STUDIES ON THE RELATIONSHIP BETWEEN CHARACTERISTICS OF RAM SEMEN AND FERTILITY

by

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Ask, and it shall be given you;
seek, and ye shall find;
knock, and it shall be opened
unto you.

For every one that asketh,
receiveth;
and he that seeketh,
findeth;
and to him that knocketh
it shall be opened.

(Mattew 7: 7, 8)

Dedicated to my wife Claudia,

to my coming first child,

to my parents and family.

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SUMMARY

This thesis examined several aspects of male reproduction in the sheep, and how these are related to fertility in the female when semen is introduced by natural mating or artificial insemination (AI). Aspects of ram semen quality were examined with the aid of computerised image analysis and can be summarized as follows: the repeatability of the estimation of semen characteristics in semen assessment; processing and freezing of ram semen and the variation of fresh and frozen-thawed semen quality; the fertility of frozen-thawed semen from normal and subfertile rams inseminated by cervical and intrauterine inseminations; some of the factors which affect semen production, quality and fertility; the correlation with fertility of semen characteristics assessed manually or by computerised image analysis, and the prediction of fertility from assessment of ram semen.

In the area of semen assessment, processing and freezing, experiments were undertaken showing that pre-freezing concentration of the diluted semen did not affect the post-thawing viability of the spermatozoa when semen was processed in straws. The size of straws also had no effect on the survival of spermatozoa. The viability of spermatozoa frozen in straws was comparable with the viability of spermatozoa frozen in pellets, but with the advantage of positive identification and easier handling. It was noted that there were large variations in semen characteristics between rams and between samples within batches within rams. Semen characteristics were significantly improved by mixing the diluted semen before drawing it into the straws and freezing, but unfortunately it was not possible to determine whether the variation between straws was reduced. In a fertility test, cervical and intrauterine inseminations with a range of doses of frozen-thawed spermatozoa were compared. Intrauterine insemination, even with the lowest dose of spermatozoa, gave much higher fertility rates than cervical insemination with large doses of spermatozoa.

There is a standard diluent for pellet freezing of ram semen which provides satisfactory cryoprotection of spermatozoa. The levels of tris and glucose were decreased and increased

respectively to see if the cryoprotective action of this diluent could be improved. It was found that the standard diluent and a second diluent which had a lower amount of tris and higher amount of glucose gave the best protection. If the osmolarity was maintained at 325 mosmol the amount of tris in the diluent could be varied between 250 to 300 mM and of glucose between 30 to 95 mM without reducing the viability of spermatozoa. A group of ewes in synchronized oestrus were inseminated by laparoscopy using semen processed with the diluents from the same experiment. There was no difference between the diluents in terms of conception rate, and the correlation between semen characteristics and fertility was low.

Two experiments were designed to estimate the repeatability of semen characteristics measured by computerised image analysis, to measure the variation in these characteristics between samples within batches of semen within rams, and the variability in semen characteristics between fresh and frozen samples. One experiment used semen frozen in straws and the second fresh semen and semen frozen in pellets. In the first experiment, due to the small number of rams, the variation between samples was much higher than the variation between rams, and also the variation between batches of semen within rams was higher than the variation between rams. This variation may contribute to differences in fertility between batches within the same ram when frozen-thawed semen is used for AI. The repeatability in the estimation of semen characteristics was low in straws due to the large variation between samples and batches, but it may be reduced by improving the methods of processing, freezing and assessment. From this repeatability study it was possible to estimate the minimum sample size required for a semen assessment in order to obtain reliable information on a batch of frozen semen or an ejaculate. It was found that one sample from a batch or ejaculate is not sufficient and the sample size and the limits of confidence should be set depending on the impact or importance of the semen characteristic on fertility.

In the second experiment, fresh and pellet-frozen ram semen were used. Semen characteristics measured in fresh and frozen-thawed semen samples from the same ejaculate

were compared by analysis of variance. All semen characteristics varied significantly due to the processing and freezing, and between rams.

When semen characteristics of fresh and frozen-thawed samples were analyzed independently, it was found that in fresh semen most of the variation was due to the rams and the repeatability of measurements was high. In frozen-thawed semen, however, the variation was higher between samples than between rams for more than half the semen characteristics. The repeatability was higher in fresh than frozen-thawed semen due to a lower variation between samples, so the sample size for an accurate estimate of semen characteristics could be smaller.

Several experiments were conducted to examine the effects of separation of the epididymis from the testis, increasing the subcutaneous scrotal temperature, and rams from different strains on semen production, semen quality and fertility. It was found that when the epididymis was separated from the testis, there was a reduction in semen quality, fertilizing capacity of the spermatozoa, and the viability of the fertilized eggs.

When the subcutaneous scrotal temperature of rams was increased about 2°C by insulation for 16 hours per day for as little as 4 days, there was an increase in the embryo mortality in ewes mated to these rams without any modification of the fertilization rate. After 21 days of heating, both the quantity of the spermatozoa in the ejaculates of rams and the fertilizing capacity of spermatozoa were affected. Thus the earliest effect of heating seemed to be on the epididymal spermatozoa by reducing their capacity to produce a viable embryo, then to reduce the capacity of spermatozoa to fertilize the egg, and finally to reduce sperm production.

When two groups of rams from different strains were used in a mating trial, it was found that spermatozoa from rams of both strains had the same capacity to fertilize eggs, but there was a significant difference between strains of ram in the production of embryos which became viable offspring. So the two strains of rams produced differences in embryo mortality when mated to ewes which were attributed to a genetic effect on embryo survival.

To conclude the present study regression analyses were conducted on semen quality and fertility data from normal and subfertile rams in an attempt to correlate semen characteristics with fertility. The confidence limits within which a change in some of the semen characteristics would predict changes in fertility were estimated, the impact on fertility of some of the correlated semen characteristics was determined, and an equation of fertility prediction was established using multiple regression analysis. Semen characteristics from subfertile rams were highly correlated with fertility. This was due to the large variation in semen quality found within the population of rams examined. When semen characteristics from normal rams were assessed, few characteristics were correlated with fertility and the correlation coefficients were low. The following semen characteristics were significantly correlated with fertility:

(1) Using natural mating and manual assessment of semen: mass motility or wave motion score, percentage of motile sperm, percentage of live cells, percentage of normal cells; a negative correlation was found between the percentage of cells without heads and fertility.

(2) Using natural mating and computerized image analysis of semen: total concentration of sperm, percentage of progressively motile sperm, percentage of motile sperm, percentage of rapid and medium motile spermatozoa; a negative correlation was found between the percentage of static spermatozoa and fertility.

(3) Using natural mating and both manual and computerised image analysis of semen: the proportion of live sperm and the proportion of normal sperm, and negatively the proportion of dead sperm and sperm with abnormal tails (manual assessment). With computerised image analysis percentage of progressively motile spermatozoa, percentage of motile spermatozoa, percentage of rapid spermatozoa, and negatively the percentage of static spermatozoa.

(4) Using intrauterine insemination with frozen-thawed semen and computerised image analysis of semen: percentage of progressively motile sperm, percentage of motile sperm, percentage of rapid, medium and slow spermatozoa; a negative correlation was found between percentage of static spermatozoa and fertility.

The semen characteristics assessed by computerised image analyses and the results of fertility from three experiments (2 chapter 3, 8 chapter 5 and 4 chapter 7) were used to derive an equation to predict fertility. All semen characteristics were included in the regression analysis to derive the equation. Some of the semen characteristics which were previously correlated with fertility did not have the same importance in predicting fertility in the presence of some other semen characteristics. In experiments 2 and 8 chapters 3 and 5 respectively in which multiple regression analysis was performed, one equation was based on two of the semen characteristics (percentage of progressively motile sperm and progressive velocity) and in the other, four of the semen characteristics were included in the equation (percentage of progressively motile sperm, percentage of motile sperm, percentage slow spermatozoa and mean lateral head displacement). In experiment 4 chapter 7, the equation for fertility prediction was based on nine semen characteristics (motile sperm concentration, percentage of progressively motile sperm, percentage of motile sperm, progressive velocity, percentage of rapid, medium and slow spermatozoa, dead spermatozoa and spermatozoa with abnormal tail). From these results, standard regression coefficients were estimated which expressed the importance for fertility prediction of each semen characteristic in standard units. The following order of importance was established for the semen characteristics presented in the equations: percentage of progressively motile sperm and progressive velocity for the first equation; percentage of progressively motile sperm, percentage of motile sperm, percentage of slow spermatozoa and mean lateral head displacement for the second equation; and percentage of rapid spermatozoa, proportion of dead sperm, percentage of motile sperm, percentage of progressively motile sperm, proportion of spermatozoa with abnormal tails, percentage of slow spermatozoa, percentage of medium spermatozoa, concentration of motile spermatozoa, and progressive velocity for the third equation. For normal rams, the confidence limits for straw-frozen semen within which an increase in semen characteristics predicted increased fertility were small and the sample size to predict such differences was reasonable. For fresh semen characteristics the confidence limits were slightly larger than for frozen semen in straws, but the sample size required was smaller. Therefore, small increases in certain semen characteristics would predict

increases in fertility. For example an increase of 7.41 % in motility would be predicted to result in an increase of 10 % in fertility, and it is necessary to assess 10 samples to enable such a prediction (straw-frozen semen). In fresh semen an increase of 20.01 % in motility would be predicted to result in the same improvement in fertility, but it is required to assess 2 samples to enable this prediction.

DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University. To best of my knowledge and belief, the thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

I consent to this thesis being made available for photocopying and loan if accepted for the award of the degree.

Pablo Ignacio Quintana Casares.

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Abstracts

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CHAPTER 1. LITERATURE REVIEW

1 Semen production, composition and collection methods; factors affecting survival of spermatozoa.

1.1 Semen description.

Semen consists of two major parts, spermatozoa which are the male gametes and contain the paternal genetic material and seminal plasma which is the medium in which the spermatozoa are suspended. The composition of semen varies between species in volume, concentration, motility, content of organic and inorganic substances. In rams, the volume is very small but the concentration of sperm cells per ml is the largest among the domestic mammals, and its activity is also considered the most vigorous; in bulls, semen is expelled in greater amounts, being less concentrated and less active. When these two types of semen are compared with that of the boar and stallion, they are very different because these last two are ejaculated in fractions and the volume is much greater, but the concentration and activity of sperm much lower (Sorensen, 1986).

1.2 Spermatozoa production.

1.2.1 Site of production.

The spermatozoa are produced in the seminiferous tubules of the testes which are set in interstitial tissue. The seminiferous tubules drain towards the center of the testis into straight tubules and from there into a network of ducts called the rete testis. The rete testis is connected to a series of efferent ducts which lead to the head of the epididymis. The seminiferous epithelium, lining the seminiferous tubules, is composed of two basic cell types: the Sertoli cells and the developing germ cells. The Sertoli cells support the germ cells (Steinberger and Steinberger, 1975; Russell and Peterson, 1984; Garner and Hafez, 1987). The Sertoli cells

have several functions including secretion of fluid, phagocytosis, the maturation and release of spermatozoa and synthesis of the intra-tubular androgen-binding protein (Fawcett, 1975). The first release of motile spermatozoa occurs after puberty or sexual maturation, but the very process of spermatogenesis starts during foetal life. The earliest germ cells, namely stem-cell spermatogonia, are developed from the primordial germ cells which migrate from the germinal crest in the foetus. Once the stem-cells are located into the seminiferous tubes, they remain static until puberty when their activity is started and incorporated in the spermatogenic cycle (Ortavant et al., 1977; Sorensen, 1986).

1.2.2 Spermatogenesis.

Spermatogonia contain the full diploid number of chromosomes and morphologically they can be divided into three types: A, Intermediate and B spermatogonia. The A spermatogonia are characterized by a large pale ovoid nucleus usually lying with its long axis parallel to the boundary tissue. Several generations of A spermatogonia are produced by successive mitoses; the first divisions occur unsynchronized with other events and the last three or four divisions are synchronized with other events in spermatogenesis.

After several generations of morphologically very similar cells, the intermediate spermatogonia and B spermatogonia are formed which contain crust-like chromatin, in nuclei which are smaller and more nearly spherical. For several generations it is difficult to distinguish between the spermatogonia types until after several mitoses they are transformed to primary spermatocytes now called 'preleptotene spermatocytes' (Bishop and Walton, 1960; Ortavant et al. 1977). They were originally called "resting spermatocytes", but the term became inappropriate as this is the stage in which the synthesis of DNA and other compounds is very active. The primary spermatocytes (4n) undergo two meiotic divisions in which the DNA is reduced and crossing over occurs between the pair of chromosomes (Setchell, 1978). The primary spermatocytes are transformed to secondary spermatocytes, and finally haploid spermatids (n chromosomes). Each primary spermatocyte yields four spermatids, but the early divisions of the spermatogonia ensures many times this number of spermatids are formed from

each stem cell. Although this process is continuous, it may be affected by physiological changes caused by disease, undernutrition, or seasonal variation (Hochereau-de Reviers et al.,1976).

The spermatids do not divide further, they are transformed into very complex spermatozoa, undergoing condensation of the nucleus, formation of the acrosome, virtual elimination of the cytoplasm, development of a tail and the arrangement of mitochondria into a helix to produce the midpiece (Setchell, 1978). This process of morphological transformation is know as spermiogenesis (Ortavant et al., 1977; Setchell, 1978; Evans and Maxwell, 1987). When this process is finished, the resulting spermatozoa are released into the lumen of the tubules and washed along to the rete testis by secretions of the Sertoli cells (Setchell, 1978).

The time elapsed from the activation of the stem-cell to the release of the spermatozoa into the tubules is about 40 days in the ram, and the time of the passage through the epididymis takes about 10-14 days (Ortavant, 1959).

1.2.3 Spermatozoon description.

Each spermatozoon consists of two major parts, the head and the tail. The shape of the sperm head is characteristic of the species, hook-shaped in rats and mice but flattened and rounded in man and the domestic animals (Setchell, 1982; Evans and Maxwell, 1987). The nucleus of the head contains a highly condensed mass of DNA-protein called chromatin which contains the paternal genetic information (Fawcett, 1975). The anterior half, or slightly more, of the head is covered with a special cap called the acrosome, which contains the enzymes necessary for the fertilization process (Fawcett, 1975; Setchell, 1982). The shape of the acrosome is also species-dependent, but is usually moulded over the nucleus, so that the acrosomal membrane can be said to have inner and outer parts. Behind the acrosome, there is a postacrosomal region which is important because it is in this region that the spermatozoon attaches and fuses to the egg (Fawcett, 1975). Like other cells, the whole spermatozoon

including the acrosome is enclosed by a plasma membrane; as a cell it has a remarkably small amount of cytoplasm (Setchell, 1982).

The tail produces the propulsion necessary for the spermatozoon to move in fluids. The head and tail are joined together by a short neck, which is known as the connecting piece, and joins the motile apparatus to the nucleus (Fawcett, 1975; Setchell, 1982; Evans and Maxwell, 1987). Under certain conditions, the neck can ~~fail~~ break causing separation of the head and tail, particularly in hot environments or other stressful conditions.

The tail or flagellum is usually divided into three parts: the midpiece, mainpiece or principal piece and endpiece. There is a common axial core which consists of a series of contractile elements, or fibrils. The contraction and relaxation of these elements results in locomotion.

The total length of a ram spermatozoon is about 60 microns. The head alone is 8-10 microns long, 4 microns wide and 1 micron thick (Setchell, 1978; Evans and Maxwell, 1987).

1.2.4 Seminal plasma.

The seminal plasma is a mixture of fluids secreted mainly by the vesicular glands, and in smaller proportions by the testes, epididymides and the rest of the accessory sex glands (ampulla, seminal vesicles and prostate). The seminal plasma consists in rams of about 75% water, and numerous organic substances; those studied so far include fructose, citric acid, inositol, ergothioneine, glycerylphosphorylcholine, glutamic acid, phospholipids, prostaglandins, proteins and certain enzymes (Setchell, 1977; Mann and Lutwak-Mann, 1981; Evans and Maxwell, 1987) and inorganic ions like sodium, potassium, calcium, magnesium, chloride, bicarbonate which make the seminal plasma an isotonic, neutral fluid and serve to protect and nourish the spermatozoa (Setchell, 1977; Evans and Maxwell, 1987). The seminal plasma has basically three functions: " (1) to act as a vehicle for the spermatozoa, transporting them from the male reproductive tract during ejaculation, (2) to serve as an activating medium for the previously non-motile spermatozoa, and (3) to provide a buffered, nutrient-rich medium

which aids survival of the spermatozoa after deposition in the female tract " (Evans and Maxwell, 1987).

1.2 Methods of semen collection.

Semen can be collected for testing and/or for artificial insemination (AI). In general terms there are two principal methods for semen collection: artificial vagina and electrical stimulation (Mattner and Voglmayr, 1962; Watson, 1978; Sorensen, 1986; Hafez, 1987; Polge, 1972; Evans and Maxwell, 1987). Borisov (1947) did not find appreciable differences in the composition of the semen obtained from rams by each of the methods, while Brady and Gildow (1939) found the characteristics of the ejaculate varied according to the method. In a study comparing both methods, Mattner and Voglmayr (1962) concluded that ejaculates collected by electro-ejaculation were greater in volume, due to a larger amount of seminal plasma, and in content of fructose than the ones collected by artificial vagina. The values for concentration and wave motion were greater for semen collected by artificial vagina than by electro-ejaculator. The method of collection did not affect the proportion of live, morphologically normal or actively progressing sperm. There was a variation in semen quality from one day to the other within the same ram with both methods. Such differences were higher using electro-ejaculation, thus it was concluded that semen collection by artificial vagina gives more consistent results for most characters. Breed, age, seasonal, environmental, and individual ram differences, and the standardization of the method applied, may also influence the results (Mattner and Voglmayr, 1962).

The artificial vagina method is preferred since it is painless, quick and simple and results in a better quality of semen, but requires that the animals are trained. It is possible to collect several times a day from the same ram by artificial vagina. Electrical stimulation may be used when semen is required from a male which cannot mount (Evans and Maxwell, 1987), or from a young or aged animal (Polge, 1972). There are disadvantages in obtaining semen by electrical stimulation; it causes considerable discomfort to the animal, semen may be

contaminated with urine during collection and it is more diluted than the normal ejaculate, but its fertilizing capacity is apparently quite normal (Polge, 1972; Evans and Maxwell, 1987).

1.2.1 Collection by artificial vagina.

This method is the most commonly used for semen collection. The artificial vagina is an imitation of the vagina and provides to the erect penis of the male the correct thermal stimulation (temperature) which varies according to species, and mechanical (pressure) stimulation which can be regulated by adding air (Polge, 1972) and lubrication (Hafez, 1987), all of which are necessary to induce ejaculation (Laing, 1955; Polge, 1972; Evans and Maxwell, 1987). Although the size and shape of the device varies among species and several modifications from the basic construction have been suggested, all are of similar design (Watson, 1978). The artificial vagina consists of an outer casing made from heavy rubber, plastic or other synthetic material with good insulation properties, and inner liner made of rubber or suitable synthetic material (Polge, 1972; Evans and Maxwell, 1987). The artificial vagina is half-filled with hot water (48-50°C) so that the correct temperature is obtained, which normally is higher than body temperature (Watson, 1978). The temperature before the semen collection should range between 42-45°C. One of the ends is lubricated with sterile lubricant to aid the passage of the penis and in the other end a sterile calibrated semen collecting tube is inserted for 1.5-2.0 cm to allow easy semen collection and volume measurement.

A teaser female or dummy is secured in a collection bail (Watson, 1978). The operator takes a crouching or kneeling position at the right side of the teaser and holds the vagina along with the collecting tube. For the collection to take place, the penis has to be guided into the vagina. A vigorous upward and forward thrust signifies that ejaculation has been accomplished. As soon as the male dismounts, the vagina is gently removed and kept with the collecting tube downwards. When all the semen has drained into the collection tube, it is covered with aluminium foil and kept in a warm place (Laing, 1955; Hafez, 1987; Evans and Maxwell, 1987).

Different behavior can be found among males, but with an experienced operator this method can achieve good results in most animals. The frequency of semen collection without disturbing semen quality, such as volume or concentration of spermatozoa, is between 3 to 5 collections a day for 4 to 5 day periods separated by 2 to 3 days rest (Evans and Maxwell, 1987). Cameron et al. (1984b) reported that only two collections per day are possible without disturbing the volume and sperm concentration, but this varies with the breed and age of the animals.

1.2.2 Collection of semen by electrical stimulation (electro-ejaculation).

Electrical stimulation is the preferred method only when males cannot be trained, refuse to serve the artificial vagina or when injuries and infirmities may make mounting impossible (Polge, 1972; Hafez, 1987; Evans and Maxwell, 1987). It is possible to use this method successfully in the bull, ram and buck, and a semen sample of low volume can be obtained from the boar.

The method was introduced for sheep by Gunn (1936) who found that when a series of stimuli was applied between electrodes placed in the lumbar musculature and in the rectum of the ram, erection and ejaculation resulted. In its original form, Gunn (1936) used separate rectal and external lumbar poles, but these appeared to give pain and produced more muscular contractions. Later on, Blackshaw (1954) introduced another apparatus which is very close to the present models with a bipolar rectal pole and a portable stimulator.With this kind of electro-stimulator it is possible to obtain 1 ml of creamy ejaculate which sometimes does not appear different from the normal ejaculate. The most commonly used electrical stimulator in Australia and New Zealand is the Ruakura Ram Probe. This is a self-contained apparatus operated by batteries giving an output of 10 to 15 volts (Evans and Maxwell, 1987).

