractions.

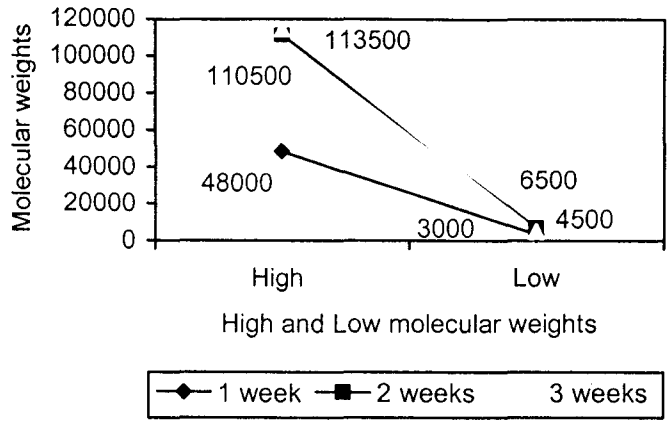
The effect of proteins with high molecular weights can play an important role in spermatic capacitation in older animals but is also a protector because of the presence of proteins such as globulins. Antibodies are possibly produced because of the presence of antigens, to which the animal is exposed in its sexual life (breeding year after year).

Other proteins will cause high viscosity (midiconet.com 2002) that present seminal plasma, which is on of the factors that immobilizes the spermatozoa for various hours *in vitro* until other enzymes are activated and the spermatozoa are slowly freed and reactivated. *In vitro* seems to be the way to avoid cellular energy loss and its exposition to an unfavorable environment.

The presence of enzymes of heavy proteins similar to phosphorilaze B and Fructose 6 phosphate quinase (SIGMA 2000) are those that have higher quantities in seminal plasma of older animals. Lower amounts of heavy proteins in younger animals could be because of low percentages of fertility and descendants that these animals can have.

4.3.2 Molecular weights between collection weeks

Graph 6 Molecular weight ranges between collection weeks.



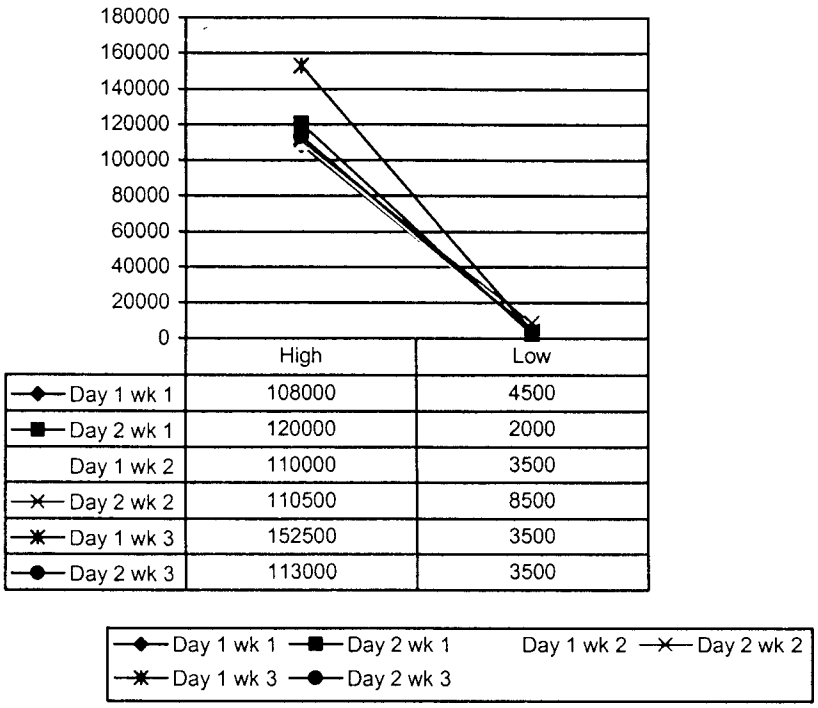
Graph 6 shows molecular weight results of a four year old animal according to collection weeks (more representative). In the first semen collection week protein fractions of 3000 to 48000 Da were found. In the second week protein fractions of 6,500 to 110,500 Da were found and in the third week, protein fractions of 4,500 to 113,500 Da were found. Results from semen collections from other ages of males are found on annexes 4 -21.

The presence of protein fractions with high molecular weights is manifested in the third collection week, which is probably due to an increase in glandular secretions and an autoimmune response to the presence of antigens and/or auto antigens. Therefore, low fertility in the first week of collection is due to being accustomed to collection and secretion of other proteins that influence the number of protein fractions.

4.3.3 Molecular weights between collection days in the week

Molecular weights, according to collection days in the week, do not how great variations. A five year old animal was considered the best representative for a description of the results. The information for other ages is presented in annexes 4-21. Graph 7 shows proteins of high and low molecular weights, which are described according to the collection day in their respective weeks.

Graph 7 Ranges of molecular weights between collection days in the week



	High	Low
Day 1 wk 1	108000	4500
Day 2 wk 1	120000	2000
Day 1 wk 2	110000	3500
Day 2 wk 2	110500	8500
Day 1 wk 3	152500	3500
Day 2 wk 3	113000	3500

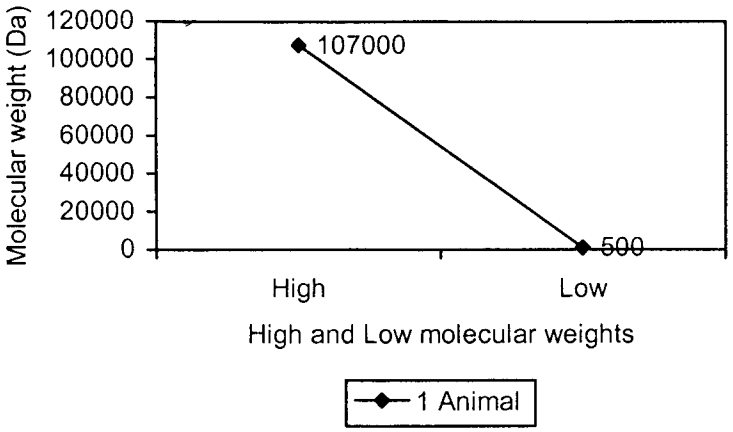
The low molecular weights are from 8500 to 2000 Da, considering all of the days in the three collection weeks. The high molecular weights are variable, going from 108,000 to 120,000 Da in the first and second collection days in the first week, from 110,000 to 110,500 Da in the first and second days in the second week and finally from 152,500 to 113,000 Da in the first and second days in the third collection week. These last results are higher with respect to the first two collection weeks.