The ram responds exceptionally well to electrical stimulation, and the response is more rapid than in the bull. Rhythmic applications of electrical stimulation to the male leads first to secretion from the accessory sex glands and , finally, to ejaculation (Gomes, 1977). It is

recommended that the stimuli be applied every seven seconds with increments of one volt (Cameron, 1977).

The rectal probe is pressed towards the dorsal part of the pelvis when the stimuli is applied (Watson, 1978). The amount of stimuli needed for ejaculation varies among the males, and apart from discomfort and muscular contractions there are no ill-effects (Evans and Maxwell, 1987).

1.2.3 Factors affecting semen viability after collection.

The spermatozoa are highly specialized cells. The head of each spermatozoon contains a haploid set of chromosomes, well-packed into the nucleus in the form of chromatin, which ultimately combines with the set of haploid chromosomes in the egg to restore the full diploid number when fertilization is accomplished. For the penetration of the egg, it is necessary that the spermatozoon is motile and possesses a full complement of acrosomal enzymes. Both the tail which provides movement, and the acrosomal membrane are susceptible to damage. It is therefore essential that the integrity of the acrosome as well as the motility of the spermatozoa is preserved during semen handling or processing (Evans, 1987).

The sperm cells of rams and bucks are particularly sensitive to changes in their environment, and such changes may affect the viability of the cells. Factors which affect the survival or integrity of the spermatozoa include the following:

(a) Temperature

Semen is discharged out of the male tract at around body temperature. Exposure to higher temperatures will rapidly kill the spermatozoa because it increases their metabolism and exhausts their energy reserves, thus reducing their life-span.

On the other hand, reduction of temperatures may decrease the metabolism of the sperm cells, and hence prolong their viability; but ram semen is very sensitive to sudden cooling, particularly to below 10°C , causing irreversible loss of their viability. This is called ' cold

shock ' and it can occur through careless over-exposure to cold air, or by using a cold collecting tube or even a cold microscope slide. However, if semen is extended with an appropriate diluent and cooled down slowly to 2-5°C the viability of spermatozoa and their capacity of fertilization may be prolonged. Semen for fresh use should be maintained at 30°C (Evans, 1987).

(b) Sunlight

Direct exposure to sunlight, ultra violet radiation or even strong fluorescent lights is detrimental and can reduce the viability of spermatozoa (Milovanov, 1962).

(c) Contact with metal

All contact with metal is harmful to spermatozoa (Milovanov, 1962). Therefore, it is recommended to use glass or non toxic plastic for semen handling, processing and freezing .

(d) Contact with water

Water reduces the osmotic pressure of the seminal plasma and may thereby kill the spermatozoa. Therefore, care must be taken that water does not come into contact with the semen and all the equipment used must be dried beforehand (Evans, 1987).

(e) Osmotic pressure

The components (solutes) dissolved in the medium surrounding the sperm cells exert osmotic pressure on the cell membrane. When the medium surrounding the spermatozoa has the same osmotic pressure as seminal plasma it is called an isotonic medium, but when it is in higher or lower concentration, it is called hyper-tonic and hypo-tonic respectively. Spermatozoa may suffer from osmotic shock when exposed suddenly to a diluent which is not exactly isotonic. However, in general terms, spermatozoa remain motile longest when suspended in slightly hyper-tonic diluents (Salamon, 1968; Salamon and Visser, 1972).

(f) pH

Seminal plasma normally is close to a neutral pH (slightly basic). Any change above or below this pH value is detrimental for the sperm cells and reduces their viability. Thus, diluents for semen contain buffers to protect against shifts in pH which can be altered by a long exposure to air, because this increases the metabolic activity of the spermatozoa and thus the production of lactic acid which reduces the media pH (Graham et al., 1978).

(h) Contaminants

Bacteria, dust, urine, hair and other materials are common materials which can get into the semen and reduce the viability of the sperm cells and may transmit some diseases if no antibacterial agent is used in the semen for AI (Pickett et al., 1978).

(i) Disinfectants

Disinfectants and antiseptics are harmful to spermatozoa and their use should be avoided. For sterilization of equipment 70% alcohol in water is suitable, but it must be dried before use. For glassware, dry heat sterilization can be used (Evans, 1987).

1.2 Methods of semen assessment.

1.2.1 Introduction; the importance of semen evaluation.

Since spermatozoa were first seen under a microscope (Spallanzani, 1776), one essential question was formulated: is this semen capable of fertilizing an ovum (Pace, 1980). Semen handling and processing have been improved with time, but new developments are hampered by the lack of reliable laboratory methods for predicting the fertilizing capacity of semen in the field (Evans, 1988). The only conclusive test of fertility is an insemination trial (fertility trial), which is both time-consuming and expensive (Watson, 1979; Hafez, 1987; Salamon , 1987). The ideal method for evaluating the fertility of a breeding male, besides a determination of his ability to produce pregnancies, is by assessing his semen. The evaluation of semen is a very important part of a physical test of a male or to identify an infertile male (Fowler and Jenkins, 1970; Hafez, 1987). The presence of infertile rams may increase the number of non-pregnant ewes. There has been some progress in the development of laboratory diagnostic tests of fertility in recent years using semen characteristics (Watson, 1979; Salamon, 1987), but to date no single criterion or group of criteria has proved to be of great value for predicting the fertilizing capacity of semen (Jones, 1971). The most important point to consider is that such tests must be highly correlated with fertility; this requires a test which is specific, precise and accurate combined with a precise and accurate collection of fertility data, complemented by a large number of observations (Amann, 1989).To develop a procedure with high repeatability is very difficult, and thus there is considerable variation in test results among laboratories and between assays within a laboratory.

Semen evaluation is important in AI programs, because the quality and concentration of spermatozoa determines the possible degree of dilution of the semen and thus the number of females which can be inseminated (Hafez, 1987; Evans and Maxwell, 1987). The success of an AI program also depends on the assessment of the potential fertilizing capacity of the spermatozoa in the semen (Emmens and Blackshaw, 1956). Estimation of the quality of semen

is usually the first attempt to predict this fertilizing capacity, but few studies have examined the correlation between the various characteristics of semen and fertility. If a characteristic is related to fertility, then the prediction will depend entirely on the precision and accuracy of the assay and the fertility test.

Recent efforts have been focused on finding a simple, reliable and rapid method of semen analysis for predicting fertility, especially for frozen-thawed semen. However, since sperm survival and transport in the female tract and its capacity of fertilization depend on more than one characteristic, a reliable test for one characteristic may not be accurate for fertility prediction. Eppleston et al. (1986) reported that subjective semen assessment is a poor predictor of fertility, therefore an objective semen assessment which involves the accurate measurement of several semen characteristics may increase the possibility of predicting fertility (Maxwell et al.,1990).

Since motility (Hulet and Ercanbrack, 1962; Hulet et al., 1965; Budworth, 1988), morphological characteristics and live and dead spermatozoa in an ejaculate (Hulet et al., 1965; Linford, 1976) and more recently percentage of motile spermatozoa, linear and straight line velocity of spermatozoa (Budworth, 1988) have been found correlated with fertility, the accuracy of predicting fertility from a semen sample may be improved by measuring these characteristics as accurately as possible.

1.2.2 Manual assessment of semen characteristics.

In the present work, manual methods were considered as those in which human bias is involved, and therefore their precision depends largely on the skills of the operator. Some methods are easy, where others are time-consuming but still depend on human bias.

The characteristics of semen after collection which are used to determine its quality by manual assessment are as follows:

colour and smell of semen, volume of ejaculate, motility (mass motility or vigor of the wave motion), percentage of motile spermatozoa, concentration of spermatozoa, consistency of semen, morphology of the spermatozoa and live and dead spermatozoa (Emmens and Blackshaw, 1956; Watson, 1979; Pace, 1980; Sorensen, 1986; Hafez, 1987; Evans and Maxwell, 1987).

1.2.2.1 Colour, smell and volume of semen.

The colour of the semen is the first feature which is examined after collection of the ejaculate. Normal ram semen is milky-white or pale cream in colour. When semen appears to be darker it is because it is more concentrated (Hafez, 1987; Evans and Maxwell, 1987).Semen also can be contaminated with blood or urine, in which case the semen will be a pink color when blood is present; when semen contains urine, it can be recognized by its typical odor. These two conditions are detrimental to the semen and reduce the life-span and viability of spermatozoa (Hafez, 1987; Evans and Maxwell, 1987).

The volume of the semen is normally assessed either in the collecting tube if this is calibrated or in a more accurate way using a calibrated pipette.When an artificial vagina is used, the average volume of ram ejaculates is 1 ml depending on several factors such as the age of the animal, its condition, the frequency of collection and the skill of the operator.Young males or those in poor condition generally produce a lower volume of semen.

Wiggins et al. (1953) found a positive correlation between the volume of the ejaculate and percentage of ewes lambing. However the predictive value of volume measurements is poor, and the reason for the relationship between volume and fertility is not clear.

1.2.2.2 Motility of spermatozoa.

Motility due to the physical activity of the spermatozoa is important in certain stages of sperm transport in the female tract (Lightfoot and Restall, 1971) or during the penetration of the cumulus cells and the zona pellucida during fertilization (Nelson, 1985). It is the basis of several methods of semen evaluation (Emmens and Blackshaw, 1956; Watson, 1979; Pace, 1980).

Spermatozoa are highly specialized cells whose sole objective is to accomplish fertilization. In order to achieve this, the spermatozoa depend on getting to the place where the ovum is at the right time. While the sperm cells are passing through the female tract, many environmental conditions can affect their survival. It is important to consider that motility is essential for fertility, but it is not necessarily indicative of fertilizing capacity.Under physiological conditions, the motility and fertilizing capacity of sperm cells are normally related. However in some situations these two functions of sperm cells may be dissociated (Mann, 1975), and therefore sperm movement alone is not a sufficient criteria for fertilizing capacity. Normal spermatozoa lose fertilizing capacity before losing motility (Hafez, 1987). Moreover, some sperm may show normal movements and yet are incapable of fertilizing an ovum. Also, motility is secondary to other transport mechanisms such as muscular contractility and ciliary motion of the female reproductive tract. Motility becomes crucial in passing the cervix and the uterotubal junction, and is very important as mentioned above, for penetration of the cumulus cells and the zona pellucida of the ovum (Nelson, 1985).

Motility can be assessed subjectively as either a wave motion characteristic of fresh semen or the proportion of progressively motile spermatozoa in a sample (Evans and Maxwell, 1987). Wave motion can be seen under low magnification in a non-diluted and dense semen sample in the bull and ram, and it is the simplest test for motility. When semen has been diluted or extended with some kind of diluent, or after freeze-thawing, assessment of the percentage of progressively motile spermatozoa has to be performed under high magnification.

The procedure for assessing wave motion is very simple; a semen drop is placed on a warm (37°C), clean, microscope slide without a coverslip, and it is observed under a microscope at low power (40x or 100x). The estimation of the wave motion is based upon its vigor or strength and generally is scored on a 0-5 scale (Bishop et al., 1954; Hulet and Ercanbrack, 1962). Evans and Maxwell (1987) described the method in detail and gave criteria for the scoring; they have suggested that an ejaculate can be used for AI if it has a good motility (4-5) but otherwise should be discarded.

The proportion of progressively motile spermatozoa is estimated in diluted semen samples, and because it is an observation of individual spermatozoa, it needs to be carried out with a greater magnification (400x). In practice for AI, or for a simple description of semen quality, subjective analysis is adequate (Evans and Maxwell, 1987). Hafez (1987) claims that the percentage of motile sperm determined by subjective analysis is positively correlated with fertility. Hulet and Ercanbrack (1962), Hulet et al.(1965) , Linford et al. (1976) and Budworth et al. (1988) also found a correlation between motility (as either the percentage of motile spermatozoa or as a wave score) with fertility. Linford et al.(1976), using the method developed by Glover (1968) named the "motility meter", also found a positive correlation between motility and fertility. Despite these findings, when motility is correlated with fertility the predictive value is poor (Watson, 1979). Furthermore, motility estimates made immediately after freezing and thawing or following 1 or 2 hours incubation of semen at 37°C, have been shown positively correlated with fertility but of low predictive value (Saacke and White, 1972; Linford et al., 1976). On the other hand, Roussel et al. (1963) found a better correlation between motility and fertility (r=+0.79) when bull semen was incubated for 5 hours at 38°C after thawing, which suggests that some kind of "stress test" may reveal features of survival of spermatozoa not seen immediately after thawing (Pickett et al., 1961).

The findings of low correlations between motility and fertility sometimes may result from the low accuracy and precision of the visual method used to evaluate motility, the influence of other sperm characteristics, the imprecision of measuring fertility from ejaculates used for AI, factors which do not concern semen quality such as the parity of the females,

season of the year (Foote et al., 1977), or the method for measuring fertility as " non-return rate " (O'Connor et al, 1981).

More recently, methods for motility estimations on an objective basis have been developed as shown on Table 1.1.

Table 1.1. Objective methods for sperm motility estimation.

Principle of the method.	Species in which study was performed.	Semen concentrated (C) or diluted (D).	Reference
Cinematography	Bull	D	Rothschild (1953)
"	Human	D	Janick and McLeod (1970)
Speed of spermatozoa crossing grid	Human	D	Harvey (1960)
Rate of return of wave motion after application of a shearing stress "motility meter"	Bull	C	Glover (1968)
Dopper effect	Human	D	Dubois et al. (1975)
Image Analysis Computer	Bull	D	Dott (1975)
Photomicrography	Bull	D	Revell and Wood (1978)

Modified from Watson (1979).

Besides the measurement of motility in a objective way to increase the precision, it is desirable to consider other semen characteristics which are also correlated with fertility but not necessarily with motility. A combination of measurements of motility and other semen characteristics might provide a better prediction of fertility (Watson, 1979).

1.2.2.3 Concentration and consistency of spermatozoa.

Accurate determination of the concentration or density of spermatozoa (number of sperm per unit volume) in an ejaculate or sample is very important. Concentration is a highly variable semen characteristic and it determines the dilution ratio which can be used with different semen extenders. When this characteristic is combined with the volume of the ejaculate, this quantity of sperm cells establishes the theoretical number of females which can be inseminated, considering the optimal number of spermatozoa recommended per insemination

dose (Hafez, 1987; Evans and Maxwell, 1987). It has been established that ram semen of good quality contains 3.5 to 6.0 thousand million spermatozoa per ml (Evans and Maxwell, 1987).

Concentration of spermatozoa can be assessed by means of a very simple and subjective methods of visual consistency or appearance of the semen (Evans and Maxwell, 1987). Alternatively this characteristic can be measured objectively by the more accurate but time-consuming method of haemocytometer counts (Willet and Buckner, 1951; Zaneveld and Polakosky, 1977), or by photocolorimetry (Colorimeter): the measurement of the optical density of a diluted sample Foote (1972), and some more sophisticated electronic machines which are expensive and not suitable for field practice (Evans and Maxwell, 1987) which will be discussed in the next section.

The assessment of consistency is a very simple and rapid method of estimating the concentration of spermatozoa and depends on the skills of the observer. This semen trait depends on the ratio spermatozoa:seminal plasma. Semen samples with thick consistency contain more spermatozoa than those of thin or watery consistency. The scoring values were used for the first time by Wiggins et al. (1953) and are widely discussed by Evans and Maxwell (1987) for practical application to semen dilution in the field during an AI program.

The haemocytometer method depends on the physical counting of the number of sperm in a standard volume of diluted semen. The blood cell counter kit consists of a counting chamber, coverglass and two mixing pipettes with a flexible tube and mouthpiece. The counter chamber consists of a thick glass slide with two counting grids scored onto a smooth area in the center. The counting chamber usually contains groups of 16 small squares divided by double or triple lines into large squares. The semen is diluted at a known dilution rate with a spermicidal solution (e.g. 3 % saline) and a small drop of this diluted semen is placed into both sides of the counting chamber covered with a special coverslip. The sample is allowed to settle for 3 to 5 minutes (some authors Willet and Buckner, 1951; Zaneveld and Polakoski, 1977 said one hour, but this is not practical) and the spermatozoa are counted following a standard pattern in 5 of the big squares. Knowing this value, the volume of the counter chamber, and the dilution rate, the concentration of sperm per ml of semen is calculated. The haemocytometer method is

fully described by Zaneveld and Polakoski (1977), Sorensen (1986), Hafez (1987) and Evans and Maxwell (1987).This method has been found more accurate than estimating the consistency, but it requires time and skill.

Salisbury and Van Demark (1961) found a standard error of ± 8 to 12 % of variation between duplicates haemocytometer counts. Therefore several counts of a same sample are needed, and the coordination of two capable and experience observers may improve the accuracy of readings (Foote, 1958).

The colorimeter method is a significant improvement on the haemocytometer method in terms of speed and consistency. This method, originally described by Emik and Sidwell (1947), is not so time consuming and there are some portable colorimeter models which are inexpensive and well suited to field conditions (Evans and Maxwell, 1987). As a prerequisite for the measurement of optical density in a colorimeter the semen must be diluted with an appropriate spermicidal solution which does not interfere with the operation of the apparatus. The principle of this method is based on the amount of light which passes through a liquid sample. At a standard rate of dilution, the percentage of light transmitted though the sample varies inversely with the number of spermatozoa present. The machine used by Emik and Sidwell (1947) and Willet and Buckner (1951) proved to be more accurate than the haemocytometer and had less variation between the readings.

The colorimeter requires a prior calibration against 10-15 haemocytometer counts to obtain a working graph. For this purpose, several semen samples with different concentrations are used, and from time to time this process needs to be repeated to check on the accuracy of the machine. The method is explained in more detail by Foote (1972), Sorensen (1986), Hafez (1987) and Evans and Maxwell (1987).

During the past two or three decades, several workers have tried to correlate density or concentration of spermatozoa with fertility. In most studies with bull semen very little correlation has been found. Ellenberger and Lohmann (1946) suggested there was little value in determining density for fertility prediction. This was confirmed by Mercier and Salisbury

(1946) who found a very low correlation (r = 0.162) between sperm concentration and fertility. In ram semen, Wiggins et al. (1953) found no correlation between semen density and fertility.

1.2.2.4 Sperm morphology.

Motility alone is not enough information for the prediction of fertility, and is not the only factor contributing the ability of the sperm to fertilize the egg. It is therefore important to study the morphology of the spermatozoa, particularly when an animal is included in an AI program (Evans and Maxwell, 1987), or during the examination of males for the detection of infertility (Sorensen, 1986; Hafez, 1987). The method for morphology evaluation is time-consuming and requires well trained staff.

Morphological examination of sperm cells is a detailed test of semen quality. Every ejaculate contains a certain percentage of abnormal cells, but poor fertility is expected from samples with a high number of these abnormal cells (Sorensen, 1986; Hafez, 1987; Evans and Maxwell, 1987). The examination for structural abnormalities of sperm cells is done by examining semen smears prepared and stained on a microscope slide (Hackett and Macpherson, 1965). Many stains have been suggested for this purpose but the one which is commonly used today is eosin/nigrosin (Swanson and Bearden, 1951; Mayer et al., 1951; Campbell et al., 1956; Hancock, 1956; Evans and Maxwell, 1987).

The morphological abnormalities of spermatozoa are classified into three groups: primary, secondary abnormalities (Sorensen, 1986) and tertiary abnormalities (Hafez, 1987).

The primary abnormalities originate in the testis during the spermatogenic process; when the acrosome is abnormal or different shapes of the head are present (pear shape, round, elongate, thin, small or large); when the neck section is affected, the abnormality is expressed as a double or twin spermatozoon, or the insertion point is out of the center; and when an abnormality is present in the tail, the spermatozoon could have a spiral, bent, double or coiled tail. The secondary abnormalities are produced during the passage of spermatozoa through the epididymis, and it is common to find heads without tails, cytoplasmic droplets on the neck or

tail, or sperm without an acrosomal cap. Tertiary abnormalities are produced during the collection, handling and processing of semen and the most commonly found is the absence of the acrosomal cap or other organelles which are vital for the life of the spermatozoa (Hafez, 1987).

The reliability of estimates of the proportion of morphologically damaged sperm cells was examined by Salisbury and Mercier (1945), who came to the conclusion that a count of 100 cells from one slide was sufficient.

Special attention has been given to the integrity of the acrosome (Saacke, 1982; Habenicht, 1987) as this is the organelle most needed for the penetration of the zona pellucida. Penetration is achieved with the aid of acrosin, a serine proteinase localized within the acrosome (Habenicht, 1987). The acrosome is a modified lysosome lying inside the plasma membrane, with the outer and inner acrosomal membrane surrounding the acrosomal content which consists of several enzymes involved in gamete fusion (Fawcett, 1975).