Rubinson (2001) describes a series of proteins with their respective molecular weights, similar to those found in llama semen. They are proteins from another species of animal or substance, which implicates different substances with similar molecular weights.

- **Cattle seminal plasma analysis**

For better information, an electrophoretic row was done for three year old cattle seminal plasma. A total of 18 protein fraction molecular weights were observed.

Graph 8 Molecular weights of protein fraction ranges in cattle seminal plasma



Graph 8 shows molecular weight ranges of proteins between 107,000 and 500 Da. The last value is the lowest compared to those of the llama. The closest approximation to the number of protein fractions is the sixth animal (five years), which has 18 protein fractions with 110,000 to 4,500 Da.

The bull has more protein fractions than the three year old llama, which are possibly related with spermatic concentration in cattle. The opposite occurs with the llama, because of its low quantity of protein fractions and greater need for protection from spermatozoa of this species.

4.4 Presence of antibodies or immunoglobulins in llama seminal plasma

For the same reasons as the prior variable, these results were not submitted to a statistical analysis. This is only a description of the results.

Antibodies have molecular weight equal or higher than 150,000 Da. In the determination of molecular weights of protein fraction seminal plasma distances in llamas, equal or superior values to those mentioned earlier have been found. This confirms the existence of antibodies in llama seminal plasma.

4.4.1 Presence of antibodies between ages.

Figure 2 Presence of antibodies between ages

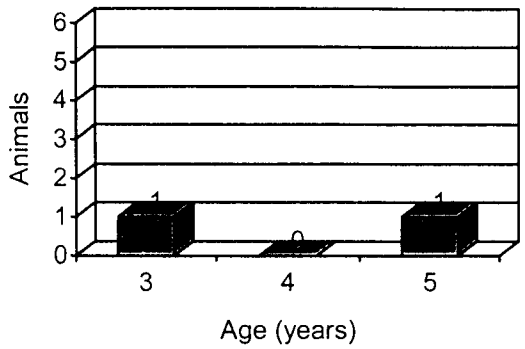
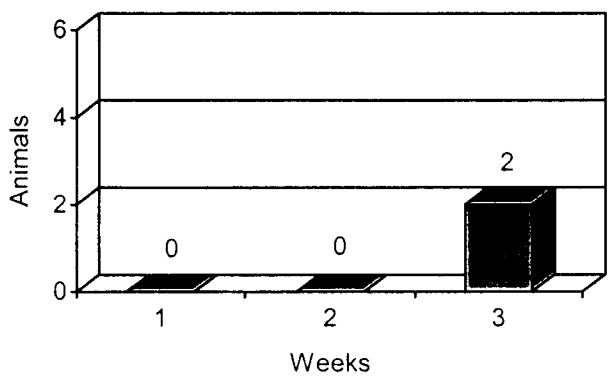


Figure 2 shows animals that have antibodies according to age. In the three year old group, 1 animal had antibodies in its seminal plasma. In the four year old group, no antibodies were detected. One animal had antibodies in its seminal plasma in the five year old group.

The presence of antibodies in seminal plasma should still be studied to determine its specific function. The presence of antibodies in plasma could be because of the reaction of the organism against itself, or against the spermatozoa as if it was an antigen (Tizard 1998, and Moreno 1996). These could be responsible for the llama's low fertility.

4.4.2 Presence of antibodies between collection weeks

Figure 3 Presence of antibodies between collection weeks



The presence of immunoglobulins or antibodies in collection weeks is shown in Figure 3. In the first and second weeks, antibodies are not detected, but in the third week, antibodies are detected in two animals.

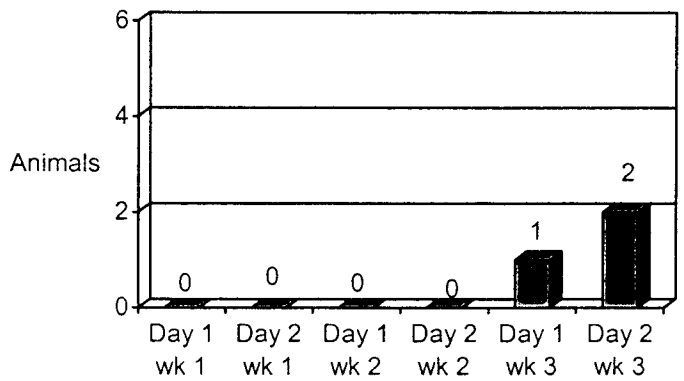
The semen samples showed jellification, which is possibly related to or caused by antibodies (possible agglutination, or protection). This confirms the existence of antibodies in llama seminal plasma as referenced by Hafez (1996). Also, the jellification could be due other proteins and enzymes that react to the variations in temperature of the environment (as observed at the moment of semen collection).

The presence of antibodies is due also to the secretion of the accessory glands and other seminal liquids like those that secrete at the level of seminiferous tubules. In this region, the effect of antibodies is important, including from the hematotesticular barrier and the development of the germinal cells (spermatogonias) as stated by Hafez (1996).