Acrosome damage is increased when semen is diluted and/or frozen and its intensity depends on the species (Healey, 1969; Watson and Martin, 1972). Some studies comparing the damage produced in semen of different species when it is frozen and thawed have shown that bull sperm have far less acrosome damage than ram sperm. This damage may involve losing the acrosomal cap, and therefore the acrosin enzymes which are needed during fertilization, or losing the whole acrosome complex (Healey, 1969). Moreover, when diluents designed to protect the semen and preserve motility during semen processing and freezing are used, alterations have been found in different species in the structure of a number of spermatozoa organelles (Healey, 1969; Tasseron et al., 1977). For these reasons, the evaluation of acrosomal morphology and function is of particular importance in the diagnosis of male fertility (Schill, 1987).

In a study of the possible relationship between the acrosome integrity and fertility, Saacke and White (1972) found that there was a correlation (r = 0.80 and 0.81) between fertility and intact acrosomes after incubation of semen for 2 and 10 hours respectively. Many other workers have related the number or percentage of abnormal cells with fertility, and it has

been established that this percentage does not affect the fertility until it is above 20% (Sorensen, 1986; Hafez, 1987) when conception rate falls. Thus it has been suggested that semen with more than 20 % of abnormalities should be discarded (McKenzie and Phillips, 1934; Flemming et al., 1976).

It has been found that fertility is related to numbers of abnormal cells better than any other characteristic (Mercier and Salisbury, 1946; Cupps et al., 1952), and it also has been suggested that there is a relationship between the number of normal cells and conception rate (Trimber and Davis, 1942). Studies with bull semen showed that a conception rate of 66.6 % was achieved when 10 bulls had between 10 to 20 % of abnormal cells, 62.2 % in two bulls with 20 to 30 % of abnormal cells, and 61 % in 5 bulls with a range between 30 to 45 % of abnormal cells (Munro, 1961).

Some workers have correlated either the number of abnormalities or the normal cells with fertility. McKenzie and Phillips (1934) and Gunn et al.(1942) found an inverse relationship between percentage of abnormal cells and fertility. Wiggins et al.(1953) found a positive correlation, but this was a low value for prediction. Hulet et al. (1965) found that not only the number of abnormal cells correlates with fertility but also the number of dead cells, which will be discussed next.

1.2.2.5 The use of supravital stains to determine the percentage of live and dead sperm cells.

This technique is based upon the finding that live sperm with functional membranes resist permeation of supravital stains whilst dead sperm take it up (Watson, 1979; Sorensen, 1986). Several supravital stains have been introduced and used with different results. Lasley et al. (1942) first introduced eosin and opal blue in isotonic phosphate buffer as a vital stain. The second component of this stain mixture provided background staining so that the unstained cells could be identified. When opal blue became unobtainable, Mayer et al.(1947, 1951) introduced a new background, fast green F. C. F. This stain was claimed to be superior to opal

blue, but the authors also put special emphasis on the correct osmotic pressure, concentration and pH of the stain for clear definition of the spermatozoa. After several studies testing different colourants like rose bengal, aniline blue (4%) or congo red, eosin/nigrosin with sodium citrate as a buffer (Mayer et al., 1951; Swanson and Bearden, 1951; Campbell et al., 1956; Hancock, 1956) and eosin with fast green, F.C.F. with sodium citrate as a buffer (Swanson and Bearden, 1951; Sorensen, 1986) became the standard vital stains for identification of live and dead spermatozoa.

When the eosin/nigrosin stain was first used, some workers found a tendency of eosin to stain hitherto unstained cells, and this was resolved by using a combination of 3% congo red and 5 % nigrosin in the citrate buffer (Emmens and Blackshaw, 1956). More recent studies have reported a number of factors which influence the efficiency of live and dead staining such as the variations between stain batches, the pH of the stain (Mayer et al., 1951) and its temperature and duration of application (Hancock, 1951; Campbell et al., 1956). Moreover, the presence of glycerol may affect the results, a problem which is solved by adding congo red to the stain (Blackshaw, 1958).

Despite the variety of results obtained with the eosin/nigrosin staining method, workers have been able to correlate the number of live and dead spermatozoa with fertility under controlled conditions (Bishop et al, 1954; Hulet and Ercanbrack, 1962; Hulet et al., 1965; Linford et al., 1976) but the predictive value of live/dead staining is low.

1.2.3 Objective semen assessment.

1.2.3.1 Introduction.

Visual estimation of the percentage of motile spermatozoa is the most common laboratory test of sperm quality. In most laboratories, the procedure used for estimating the percentage of motile spermatozoa and the relative velocity of progressively motile spermatozoa has not progressed much (Amann and Hammerstedt, 1980). This usual estimation is a poor

predictive test of fertility (Saacke and White, 1972; Linford et al., 1976), and both the subjective nature of visual estimation and human bias are undesirable.

It has been estimated that the 95% confidence interval for a single subjective estimation of the percentage of motile sperm is ± 30-60 percentage units (Deibel et al., 1976; see Amann, 1979). Precise and accurate objective methods, free from human bias, for measuring the percentage of motile sperm or velocity are desirable for clinical use, for research to train personnel and to establish standards among laboratories (Amann, 1979; Amann and Hammerstedt, 1980). Objective methods for semen evaluation should give better prediction of fertility than visual estimates (Saacke, 1982), but until recently most methods were labour intensive.

During the last forty years an intensive effort has been made to develop an objective method for evaluating sperm motility (Amann and Hammerstedt, 1980). The different methods used over this period may be classified in chronological order as follows: impedance change frequency developed by Rothschild (1948); photoelectric sensing (Rikmenspoel, 1957); visual tracking (Harvey, 1960); the "track motility" method suggested by Rothschild (1953) applied to assessment of motility of bull spermatozoa (Revell and Wood, 1978; Amann and Hammerstedt, 1980) and human spermatozoa (Janick and MacLeod, 1970; Makler, 1978). The latter method is based on multiple photography to measure the motility. The tracks left by motile sperm cells on a photographic negative made by a 1 or 2 second time exposure, usually using dark-field illumination, are evaluated manually.

In an attempt to develop an objective, reliable and inexpensive system from the "track motility" method, Katz and Overstreet (1981) developed videomicrography analysis of semen, which eliminated the necessity of developing the 35 mm negatives used for tracking analysis. This approach, nevertheless, did not eliminate the human evaluation of video tapes. More recently another method similar to the tracking motility and videomicrography methods, namely microcomputer-photographic analysis, was developed by Budworth et al. (1987) for determination of motility and velocity of bull sperm. This semiautomatic computerized system assessed sperm tracks on 35 mm negatives obtained by dark-field microscopy.

The laser-doppler spectroscopy method first studied by Berge et al. (1967), has been used by Jouannet et al.(1979) to assess the motility of human sperm. This method is based on the determination of the velocity of microscopic particles from the doppler shift of scattered light. The method can provide objective information on two aspects of sperm motion: percentage of motile sperm cells and velocity distribution.

Katz and Dott (1975) developed the Quantimet 720 image analysis computer (Imanco Systems, Melbourn, Cambridgeshire) in an early attempt to use a computer in the estimation of sperm motility. This machine scans the field of a microscope using phase-contrast optics and is capable of counting the number of spermatozoa in a specified rectangular area of the field at specific successive instants of time. With certain adjustments it is possible to count either the total number of spermatozoa or the number of immobile spermatozoa. When this system was tested against photomicrography it provided satisfactory estimates of the surface density and the percentage of motile spermatozoa with an 11% standard error (Katz and Dott, 1975).

Compared with the photographic method the Quantimet method has the advantages of being quicker, and more spermatozoa can be counted in each field without losing accuracy. This means that more reliable estimations of the percentage of motile spermatozoa can be obtained because more fields can be counted (Dott, 1975). Thus, mean speed, total number density, and motility percentage could be simultaneously and automatically determined.

The first validated system for computerized evaluation of sperm motion was developed by Liu and Warme (1977) and validated by Amann (1979) and Amann and Hammerstedt (1980). Because of the cost of the hardware to capture information in real time, the system used a cinematography-to-video interface. With this method, computer evaluations were precise and accurate in samples containing ≥ 50 % of motile sperm, since they agreed with both manual cartography and track motility. However , the evaluations were inaccurate for samples with ≤ 25 % motile spermatozoa. This problem was later overcome by increasing the number of pixels per spermatozoon (Amann and Hammerstedt, 1980). Despite the latest achievements, no objective method has been carefully validated and accepted by the AI industry. Furthermore, no objective approach for estimation of sperm motility has been demonstrated to predict accurately

the fertility of a given sample of semen (Budworth et al., 1988). With the microcomputer-photographic method, good correlations between motility and velocity with fertility were obtained when semen from 9 bulls was evaluated 0 and 1.5 hours after thawing (Budworth et al., 1987). This method is less expensive than the fully automated method and the results are more useful for fertility prediction than those obtained from the track motility or videomicrography. Although repeatable and precise (O'Connor et al., 1981), evaluations by track motility are only slightly more reliable in predicting fertility of spermatozoa than the technique of replicated visual estimations of sperm motility (Saacke et al., 1980).

Recent advances in electronics have permitted the fabrication of moderately priced hardware to capture, in real time, a series of video images in digital form. Software has been developed to extract information on sperm location in successive video frames and process it further to provide comprehensive data on sperm motion (Knuth et al., 1987). This software has been applied to the development of a research system called computer-automated sperm motility assay (CASMA) (Stephens and Hoskins, 1988) which provides a rapid and accurate analysis of multiple sperm movement parameters, and a new measure of linearity, the linear deviation angle. Two commercial systems have also been developed referred to as computer automated semen analysis instruments which have been described, tested and validated for the analysis of semen from humans and/or other species. The commercial systems are the Cellsoft automated semen analyzer (CRYO Resources, Ltd., New York, N. Y.) and the Hamilton Thorn Motility Analyzer (HTM) (Hamilton Thorn, Research Inc., Danvers, MA.) (Mathur et al., 1986; Katz and Davis, 1987; Knuth et al., 1987; Working and Hurtt, 1987; Gill et al., 1987; Budworth et al., 1988; Ginsburg et al., 1988; Knuth and Nieschlag, 1988; Mack et al., 1988; Mahony et al., 1988; Mortimer, et al., 1988; Vantman et al., 1988; Amann, 1989; Pedigo et al., 1989; Vantman et al., 1989; Jasko et al., 1990; Olds-Clarke et al., 1990).

1.2.3.2 Computer assisted analysis of semen characteristics.

Evaluations of male fertility are based predominantly on results from semen analysis (Working and Hurtt, 1987; Vantman et al., 1989). Determination of semen concentration, percentage of motile spermatozoa, and proportion of normally shaped sperm represent the main traits of semen assessment (Knuth and Nieschlag, 1988). These traits can be measured as well by a skilled technician but the measurements are time consuming and affected by human error or variation. The use of computer assisted semen evaluation is a promising alternative to this routine approach of microscopic examination of sperm movement, and haemocytometer evaluation of sperm concentration (Amann, 1979; O'Connor et al., 1981; Mathur et al., 1986).These computer systems not only assess the concentration and motility of sperm, but also measure more sophisticated characteristics of sperm motion, which may be more useful in predicting fertility, such as velocity, linearity of movement (ratio of curvilinear versus straight line velocity), lateral head displacement, and beat frequency (Katz and Overstreet, 1981; Mathur et al., 1986; Knuth et al., 1987: Mahony et al., 1988; Knuth and Nieschlag, 1988).

These developments also have required that standards should be developed which can be used in various laboratories to reduce the variation in results particularly in human semen assessment (Eliasson, 1971).

1.2.3.3 Advantages of computer assisted semen analysis.

Computer assisted systems allow studies on the potential and limitations of laboratory tests for predicting fertility (Amann, 1989) and provide objective data for each individual spermatozoon with good precision and accuracy (Amann and Hammerstedt, 1980; Pedigo et al., 1989). They may also reduce or eliminate the variation in results which is often found among technicians or between laboratories (Mahony et al., 1986). The direct access to computers in semen evaluation allows results to be stored in data bases, creating banks of information, and integrating information between laboratories and networks (Mahony et al.,

1986). Budworth et al.(1988) presented the following advantages which are more related to the system itself:

a) Unbiased and objective data on the percentage of motile spermatozoa.

b) The distribution of the values, or the mean, for sperm velocity

c) Data on linearity of motile spermatozoa

d) The percentage of spermatozoa with circular motion.

1.2.3.4 Prediction of fertility from semen analysis.

The ultimate goal of semen evaluation is to predict the fertility achievable with a given sample (Pace, 1980; Saacke, 1982; Amann, 1989). A problem in achieving this goal has been the lack of understanding of the biological mechanisms important to the relationship between semen quality and fertility. This relationship has been masked or poorly estimated due to the poor repeatability of many laboratory tests, the inaccuracy of fertility estimation, and insufficient variation in fertility (Foote et al., 1977; O'Connor et al., 1981; Saacke, 1982). Some biological limitations include the lack of understanding of the interaction between semen quality and quantity and fertility, the effect of the female tract on selective sperm transport and retention, and the influence of semen characteristics on fertilization of the ovum and further embryonic development (Saacke, 1982). Many authors have emphasized the importance of the number of semen samples assessed, and the number of inseminations required to have confidence in the fertility obtained (Oltenacu and Foote, 1976; Pace, 1980). Linford et al. (1976) demonstrated the importance of variation in fertility by comparing two populations of bulls. In this study, all the correlations obtained between semen characteristics and fertility were improved when the variation in fertility among the males was greater. Thus, correlations of semen characteristics with fertility can be measured more accurately when there is more variance in fertility among the males (Saacke, 1982). In addition to this, more variation has often been found among the males than from ejaculate-to-ejaculate within males (Saacke and

White, 1972). The latter authors suggested that data from at least three samples per male were desirable so that the variation associated with samples within males can be separated from that between the males (Amann, 1989).

In a different approach to the subject, it has been reported that the relationship of semen quality and fertility in AI is particularly dependent upon the number of spermatozoa deposited. Salisbury and Van Denmark (1961) proposed an asymptotic model describing this relationship, where maximum fertility for a given population of females is attained with a given (threshold value) number of spermatozoa. Deposition of further numbers of sperm would not improve fertility. Assuming this hypothesis is correct, no correlation would be found between number of spermatozoa and fertility in experiments where numbers of sperm greater than the threshold were used. Conversely, experiments using lower sperm numbers than the threshold would give higher correlations between number of sperm and fertility (Saacke, 1982). Sullivan and Elliot (1968) and Pace et al. (1981) found that the threshold theory is also true for other semen characteristics such as motility, proportion of intact heads, swelling of sperm in hypotonic media, and number of sperm passing through a Sephadex filter.

For prediction of fertility from semen analysis, the data collected has to be precise and accurate: precision refers to the exactness of a measured value, whilst accuracy refers to the correctness of a value relative to an absolute standard (Amann, 1989). Measurements can be precise but inaccurate and vice versa. Even when all these points are considered, the test or tests might not be useful for predicting fertility. Attaining that goal requires:

a) Development of a predictive equation derived from a set of samples

b) Application of that equation prospectively to a different set of samples

c) Subsequent validation that the predicted results with the second series of samples were reasonably correct (Amann, 1989).

The prediction of fertility is most likely impossible in humans due to ethical and biological reasons, but in other species like cattle or sheep this will be possible and may be used to extrapolate predictions to humans (Amann, 1989).

1.2.3.5 The Cellsoft computerised semen analysis system.

1.2.3.5.1 Description of the system.

The system consists on a IBM PC computer (International Business Machines Corporation, Boca Raton, Fl.), Okidata dot Matrix Printer (Okidata, Tokyo, Japan), Olympus BH-2 phase-contrast microscope (Olympus Optical Co., Ltd., Tokyo, Japan) with 10x objective, high resolution video camera, two monitors (Quadram Corp., Norcross, Ga.), video recorder panasonic (Panasonic, Denver, Co.), Makler chamber (Sefi-Medical Instruments, Rehovot, Israel) and the Cellsoft software (CRYO Resources, Ltd.) (Mathur et al., 1986; Knuth et al., 1987; Working and Hurtt, 1987; Ginsburg et al., 1988; Budworth et al., 1988; Vantman et al., 1988; Toth et al., 1989).

1.2.3.5.2 Principles of the system.

The Cellsoft semen analyzer is able to recognize sperm cells and distinguish them from the other semen constituents based on their size, luminosity and motion. Live or videotaped images are analyzed, and concentration, motility, linear velocity, amplitude of lateral head displacement (ALH) and beat cross frequency (BCF) data are recorded (Working and Hurtt, 1987; Budworth et al., 1988; Gill et al., 1988; Mack et al., 1988). This data is available on the screen, a print out, or can be stored in a computer file.

The computerized analysis of the Cellsoft system is performed using a digitized gray-level image. Particles which do not appear luminous through the phase contrast microscope will not be "seen" by the computer. Adjustment of thresholds for luminosity and size is part of the setting up process for the machine and is a subjective procedure which helps to eliminate most of the debris without affecting the sperm determination (Gill et al., 1988; Knuth et al., 1988).

To evaluate whether a cell is moving, a change in the position on consequent video frames is used. The computer can be instructed to consider only those motile cells that change their position in a specified number of frames e.g. 30 frames per second for bull semen

(Budworth et al., 1988). A Makler chamber of 10 μm depth is used to ensure that only one layer of sperm cells will be present and the cells will not move in and out of focus (Gill et al., 1988). A certain threshold velocity is selected (e.g. human semen 10 μm/sec, see Knuth et al., 1987) which defines the minimum velocity at which a sperm cell is considered motile. Any cell moving slower than the threshold velocity is considered non motile. This limit helps to exclude nonspecific movement of debris due to the collision between motile cells or flow effects (Knuth et al., 1987). The same kind of adjustments (thresholds for speeds, lateral head displacement, size of the cells, brightness, etc.) are required for the rest of the semen characteristics, and these are recommended by the manufacturer and differ among species.

For assessment of a semen sample, the optical system acquires video data at a certain number of frames per second in real, time and the computer digitizes such data. The rate of frames can vary and thus the time of acquisition can be made shorter to avoid the sample drying and to increase the precision. The computer converts the video image into digitized image (256-point grey scale) with a resolution of 512 x 512 pixels (picture elements). Objects analyzed are those within the central portion of this image, consisting of a 320 x 400-pixel matrix representing the area of 215 x 265 μm (pixel spacing= 0.63 μm); this central field represents 128,000 pixels. A threshold grey-level is arbitrarily set for each field of view to erode the image until the middle piece and principal piece of each spermatozoon are no longer visible. This slightly reduces the dimensions of the head without eliminating the sharp contrast with the background. Upper and lower limits for the area of objects to be considered as sperm heads must be set by the user (Katz and Davis, 1987; Budworth et al., 1988; Vantman et al., 1988).

The digitation of the video image is performed by an analog to digital converter (the video signal processor) which produces X and Y coordinate values for objects in a given video line; these are buffered within the hardware of the processor prior to input to the computer. The video signal processor also produces codes necessary to specify parameters of the picture, such as frame start, frame rate, stimulus on/off and others (Jecht and Russo, 1973). To complete identification of an individual spermatozoon, a group of programs within the computer manages the input and storage of raw digital data as it comes out of the video processor. The data is core buffered from the digitizer to magnetic tape with no additional computations. Then

the computer evaluates the digitized image of any object in the central field. From pixel area and luminosity (256-point grey scale), the computer determines if the object is to be considered as a spermatozoon or debris (see Budworth et al., 1988). If the object is considered as a spermatozoon, the centroid is calculated and its X and Y coordinates are stored. The image is tracked at a certain number of frames per second, and based on the information of the coordinates of the centroid, and the velocity of the spermatozoon, a circular area of possible location in the next frame is determined. The spermatozoon is recognized if it is the same cell in the next frame (Working and Hurtt, 1987; Budworth et al., 1988; Mack et al., 1988).

1.2.3.5.3 Terminology of semen characteristics measured by the Cellsoft system.

a) **Percentage of motile spermatozoa:** is the percentage of all spermatozoa analyzed with a linear velocity ≥ V μm/sec, where V is set by the user. The linear velocity is calculated by summing the distances of the linear path between the position of a sperm centroid in all successive frames and dividing by the interval during which the cell was tracked.

b) **Straight line velocity:** is calculated from the straight line path between the first and last positions of a centroid and always is equal to or less than linear velocity.

c) **Linearity:** is the ratio of the straight line and linear velocity multiplied by 10.

d) **Amplitude of lateral head displacement (LHD):** is twice the distance the centroid deviates from the average path . The lateral displacement of the centroid in each frame is multiplied by 2 (to transform data from half to a full cycle) and the values are averaged for each spermatozoon.

e) **Beat cross frequency:** is the frequency in which the linear path of the centroid crosses the average path.

To determine the circular motion of cells, tangents to the average path are calculated for each frame. If all the tangents fall on the same side of the average path, and the radius of the average path is ≤ to a certain value, then the spermatozoon is considered to be swimming

circularly. The Cellsoft system does not compute the percentage of progressively motile spermatozoa, which could be determined by subtracting the percentage of cells with circular motion from the percentage of motile cells. It may be important to estimate this value and its correlation with fertility (Budworth et al., 1988; Ginsburg et al., 1988; Mack et al., 1988).

1.2.3.6 Hamilton Thorn Motility Analyzer (HTM): description and principles.

1.2.3.6.1 HTM system description

The HTM analyzer is a self contained system for analyzing cell motion. It consists of a stroboscopic optical system, memory, microprocessor, screen and printer. The optical system produces dark field images of the specimen so that sperm cells appear as bright objects on a dark background. The system has a variable temperature-controlled stage, and includes a playback system that allows the user to view a particular field and evaluate whether the motile and non-motile cells have been counted (Mahony et al., 1988; Hamilton Thorn Research, 1989). The system has been designed to minimize user training, and maximize convenience and speed.

The HTM uses four different sample chambers: self-filling slide, cannula (flat capillary), Makler, and the ABS (Petroff Hauser) slide. The type of chamber depends on the concentration of the sample.

The abbreviations used for the semen characteristics measured by the system are listed below:

TOTAL: Total concentration of all cells

MOTILE: Motile cell population

PROG: Progressive cell population

PROGR: Progressive fraction of cells expressed as a percentage

MOT: Percentage of motile cells

RAP: Number of rapid cells

RAP %:Percentage of rapid cells

MED: Number of medium cells

MED %:Percentage of medium cells

SLOW: Number of slow cells

SLOW %:Percentage of slow cells

STATIC: Number of static cells

STATIC %:Percentage of static cells

VSL: Straight line velocity or progressive velocity

VCL: Track speed, or curvilinear velocity

VAP: Average path velocity

LVV: Low VAP velocity

MVV: Medium VAP velocity

ALH: Amplitude of lateral head displacement

STR: Straightness (or linear Index)

So: Threshold straightness

LIN: Linearity

For a standard deviation of any of these abbreviations, an SD is added at the end of the abbreviation.

1.2.3.6.2 Principles of the system.

A) Introduction.

The system utilizes a combination of components to automatically determine the sperm motility and concentration in a specimen. A stage whose temperature and position are computer controlled holds the specimen. An optical subassembly provides a strobed visible or infra-red beam, focussed by an imaging lens on to a CCD (Charge-Coupled Device; Sony XC 37/47) sensor, to acquire images of the sample. Following conversion into a digital format, the images are analyzed by processing algorithms which quickly and accurately determine the properties of the sperm motion (Gill et al, 1988; Mahony et al., 1988: Hamilton Thorn Research, 1989).

B) Image acquisition.

Light is provided by a stroboscopic source which illuminates the specimen with a series of phase-locked light flashes of 5 ms duration, at a rate of 30 Hz. This effectively freezes the sperm image during acquisition, removing error due to image blurring from the motion of spermatozoa. There are three kinds of illumination in the system:

1. Bright field illumination

2. Negative phase-contrast illumination

3. Infra-red illumination.

1. Bright field illumination. When the beam from the optical source strikes the specimen, part of the beam is transmitted and part is scattered by the objects (e.g. sperm cells) in the specimen. The transmitted and the scattered light are recombined at the image plane of the camera, by the image lens, and a bright field image is produced. Thus the spermatozoa appear as black cells on a white background.

2. Negative phase-contrast illumination. A much clearer and more accurate image is produced by phase-contrast illumination. A negative phase-contrast image is produced by retarding the phase of the transmitted ray by a quarter wavelength, and simultaneously decreasing the intensity. When this is done and the rays recombine at the image a negative phase-contrast image is produced. The image brightness depends on the degree of retardation of the transmitted ray caused by the specimen. In this way a "phase object" (an object with negligible absorbance whose only effect on the beam is caused by its (small) refractive index difference from the surrounding medium) is finally clearly visible. Since most of the biological objects, including sperm, are "phase objects" this allows the detection and measurement of cells which are otherwise very difficult to see.

In the HTM system, phase-contrast illumination is produced by a phase plate inserted in the beam. The illumination used is 660 nm. The function of the phase is to retard and attenuate the transmitted beam of the optical system.

3. Infra-red illumination. A third form of illumination available in the analyzer is dark-field infrared illumination. In this case scattered infrared light at 882 nm is focussed on to the image plane by the imaging lens. No direct beam reaches the image plane, so the image is formed entirely in scattered light and is bright on a dark field.

The resultant image is useful for certain large irregular cells such as rat, mouse and hamster sperm, as well as the sperm of turkeys and cocks. It can also be used for human cells. The advantage of this kind of illumination is the greater depth of focus provided by the longer wavelength. It permits the use of a 200 micron depth cannula specimen chamber, in which all objects are effectively in focus simultaneously.

C) Image processing.

A sample analysis starts with the collection of successive images at equally spaced time intervals. The number of frames (from 5 to 20) and the time intervals (from 7 to 30 frames per second) are specified by the operator in the main gates (main numbers controlling the image acquisition) menu. In most cases 30 frames/sec are assessed. For sperm concentrations at less than 50 x 10 million per ml, 20 frames/sec is recommended. For sperm concentrations above 150 million per ml, the manufacturer recommends 5 frames/sec. The analog signals produced by the camera are transmitted to the image processing section of the analyzer. The image is sampled and digitally encoded in an array, each of which corresponds to a pixel with one of 64 levels of light intensity. The resulting digitized images are then stored in memory for further analysis.

The next step in the analysis is to remove the artifacts from the background. Objects which are motile or stationary are located and separated. Objects smaller in size and lower in contrast than the operator-specified minimum thresholds are eliminated. This is important because the nonmotile concentration of spermatozoa is required for the analysis, and therefore a discrimination is required between nonmotile cells and debris. This is derived from the mean

size and mean optical intensity obtained from an analysis of all moving objects. It is assumed that all moving objects above minimum limits of size and brightness are sperm.

Assessment of the numbers of both motile and non-motile objects then provides the basis for the motility analysis. Cells with velocities less than the low path velocity (VAP) threshold may be also counted as static for motility calculation if the user selects "slow cells static"(see Hamilton Thorn Research, 1989). Cells moving with VAP (average path velocity) less than the lower threshold velocity are excluded from the calculation of average cell velocity. This lower limit on cell velocity provides a method of avoiding counting cells moved by others.

D) Analysis.

1. Positions.

The position of the spermatozoa on each frame are determined by finding the center-of-brightness (CB) position for each sperm image. This is the average of the pixel positions, weighted by pixel intensity. The pixel within which the CB falls is then illuminated on the play back screen with a red colour for motile cells and a blue colour for non-motile to allow a visual check of algorithm accuracy. For the negative phase-contrast illumination, the CB corresponds closely to the center of mass of the cell.

2. Velocities.

Track speed (VCL) is calculated by summing the total distance travelled by the sperm head CB during frame acquisition, and dividing by the elapsed time. It is a measure of the "curvilinear" velocity and its value depends on the acquisition rate, and the results are expressed in microns/sec.

Path Velocity (VAP) of individual sperm is computed from displacement data and time intervals between adjacent images. A five-point smoothing is used to filter out high-frequency lateral head motion and obtain a path more representative of the sperm average position along the track. The path velocity is the rate of change of distance along the path in microns/sec.

Progressive velocity (VSL) is the straight-line distance between the beginning and the end of the track, divided by the time taken, in microns/sec.

3. Track Dimensions and frequency.

Lateral head displacement (ALH) is measured in the analyzer by computing twice the distance between CB and the average position of the CB and its four neighboring values. The maximum value of this quantity is taken as ALH. This five-point running average gives a good correlation with measured lateral head displacement amplitude. Beat Cross Frequency (BCF) is determined by measuring the frequency with which the track crosses the path in either direction: it is therefore twice the full wave frequency.

1.2.3.6.3 Terminology of the output data or semen characteristics measured by the HTM.

The data summary is presented in Table 1.2.

Table 1.2. Terminology of the output data or semen characteristics measured by the HTM.

Semen characteristic	Definition
Velocity VAP (mean VAP)	The five-point running average path velocity, averaged over all cells for which VAP > LVV see below. The standard deviation of VAP is shown in squared parentheses.
Medium VAP velocity (MVV)	Medium value of VAP velocity pre-set as a threshold by the user.
Low VAP velocity (LVV)	Low value of VAP velocity pre-set as a threshold by the user.
Mean track speed (VCL)	Average value of the track speed over all cells for which VAP > LVV. This is computed by taking the total distance covered by a cell in its track, taking straight lines for each cell between the successive 5 to 20 points acquired, summing the distances and dividing by the total elapsed time.
Mean straightness (mean STR)	Ratio VCL/VAP. Measures departure of the cell path from straight line.
Threshold of STR (So)	The straightness threshold value pre-set by the user.
Total sperm (TOTAL)	The sum of all motile and non-motile spermatozoa. The value is given as the total concentration of all cells, the total number of cells in an ejaculate and the total number of cells counted.
Motile sperm (MOTILE)	The motile cell population, given as motile cell concentration, number of motile cells in an ejaculate, and number of motile cells counted.
Progressively motile sperm (PROG)	The progressive cell population. This is composed of all cells with average path velocity (VAP) see above greater than the set value for medium velocity (MVV) see above and the mean straightness (STR) greater than a set threshold value (So) see above. Both MVV and So are set by the user on the main gates screen. Prog is given as concentration, number of progressive cells in sample, and number of progressive cells counted see below.

Motility (MOT)	The fraction of total cells for which path velocity VAP > 0 (if the slow cells motile: yes option is taken); or for which VAP > a pre-set value for low velocity (LVV) (If slow cells motile : no option is taken). Therefore the user can choose whether to count all moving cells as motile, or only those moving faster than a chosen velocity.
Progressive (PROGR)	The fraction (percentage) of all cells moving with VAP > MVV and STR > So, in a sample see PROG above.
Rapid (RAPID RAPID%)	The fraction of all cells moving with velocity VAP > MVV.
Medium (MEDIUM, MEDIUM %)	The fraction of all cells moving with velocity between LVV and MVV: i. e. MVV > VAP > LVV.
Slow (SLOW, SLOW %)	The fraction of all cells moving with velocity bellow LVV i.e. LVV > VAP > 0.
Static (STATIC, STATIC %)	The fraction of all cells which are not moving at all; i.e. VAP= 0
Mean progressive velocity (mean VSL)	The velocity measured in a straight line from beginning to end of track, averaged over all cells for which VAP > LVV.
Mean linearity (mean LIN)	Ratio VSL/VCL. Measures departure of the cell track from a straight line.
Mean lateral head displacement (mean ALH)	displacement measured in the cell track, averaged over all cells for which the straightness exceeds the threshold straightness (STR > So), and for which VAP > LVV.

Modified from Hamilton Thorn Research (1989). For some of the characteristics, the value is also expressed as a percentage (Rapid, Medium, Slow, Static) and standard deviation is calculated as well and 'sd' is added at the end of the abbreviation.

1.2.3.7 Computer assisted semen analysis: some individual and comparative studies on the precision and accuracy of the Cellsoft and HTM systems.

1.2.3.7.1 Cellsoft system.

There are differences of opinion about the usefulness of this system for the evaluation of semen concentration and motion characteristics.

Firstly, it is important to consider that because of the subjective setting up procedure which involves human bias, there are differences in analysis results between operators and

laboratories (Knuth and Nieschlag, 1988; Mortimer et al., 1988). Furthermore, the accuracy of the results depends on the setting up procedure (Mahony et al, 1988). Specifically the version 3.02 of the Cellsoft system has been reported to have limitations as an automated semen analyzer because of the variations found among results (Knuth and Nieschlag, 1988; Mortimer et al., 1988; Vantman et al., 1988).

A number of studies have described some of the limitations of the system. The system is inaccurate when the semen is less concentrated i. e. when the counts are ≤ 50 million/ml (Gill et al., 1988; Mortimer et al., 1988; Vantman et al., 1988), particularly when counts are ≤ 5 million sperm per ml (Knuth and Nieschlag, 1988) causing an overestimation. Inaccuracy also occurs when the sample contains large amounts of debris, or when the sample contains more than 100 million spermatozoa per ml, probably because the spermatozoa overlap (Knuth et al., 1987; Knuth and Nieschlag, 1988). Along with these observations, the accuracy of analysis seems to be concentration dependent for the rest of the semen characteristics measured (Vantman et al., 1988).

There are some general findings about this system which could help to increase its accuracy. Regardless of the number of frames analyzed, approximately 10 % of variation is found between the males and 90 % within the fields (Budworth et al., 1988). It is important to consider the number of frames for image acquisition and the grey scale to optimize the results (Knuth et al., 1987). The accuracy can be improved by increasing the number of frames assessed up to a certain value according to the concentration and motility in the sample and by increasing the grey scale. Moreover, as the number of fields and cells analyzed is increased, the precision is increased (Knuth et al., 1987; Budworth et al., 1988; Vantman et al., 1988; Blach et al., 1989)

Ginsburg and Abel (1987) studied the reproducability and accuracy of the Cellsoft system in fresh human semen. They used three different protocols to evaluate the concentration, percentage of motile spermatozoa, linearity, velocity, amplitude of lateral head displacement (ALH) and the beat cross frequency (BCF). These were designed to study 1) reproducibility, and 2) the effect of number of cells analyzed on the accuracy of the

measurements. A high correlation was found for concentration, motility percentage, and velocity between measurements ($r= 0.851, 0.835$ and 0.899, $P < 0.01$ respectively). Linearity and beat cross frequency were not well correlated, indicating significant variation for some cells and movement measurements can vary on different areas of slide. It was also found that after assessing 200 cells, measured values did not vary even when more cells were assessed. The reproducibility of the machine using videotaped images was high with average within-sample standard errors of 1.7, 2.7, 3.2, 3.3, 4.9, 8.2 and 4.4 % for concentration, motility percentage, velocity, linearity, maximum of ALH, mean ALH and BCF respectively. The same team of workers (Ginsburg and Abel, 1987) found the accuracy of the system was optimized by setting at 20 frames with a rate of 30 Hz and counting ≥ 225 cells. This was supported by Budworth et al. (1988) who estimated the optimum accuracy of the system, with a coefficient of variation of 6% based on triplicate analyses of the same sample.

Gill et al. (1988) found a 35 % variation between the Cellsoft system and haemocytometer counts. These authors analyzed the variation in sperm count and motility according to the sperm density. When the concentration of spermatozoa was < 30 million per ml, 30-50 million per ml and > 50 million per ml, the variation between the Cellsoft system and haemocytometer counts was + 95 %, + 63 % and + 11 % respectively.

More observations about the accuracy of the Cellsoft system in measuring semen traits are shown in Table 1.3.

Table 1.3. Some observations on the coefficients of variation(%) of concentration, percentage of motile spermatozoa, velocity and linearity of spermatozoa measured by the Cellsoft system.

	Coefficients of variation			
Author/ species	Concentration	Motility %	Velocity	Linearity
Working and Hurtt (1987)/ Rat	---	15	9	10
Ginsburg et al. (1988)/ Human	6.2	8.3	10.9	10.4
Jasko et al. (1988)/Stallion	---	7.8	6.4	1.9

1.2.3.7.2 HTM system.

Pedigo et al.(1989) performed a series of studies to validate the HTM and estimated the optimal setting up for human semen (Table 1.4.)

Table 1.4. Coefficients of variation (CV) found intra and intersample of human semen characteristics measured using the HTM.

Semen characteristic	CV % Intrasample	CV % Intersample
Concentration	9.5	25
Motility (%)	18.4	28.9
ALH	16.5	19.9
Path velocity	6.8	13.9
Progressive velocity	4.5	9.9
Linear index	2.5	4.2

Modified from Pedigo et al. (1989)

Table 1.5. Coefficients of variation (CV) for semen characteristics in video-recorded sperm and fresh semen measured by computerized semen analysis

Video-recorded semen characteristics	CV %	Fresh semen characteristics	CV %
Sperm concentration (x 10^6/ ml)		Sperm concentration (x 10^6/ ml)	
20.0 (40)	8.3	20.0 (40)	24.0
55.0 (110)	6.2	48.0 (96)	21.8
125.0 (250)	2.0	125.0 (250)	16.0
Motility (%)		Motility (%	
30.0	13.3	30.0	21.1
85.0	4.0	82.0	7.6
100.0	1.4	100.0	2.9
ALH (µm)		ALH (µm)	
3.0	11.1	3.0	14.6
4.5	8.5	5.8	9.7
8.0	2.1	8.0	5.8
Path Velocity (µm/s)		Path Velocity (µm/s)	
30.0	5.0	30.0	11.0
55.0	3.5	56.0	8.5
90.0	1.4	90.0	5.2

Values in parentheses are the actual numbers of spermatozoa analyzed in the HTM at the given concentration. Modified from Pedigo et al.(1989).

Table 1.5 presents four semen characteristics assessed from video-recorded sperm values in which the coefficients of variation were determined. The HTM was more accurate when semen was assessed from video-recorded sperm than when fresh semen was assessed.

In an earlier study, Gill et al. (1988) validated the system by comparing it with the Makler method for evaluation of sperm concentration. The HTM had 2%, 12% and 16 % of variation compared with Makler when the counts were less than 30 million spermatozoa per ml, 30 to 50 million spermatozoa per ml and more than 50 million spermatozoa per ml respectively. In specimens with high concentrations of debris, the HTM had 29 % of variation compared with the Makler and the variation within the same sample done in triplicate was found to be 10 %. These levels of variation are within acceptable limits and are lower than that observed with the Cellsoft system.

1.2.3.8 Correlation of some semen characteristics and fertility as measured by the computer assisted systems.

O'Connor et al. (1981) compared a computer assisted system with standard laboratory tests in assessing some semen characteristics and their correlation with fertility. In a preliminary experiment, no correlations were found except for one between visual motility and fertility, mainly because of the lack of accuracy in measuring fertility (66 day non-return rate) and the poor range of semen quality and fertility among the males. In a second attempt, some corrections were made in setting up the system, competitive fertility indexes were used, and heterospermic semen was used for insemination. In this experiment most correlations with the competitive fertility indexes were significant regardless of the incubation period before semen evaluation. No significant differences were found between either methods of evaluation, but computer evaluations had 26 to 67 % of variation, while laboratory assessment had 61 to 86 % of variations in determining the values (O'Connor et al., 1981). These results were supported by Budworth et al. (1987) who compared similar systems of semen assessment. These authors found that motility and velocity were correlated positively with a fertility index from 9 bulls.

They concluded that the computer assisted evaluation of semen is accurate and precise compared with the traditional semen analysis.

Budworth and Amann (1987) and Budworth et al. (1988) also reported a positive correlation between the semen characteristics evaluated and fertility. In a fertility trial, a competitive fertility index (a measure of relative fertility) from a known bull population (n= 9) was correlated with percentage of motile spermatozoa (r=0.86, 0.78), progressively motile spermatozoa in percentage (r= 0.87, 0.80), linear velocity (r= 0.68, 0.77), straight line velocity (r= 0.70, 0.73) and track velocity (r= 0.84, 0.81) at 0 and 1.5 hours of incubation and linearity (r= 0.66 $P < 0.05$) after 1.5 hours of incubation (Budworth et al., 1988). In this study, the ALH and BCF were not found to be correlated with fertility.

1.3 Some physiological, environmental and management factors which affect semen quality and fertilizing ability of the spermatozoa.

1.3.1 The role of epididymis in semen quality.

1.3.1.1 Introduction

On emerging from the efferent ducts the spermatozoa enter the epididymis (Hamilton, 1975; Setchell, 1977; Cooper, 1986; Sorensen, 1986) an organ consisting of a highly convoluted duct system reputed to cover a total length of about 20 meters in man, 40 in the bull, 60 in the boar (Mann and Lutwak-Mann, 1981). In the ram the epididymis is about 60 meters long and it takes between 10 to 14 days for the spermatozoa to pass along the epididymal duct (Ortavant, 1959). The epididymis is dependent upon androgens for the maintenance of its structure as well as secretory and others activities (Mann and Lutwak-Mann, 1981; Cooper, 1986; Amann, 1987; Robaire and Hermo, 1988). The actions of the epididymis in influencing spermatozoa is thought to be mediated by the fluid it secretes which surrounds the cells (Jones and Glover, 1975). The composition of this fluid varies along the length of the duct due to modification of the prevailing current of testicular exocrine secretion. This is achieved by secretion into and resorption from the lumen, across and mediated by the epithelium (Mann and Lutwak-Mann, 1981). Fluid in the cauda epididymis (Jones and Glover, 1975) is more likely to influence the maintenance of sperm fertilizing capacity (sperm storage) than sperm maturation, which occurs more proximally (Mann and Lutwak-Mann, 1981).

Sperm maturation and storage are clearly different processes in that the storage is more susceptible to inhibition by progestins in hamsters (Lubicz-Nawrocki, 1973) and by the effect of abdominal temperature in cryptepididymal rats (Bedford, 1978; Foldesy and Bedford, 1982) and rabbits (Bedford, 1978).

Testicular spermatozoa are infertile (Amann and Griel, 1974) whereas spermatozoa from the distal cauda epididymis have a fertilizing potential comparable to that of ejaculated

spermatozoa. Spermatozoa should not be considered as mature until they have acquired normal motility, fertility, and the ability to induce normal embryonic development (Amann, 1987).

The maturation of the spermatozoa involves a series of complex changes which result from a sequence of events occurring at different points within the caput and the corpus epididymis (Amann, 1987). Spermatozoa from the cauda epididymidis, which apparently are functionally mature, are further altered by admixture with seminal plasma (Killian and Amann, 1973). Spermatozoa from the distal caput bind to about 50 % of sheep oocytes during in vitro incubation, but are non-motile and they do not fertilize following AI into the uterus (Fournier-Delpech et al., 1979). However, the motility and fertility are greater using semen from the distal corpus of the epididymis; the majority of the eggs fertilized result in lambs (69 %) whereas oocytes fertilized by spermatozoa from the central part of the epididymis result in embryonic death (Fournier-Delpech et al., 1981). The unusually high embryonic mortality associated with oocytes fertilized by spermatozoa from the distal corpus epididymis is a consequence of delayed early cleavage divisions which presumably is an intrinsic cause of embryo death or leads to asynchrony between embryo development and the environment afforded by the oviduct and the uterus (Fournier-Delpech et al., 1981).