In the first and second weeks, there is no trace of antibodies, which does not confirm the absolute absence of antibodies. They could just be in low concentrations and not able to be seen with the human eye or our technology.

4.4.3 Presence of antibodies between collection days in the week

Figure 4 Presence of antibodies between collection days in the week



In the two days of the first two collection weeks there was no presence detected of antibodies. But in the third week on the first day one animal (five years old) showed the presence of antibodies in its seminal plasma in the second day of the same week two animals (three and five years old) (figure 4) showed the presence of antibodies. As described earlier the presence of antibodies is not expressed in the first collections (days and weeks).

The results show that surely there are antibodies that are produced by the male genital tract as well as confirmed by Mediconet.com (2002) and Sanz (2002). It could possibly be responsible for spermatic inactivity, which is produced by the accessory glands.

- Radial immunodiffusion test

The statistics on this type of test (annex 3) show that three year old animals and one four year old animal had immunoglobulin G. The presence of Ig G probably immobilizes the spermatozoa, which adheres to the surface (Fecunditas 1998), impeding the completion of its function of fertilizing the egg.

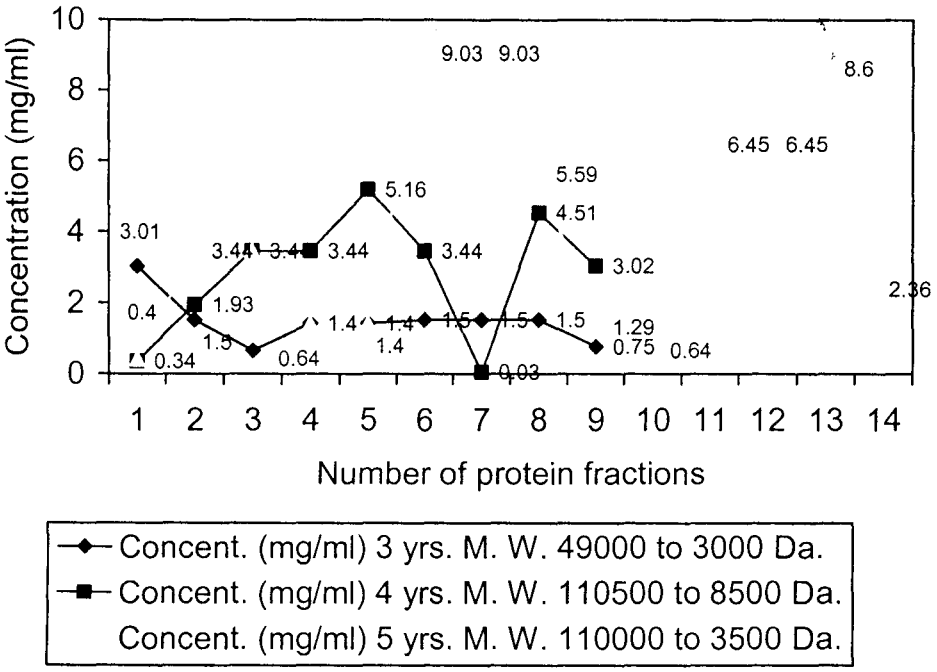
In human semen, Igs. A, G and M was found, which cause infertility or low fertility (Yepe and Gonzales 2002). This would show that the antibodies found in llama seminal plasma are causing low fertility in the males. In this last test, Ig G was found, which effects spermatozoa physiology.

4.5 Protein fraction concentrations in llama seminal plasma

For reasons previously explained in terms of molecular weight as well as antibodies, and for the specificity of each protein fraction, it is impossible to group their concentrations. This impedes further statistical analysis. Therefore, only a description of the most representative animals is done.

4.5.1 Protein fraction concentrations between ages

Graph 9 Protein fraction concentrations between ages



Graph 9 shows the variations in 3, 4, and 5 year old llama seminal plasma. Five year old animals show higher quantities of protein fractions and proportionally, these have higher concentrations in their protein fractions. Three and four year old animals have lower concentrations.

The higher values in different animals range from 9.03 mg/ml to 0.34 mg/ml in five year old animals. Four year old animals have concentrations that range from 0.03 mg/ml to 5.16 mg/ml. Three year old animals have concentrations that range from 0.75 mg/ml to 3.01 mg/ml of protein fraction concentrations in seminal plasma.

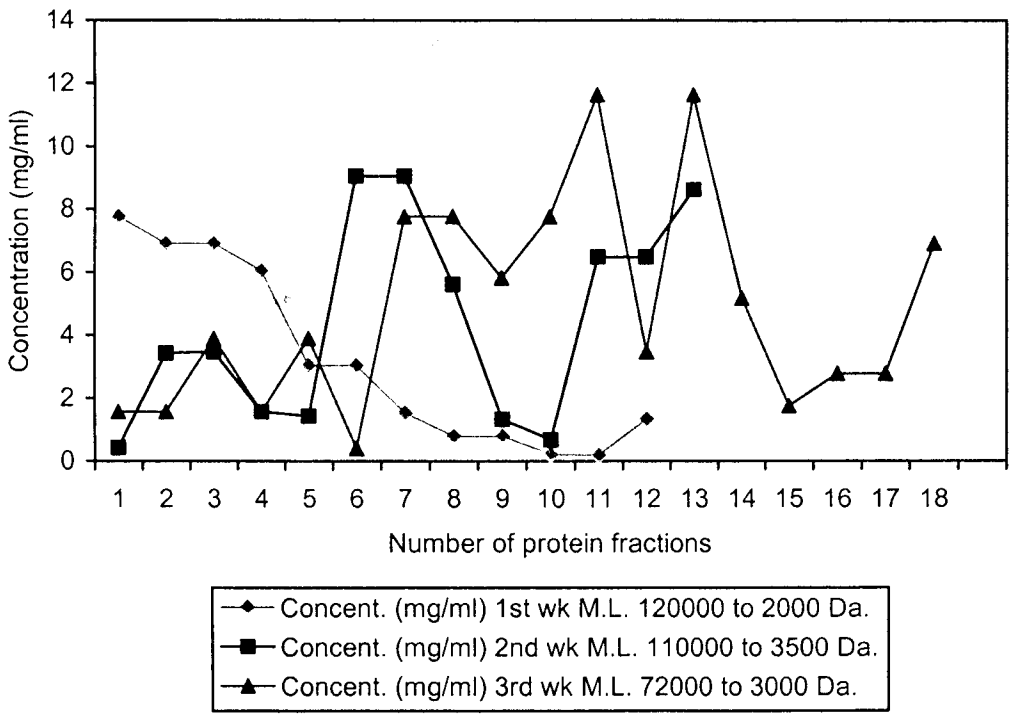
This behavior could be related to the amount of secreted fractions in seminal plasma, and those responsible for older animals having spermatic viability because the have proteins