1.3.1.2 Epididymal structure.

This is a compact fibrous organ closely applied to the posterior or superior border of the testis, and it consists basically of a single very convoluted duct (Hamilton, 1975). Usually the epididymis is divided anatomically into a "head" which receives spermatozoa and fluid from the testicular efferent ducts, a narrower "body" in which the sperm mature and a "tail" which stores sperm before ejaculation (also called the caput, corpus and cauda epididymidis). However, the microscopic anatomy of this duct varies appreciably along its length, as the functions of the epididymis change (Hamilton, 1975). On a histological and cytological basis, the epididymis can be divided into a 'initial' segment, a 'middle' or 'intermediate' segment and a 'terminal' segment (Glover and Nicander, 1971). The first two segments might be concerned with sperm maturation, and the terminal segment is probably the sperm store (Glover and Nicander, 1971).

These segments do not necessarily correspond to the anatomical areas described above, which are used for reference and orientation. Histologically, the initial segment has a high epithelium and a narrow lumen containing very few spermatozoa; its ~~most likely~~ best-known function is the re-absorption of fluid leaving the testis. The middle segment has a slightly lower epithelium and a wider lumen with many spermatozoa; in this segment the spermatozoa "mature". The terminal segment has a low epithelium and a very broad lumen packed with spermatozoa; this is the storage site for spermatozoa awaiting ejaculation (Glover and Nicander, 1971).

1.3.1.3 Epididymal function.

The epididymis performs a series of functions in relation to spermatozoa: maturation of spermatozoa which is a prerequisite for sperm motility and fertilizing ability achieving the maximal potential for fertilization of oocytes with embryonic viability (Amann, 1987), maintenance of the mature spermatozoa in good condition until ejaculation (reservoir) (Amann, 1987), transport of the spermatozoa through the epididymal duct (Mann and Lutwak-Mann, 1981; Amann, 1987), and assistance in the disposal of ageing and superfluous spermatozoa (Mann and Lutwak-Mann, 1981), although many sperm are voided in the urine (Lino and Braden, 1972)

The role of the epididymis in developing the fertilizing capacity of spermatozoa was established in experiments in which the spermatozoa were retained in various parts of the epididymis with ligatures. The sperm held in the most proximal parts never became fertile, whilst those held in the body and tail became fertile (Dacheux and Paquignon, 1980).

Sperm maturation is equated by some authors with the final stage of spermiogenesis, but there is no reason for not including all the processes developed in the epididymis which conclude with the release of motile and fertile spermatozoa. The maturation processes could be extended beyond the departure of the spermatozoa from the epididymis, in the light of evidence that sperm maturation continues after deposition in the female reproductive tract, as a part of the capacitation process which leads to the acquisition of full fertilizing capacity (Bedford, 1970). Considering capacitation as an extension of maturation, it may be that suitable preparation of

epididymal spermatozoa suspensions may enhance fertility following AI. The delayed fertilization which results from insemination with epididymal spermatozoa perhaps arises from the need for a longer period of capacitation within the female tract (Overstreet, 1970).

The epididymis is very efficient as a storage organ, enabling the spermatozoa to preserve their motility and fertility potential for several days or weeks (Mann and Lutwak-Mann, 1981). However, some observations in the rabbit have shown that the spermatozoa held by a ligature in the cauda, retain the capacity of motility for 30 to 60 days, whereas the motility of those held in the caput persist for only a few days (Paufler and Foote, 1968). The latter authors observed that motility of spermatozoa in the caput was never as vigorous as in the cauda, suggesting that either the cauda provides an environment more favorable to the spermatozoa or that those which matured while migrating to the cauda were more capable of survival. Thus, it is possible to differentiate the development of motility and the capacity of fertility (Mann and Lutwak-Mann, 1981). It is generally agreed that the caput and corpus are related to sperm maturation with the cauda as a reservoir. It is important to note that although sperm maturation, survival and storage are processes performed within the epididymis, they are controlled by different mechanisms (Mann and Lutwak-Mann, 1981). This is supported by the observation that when the epididymis of rats or rabbits are placed into the abdomen, while maintaining their continuity with the functional scrotal testes, the spermatozoa can undergo maturation but the epididymis fails to store the spermatozoa (Bedford, 1978).

The precise extent of sperm elimination in the epididymis and the mechanism of disposal are both unknown. Uptake by epithelium cells and intraluminal destruction by macrophages are thought to be responsible for the removal of dead or deteriorating (ageing) spermatozoa in the epididymal duct, but their relative contribution in the elimination of the superfluous spermatozoa has not been determined (Hoffer et al., 1975; Paufler and Foote, 1969).

The survival of the spermatozoa passing through the epididymis depends on normal androgen production by the testis; conditions such as castration or hypophysectomy rapidly cause death of the spermatozoa but they can be kept motile and fertile by injecting testosterone. Other androgens, such as 5 α-androstan-3α 17 ß-diol, which have lower androgenic potency

than testosterone on the accessory sex glands, may be more effective than testosterone in maintaining the spermatozoa in the epididymis (Bedford, 1975; Orgebin-Crist et al., 1975; Cooper, 1989).

1.3.1.4 Structural and morphological changes in epididymal spermatozoa.

The spermatozoa exhibit no progressive movements and have no fertilizing ability as they emerge from the testis and begin to appear in the caput. They acquire both properties gradually during the transit through the epididymis. In the rabbit, the fertilizing capacity of spermatozoa is first recognizable in the corpus of the epididymis, but the fertile sperm are not in large quantities, as they appear to be in the cauda. Once they completely acquire such capacity in the cauda, they do not differ from the ejaculated sperm (Orgebin-Crist, 1967), both in fertilizing capacity and the ability to sustain embryonic development (Overstreet and Bedford, 1976). The epididymal spermatozoa of the hamster do not acquire the fertilizing ability until they reach the proximal part of the cauda (Horan and Bedford, 1972). In the pig, fertilizing capacity is initiated in the caput but it goes up significantly in the cauda (Holtz and Smidt, 1976).

The acquisition of full motility by epididymal spermatozoa is also a gradual process, subject to considerable species variation, and can be stimulated by several substances. In bulls the inclusion in diluents of phosphodiesterase inhibitors stimulates motility, as does ionic calcium or cyclic nucleotides in hamsters (Mann and Lutwak-Mann, 1981). In the rabbit, some rather feeble tail movements can be observed in spermatozoa removed from the caput, whereas in man and bull this is rarely found. In the hamster, rat and mouse the acquisition of motility is even slower (Mann and Lutwak-Mann, 1981). In rams, the capacity of fertilizing and motility are acquired in the distal part of the corpus at the same time (Dacheux and Paquignon, 1980).

Structural changes in spermatozoa undergoing maturation include migration of the kinoplasmic or cytoplasmic droplet along the middle piece and its eventual loss (Setchell, 1978). In the stallion and bull this occurs in the middle of the caput; in the ram and boar it

occurs immediately distal to the caput (Mann and Lutwak-Mann, 1981). The droplet normally is found at the proximal end of the midpiece, close to the sperm head. When the droplet remains in the midpiece, it is an indication of infertility. Some other changes include a reduction in the amount of cytoplasm, and alterations in the size, shape and contents of organelles such as the acrosome, the midpiece and the sperm membranes (Setchell, 1978; Mann and Lutwak-Mann, 1981).

1.3.2 The effect of unilateral castration (hemicastration) on semen production.

The number of spermatids supported by each Sertoli cell varies between species of mammals (Russell and Peterson, 1984). In some species, the number of spermatids produced per testis is correlated with the number of Sertoli cells present to support them (Berndtson et al., 1987). Proliferation of the Sertoli cells in immature rams lambs ceases at approximately 25 to 40 days after birth (Monet-Kuntz et al., 1984) and the numbers appear to remain constant in adult rams even under induced cryptorchidism (Hochereau-de Reviers et al., 1979) or seasonal changes (Hochereau-de Reviers et al., 1985). When unilateral castration (UC) is performed, an increase in the cross-section area of Sertoli cell nuclei is observed and it is associated with a compensatory hypertrophy, suggesting that the Sertoli cell number is always maintained (Hochereau-de Reviers et al., 1976). Further suggestions are that changes in size of the testis, as well in a germ cell population, are associated with the changed size of the Sertoli cells. Waites et al. (1983) reported an increment in Sertoli cell mass in UC ram lambs, but did not determine whether the change was due to hypertrophy or hyperplasia. Mirando et al. (1989) in similar experiments found that 150 days after UC, the mean testis weight of rams was increased by 56 % and the mean epididymal weight by 15 %; furthermore, the mean testis volume was 146 % greater for UC rams than controls. Thus, UC in rams results in a significant compensatory testicular hypertrophy (Hochereau-de Reviers et al., 1976; Walton et al., 1978). The epididymal weight is also increased (Brown et al., 1987; Mirando et al., 1989) and thus, a

larger epididymis is associated with an increase in sperm production in rams (Mirando et al., 1989) and bulls (Barnes et al., 1980a).

Within the testis of UC rams, it has been reported there is an increment in the seminiferous epithelium volume which is associated with an increase in the diameter of the seminiferous tubules. This is probably indicative of an increase in spermatogenic activity as is known to occur in UC adult rams (Hochereau-de Reviers et al., 1976; Mirando et al., 1989), and it is possible to detect a larger number of round spermatids in such rams apparently coming from the larger population of spermatogonia present (Mirando et al., 1989).

1.3.3 The influence of the age of the ram on semen production and quality.

Fertility in the sheep depends on sperm production of the ram as a large number of spermatozoa is required for successful pregnancy in any breeding program. The sperm production varies with the testicular development. The first spermatozoa can be obtained from young lambs at the age of 5 to 6 months (Courot, 1979). Sperm production increases in quality and quantity with age, and achieves good fertility at 7.5 to 9 months of age depending on the breed (Watson et al., 1956; Courot, 1979), as the testicular weight seems to be more correlated with body weight than chronological age (Skinner and Rowson, 1968; Courot, 1979). Many studies have shown that the initial ejaculates of young rams contain a great number of abnormal cells with very low motility (Dun, 1955; Skinner and Rowson, 1968). These abnormalities include head malformations and proximal cytoplasmic droplets which indicate incomplete spermatogenic activity and epididymal maturation (Courot, 1979). After puberty, testicular growth continues for some time, and it is influenced by photoperiodism and it varies with the breeds (Carr and Land, 1975). In addition, the season of birth has an influence on reproductive development in ram lambs. For example, the reproductive development of lambs born in late summer is delayed (Skinner and Rowson, 1968).

Lambs which have been submitted to semen collection by early training at 5 to 6 months of age, are much better semen producers at 1.5 years old than animals of the same age but without previous sexual experience (Colas et al., 1975). The production of semen increases with the age regardless sexual experience. Lightfoot (1968) demonstrated that animals 2.5 years old produce more semen than animals 1.5 years old. The change in quantity with age could be related to several factors: one is a better efficiency of spermatogenesis with testicular development and another a better epididymal maturation which is due to the increase in androgen secretion with age (Courot, 1979).

1.3.4 Influence of environmental factors on sperm production and quality.

1.3.4.1 Introduction.

Several studies have shown that within the same breed, a wide variation exists in the fertility of rams (Dun et al., 1960; Salamon and Robinson, 1962), and within the same animal the quality of semen does not remain constant throughout the year (Colas, 1981). Fertilization success depends on the interrelationship of a range of factors influencing semen production and its characteristics and the behaviour of the ram. As with ewes, rams are influenced by the environment, genotype, latitude, climate (heat), age, nutrition and management procedures (Doney et al., 1982).

The understanding of such environmental factors will allow rams to be kept under optimum conditions for sperm production and to eliminate those which produce poor quality semen. This is more important with the use of AI following oestrus synchronization, where it is desirable to use the same ram for a large number of females, at any time of the year, especially the non-breeding season (Colas, 1983).

1.3.4.2 Daylight (photoperiodism).

In temperate latitudes photoperiodism is the main factor controlling the whole reproductive process in the male (Lincoln, 1976). The testes of the rams of most breeds increase in size during autumn and decrease in spring (Setchell, 1978; Haynes and Schanbacher, 1983), which can be induced by artificial light regimens (Lincoln and Davison, 1977; Lincoln et al., 1977).

These variations in the size of the testis produce a change in semen quality and quantity, particularly the concentration of spermatozoa in ejaculates, seminal plasma constituents and sexual behaviour (Colas and Courot, 1977; Colas, 1979; Courot, 1979). Dun et al. (1960) observed that the fertilizing ability of spermatozoa is reduced during spring. A similar result was reported by Salamon and Robinson (1962), who found that inseminations with semen collected in autumn produce more conceptions than inseminations with semen collected in spring. Despite these observations, training the ram for collection all year-round may overcome these limitations (Doney et al., 1982).

There appears to be a direct effect of season on the onset of puberty and the maturity in ram lambs. Short daylength increases testicular growth and sperm output, while long daylength has the opposite effect. Thus, the application of suitable light regimens has been shown to advance the development and use of young rams (Doney et al., 1982).

1.3.4.3 Temperature (heat).

Heat has several effects on male performance and behaviour. In early reports, it was established that high temperatures depress testicular activity and semen quality of the ram (Phillips and McKenzie, 1934; McKenzie and Berliner, 1937; Bogart and Mayer, 1946). Dun and Restall (1961) reported a diminution in sexual activity in Australian Merino rams during summer. Lindsay (1969) also showed that the sexual activity of rams, measured by ability to

ejaculate hourly, is reduced by high temperatures, but this reduction in activity varies between breeds.

Exposure of the scrotum alone or of the whole animal to hot temperatures also results in changes in semen quality particularly affecting morphology of spermatozoa (Courot, 1976, 1979; Colas, 1983). A number of authors have demonstrated an increase in the proportion of abnormal sperm following exposure of males to high temperatures (Dutt and Hamm, 1957; Dutt and Simpson, 1957; Moule and Waites, 1963; Rathore, 1968; Howarth, 1969; Braden and Mattner, 1970; Rathore, 1970a, 1970b; Smith, 1971; Bornman et al., 1989), but this effect shows considerable variation between individuals (Dun, 1955) and breeds (Lindsay, 1969). These morphological abnormalities involve every part of the cell, but mainly concern the sperm head particularly the acrosome (Rathore, 1968, 1970a, 1970b; Colas, 1983). The damage to the tail produces lost or coiling tails (Moule and Waites, 1963; Rathore, 1968; Braden and Mattner, 1970; Colas, 1983). The length and severity of exposure to high temperatures determines the extent of damage (Dutt and Hamm, 1957; Smith, 1971) and there are variations between breeds (Lindsay, 1969) due to differences in sensitivity to heat conditions and the degree of development of the scrotal cooling system. The exposure of the whole animal to as little as 12 hours of heat (Moule and Waites, 1963) and much shorter periods of scrotal heating can have a detrimental effect (Waites and Setchell, 1964).

Spermatozoa take up to 14 days to pass through the epididymis of the ram (Ortavant, 1959). Abnormalities found 14 and 21 days after heat treatment and returning to normal on days 30-31 suggested that the spermatozoa in the epididymis are damaged at early stages of heat exposure (Ortavant, 1959). This was supported by the finding of some abnormal cells as early as 4 to 5 days after heating (Braden and Mattner, 1970; Rathore, 1968, 1970a; Smith, 1971). These observations suggest that the spermatozoa can be damaged during passage through the epididymis. Other reports suggest that the pachytene spermatocytes and B-spermatogonia are the most sensitive to heat stress. Thus, abnormalities of sperm cells and a low concentration of spermatozoa have been found from 30 up to 50-60 days after exposure to heat (Moule and Waites, 1963; Waites and Setchell, 1964; Braden and Mattner, 1970).

The motility of sperm is also adversely affected by heat stress (Dutt and Hamm, 1957; Dutt and Simpson, 1957; Lindsay, 1969; Smith, 1971). Spermatozoa confined by ligatures in the cauda epididymis, and exposed to abdominal temperatures, only retain the potential for motility up to five or seven days in rats and rabbits respectively (Bedford, 1978). Sperm concentration or density is also decreased when scrotal temperature is elevated (Dutt and Simpson, 1957; Moule and Waites, 1963; Waites and Setchell, 1964; Rathore, 1968; Howarth, 1969; Braden and Mattner, 1970; Rathore, 1970; Smith, 1971). There are some reports that volume is affected by an increase in temperature: some authors have reported a decrease in volume (Dutt and Simpson, 1957; Lindsay, 1969), while other suggest that volume remains constant during the summer when temperature is raised (Bornman et al., 1989). In the studies developed by Dott and Hamm (1957), simple shearing of the rams would reduce the effects of heat exposure.

Of major importance, the fertility measured as conception rate and embryo survival is affected by temperature. The fertilizing capacity of sperm is significantly reduced by exposure to heat (Dutt and Simpson, 1957; Fowler and Dun, 1966; Fowler, 1968; Howarth, 1969; Braden and Mattner, 1970; Rathore, 1970b). This is apparent in ewes mated with heat-treated rams as early as 3 or 4 days post-treatment and the duration of fertilization failure depends on the duration and intensity of the treatment (Rathore, 1968; Colas, 1983).

Several reports have shown that high temperatures affect embryo survival (Howarth, 1969; Rathore, 1970b). Dutt and Simpson (1957) reported higher embryo loss from ewes mated to heat stressed rams, but the results were not significantly different from control rams. Rathore (1968) confirmed this finding of higher embryo mortality in similar experiments and suggested that increasing temperatures affect embryo survival due to damage to cell morphology. Thus gametes capable of fertilizing an egg may not be able to maintain the pregnancy. The above reports had poor precision of measuring embryo mortality due to the number of observations, but still proved that the embryo mortality can be increased by heat treatment of the ram (see Setchell et al., 1988).

The same situation has been reported in other species. Wetteman et al.(1976) reported the same effect when gilts were joined to heat stressed boars. Setchell et al. (1988) reported that before male rats became infertile due to heat stress (judged by the percentage of females becoming pregnant), the embryo mortality is decreased (judged by the foetus to corpora lutea ratio). These and other authors have concluded that the male can affect the fertility and fecundity of the female by increasing the embryo mortality (see also Dutt and Simpson, 1957; Howarth, 1969; Rathore, 1968, 1970b).

1.3.4.4 The impact of nutrition on semen quality and fertility.

There is ample evidence of the importance of protein and energy intake on the production of spermatozoa in Australian Merino rams (Cameron and Tilbrook, 1990). The testes of rams seem to be very sensitive to nutrition, gaining or losing weight in the same direction as the body but at a greater rate (Setchell et al., 1965: Oldham et al., 1978; Lindsay et al., 1979; Masters and Fels, 1984). Oldham et al. (1978) found that rams with high levels of feed for 9 weeks increased liveweight by 32 % and testicle volume by 67 %. For Merino rams grazed at pasture in Australia, annual variation in the quantity and quality of available feed has much more influence on testis size than photoperiod. Thus, testicular weights are greater in spring than autumn due to better quality and quantity of feed (Masters and Fels, 1984). Each gram of testicular tissue produces 20 to 24 million spermatozoa per gram of testis per day (Cameron et al., 1984a). When rams are given diets that reduces testicle size, their sperm production is reduced to 18 million spermatozoa per gram of testis per day, and when the diet increases testis size, the sperm production is increased up to 26 million spermatozoa per gram of testis per day (Oldham et al., 1978). Thus, it is possible to calculate the daily production and also appreciate that when the testis reduces in weight, the sperm production is also reduced (Lindsay, 1976).This also means that rams with larger testes produce more spermatozoa than rams with smaller testes.

The testes respond rapidly to changes in nutrition (Lindsay et al., 1979). Such rapid changes in weight are also accompanied by a change in the output from the seminiferous tubules (Oldham et al., 1978) so that the capacity of spermatozoa production can be increased by high protein and energy supplementation (Braden et al., 1974; Cameron and Tilbrook, 1990), with energy being more important than the level of protein (Braden et al., 1974).

Undernutrition of previously well-fed adult rams causing losses of 30 % of body weight over three months led to some tubular degeneration, a decrease in tubular diameter and a reduction in testicular size of about 50 %, and led to a reduction of the epididymal sperm reserves (Setchell et al., 1965). This reduction in epididymal sperm reserves was also reported by Braden et al. (1974) who also found a significant variation between strains of rams. Moreover, restricted levels of intake will limit the growth rate of young rams and will delay the sexual maturity and may reduce the efficiency of sperm production in later life. During the mating season, it is possible to obtain a rapid loss of weight due to the high nutritive cost of sexual activity and to the behavioural effects on food intake. High levels of nutrition during mating enhance sperm production and quality by maintaining body weight and thus the weight of the testes (Courot, 1976; Lindsay, 1976).