that positively act in metabolism and spermatozoa capacitation. This way they can penetrate the egg oophoros as stated by Fecunditas (1998).

The concentration of these protein fractions increases as the animal's age increases. This behavior of the fractions implies that older animals have proteins that nourish the spermatozoa more efficiently (Illera 1994). Enzymes probably exist that nourish other proteins like the [semenogueilina?] (mediconet 2002) that provokes jellification of the semen and therefore the decrease in spermatozoa motility.

4.5.2 Protein fraction concentrations between collection weeks

Graph 10 Protein fraction concentrations between collection weeks



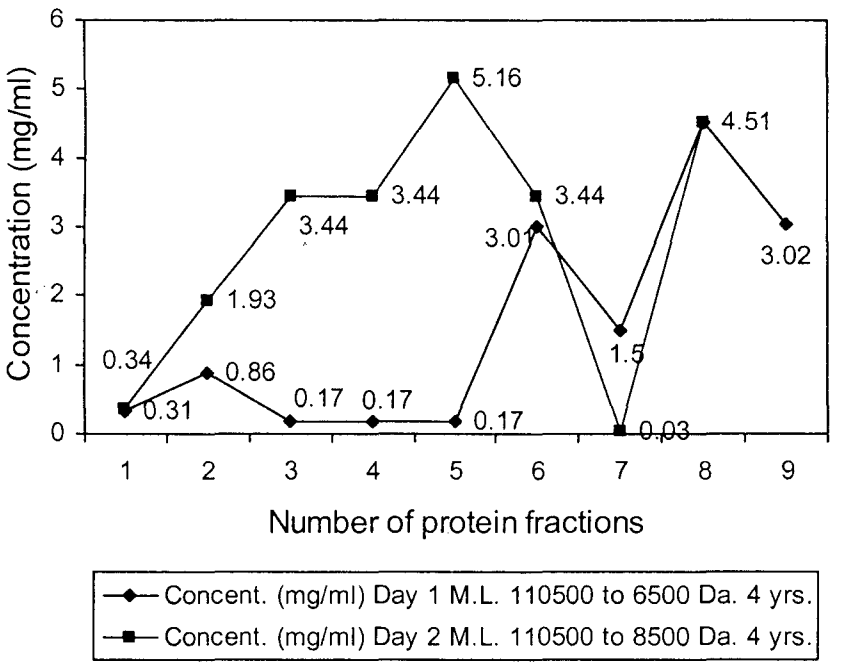
Graph 10 shows the concentration variations in the protein fractions according to the collection weeks. The third collection week had protein fractions with the highest concentrations, while the lowest concentrations of protein fractions were observed in the first week in five year old animals.

In the third week protein fractions of 11.61 mg/ml to 0.38 mg/ml were seen. The second week showed protein fraction concentrations of 9.03 mg/ml to 0.64 mg/ml. The first week showed lower concentrations of 7.74 mg/ml to 0.16 mg/ml.

Concentrations of immunoglobulins found in blood serum have values of 11.0 mg/ml and 0.59 mg/ml in the milk (Blood 1983). The concentration of antibodies found in five year old animals is 0.5 mg/ml (annex 21) bringing it nearer to the milk. Therefore, it could possibly be related to the volume of the substances that it secretes. This requires attention for research.

4.5.3 Concentration of protein fractions between collection days in the week

Graph 11 Concentration of protein fractions between collection days in the week



Graph 11 shows results from the protein fractions concentrations between collection days in the week. The first collection day shows protein fraction concentrations in the ranges of 0.17 mg/ml to 4.51 mg/ml. The second day shows ranges of 5.16 mg/ml to 0.03 mg/ml.

The protein fraction concentrations are variable between ages, collection week and days of the week, including animals of the same age. These variations could be linked to other intrinsic and extrinsic factors not considered in this investigation.

Apparently, all of this behavior describes that the male llama, during breeding that lasts from December to March each year, experiments a series of events in their organism that help it reach a physiological state such that its levels and quality of components of its semen are optimal. Upon beginning the breeding season, the first ejaculations will be low quality, but will improve until the third week.

The low value protein fractions and their concentrations influence sexual activity of younger animals. This can also be somewhat related to the decrease in the quality of food the animal consumes.

The presence of immunoglobulins is associated with an autoimmune process that can be developed in animals. It is affected by the number of previous pregnancies or a constant fight for group supremacy and a lesion in the hematotesticular barrier and an autoimmune reaction is created and the organism responds against its own spermatozoa. This explanation is held to the assertion of Fecunditas (2002), which causes a decrease in fertility.

The presence of Immunoglobulin G is an important element. Its function is first to protect, and second to give a negative effect. Its presence is probably due to constant copulations that, because of mechanical effects and contact with diverse and foreign antigens, lead to the stimulation of the immune system. It then responds with the production of antibodies, as stated by Gonzales (2002).

The smaller protein fractions can indicate the presence of complete structures. When more fractions appear during electrophoresis it can be concluded that it is because of the enzymatic action, which divide into their respective units. This is to say that the presence of protein fractions is made up of dimer or trimer. With this possibility, it is necessary to

do an electrophoretic run with B- mercaptoethanol, which will affirm or negate the previous explanation to avoid further denotations.

Four and five year old animals would be the best to work with in obtaining semen and assuring high amounts and high quality semen. It is necessary to give special attention to these animals to provide better conditions for germplasm conservation. However, one should be careful of animals older than seven years of age because they could have more antibodies and a decrease in protein fraction concentrations.

This research work indicates that the best moment for semen collection is after the first week or the breeding activity for the males. This is because protein concentrations indicate better spermatozoa protection (protein tampon effect and presence of immunoglobulins). This will also facilitate better viability time and support for cryopreservation techniques, which could implicate negative and positive effects for spermatozoa physiology.

5. Conclusions

According to the results obtained in the determination of the protein profile and presence of antibodies in llama seminal plasma, the following conclusions have been made.

1. The number of protein fractions in seminal plasma show averages of 10.08, 11.5, and 12.75 in 3, 4, and five year old animals, respectively.
2. Seminal plasma protein fractions have averages of 9.58, 10.91 and 13.83 in the first, second and third weeks of collection, respectively. Between days, the first presents and average of 12 and the second, 10.8 protein fractions.
3. The molecular weights of protein fractions between days range from 108000 to 4500 Da, 60000 to 5000 Da and 152000 to 3500 Da in 3, 4, and 5 year old animals, respectively.
4. Molecular weight ranges of protein fractions are from: 48000 to 3000 Da, 110500 to 6500 Da, and 113500 to 4500 Da in the day of the first, second and third

collection weeks respectively for a five year old animal. Collection days show 108000 to 4500 Da, 110000 to 3500 Da and 152000 to 3500 Da in the first, second and third week, respectively. The second day shows 120,000 to 2,000 Da; 110,500 to 8,500 Da and 113,000 to 3,500 Da in the first, second and third week, respectively.

5. There are 2 three and five year old animals with antibodies in their seminal plasma (IgG).
6. Only 2 animals were found to have antibodies in the third weed of collection. On those days, 1 animal was detected on the first day and 2 animals were found to have them on the second day of the same week.
7. Protein fraction concentrations in llama seminal plasma range from 3.01 to 0.64 mg/ml, 5.16 to 0.34 mg/ml, and 9.03 to 0.4 mg/ml in three, four, and five year old animals respectively.
8. In terms of collection times, protein fraction concentrations range from 7.74 to 0.16 mg/ml, 9.03 to 0.4 mg/ml and 11.61 to 0.38 mg/ml in the first, second, and third week for five year old animals. Collection days show concentrations that range from 4.51 to 0.17 mg/ml, and 5.16 to 0.03 mg/ml in the first and second day respectively for four year old animals.

Four and five year old animals in the third week of collection are the best for obtaining semen because they have the best quality and quantity of protein fractions and the best conditions for conserving germplasm.

The presence of immunoglobulins G is very important for spermatozoa because of it protecting function and because it influences the llama reproduction cup.

6. Recommendations

- Corroborate the results obtained from the study and work with animals between the ages of 3 and 8 to compile a complete protein profile for seminal plasma and its variations with time.

- Purify the antibodies present in seminal plasma to establish complete production tests of antispermatozoa antigens and test them with dilutions of semen.
- Determine the presence of proteolytic enzymes and their relation to capacitation and/or spermatozoa activity for isolation.
- To obtain more information and compliments to these preliminary results, it is necessary to work in evaluating the female llama immune response in light of semen according to age and possible auto immune responses of the male against its own spermatozoa.
- For semen collection and cryopreservation it is recommended to use 4 and 5 year old animals. They should be used after the first month of breeding or sexual activity (ejacualation) because these conditions facilitate more protein fractions with higher concentrations.

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8. Annexes

Annex No. 1

Protein patterns of high and low molecular weights

Proteins	High Daltons	Low Daltons
Rabbit Muscle myosin	205,000	-
Beta galactosidase of E. Coli	116,000	-
Rabbit muscle phosphorilaze B	97,000	-
Rabbit muscle Fructose-6 phosphate quinoa	84,000	-
Cattle albumin serum ASB	66,000	-
Glutamic dehydrogenate of cattle liver	55,000	-
Albumin ovum of the chicken egg	45,000	45,000
Rabbit muscle dehydrogenate phosphate Gliseriraldehido-3	36,000	36,000
Carbonic Anhydrase of cattle erythrocytes	-	29,000
Trypsinogen of cattle pancreas	-	24,000
Trypsin inhibitor (soybean)	-	29,000
Alfa lacto albumin of cattle milk	-	14,000
"Aprotrymine" (lung) cattle	-	6,500

Annex No. 2

Solution preparation protocol (SIGMA)

a) Acryl solution – bis acrilamida:

Acrilamida	30 g.
N, N' – metilen – bis-acrilamida	0.79 g.
Distilled water c.s.p.	100 ml.

This solution passed through a milipore filter (0.45 um.) and was later conserved in a dark amber colored bottle at 4°C.

b) Crack Buffer 0.5 M. pH 6.8 (Buffer stacking):

Trys base	6.055 g.
SDS (dodesil sodium sulfate) 0.4%	4 ml. of SDS 10%
Distilled water c.s.p.	100 ml.