1.3.5 The influence of semen technology and management on semen quality and fertility.

1.3.5.1 Introduction.

The efficiency of storage of ram semen by freezing or chilling generally is low (Salamon and Robinson, 1962) and it is affected even more when semen is processed at high concentrations (Allison and Robinson, 1972). The low preservation achievable in ram semen has an additive negative effect on fertility with the method of insemination used, because normally AI is performed by cervical insemination which uses a small volume and high density (Allison and Robinson, 1972) with low fertilization and conception rates (First et al, 1961; Salamon, 1967; Tervit et al., 1984; Maxwell and Hewitt, 1986). However, the poor results

with ram semen storage have been improved by enhancing its fertilizing capacity with the introduction of intrauterine AI by laparoscopy (Killeen and Caffery, 1982), which reduces the required volume and concentration of the inseminate dose, and improves the fertilization and conception rate.

The fall in quality observed during preservation is thus always the result of the interaction between the effect of dilution rate and other aspects of the technique, mainly the composition of diluents and the temperature at which the semen is stored (Colas, 1983).

1.3.5.2 Fresh diluted semen.

A decline in fertility is evident as early as a few hours after dilution of fresh semen (Entwistle and Martin, 1972a; Watson and Martin, 1976). However, when diluted semen is to be used within 10-12 hours of collection, reducing its concentration to 1.6 thousand million spermatozoa per ml and storing it at 15°C in skim milk diluent, gives the best maintenance of fertilizing ability (Smyth et al., 1978). Maxwell (1978) reported that after longer periods of storage (24 or more), the fertility is rapidly reduced, and embryonic loss is increased ~~These data also demonstrate the effect of sperm storage on embryonic loss~~. In practice fresh diluted semen is not used more than 10-12 hours after its preparation (Colas, 1983).

1.3.5.3 Frozen semen.

Presently, all the literature dealing with this subject has shown that freezing at very low temperatures greatly reduces the survival and fertilizing capacity of ram spermatozoa (Colas, 1983). Factors which affect freezability of ram semen include freezing concentration of spermatozoa (Lightfoot and Salamon, 1969a), the level of egg yolk in the diluent (Salamon and Lightfoot, 1969; Watson and Martin, 1975), the level of glycerol in the diluent (Lightfoot and Salamon, 1969; Colas, 1975), and the osmolarity of the extender (Salamon and Lightfoot, 1969; Colas, 1975; Fiser et al., 1981). The crystallization phase of cooling must be rapid and

consequently, the post-thawing viability of ram spermatozoa is better when the rate of freezing is rapid than when it is slow (Colas, 1983). Moreover, some studies have shown that rapid thawing reduces freeze-thawing injury to spermatozoa and preserves the fertilizing ability reflected in higher conception rates (Aamdal and Andersen, 1968; Colas, 1979). Colas and Guerin (1981) reported an increase in the fertilizing capacity of spermatozoa when the thawed semen was re-diluted before insemination. With this practice, the differences in fertility obtained after using fresh diluted and frozen-thawed semen for AI were diminished (Colas, 1983).

1.4.Embryo mortality.

1.4.1 Introduction.

Early embryo mortality combined with fertilization failure is reflected in the proportion of ewes returning to service within 19 days of mating or AI (Edey, 1966, 1967; Cumming, 1972a; MacKenzie and Edey, 1975a,b; Sawyer and Knight, 1975), and it is different from foetal loss or later embryo mortality which occurs in the later stages of pregnancy (Morley, 1954). Edey (1966) showed that when embryo mortality occurs during the first week after mating, the ewes returned to oestrus at the normal time; whereas mortality occurring during the second and third weeks after mating resulted in delayed return to service. This is explained chronologically by Hafez (1967) according to the embryo stage:

(1) the fertilized egg develops to morula or early blastocyst stage, but degenerates before the middle of the oestrous cycle; the corpus luteum then regresses as in a normal cycle when fertilization has not taken place, and the animal returns to a normal oestrus; and

(2) the blastocyst degenerates beyond the middle of the cycle prior to or following implantation; in this form the regression of the corpus luteum is thus delayed for a period and the oestrous cycle is prolonged.

During early pregnancy the size of the embryo increases very slowly up to day 10-11, followed by a rapid increase and the commencement of implantation (Kelly, 1984). In non-pregnant ewes, the corpus luteum starts to degenerate about day 14 after the commencement of the oestrous cycle (Warbritton, 1934). Moor and Rowson (1964) determined, in their work on ova transfer in sheep, that the presence of the embryo gives a signal for the maintenance of the corpus luteum, beginning on day 12-13 of the cycle, since removal of embryos up to, but not after this time, results in cycles of normal length. This is supported by similar findings reported by Edey (1966).

There are several reports about the timing of early embryo loss. Edey (1979) reported that the majority of the embryo loss occurs before day 18; the same author previously reported that the normal basal level of embryo mortality is between 20 and 30 % due to environmental and genetic reasons (Edey, 1969). Blockey et al. (1975) suggested that there is little loss of embryos prior to day 12 after mating, by comparing return rates up to day 20 with fertilization rates. However, Moore et al. (1960) and Mattner and Braden (1967) had shown that such losses do occur before day 12. Moreover, Lawson and Findlay (1977) reported that embryo development can proceed despite defects until about the time of commencement of endometrial attachment, and this is supported by Robinson (1951) who suggested that losses after commencement of implantation may be a continuation of a process initiated earlier. It is known that the pure morphological examination of embryos prior to day 12 of pregnancy may not necessarily be a good indicator of embryo viability (Killeen and Moore, 1971). However, Maxwell et al (1990) obtained a good correlation between visual classification of morula and blastocyst stage sheep embryos and pregnancy in embryo transfer programs.

Embryo wastage can be increased above the basal level (Edey, 1969) by environmental factors such as high temperatures, variation in the plane of nutrition around mating and early in pregnancy, diseases and perhaps several stress syndromes (Edey, 1979). The basal embryo mortality in normal conditions when no environmental factors are involved, can be described as a mortality due to inate failure of the cleaving ovum or of embryo development, or alternatively, a failure of the maternal utero-tubal system to support embryo development. Thus, when an increase in embryo mortality is noted due to environmental or genetic factors, it can be termed an induced embryo loss (Edey, 1979). Bishop (1964), has reported that basal embryo loss is unavoidable, and should be considered as a normal way of eliminating unfit genotypes in each generation. Furthermore, this failure is inherent in the male and female gametes or arises by mutation during development, and can not be just attributed to the maternal environment (Bishop, 1964).

In cattle, the majority of embryo mortality occurs between day 16 and 25 of single pregnancies (Hawk et al., 1955). During this period the embryo depends for its survival on the

absorption of nutrients contained in the uterine milk (Greenstein and Foley, 1958) until the foetal-maternal union about day 36 of pregnancy (Foley and Reece, 1953). Thus deaths of embryos during this period may be due to an unfavourable uterine environment or to a defect in embryo development.

The diagnosis of early embryo mortality is sometimes difficult to assess because fertilization failure occurs at approximately the same time. There is a need for better methods for detection of mortality which are also practical (Courot and Colas, 1986). The slaughter of a sample of sheep about day two or collection of eggs following laparatomy are good but impractical methods in field conditions (Edey, 1979). There are other methods which can evaluate the fertilization and embryo mortality rates at different stages of pregnancy. These include fertilization and cleavage at 38 to 48 hours by egg collection, diagnosis of pregnancy by progesterone assay in the peripheral blood at day 17 (Robertson, 1977) or 18 days after mating or AI (Fournier-Delpech et al., 1981); ultrasound at 60 to 65 days of pregnancy (Lyndahl, 1976) or ≥ 80 days (Jefferies et al., 1988) and the observation of lambing (Fournier-Delpech et al., 1981; Jefferies et al., 1988).

1.4.2 Normal embryo development until implantation in sheep.

Considering the day of onset of oestrus as day 0, ovulation and fertilization usually occur on day 1 and the cleaving ovum arrives in the uterus on day 4 (Edey, 1979). Migration of embryos between the horns of the uterus apparently occur after day 10 but not later than day 14 (Abenes and Woody, 1971). On Day 11, blastocysts are readily visible to the naked eye and from day 12 rapid elongation of the chorionic sac occurs to a mean of about 4 cm on day 13 and 10 cm on day 14 (Hafez, 1987). By day 20 the fluid-filled chorion, allantois and amnion distend the uterus and a 1 cm embryo with a visible heart beat is present (Edey, 1979).

Moor and Rowson (1966) reported that the presence of a developing embryo on day 12 prevents regression of the corpus luteum and permits establishment of a corpus luteum of pregnancy. However, as mentioned above, if an embryo dies before day 13 the corpus luteum

regresses and the ewe comes into oestrus again about day 17 (Edey, 1967), giving no evidence of pregnancy.

1.4.3 Factors influencing embryo mortality (female and environment).

1.4.3.1 Ovulation rate.

It has been determined that ovulation rate is one of the most important factors affecting embryo mortality and it is influenced by the female and the environment (Kelly, 1984). However, the losses in early pregnancy can be due to fertilization failure or embryo mortality. There are several opinions on this subject: Geisler et al. (1977) and White et al. (1981) reported that embryo survival for a single ovum is higher than for a twin-ovulated ovum and is inversely proportional to ovulation rate, as more ova are shed. Despite this relationship, the pregnancy rate for twin ovulations has been reported greater than for single ovulations since embryo loss generally occurs at random (Restall et al., 1976). Wilkins (1989) found in numerous observations that pregnancy failed in 10 % of single ovulating ewes compared with 4 % in flocks of females with twin ovulations. A more accurate estimation of the losses due to early embryo mortality can be obtained by examining the extent of partial failure of multiple ovulations, that is the proportion of ewes lambing that have fewer lambs than number of corpora lutea recorded at mating. This is possible since fertilization is generally regarded as an all or none process in multiple ovulating ewes (Restall et al., 1976). To further test the dependence of embryo survival on ovulation rate, Restall et al. (1976) used a statistical approach which compared ovulation rate and fertilization rate. This method considered the proportion of eggs shed by the ewes (the ovulation rate), the proportion of eggs fertilized (the fertilization rate), and the proportion of fertilized eggs implanted (embryo survival). These observations were used to classify the ewes according to the number of ova they have shed and the number of fertilized eggs surviving, and this classification was displayed in a matrix. Then an hypothesis of independence was tested by comparing the observed number of ewes in each cell of the matrix with the expected number assuming a binomial distribution. With this

statistical approach, it was concluded that embryo survival depended directly on ovulation rate. This supports the conclusion of Edey (1969) and Geisler et al. (1977).

1.4.3.2 Age.

Embryo mortality is greater in young than older ewes. Significant differences in embryo mortality between maiden 1.5 year old and older parous ewes were reported by Edgar (1962). Allison and Kelly (unpublished data; see Kelly, 1984), reported that although no differences in ovulation rate were found between mated lambs (7-9 months of age) and older ewes, the proportion of ewes lambing following transfer of cleaved ova was markedly lower in lambs, indicating a substantial embryo wastage. Quirke and Hanrahan (1977) recovered 33 % of cleaved ova from ewe lambs compared with 73 % of cleaved ova from adult ewes following their transfer to adult ewes, suggesting that either the cleaved ova from lambs have lower survival capacity inherent in the ovum itself or there are important conditions in the donors prior to collection and transfer. Significant but less pronounced differences in embryo mortality were estimated between maiden 1.5 year old and older parous ewes (Blockey et al., 1975). More recently, Maxwell and Wilson (1991) reported higher ovulation rates for adult than maiden ewes, whereas better embryo recovery was obtained from maiden than adults ewes, but there were no differences in embryo survival.

1.4.3.3 Nutrition.

There is considerable variation in the published work on this field. It is important to establish that nutritional treatments have a short-term effect which may be reflected in blood nutrient levels, but also a long-term effect represented by changes in live weight (Edey, 1976). In a review of nutritional influences on embryo survival in cattle, sheep and pigs, Robinson (1986) concluded that undernutrition may cause a significant reduction in the growth and survival of embryos (in these three species), but only when the undernutrition is severe and experienced for a long period.

With regard to high-gain rations, Cumming et al.(1975) concluded that Merino ewes showed higher embryo mortality when fed 200 % compared with 100 % of maintenance rations from day 2 to 16 post-mating. Parr et al.(1987) also reported that high-plane nutrition in early pregnancy reduced lambing rates in ewes, which could be avoided by the administration of exogenous progesterone. The reason for this "over-nutrition" effect seems to be related to a stimulatory effect on both hepatic blood flow and the metabolic clearance rate of progesterone (Bensadoun and Reid, 1962, ruminants; Symonds and Prime, 1989, gilts), which causes a decrease in plasma progesterone to levels that may compromise embryo growth and survival. This hypothesis is supported by an improvement in embryo survival in ewes following the administration of progesterone (Parr et al., 1987).

Both short and long-term effects of undernutrition have been reported, and treatments have been applied before and during mating, in early pregnancy or during the first stages of embryo development (Edey, 1979). Edey (1966), Cumming (1972b), Blockey et al. (1975) and MacKenzie and Edey (1975a, b) reported that severe short-term undernutrition early in pregnancy can cause a measurable level of embryo mortality. The same authors reported that significantly more ova shed are lost following multiple ovulations, and multiple-ovulating ewes are more likely to suffer complete rather than partial loss of both embryos. Edey (1966) also concluded that the loss is proportionately greater for twin ovulations than for single ovulations. On the other hand, Bennett et al.(1984) reported that severe undernutrition from the day of mating until 60 days of pregnancy (resulting in a 30 % loss of body weight) or from day 21 to 60 of pregnancy (losing a proportional amount of weight), had no effect on the number of pregnant ewes. McKelvey and Robinson (cited by Robinson, 1990) reported from two experiments on embryo transfer that a high plane of nutrition in the pre- and peri-ovulatory periods may be more important to embryo survival than during early pregnancy, when high-plane nutrition seems to be detrimental.

In different studies, it was found that high or low nutrition a few weeks before or after mating affected embryo mortality to a different degree. Cumming (1972a) and MacKenzie and Edey (1975b) found that pre-mating nutrition had little effect on embryo survival, whereas in

ewes which lost weight after mating the rate of embryo mortality was increased. Gunn et al. (1972) and Edey (1970a,b) reported that regardless of post mating treatment, low body condition at mating was detrimental to embryo survival.

There are some reports about the effect of low levels of selenium in the diet, which lead to embryo mortality. Hartley (1963) reported that fertility was improved in some regions with selenium deficiency when selenium was added to the diet. In Scotland, in areas affected by selenium deficiency, the addition of selenium to the diet or the supplementation of ewes with vitamin E reduced the embryo mortality (Mudd and Macke, 1973).

Also, it has been observed that the response to nutritional treatments is influenced by breeds (Foote et al., 1959; Cumming et al., 1975).

1.4.3.4 High temperature.

Dutt et al. (1959) and Alliston and Ulberg (1961) reported that continued high temperatures wasted a high proportion of embryos in early pregnancy, and the peak of incidence occurred during the first week after mating. Thwaites (1969), using rooms with controlled temperature and light regimes, showed that constant heat caused embryo mortality of up to 83 %, compared with 33 % in control ewes. Cold temperatures during the night in the field, or lowering the controlled room temperature, reduced the detrimental effects of high temperatures on embryo mortality. Reducing the time of exposure to heat stress from 6 to 3 days still resulted in considerable mortality particularly when treatments commenced the day after mating (Thwaites, 1971). The latter author concluded that this induced embryo mortality by diurnal variation of temperature is of minor importance as a source of reproductive wastage in the sub-tropical sheep breeding areas of Australia.

Lindsay et al. (1975) found a negative correlation between ambient temperature and lambing performance in South-west Western Australia, suggesting that the main problem was associated with the survival of embryos. Moreover, this correlation was seen only when high

temperatures coincided with mating, or for a few weeks after mating. Therefore, these findings suggest the hypothesis that heat stress at the time of mating may be affecting embryo survival.

1.4.4 The role of the male as a factor in embryo mortality.

1.4.4.1 Introduction.

Embryo mortality during the early part of pregnancy is an important contributor to reproductive wastage, but it is usually attributed to physiological or environmental factors affecting the female at or about the time of implantation (Kelly, 1984). However, Courot and Colas (1986) suggested that the male also contributes to reproductive wastage by affecting embryo survival.

The male contributes not only to the fertilization of females, but also through the quality of spermatozoa to the capacity of embryos to survive and become viable offspring (Courot and Colas, 1986). When the quality of semen is low, the embryo may degenerate, resulting in embryo mortality which normally occurs soon after fertilization.

Factors involved in this process are the **genetic make up**: breeds (Barker and Land, 1970; Doney and Smith, 1968), strains (Burfening et al., 1977; Jefferies et al., 1988), chromosomal abnormalities (Bishop, 1964; bulls: Gustavsson, 1979; pigs: Ackerson and Henricson, 1972); the **environment:** photoperiod or light seasonal variation (Dutt, 1954; Dutt and Simpson, 1957; Skinner and Rowson, 1968; Rathore, 1968; Howarth, 1969; Rathore, 1970b; Lincoln and Davidson, 1977; Pelletier et al., 1981; Colas, 1983); the **management of semen** (Salamon and Robinson, 1962; Salamon and Lightfoot, 1967; Maxwell, 1978).

Most of the male contribution to embryo loss operates just after fertilization (as mentioned above) and therefore good tools are needed for the diagnosis of early pregnancy, and differentiation of fertilization failure from embryo mortality (Courot and Colas, 1986).

1.4.4.2 The existence of male contribution to embryo mortality.

To give a clear example of its existence, Fournier-Delpech et al. (1979, 1981) in studies on epididymal sperm maturation in the ram (mentioned in para 3.1.1.), collected spermatozoa from different parts of the epididymis (head, body and tail) and compared them with ejaculated spermatozoa in their ability to fertilize the egg and in their influence on embryo mortality after intrauterine insemination of ewes. The fertility results were analyzed at different intervals after insemination in an attempt to identify different factors involved and to define the difference between fertilization failure and embryo mortality due to the influence of the male. Thus, the stages studied were fertilization and cleavage of eggs 38 and 48 hours after AI, diagnosis of pregnancy by progesterone assay of peripheral blood concentration on day 17 to 18 after insemination, and lambing.

Some eggs were fertilized by immature spermatozoa from the body of the epididymis, but at a lower rate than the mature ejaculated spermatozoa (30 vs 85 %). However, in this case the kinetics of first cleavages of eggs was abnormal, and the fertilization with immature spermatozoa resulted in delayed segmentation and poor synchronization between the embryo stage and the uterine environment (Fournier-Delpech et al., 1981). No normal pregnancies were established since none of the ewes inseminated with these immature spermatozoa lambed (Fournier-Delpech et al., 1979). With mature spermatozoa most if not all ewes presented positive pregnancy (progesterone level at 17-19 days after insemination) and produced normal lambs at birth. Thus, this study provides evidence of the male effect on embryo survival and mortality. A similar study in rabbits reported similar findings (Orgebin-Crist and Jahad, 1977).

In field conditions, Kidder et al. (1954) reported significant differences in fertilization rates of bulls known to have high (100 %) or low fertility (71 %). When cows where inseminated with semen from such bulls, they had more embryo mortality from bulls of low fertility than bulls of high fertility (19.2 vs 10.5 %) evaluated at 33 days post insemination (Bearden et al., 1956).

In sheep, Edey (1969) reported a basal mortality of 20 to 30 % with a certain amount of individual variation (Blockey et al., 1975). Rathore (1968, 1970b), Howarth (1969) and Braden and Mattner (1970) suggested that embryo mortality was increased in ewes mated to subfertile rams. Such an increase in mortality was also observed in ewes mated to rams selected for low prolificacy (Burfening et al., 1977). An increase in embryo mortality was also observed when ewes were inseminated with aged semen or with semen collected from rams in the non-breeding season (Colas, 1983; Courot and Colas, 1986). This was supported by Salisbury et al. (1976) who associated embryo mortality with fertilization by aged spermatozoa and by Salamon et al. (1979) who concluded that there is a linear relationship between time of chilled storage of ram semen and fertility, and storage of chilled semen for longer than 24 hours increasing the embryo loss. Maxwell et al. (1984) reported that when frozen-stored semen is deposited by uterine insemination relatively early before the time of ovulation, the embryo loss is greater due to the further ageing of the spermatozoa in the female reproductive tract.

Setchell et al. (1988) suggested that one of the possible causes for such effects is that aged spermatozoa or spermatozoa from subfertile males can penetrate the zona pellucida and prevent other spermatozoa from doing so, without completing fertilization. These authors concluded from their experiments on induction of subfertility in rats by means of heat, unilateral castration, irradiation, or arterial ligation, that the male, while still fertile, can affect the fecundity of the female and the rate of embryo mortality.

1.4.4.3 Factors involved in the male effect on embryo mortality.

1.4.4.3.1 Genetic factors.

Bishop (1964) reviewed the paternal contribution to embryo mortality, and mentioned that lethal factors which are carried by the spermatozoon or egg or both cause some of the embryo deaths. He also concluded that such basal embryo loss is unavoidable, and should be considered as a normal way of eliminating unfit inherent genotypes in each generation.