The initial pH of the solution was not what was desired, which is why the same hydrochloric acid had to be calibrated until it reached the indicated pH level.

c) Crack buffer 1.5 M. pH 8.8:

Crack base	18.18 g.
SDS (dodesil sodium sulfate) 0.4%	4 ml. of SDS 10%
Distilled water c.s.p.	100 ml

The initial pH of the solution was not what was desired, which is why the same hydrochloric acid had to be calibrated until it reached the indicated pH level.

d) Line Buffer

Crack base 0.025 M.	3 g.
Glycine 0.19 m.	14.4 g
SDS (dodesil sodium sulfate) 0.1 %	1.0 g
Distilled water c.s.p.	1000 ml

e) Sample buffer: (without- B-mercaptoethanol)

Crack0.14 M. pH 6.8	2.8 ml. of 0.5 M. crack
Glycine 10%	1.0 g
SDS (dodesil sodium sulfate) 2%	2 ml of SDS 10%
EDTA 2 mM. (ethylenediaminetetraacetic acid)	7.45 mg.
Blue of phenol bromine (piscas)	
Distilled water c.s.p.	10.0 ml

f) Solution tinted with Coomassie blue:

Bright blue commassie R_250 0.25%	0.25 g
Methanol 50%	50.0 ml
Glacial acetic acid 10%	10 ml
Distilled water c.s.p.	100 ml

The color was dissolved in the methanol, acid and finally the water. It is important to follow the order. It is not necessary to filter this solution and it is better to use fresh.

g) Non-tinted solution

Glacial acetic acid 10%	100 ml
Methanol 10%	100 ml
Distilled water c.s.p.	1000 ml

* Sample and pattern preparation

- The molecular weight patterns were prepared in a concentration of 1 mg. in ml. this applied to both those that had now molecular weights as well as those dissolved in sample tampons
- The llama seminal plasma samples were dissolved in sample tampons with a proportion of 1:2.

These solutions passed through a milipore filter (0.45 um) and was later conserved in a dark amber colored bottle at 4°C.

Annex No. 3

Radial immunodiffusion reaction

Animals	Age (years)	Immunoglobulins		
		Ig A	Ig M	Ig G
Clear	3	Negative	Negative	Positive
Dark	3	Negative	Negative	Positive
L. M.	4	Negative	Negative	Positive
Spots	4	Negative	Negative	Negative
Scruffy	5	Negative	Negative	Negative
Black	5	Negative	Negative	Negative

MOLECULAR WEIGHTS AND PROTEIN FRACITON CONCENTRATIONS

Annex No. 4

Clear: 3 years, 1st week

Day 1		Day 2	
M.W. (Daltons)	Concentration (mg/ml)	M.W. (Daltons)	Concentration (mg/ml)
47000	1.72	47000	1.72
35500	0.21	35500	0.21
28000	0.21	28000	0.21
20000	0.03	20000	0.03
6500	0.03	6500	0.03
3500	0.86	3500	0.86

Annex No. 5

Dark: 3 years, 1st week

Day 1		Day 2	
M.W. (Daltons)	Concentration (mg/ml)	M.W. (Daltons)	Concentration (mg/ml)
50500	0.86	49000	3.01
38000	0.34	35500	1.50
29000	0.75	32000	0.64
24000	0.30	28000	1.40
21000	0.30	21500	1.40
8500	0.86	20000	1.50
400	0.72	6000	1.50
		4000	1.50
		3000	0.75

Annex No. 6

L.M.: 4 years, 1st week

Day 1		Day 2	
M.W. (Daltons)	Concentration (mg/ml)	M.W. (Daltons)	Concentration (mg/ml)
77000	1.72	48000	0.86
66000	0.86	35000	3.44
53000	0.86	32000	10.32
50500	1.72	28000	6.88
36000	4.51	20000	0.86
32500	5.59	6500	1.72
29500	8.38	4000	0.43
24500	8.38	3000	1.72
20500	7.78		
6500	6.02		
4500	8.60		

Annex No. 7

Spots: 4 years, 1st week

Day 1		Day 2	
M.W. (Daltons)	Concentration (mg/ml)	M.W. (Daltons)	Concentration (mg/ml)
50500	0.86	50500	0.86
49000	0.03	49000	0.03
28000	0.03	28000	0.03
22500	0.03	22500	0.03
20000	0.32	20000	0.32
7000	0.13	7000	0.13
6000	3.44	6000	3.44
5000	2.42	5000	2.42
4000	3.44	4000	3.44

Annex No. 8

Scruff: 5 years, 1st week

Day 1		Day 2	
M.W. (Daltons)	Concentration (mg/ml)	M.W. (Daltons)	Concentration (mg/ml)
108000	0.03	120000	7.74
73500	0.52	50500	6.88
54000	0.49	45000	6.88
50000	1.20	35500	6.02
48500	0.86	28000	3.01
40000	3.44	22500	1.50
36500	3.44	21500	0.75
35500	0.04	20000	0.75
33000	2.15	6500	0.18
31000	6.45	4500	0.16
24500	4.30	2000	1.29
22500	6.45		
21000	4.30		
9000	4.30		
5500	2.15		
4500	1.93		

Annex No. 9

Black: 5 years, 1st week

Day 1		Day 2	
M.W. (Daltons)	Concentration (mg/ml)	M.W. (Daltons)	Concentration (mg/ml)
90000	2.15	120000	7.74
80000	2.15	50500	6.88
60500	4.30	45000	6.88
54000	6.45	35500	6.02
54000	0.97	28000	3.01
42000	12.90	22500	1.50
36000	8.60	21500	0.75
34000	8.60	20000	0.75
30000	8.60	6500	0.18
25500	8.60	4500	0.16
22000	8.6	2000	1.29
21000	4.30		
9000	3.44		
5500	5.16		
4500	6.88		

Annex No. 10

Clear: 3 years, 2nd week

Day 1		Day 2	
M.W. (Daltons)	Concentration (mg/ml)	M.W. (Daltons)	Concentration (mg/ml)
108000	0.14	94000	10.32
52500	5.16	55000	0.03
47500	6.88	46000	0.03
41600	0.43	42000	3.44
23500	0.22	38000	10.32
21000	0.22	29000	3.44
19000	0.22	24000	3.44
9000	0.22	23000	1.72
6500	6.02	22000	3.44
5000	3.01	20000	6.88
4500	0.31	13000	6.88
		11000	3.44
		5000	1.72
		3500	0.34
		3000	3.44