Furthermore, this failure was inherent to the male and female gametes, or could arise by mutation during development rather than as a result of the maternal environment. These genetic defects may be in the form of point mutations, deletions, replications, inversions or translocations. They may vary in the degree of penetrance (i.e. frequency of manifestation) and their degree of expression (e.g. strength of manifestation), and to an extent they are dependent upon the internal and external environment. According to the way they act, the conditions in which they exert their effects, or the individuals in which they are present, these factors are called conditional lethal factors, sex-limited lethal factors, or sex-linked lethal factors respectively. The lethal factors often exert their effect at a particular phase of development which is characteristic for each factor. Many are known to lead to death of early embryos and almost certainly the majority operate during the early phases of development (Bishop, 1964).

The genetic factors which are transmitted by the male to the embryo may be classified according to the time in life cycle when they are acquired (Bishop, 1964):

(1) factors that are inherited by the male from its parents,

(2) factors that arise in the spermatogenic tissue, and

(3) factors that arise in the spermatozoa after their release from the testis.

Some of these factors are lethal recessive factors, some are induced by external agents, arise spontaneously, or simply by ageing of the spermatozoa. The inherited genotype of the male may include other genetic factors of various types that lead to incompatibility and reproductive loss in certain mating systems. The resulting types of incompatibility may be divided into :

(1) incompatibility between spermatozoa and dam,

(2) incompatibility between spermatozoa and egg, and

(3) incompatibility between zygote and dam.

Category (1) is expected to lead to infertility caused by fertilization failure; category (2) when the gametes from the male and female are genetically incompatible due to difference in species (hybridization); the best example known of a category (3) incompatibility is provided by the Rhesus factor associated with haemolytic disease of newborn human beings (Bishop, 1964).

Some studies which support the above observations are in bulls (Gustavsson, 1979) and pigs (Ackerson and Henricson, 1972). If the same situation occurs in rams it is not yet well documented (Courot and Colas, 1986).

Genetic origin of strain seems to modify embryo mortality in the sheep. Burfening et al. (1977) obtained embryo mortality of 11 and 19 % respectively from ewes mated to rams born from dams selected for high and low prolificacy; Barker and Land (1970) did not find such differences in similar studies. Jefferies et al. (1988) found no differences in pregnancy in ewes mated to rams from different strains. However, these authors suggested that the rams from different strains caused differences in the numbers of foetuses present at day 80 of pregnancy, and the number of lambs surviving to marking. There is a suggestion by Doney and Smith (1968) that inbreeding of males increases the loss of embryos. There are other reports of differences between breeds in fertility and embryo survival (Dun et al., 1960; Salamon and Robinson, 1962).

1.4.4.3.2 Environmental factors.

a) Photoperiodism.

Numerous studies have demonstrated the influence of photoperiodism on sexual activity in the ram (e.g. Ortavant, 1958; Salamon and Robinson, 1962; Lincoln, 1976; Lincoln and Davidson, 1977; Pelletier et al., 1981) with large seasonal variations in fertilizing capacity, semen quality and embryo mortality rates after mating with females (Salamon and Robinson, 1962; Colas and Courot, 1977; Colas, 1979; Courot, 1979; Courot and Colas, 1986).

When ewes were inseminated at the same time with semen from rams exposed to either short or long days, embryo mortality (estimated as the difference between pregnancy rate at 18 days after AI and lambing rate) was higher in ewes inseminated with semen from rams exposed to long days (Colas, 1983). Moreover, the same author reported that semen quality was worse in animals exposed to long days, suggesting a relationship between embryo survival and the quality of the gametes. Fournier-Delpech et al. (1981) similarly found a relationship between fertilization rate with immature and mature spermatozoa and embryo mortality. The embryo mortality was increased when eggs were fertilized by immature spermatozoa. Insemination with semen from very young ram lambs, which contains large numbers of morphologically abnormal spermatozoa, also results in elevated embryo loss (Skinner and Rowson, 1968).

b) Temperature (ambient or heat induced).

The fertilizing capacity of spermatozoa is severely reduced after heat exposure or high environmental temperatures (Dutt and Simpson, 1957; Fowler and Dun, 1966; Fowler, 1968; Rathore, 1968; Howarth, 1969; Rathore, 1970b); and it decreases linearly with increased duration of heat exposure (Rathore, 1968). Several reports have suggested that exposure of the male to high temperatures increases embryo mortality (Rathore, 1968; Howarth, 1969; Braden and Mattner, 1970; Rathore, 1970b). Moreover, in normal Merino ewes mated to heat-stressed rams from a strain susceptible to heat, only 34 % of twins were reported compared with 57 % in control ewes mated to a less susceptible strain of ram (Dun and Hamilton, 1965; Fowler and Dun, 1966).

1.4.4.3.3 Semen quality and handling.

Martin-DeLeon and Boice (1985) showed that aged spermatozoa produce an increase in the number of genetically abnormal embryos, many of which do not survive, and the same occurs when subfertile males are used for mating. Bishop (1964) related lethal genetic factors

carried by the spermatozoa to embryo loss (para 4.3.5.3.1.). The importance and the effect of semen quality (semen characteristics) on fertility was discussed extensively in para 2.1.

Salamon and Lightfoot (1967) have reported that the use of frozen semen increases embryo mortality, and this was supported by Maxwell (1978) and Salamon et al. (1979) who demonstrated that the proportion of embryo mortality increases with the period of storage of chilled semen at 5°C. This is probably due to the fertilization of ova by aged spermatozoa which increases embryo mortality (Salisbury et al., 1976), a conclusion supported by the findings of Maxwell et al. (1984).

1.4.4.3.4 Stress and surgical interference.

In the literature relating to embryo mortality, the term "stress" has been used somewhat loosely to refer to heat stress, nutritional stress, climatic stress, etc., sometimes not considering the mode of action of the treatment to induce such stress. The treatments are often confounded with the effects of factors such as intensive handling, crowding and minor surgery (Edey, 1979). Many authors have used these stressful experiences to influence the adrenal cortex (Griffiths et al., 1970; Thwaites, 1970). Stress factors can increase pituitary-adrenal activity and thus depress embryo survival when applied after mating in sheep (Howarth and Hawk, 1968), and in rats (Yang et al., 1969). Doney et al. (1976) designed experiments which examined the effects of both injections of ACTH (adrenocorticotrophic hormone) and subjective conditions of environmental stress (management stress: loading into transporters and movement by road, handling in yards with dogs, handling just by man, and transfer between paddocks for grazing) at different times during the first 20 days after mating. None of these procedures are normal practice in standard husbandry, or not at this intensity, but they are capable of increasing the adreno-cortical activity (Reid and Mills, 1962). Doney et al. (1976) concluded that post-mating exposure of ewes to environmental conditions which can be subjectively described as stressful, will induce excessive adrenal activity, which may result in some increase in embryo loss. The injections of ACTH had a greater effect than environmental stress alone, thus supporting the findings of Howarth and Hawk (1968) and Yang et al.

(1969). The response to stress is also influenced by the nutritional state and genotype of the ewe (Reid and Mills, 1962).

In other kinds of stress or interference with fertilization or embryo survival, it has been reported that surgical insemination of the ewe, involving the direct injection of semen into either the fallopian tubes or uterine horns, gives a high rate of fertilization but subsequent poor lambing performance (Salamon and Lightfoot, 1967; Mattner et al., 1969; Killeen, 1969; Maxwell et al., 1984), and this was related to a high incidence of embryo mortality. There are two hypothesis explaining this effect. Killeen (1969) suggested that the number of abnormal fertilized eggs resulting from intrauterine insemination is related to their content of small anucleate particles, but he did not provide evidence of the viability of the abnormal embryos. The second explanation makes the assumption that surgery and the consequent manipulation of the tract results in failure of the eggs to enter the fallopian tubes or in rapid transport of fertilized eggs to the uterus, a site known to provide an environment unfavourable for underdeveloped eggs (Averill and Rowson, 1958; Moore and Shelton, 1964). Killeen and Moore (1971) found the same results in terms of the high fertilization achievable by intrauterine insemination (Salamon and Lightfoot, 1967; Mattner et al., 1969; Killeen, 1969) and concluded that the low lambing rate they obtained was not due to failure of fertilization. In their experiment using natural mating and intrauterine AI, no differences were found in fertilization rates or in embryo survival. That AI was not responsible for the development of abnormal embryos, was proved because a greater number of abnormal embryos was obtained from the ewes subjected to a natural mating. Killeen and Moore (1971), however, reported a lower recovery of eggs from surgical inseminated ewes. Thus it seems that the low lambing performance observed following surgical insemination was due to abnormal transport of eggs resulting from "surgical interference". Such loss of embryos due to "surgical interference" does not appear to occur following intrauterine insemination of ewes by laparoscopy (Maxwell and Buttler, 1984).

1.5 Processing and methods for freezing of ram semen.

1.5.1 Introduction.

The first recorded observation on semen preservation has been attributed to Spallanzani (1776), who noted that when human, stallion or frog semen were cooled in snow for up to 30 minutes they became inactive, but could be revived by warming. Thus, cold temperatures produce a depression of metabolism and prolong the active life of spermatozoa. Since then, it has became apparent that cell damage is produced as a result of processing, freezing and thawing semen, and much research has been focused on minimizing this damage (see review by Watson, 1979).

In 1866, Mantegazza had this future vision which nowadays had been fulfilled, particularly in animals of domestic importance:

" If the human sperm can be preserved for more than four days at the temperature of melting ice without undergoing any change, then it is certain that scientists of the future will be able to improve breeds of horses and cattle without having to spend enormous sums of money in transporting thoroughbred stallions and bulls. It will be possible to carry out artificial insemination with frozen sperm sent rapidly from one locality to another. It should also be feasible for a husband who dies on the battlefield to fertilize his wife and thus to have legitimate sons even after his own death" (Mantegazza, 1866).

The most significant discovery concerning the freezing of mammalian semen was made by Polge et al. (1949) who during their studies on low temperature storage of fowl spermatozoa, demonstrated the cryoprotective properties of glycerol. After this finding, the development of techniques were carried out all over the world. The achievement of freezing bull semen (Polge and Rowson, 1952) represented a big step, heading to the development of the pellet freezing method for bull semen (Nagase and Niwa, 1964) and for ram semen (Salamon and Visser, 1972) or the straw method (plastic straws) for bull semen (Cassou, 1964; Jondet, 1964) and ram semen (Colas, 1975).

Artificial insemination (AI) of sheep has been widely used throughout Australia, particularly Western Australia. In recent years this has been characterized by a movement away from on-farm AI using "home grown" rams, to the use of "top" sires in studs or commercial artificial breeding centres. The preservation of semen has provided the facility of using sires without having to transport them to other places, avoiding the risk of death, injury or stress that probably occurs during transport, and allowing more widespread use of individual sires and the preservation of genetic material for the future (Maxwell, 1984).

In the area of wildlife conservation, the potential benefits of semen preservation, especially in preserving genetic material from selected captive stock for future use, are obvious, the only limitation being the financial resources for applying the techniques as discussed by Watson (1978).

The responses of spermatozoa to freezing and thawing methods vary between species (Healey, 1969; Jones, 1971; Watson and Martin, 1972) and this is reflected in the extent of damage, particularly to the morphology of the cell, which enable the spermatozoa to fertilize eggs. This difference among the species is probably due to differences in membrane composition, which is reflected in different requirements for freezing diluents (Darin-Bennett et al., 1973).

1.5.2 Diluents for deep freezing of ram semen.

The reasons for dilution and the role of its components for protection of spermatozoa.

Numerous solutions have been used as diluents or extenders for semen. Although the extenders vary in the amount and kind of components, a good diluent must have the following characteristics: the diluent should supply an energy source, should not deteriorate during storage prior to use, should provide protection for spermatozoa against the adverse effects of

cooling, freezing and thawing, and should resist bacterial growth (Melrose, 1962; Mann, 1964).

There are two main reasons for diluting the semen, a) technical and b) biological. The technical reason is because one of the advantages of AI is to inseminate a greater number of females with selected rams, and the dilution of semen allows this. When natural mating is used, the male deposits several thousand million spermatozoa, but only about 100-140 million penetrate the cervix. With AI, both the volume and the numbers of spermatozoa can be reduced to make it much more efficient (Evans and Maxwell, 1987). The biological reason is that the spermatozoa need nourishment and protection. Thus diluents provide nutrients for the spermatozoa, buffers against changes in pH, and a correct tonicity in the environment. In addition they should protect against cold shock which can occur when the semen is cooled too rapidly and during storage in a chilled state, or against freezing and thawing injury when the semen is frozen and thawed (Evans and Maxwell, 1987).

There are two kind of diluents: synthetic and natural.

1) Synthetic diluents:

These diluents normally contain **sugars** as the energy source such a glucose (Salamon, 1967, 1968, 1970; Watson, 1981; Visser and Salamon, 1973; Watson and Martin, 1973, 1975b; Maxwell et al., 1984; Maxwell, 1986a; Maxwell and Hewitt, 1986; Hunton et al., 1987), fructose (Blackshaw, 1955; Jones and Martin, 1965; Salamon, 1968; Salamon and Visser, 1972), raffinose (Salamon, 1968; Lightfoot and Salamon, 1969a,b,; Salamon and Lightfoot, 1969, 1970; Lightfoot and Salamon, 1970; Salamon, 1971; Salamon and Visser, 1974), lactose (Colas, 1975; Jones and Martin, 1965), and arabinose (Blackshaw, 1955; Emmens and Blackshaw, 1955; First et al., 1961). They also contain **buffers** to control the pH such as sodium or potassium citrate (Blackshaw, 1960: Salamon, 1968; Lightfoot and Salamon 1969a, b; Salamon and Lightfoot, 1969), phosphates (Blackshaw, 1954; Enwistle and Martin, 1972b), and more recently the organic buffers such as tris (Lapwood and Martin, 1972; Salamon and Visser, 1972; Maxwell and Hewitt, 1983; Maxwell et al., 1984b; Maxwell, 1986a; Hunton et al., 1987) and other organic buffers such as tricine, TES, MES,

PIPES and HEPES , but as yet few studies of their use in semen diluents have been published (bull- Pace and Graham, 1970; boar- Crabo et al., 1972; Stallion- Ellery et al., 1971; ram- Martin and Richardson, 1976); **egg yolk** to prevent temperature shock (Salamon, 1968, Salamon and Lightfoot, 1969; Enwistle and Martin, 1972b, Salamon and Visser, 1972; Visser and Salamon, 1973; Pace and Graham, 1974); and cryoprotective agents such as **glycerol** (Salamon, 1968; Lightfoot and Salamon, 1969a; Enwistle and Martin, 1972b; Salamon and Visser, 1972; Visser and Salamon, 1973). **Antibacterial** agents are included to prevent bacterial growth: generally a combination of Penicillin (500 to 1000 I. U./ ml) and Streptomycin (500 to 1000 μg/ ml) has been used provided a broad spectrum of antibacterial activity (Melrose, 1962; Enwistle and Martin, 1972b; Watson and Martin, 1975b).

2) Natural diluents.

The main natural diluent for ram semen is cow **milk**, in whole, skimmed, or reconstituted (powder) form (Salamon, 1962; Salamon and Robinson, 1962; Jones, 1965a,b, 1969). Whole, skim or powdered milk should be heated to 92-95°C in a water bath for 8-10 minutes to inactivate the toxic factors in its protein fraction. When whole milk contains appreciable amounts of fat, it is recommended to skim off the fat, because it causes problems viewing the semen under the microscope (Evans and Maxwell, 1987). Milk has also been used as a base in diluents for deep freezing of ram semen (Blackshaw, 1960; Jones, 1965a,b, 1969; Jones and Martin, 1965; Colas, 1975; Wilson, 1988; Hunton, 1988).

1.5.2.1 The use of sugar in diluents for frozen semen.

The use of sugars in diluents for frozen ram semen depends on a series of factors which involves the type and amount of buffer used, the use of egg yolk, and the type of freezing method.

Watson (1979) summarized the functions of sugars as providing an energy source for the nourishment of the spermatozoa, promoting sperm motility after freezing or during incubation, provision of part of the osmotic pressure (which varies according to temperature

and permeability coefficients) and acting as an extracellular cryoprotective agent, particularly the high molecular weight sugars which cannot penetrate the cell (Watson, 1979).

Only certain sugars can be used as energy sources in diluents. Glucose, fructose, and mannose are glycolysable by spermatozoa (Mann, 1964) while arabinose can be metabolized by oxidation (White et al., 1954). Some sugars may provide protection against cold shock from the outside of the cell through their action on cell membranes (Nagase et al., 1964). Nagase et al. (1964) reported that sugars with higher molecular weight provide better protection during fast freezing than those with lower molecular weight, especially when lower temperatures are used for pellet freezing of semen (below - 79 °C) (Salamon, 1970). Moreover, when sugars have been used without buffers, sugars with higher molecular weights such as lactose and raffinose resulted in better sperm protection than glucose and fructose (Salamon , 1968).

1.5.2.2 Hydrogen ion concentration and buffering capacity.

The purpose of using buffers such as citrates, phosphates and organic buffers is to control changes in pH which may affect the spermatozoa viability in fresh diluted semen or frozen-thawed semen. Regardless of the buffer used, there is a common agreement that pH around 7.0 has been the best for either fresh or frozen ram semen. The value of pH varies according to the rest of the diluent components such as sugar or egg yolk, but any combination of these components need to maintain an osmolarity similar to the seminal plasma (Lapwood and Martin, 1972).

The pH can also be adjusted by the addition of the right amount of citric acid to either of the buffers (Salamon and Visser, 1972; Watson, 1979).

1.5.2.3 Osmolarity.

When ram semen is frozen slowly, there is some evidence that hyper-tonic diluents are better than the hypo-tonic diluents (Loginova, 1961, Jones, 1965b). Jones (1965b) also found that ram semen responds better to freezing with hyper-tonic than isotonic diluents. However, Blackshaw (1960) reported that isotonic diluents were more suitable for freezing ram spermatozoa. With the fast freezing method (pellet method) it has been concluded that hyper-tonic diluents are less harmful to spermatozoa than hypo-tonic diluents (Salamon, 1968; Salamon and Visser, 1972). With bull semen on the other hand it has been demonstrated that isotonic diluents in fast freezing give the best results (Nagase and Niwa, 1964).

Salamon and Lightfoot (1969) reported that although relatively hyper-tonic diluents were best for freezing ram spermatozoa by the pellet method, the optimal freezing point depression varied according to the diluent composition. They concluded that when a sugar is included in the diluent, a higher proportion of spermatozoa survived after deep freezing at all tonicities, in agreement with Emmens and Blackshaw (1950), Blackshaw (1955) and First et al. (1957).

Watson (1979) suggested that the hypo-tonic diluents probably damage cells because they lead to a gain in intracellular water and a redistribution of ions, which may have important consequences during freezing. On the other hand, spermatozoa in a hyper-tonic solution would lose water and gain osmotically active electrolytes or non-electrolytes. Hyper-tonic diluents may act to reduce the intracellular water content prior to freezing which may, in turn, reduce the likelihood of intracellular ice formation.

1.5.2.4 Egg yolk in freezing diluents.

1.5.2.4.1 Protective action.

Since its protective role was demonstrated by Phillips (1939) and Phillips and Lardy (1940), egg yolk has been a common constituent of diluents for chilling and freezing

spermatozoa. It has been demonstrated that egg yolk interacts with glycerol (additive effect) in the cryoprotection of spermatozoa (Nagase et al., 1964; Gibson and Graham, 1969; Berndtson and Foote, 1972; Pace and Graham, 1974). However egg yolk alone has a protective action (Blackshaw, 1954). Pace and Graham (1974) in a series of experiments reported that neither buffer nor buffer plus glycerol protected spermatozoa motility in absence of egg yolk, while sperm cells were protected with egg yolk or egg yolk combined with a buffer. Watson and Martin (1975b) reported that the inclusion of egg yolk reduces acrosome damage to spermatozoa and improves their motility, and that the high molecular weight of some parts of the egg yolk may stabilize cell membranes, making the cell more resistant to damage.

1.5.2.4.2 Components of egg yolk with the cryoprotective property.

Several studies had revealed two components of egg yolk with a cryoprotective action. The first is the low-density lipoprotein fraction of the egg yolk (Watson and Martin, 1973; Pace and Graham, 1974) which provides protection in frozen semen and in chilled semen (Watson and Martin, 1975a). The second is the phospholipid lecithin which can be isolated from the egg yolk and acts at much lower concentrations (as low as 0.12 %) than whole egg yolk. The protective action of lecithin is limited by the pH of the diluent, it being best at pH 6.5 rather than neutral or alkaline pH (Blackshaw, 1954; Mayer and Lasley, 1944).

In other observations, Foulkes and Stewart (1977) have shown in field trials that a lipoprotein prepared from the egg yolk is capable of protecting spermatozoa during the freezing and thawing procedures when used at a diluent level equivalent to that in whole egg yolk. Martin (1963) reported higher survival of spermatozoa in diluents containing 1% (w/v) of lecithin than those with 25 % (v/v) of egg yolk and the best record of unstained cells was found in lecithin-containing diluents after 4 hr storage at 5°C. Watson (1981) reported that either the low-density lipoprotein fraction or sonicated lecithin liposomes provided an equal measure of protection for ram spermatozoa against cold shock, but the lipoprotein fraction was superior during cold storage at 5°C.