Annex No. 11

Dark: 3 years, 2nd week

Day 1		Day 2	
M.W. (Daltons)	Concentration (mg/ml)	M.W. (Daltons)	Concentration (mg/ml)
110000	1.72	49000	3.01
53000	0.43	35500	0.15
50500	0.43	32000	0.64
45000	0.43	28000	1.74
40000	0.75	21500	1.4
28000	4.19	20000	1.5
23000	3.87	6000	1.5
21500	4.19	4000	1.5
20000	4.19	3000	0.75
9500	3.01		
4500	0.75		
4000	6.45		

Annex No. 12

L.M.: 4 years, 2nd week

Day 1		Day 2	
M.W. (Daltons)	Concentration (mg/ml)	M.W. (Daltons)	Concentration (mg/ml)
60000	0.01	110500	1.72
52500	0.17	54000	0.17
46000	0.03	52500	0.17
41000	2.41	50000	0.17
37500	2.41	42500	5.16
31500	3.44	40000	5.16
22000	1.50	38000	0.17
9500	0.45	34500	0.17
7500	0.45	32500	3.44
5500	0.45	24000	3.44
5000	0.45	22500	3.44
		21000	2.41
		11000	0.11
		7000	0.03
		6500	5.16

Annex No. 13

Spots: 4 years, 2nd week

Day 1		Day 2	
M.W. (Daltons)	Concentration (mg/ml)	M.W. (Daltons)	Concentration (mg/ml)
110500	0.31	110500	0.34
66000	0.86	66000	1.93
54000	0.17	54000	3.44
24000	0.17	51500	3.44
22000	0.17	23500	5.16
20500	3.01	22500	3.44
10500	1.5	21000	0.03
6500	4.51	10500	4.51
		8500	3.02

Annex No. 14

Scruffy: 5 years, 2nd week

Day 1		Day 2	
M.W. (Daltons)	Concentration (mg/ml)	M.W. (Daltons)	Concentration (mg/ml)
110000	0.40	110500	0.34
50500	3.44	66000	1.93
45000	3.44	54000	3.44
28000	1.40	51500	3.44
24000	1.40	23500	12.43
22500	9.03	22500	3.44
21500	9.03	21000	0.033
20000	5.59	10300	4.51
11500	1.29	8500	3.16
9500	0.64		
6500	6.45		
6000	6.45		
5000	8.60		
3500	2.36		

Annex No. 15

Black: 5 years, 2nd week

Day 1		Day 2	
M.W. (Daltons)	Concentration (mg/ml)	M.W. (Daltons)	Concentration (mg/ml)
110500	1.72	110500	1.72
54000	0.86	54000	0.86
52400	0.34	52400	0.34
22500	3.44	22500	3.44
21000	1.72	21000	1.72
13500	0.03	13500	0.03
11000	3.44	11000	3.44
8500	2.75		

Annex No. 16

Clear: 3 years, 3rd week

Day 1		Day 2	
M.W. (Daltons)	Concentration (mg/ml)	M.W. (Daltons)	Concentration (mg/ml)
113000	0.03	130000	0.03
46000	0.03	50500	0.03
43000	1.72	48500	0.03
32500	0.03	47000	0.75
29500	2.75	43000	0.75
26500	2.75	40000	0.75
22500	3.44	20000	2.20
16000	2.40	18000	0.45
11500	2.41	5500	0.03
5000	0.46	5000	5.16
4000	0.46	4500	0.75
3500	0.75		

Annex No. 17

Dark: 3 years, 3rd week

Day 1		Day 2	
M.W. (Daltons)	Concentration (mg/ml)	M.W. (Daltons)	Concentration (mg/ml)
150000	0.02	150000	0.02
100000	0.64	100000	0.64
75000	0.75	75000	0.75
48000	0.12	48000	0.12
45000	0.75	45000	0.75
43000	0.86	43000	0.86
20000	0.02	20000	0.02
13000	0.02	13000	0.02
6000	0.02	6000	0.02
4500	0.02	4500	0.02
3500	0.02	3520	0.02

Annex No. 18

L.M.: 4 years, 3rd week

Day 1		Day 2	
M.W. (Daltons)	Concentration (mg/ml)	M.W. (Daltons)	Concentration (mg/ml)
113000	0.03	113000	0.03
50500	0.03	50500	0.03
48500	0.03	48500	0.03
47000	0.75	47000	0.75
43000	2.75	43000	2.75
40000	0.75	40000	0.75
20000	0.22	20000	0.22
18000	0.45	18000	0.45
5500	0.03	5500	0.03
5000	5.15	5000	5.15
4500	0.75	4500	0.75

Annex No. 19

Spots: 4 years, 3rd week

Day 1		Day 2	
M.W. (Daltons)	Concentration (mg/ml)	M.W. (Daltons)	Concentration (mg/ml)
72000	1.54	72000	1.54
54500	1.54	54500	1.54
47000	3.87	47000	3.87
45000	1.54	45000	1.54
42000	3.87	42000	3.87
38000	0.38	38000	0.38
29000	7.74	29000	7.74
24000	7.54	24000	7.54
23000	7.54	23000	7.54
22000	5.80	22000	5.80
20500	7.74	20500	7.74
20000	11.61	20000	11.61
13000	3.44	13000	3.44
11000	0.05	11000	0.05
6500	5.16	6500	5.16
5000	1.72	5000	1.72
3500	2.75	3500	2.75
3000	6.08	3000	6.08

Annex No. 20

Scruffs: 5 years, 3rd week

Day 1		Day 2	
M.W. (Daltons)	Concentration (mg/ml)	M.W. (Daltons)	Concentration (mg/ml)
72000	1.54	113000	0.06
54500	1.54	46000	0.06
47000	3.87	43500	0.75
45000	1.54	42000	0.27
42000	3.87	20000	0.27
38000	0.38	17000	0.02
29000	7.74	14000	0.12
24000	7.75	5500	1.72
23000	5.80	4500	1.72
22000	7.74	4000	0.04
20500	11.61		
20000	3.44		
13000	11.61		
11000	5.16		
6500	1.72		
5000	2.75		
3500	2.75		
3000	6.88		

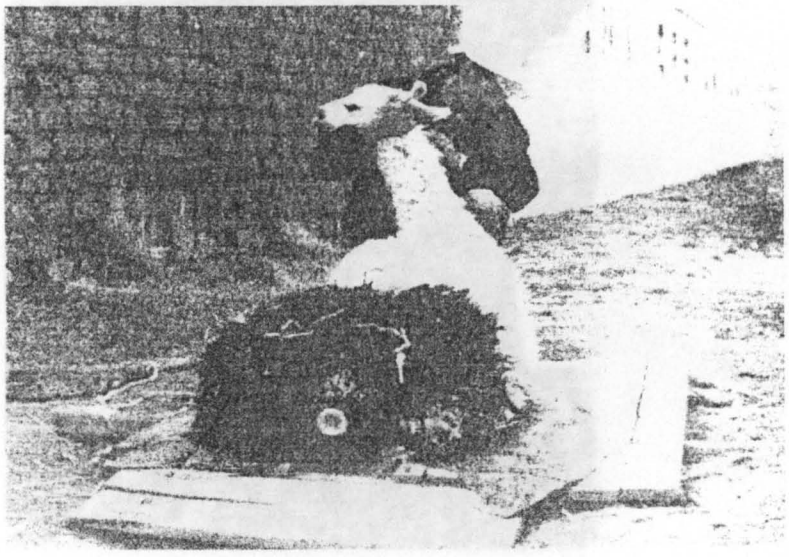
Annex No. 21

Black: 5 years, 3rd week

Day 1		Day 2	
M.W. (Daltons)	Concentration (mg/ml)	M.W. (Daltons)	Concentration (mg/ml)
152500	0.5	100000	0.31
94000	0.09	90000	0.02
47000	0.86	74500	0.02
43500	5.16	44500	0.02
39000	0.37	42000	1.5
32000	0.68	37000	0.05
28000	0.41	31500	4.25
23500	0.03	29000	4.25
23000	0.02	27000	0.11
22500	0.64	23500	0.11
20000	0.86	23000	4.25
14000	0.86	20500	5.8
12500	0.2	14000	7.75
10000	0.02	12500	5.8
5500	0.72	5500	0.02
4500	0.49	4500	0.02
4000	1.22	3500	3.87
3500	2.01		

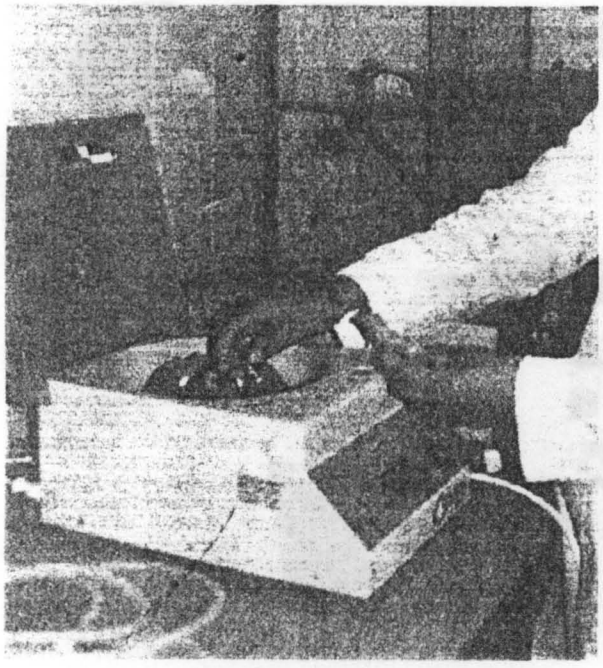
Photographs

1. Semen collection platform and group mannequin

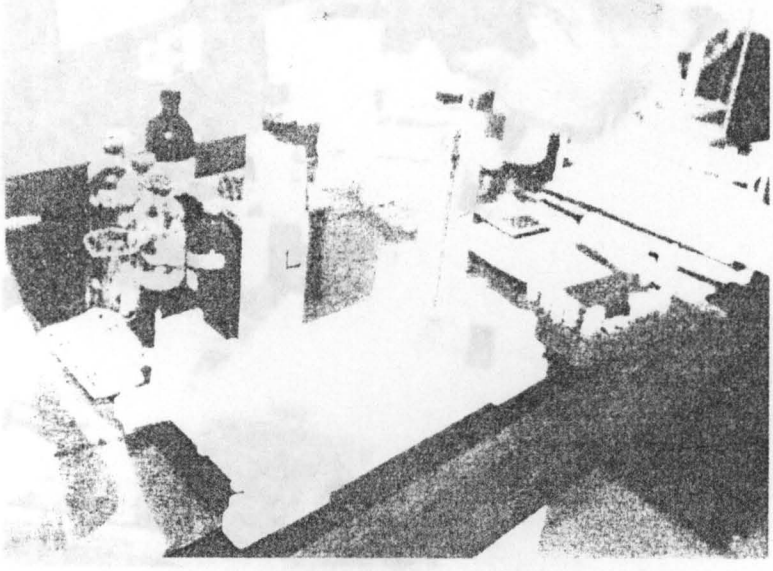


2. Mount and ejaculation

3. Separation of seminal plasma



4. Gel preparation



5. Samples charged in electrophoretic chamber

6. Radial immunodiffusion

c/mark/Bolivia/proteinprofile/mro2/012804