1.5.2.4.3 Level of egg yolk in the diluent.

The amount of egg yolk in diluents varies considerably and depends upon the kind of diluent. Variation can be from 3 to 6 % (v/v) (Enwistle and Martin, 1972b; Watson and Martin, 1973, 1975a) or as high as 15 % as reported by Salamon and Lightfoot (1969) and Salamon and Visser (1972). Higher levels could be toxic (First et al., 1961; Salamon, 1971). Many workers have adopted the level of 15 to 18 % particularly when semen is frozen in pellets (Maxwell et al., 1984; Maxwell, 1986a; Maxwell and Hewitt, 1986; Hunton et al., 1987). The amount of egg yolk may also depend on the presence of glycerol (First et al., 1961; Salamon and Lightfoot, 1969; Watson and Martin, 1975b) because of the additive effect mentioned previously.

1.5.2.5 Cryoprotective agents.

The cryoprotective agents are generally divided into those which penetrate the cell and those which remain extracellularly. Some of the most common non-penetrating cryoprotective agents in diluents for freezing semen are sugars of high molecular weight such as lactose and raffinose which act on the cell membrane (Nagase et al., 1964; Salamon, 1968) or polyvinyl pyrollidone (PVP) (see Watson, 1979), and penetrating agents such as glycerol (Lovelock, 1953; Salamon and Robinson, 1962; Salamon , 1968; Salamon and Lightfoot, 1969a,b; Pace and Graham, 1974; Colas, 1975), ethylene glycol (Salamon, 1968) and dimethyl sulphoxide (DMSO) (Jones, 1965a,b; Salamon, 1968).

Glycerol has been the most common agent used for cryoprotection of spermatozoa since its introduction by Polge et al.(1949). It has been compared with ethylene glycol (Salamon, 1968) and dimethyl sulphoxide (Jones, 1965a,b; Salamon, 1968) but none of them were better than glycerol. Being a penetrating agent, glycerol needs a period of "equilibration" or time of reaction in which presumably the glycerol penetrates the spermatozoa (Watson, 1979). Many reports about this are controversial. Blackshaw (1955) reported that a period of 15-20 hours is required; Hill et al. (1959) reported that a period of 0.5 hours of equilibration

was superior to 18 hours, and Jondet (1972) obtained promotion of motility and fertility of bull spermatozoa with exposure to glycerol for periods as short a period as < 1 second to 2 hours and maximal survival was achieved with just 10 seconds of equilibration with glycerol. Berndtson and Foote (1972) reported that glycerol has been observed to penetrate the bull sperm cells at 25 or 5°C in 3-4 minutes. Much of the variability in results depends to a great extent on the glycerol concentration, dilution rate and rate of cooling (Lightfoot and Salamon, 1969a).

1.5.2.5.1 Mechanism of action of glycerol.

The possible mechanism of action of glycerol was studied by Lovelock (1953) working with human erythrocyte freezing and generalized for living cells. Lovelock suggested that the damage caused by freezing is due to the high salt concentrations found in the cell at temperatures around -15°C to -40°C. Glycerol is believed to act as a "salt buffer" preventing the rise in concentration of salts. A second way of protection and theory of cryoinjury is the binding action of glycerol to water. This would not only reduce cell damage from ice formation, if that is a significant source of damage, but would also reduce the otherwise high salt concentrations to harmless levels.

1.5.2.5.2 Level of glycerol to be used in diluents.

Blackshaw (1955) reported that a level of glycerol of 7.5 % combined with 1.5 % of arabinose or fructose gave good survival of bull and ram spermatozoa much superior than glycerol alone. Hill et al. (1959) found good survival of ram spermatozoa when glycerol was added at 3.5 to 7 % (v/v) with 0.5 hours of equilibration, and a level of 14 % was detrimental by decreasing the motility. The level of glycerol reported by First et al. (1961) for good results on ram sperm survival and preservation of motility was 6-8 %, and there appeared to be no significant difference between these levels. Lightfoot and Salamon (1969) and Salamon and Visser (1972), found that the best level of glycerol for preservation of ram spermatozoa during

pellet freezing was 3-4 % and 5 % respectively. Many workers have adopted 5 up to 8 % of glycerol as a general level (Visser and Salamon, 1973; Watson and Martin, 1973; Watson and Martin, 1975b). Most recently 6 % (v/v) has been commonly used in Australia for pellet and straw freezing of ram semen (Maxwell et al., 1984; Maxwell, 1986a,b; Maxwell and Hewitt, 1986; Hunton et al., 1987).

1.5.2.5.3 Time of addition of glycerol.

Lightfoot and Salamon (1969a) have reported good results by adding the glycerol at 30°C, cooling down to 5°C over 2 hours and then standing for 1 hr more for further equilibration. This was supported by Salamon (1968) and Salamon and Visser (1972) who reported no difference in adding the diluent at 5°C with the time of equilibration specified. Equilibration for 2.5 hours was better than no equilibration and the effect was more beneficial when glycerol was added at 5°C. Blackshaw (1960) reported no difference in adding the glycerol either at 30°C or 5°C. On the other hand, Colas (1975) reported that the optimum level of glycerol in frozen ram semen in straws is 4 % and it should be added at 4°C. Although this is contrary to earlier reports, it may be because of the difference in method of freezing. Colas (1975) also reported that ice crystallization during freezing did not occur until -6 °C to - 10°C, so this author suggested it was not logical to add glycerol at 30°C but rather at 4°C. Moreover, he reported a lower proportion of motile spermatozoa after thawing, when the glycerol was added at 30°C. Sherman (1963) reported that glycerol penetration is more complete and faster at 30°C than at 4 °C. Despite this, Martin (1963) found that the addition of glycerol to bull semen at 5°C and an equilibration time of 4 hours gave better results for survival, motility score and live-normal cells than non incubation at all and incubation for 1 hour.

1.5.3 Methods of freezing ram semen.

There are two main practical methods for freezing ram semen. The pellet method developed by Salamon and Visser (1972) and the straw method developed by Colas (1975).

1.5.3.1 The pellet method for freezing ram semen.

The pellet method for freezing semen was first reported by Nagase and Niwa (1964) for bull semen, in which drops of semen (0.06 ml) were frozen on dry ice.

The pellet method was first described for ram semen by Salamon (1967), and can be described as follows: semen is collected by artificial vagina and diluted with an appropriate diluent containing a cryoprotective agent generally at 30°C. The diluted semen is cooled slowly over 2 hours to 5°C and sometimes held for another hour at this temperature before the diluted semen is pelleted (0.06 ml) on dry ice. The semen can be kept for 3 minutes on the block of dry ice and then the frozen pellets are transferred to liquid nitrogen (-196°C). The frozen pellets may be thawed either in dry tubes (Killeen et al., 1982; Maxwell et al., 1984; Maxwell, 1986b) or using a thawing solution (Salamon, 1968; Lightfoot and Salamon, 1969a; Salamon and Visser, 1972).

With this method semen can be preserved indefinitely. Salamon and Visser (1974) reported that storage of semen for 5 years did not change its fertilizing capacity, resulting in high lambing rates comparable with semen frozen-stored for 2 weeks. Salamon et al. (1985) reported no differences in lambing rate between fresh ram semen and semen frozen-stored for 16 years following intrauterine insemination.

1.5.3.1.1 Factors affecting the survival of pellet-frozen spermatozoa.

1.5.3.1.1.1 Size of pellet.

Lightfoot and Salamon (1969b) showed that the freezing rate within a pellet was not uniform and the freezing profile was different depending the size of the pellet. This is because about 50 % of the pellet is in contact with the dry ice, so the lower part is frozen more rapidly. No significant differences were found in survival of spermatozoa when the pellet volume varied from 0.03 to 0.86 ml. Moreover, there was no interaction between pellet volume and either diluent tonicity or glycerol concentration on survival. But sperm survived better during incubation with a bigger pellet size, and these authors recommended bigger pellets (0.5 ml) which yield sufficient spermatozoa to perform a single cervical insemination. This is supported by Leipnitz (1966) and Hunton et al. (1987) who found that 0.2 ml pellets are slightly superior to 0.1 ml pellets in terms of fertility following AI. Nagase and Niwa (1964) and Peskovotskii and Habibullin (1968) reported that increasing the volume of the pellets from 0.013 to 0.20 ml and from 0.1 to 0.5 respectively, had no effect on the revival of bull spermatozoa after pellet freezing.

1.5.3.1.1.2 Temperature of freezing (freezing rate).

Salamon (1970) worked on the effect of increasing the freezing rate on the survival of pellet-frozen ram semen by forming pellets either on dry ice at -79°C or on a stainless steel plate below that temperature. No differences were found within the range of -100 to -160°C but poor results on survival were found when pellets were frozen at -180°C or by plunging directly into liquid nitrogen (-196°C) (Salamon, 1970). Salamon (1971) compared pellets frozen at -79°C and -140°C and found no differences in terms of survival of spermatozoa and lambing rates following AI.

1.5.3.1.1.3 Thawing methods.

There are two types of thawing: in dry tubes or with a thawing solution. The first method is normally used for intrauterine AI because of the small amount of spermatozoa and volume needed to achieve good fertilization and lambing rates (Killeen and Caffery, 1982; Killeen et al., 1982; Maxwell et al., 1984; Maxwell, 1986b). This method is very simple, the frozen semen is thawed in test tubes (two or three pellets in each) which are shaken vigorously in a water bath at 37°C, and kept at 30°C to be used normally within 10 minutes (Maxwell et al. 1984b; Maxwell, 1986a; Maxwell and Hewitt, 1986; Evans and Maxwell, 1987; Hunton et al., 1987). The second method was used principally with cervical insemination, which gives poorer fertilization rates with frozen ram semen. Thawing with a thawing solution has been reported to enhance the survival and fertilizing capacity of the spermatozoa in two ways: just as fast freezing helps to avoid damage, rapid thawing is needed in order to obtain maximum recovery. The improvement in recovery, particularly when larger volumes of solution are used, could be attributed to a more rapid warming and thawing process (Salamon, 1968). The thawing solution may also contribute to maintenance of the viability of spermatozoa (Lightfoot and Salamon, 1969a; Salamon and Visser, 1972). There is a big disadvantage in using such solutions when semen is to be used for cervical insemination in sheep (Mattner, 1963, 1966; Mattner and Braden, 1963). The cervix is very important in the physiology of the transport of ovine spermatozoa as it constitutes the first barrier to the transport through the genital tract, provides favourable environmental conditions for the survival of spermatozoa and acts as a reservoir from which spermatozoa continuously progress to the site of fertilization. Therefore, it is important to establish a good cervical population of spermatozoa for cervical or vaginal AI, and the concentration of motile spermatozoa in the inseminate should be as high as possible, probably 200 million spermatozoa per ml (Lightfoot and Salamon, 1970). Therefore, when ram semen is frozen in pellets and thawed in a thawing solution, this necessitates centrifugation immediately after thawing to re-concentrate the spermatozoa (Lightfoot and Salamon, 1970; Salamon and Lightfoot, 1970). The use of very low dilution rates both before freezing and at thawing is associated with low revival rates and subsequent poor viability of the thawed spermatozoa (Lightfoot and Salamon, 1969a,b); therefore freezing and thawing

semen at low dilution rates is not a solution. A second option to overcome the problem with frozen-thawed semen with low concentrations, is to perform double inseminations (Salamon and Lightfoot, 1970).

The most common thawing solutions used are glucose-sodium citrate (Lightfoot and Salamon, 1969a,b; Salamon and Lightfoot, 1969; Lightfoot and Salamon, 1970; Salamon and Lightfoot, 1970), sodium citrate (Salamon, 1968), and tris-fructose (Salamon and Visser, 1972; Visser and Salamon, 1973).

1.5.3.1.1.4 Temperature of thawing.

With either of the thawing methods, the frozen semen is normally thawed at 37°C for 30-60 seconds (Lightfoot and Salamon, 1969a,b; Salamon and Lightfoot, 1969; Salamon, 1970, 1971; Lightfoot and Salamon, 1970; Salamon and Lightfoot, 1970; Visser and Salamon, 1973; Salamon and Visser, 1972, 1974; Maxwell et al., 1984; Maxwell, 1986a; Maxwell and Hewitt, 1986; Evans and Maxwell, 1987; Hunton et al., 1987). Salamon (1968) compared different temperatures of thawing: 5, 10, 15, 20, 30, 40 and 45°C. He found that temperatures from 30 to 45°C gave superior results (using sodium citrate thawing solution) than temperatures in the range between 5 and 20°C. Tasseron at. al. (1977), using a thawing solution of glucose-sodium citrate, reported that thawing at 70°C for 15 seconds gave superior results to the combinations of 60°C for 20 seconds, 50°C for 25 seconds or 37°C for 30-60 seconds.

1.5.3.1.1.5 Advantages and disadvantages of the pellet method.

The advantages of this method are:

* rapidity and low cost (Sorensen, 1986; Evans and Maxwell, 1987), and

* good fertility may be achieved following intrauterine AI (Maxwell et al., 1984; Maxwell, 1986b).

The disadvantages of the method are:

* no positive identification of the sample (Sorensen, 1986).

* the need for centrifugation to re-concentrate the semen or double insemination when a thawing solution is used (Lightfoot and Salamon, 1970; Salamon and Lightfoot, 1970), and

* the storage is less efficient (Sorensen, 1986).

1.5.3.2 Straw method for freezing ram semen.

This method was first described by Cassou (1964) using bull semen and then by Colas (1975) using ram semen. This method involves first the dilution of fresh collected ram semen with an appropriate diluent to a constant concentration or dilution rate. The dilution can be done in two ways: in one step at 30°C by a single addition of diluent containing lactose-egg yolk-glycerol (Nagase and Graham, 1964), tris-based (Wilson, 1988) or milk-based extender (Hunton, 1988) or in two steps, the first at 30°C with no glycerol included in the diluent, and a second addition added at 4-5°C with the rest of diluent (lactose-egg yolk or skim milk or the INRA diluent) containing glycerol (Colas, 1975; Hunton, 1988; Wilson, 1988). With either method the concentration of glycerol is between 2 and 4 %. The semen is thawed at 37-38°C for 30-60 seconds and may be suspended in trisodium citrate or used undiluted (Evans and Maxwell, 1987).

Colas (1975) concluded that the addition of glycerol at 4°C had significant advantages over the addition at 30°C in motility of spermatozoa immediately after thawing and a higher percentage of pregnant ewes. An initial freezing temperature at -55°C gave a low revival of spermatozoa but there were no differences over the range of -75 to -125°C. The best revival was obtained when semen was frozen at -75°C regardless of the time of glycerol addition (at 30 or 4°C). With 4 % of glycerol the fertility results were superior to 2 % glycerol, regardless of the time of equilibration (20 or 150 minutes).

Hunton et al. (1987) and Hunton (1988) reported good results with a "one-step" method for freezing ram semen in straws. They used a tris-glucose-citric acid-egg yolk-glycerol diluent to extended the semen 12 and 24 fold. The authors obtained similar fertility following intrauterine insemination using semen frozen in straws or at a lower dilution rate by the pellet method. Wilson (1988), freezing semen using a tris-based diluent or a milk-based two-step extender, reported post-thawing motility of spermatozoa was better for the semen frozen in the tris than the milk diluent. Moreover, the variability between straws was higher when semen was frozen in the milk-based diluent, and when the author compared two temperatures for freezing (-79°C and -120°C), -79°C gave slightly better post-thawing motility of spermatozoa (Wilson, 1988).

The normal procedure for thawing straw frozen semen is at 37°C, in a water bath (Colas, 1975; Hunton et al., 1987).

1.5.3.2.1 Advantages and disadvantages of the "straw" method.

This method offers:

* positive identification of semen (Hunton et al, 1987; Hunton, 1988; Wilson, 1988),

* easy and efficient storage (Hunton et al, 1987; Hunton, 1988),

* fast thawing (Hunton, 1988),

* accurate measurement and delivery of the semen dose (Hunton et al, 1987),

* compliance with Australian ram semen export regulations (Hamilton and Killeen, 1987; Wilson, 1988), and

* the possibility of establishing freezing rates more accurately within the sample (Watson and Martin, 1975b).

The straw freezing method has some disadvantages:

* the cotton or sealing powder sometimes can be lost (Sorensen, 1986),

* the equipment is expensive and the method is slower than the pellet method (Sorensen, 1986), and

* the survival of spermatozoa may be more variable than with pellet-frozen semen (Hunton, 1987; Hunton et al., 1987).

1.6 Artificial insemination with frozen semen.

1.6.1 Introduction.

Artificial insemination (AI) in the sheep is not a new technique. It has been practiced for over 50 years, originally in the Soviet Union (Evans, 1988) where for many years it has played an important role in flock improvement (Dun and Restall, 1961). Many factors in management are important to consider such as disease control, time of insemination, nutrition of the male and female, the selection of ewes for insemination, semen collection and handling, and the insemination technique itself (Evans, 1988). The use of AI in commercial programs has been limited by the cost involved, the difficulty of identifying superior sires, and low conception rates particularly with frozen semen (cervical insemination) (Salamon, 1976; Maxwell and Hewitt, 1986). The latter problem is probably due to inability of frozen-thawed semen to penetrate the cervix and has been overcome by the use of intrauterine insemination by laparoscopy (Killeen and Caffery, 1982; Maxwell et al., 1984; Maxwell, 1986b; Maxwell and Hewitt, 1986).

Since the efficiency of using frozen-thawed semen has until recently been low, inseminations have been performed with fresh diluted semen, using the vaginal and cervical methods. The development of intrauterine insemination by laparoscopy has now made possible the use of frozen-thawed ram semen, increasing conception rates (Walker, 1984) and making its use very efficient and economical (Maxwell et al., 1984).

Some of the advantages for the use of AI are:

1) AI can increase the rate of genetic improvement in the production of livestock by allowing more widespread use of superior sires and expediting progeny testing to increase the accuracy of identification of superior sires (Tervit et al., 1978; Davis et al., 1984a; Maxwell and Ponzoni, 1987).

2) AI makes possible better use of crossbred livestock; e.g. in pigs this is important to achieve heterosis to improve grade livestock (Jones, 1971).

3) AI permits hybridization between species which do not mate normally (Jones, 1971).

4) AI allows the use of incapacitated or oligospermic males (Dun and Restall, 1961), although this has been limited because infertility or subfertility is rarely due to a single factor (Sherman, 1965).

5) AI has allowed the prevention and eradication of several venereal infectious diseases (Maxwell, 1984).

1.6.2 The evolution of AI: some methods used before intrauterine insemination.

Several techniques were used for AI in sheep with fresh and frozen-thawed semen before the development of laparoscopy. Such methods include the vaginal method or "shot-in-the-dark" (Fairnie and Wales, 1982), and the cervical method known as the"over the rail" technique (Salamon, 1976).

1.6.2.1 Vaginal insemination or "shot-in-the-dark".

This was developed in the U.S.S.R. as the most simple AI method (see Maxwell, 1984). It involves the simple deposition of fresh diluted semen (usually in large amounts) deep into the vagina without attempting to locate the cervix (Maxwell, 1984) and gives relatively good results with fresh diluted semen (Fairnie and Wales, 1982; Tervit et al., 1984; Maxwell and Hewitt, 1986). However, there have been some reports that conception rates are lower than for cervical insemination, even with large doses of semen (Kerton et al., 1984; Rival et al., 1984). The method is deficient using frozen-thawed spermatozoa (Tervit et al, 1984; Maxwell and Hewitt, 1986).

1.6.2.2 Cervical or "over the rail" insemination .

Cervical insemination probably is the most widely used method of AI and involves the deposition of fresh or frozen-thawed semen into the first fold of the cervix using a plastic pipette, speculum and head lamp. It is called also "over the rail" because the ewes usually are suspended by the hindquarters over an elevated rail (Salamon, 1976). This technique is usually more efficient with fresh than with frozen semen (Evans, 1988). The problem with cervical insemination with frozen-thawed semen has been studied by Lightfoot and Salamon (1970), who concluded that the cervix is the first barrier in the spermatozoa transport to the site of fertilization. Thus they found that it was important to establish a good initial cervical population of spermatozoa, from which spermatozoa progress to the fertilization site. This requirement is particularly fulfilled by using a high inseminate dose or double insemination (Lightfoot and Salamon, 1970; Salamon and Lightfoot, 1970).

Although good results have been obtained on occasions following cervical insemination of ewes with frozen-thawed semen (Salamon, 1962; Salamon and Lightfoot, 1970; Salamon, 1971; Visser and Salamon, 1974; Colas, 1975; Maxwell et al., 1980; Mekonnen, 1988), these have been obtained by preselection of semen, reconcentration and the use of very high doses of spermatozoa and/or double inseminations. Conception rates of 10 to 40 % are much more common (First et al, 1961; Salamon, 1967; Salamon and Lightfoot, 1970; Tervit et al., 1978; Maxwell and Butler, 1984; Tervit et al., 1984; Maxwell and Hewitt, 1986) which confirms that cervical insemination with frozen semen is less efficient than intrauterine insemination. The minimum cervical inseminate dose for maximum fertility is believed to be about 125×10^6 spermatozoa (Salamon, 1962). Some of the values achieved by this method with frozen semen are presented in Table 1.6.

Table 1.6. Some results of pregnancy rates following single cervical insemination with frozen-thawed semen.

No. of Sperm x 10^6	Time of insemination hours after onset oestrus	Fertility rate (%).	Reference
60 to 75 **	48 to 54*	21d	Tervit et al. (1978)
60 to 75**	48 to 54*	18d	Tervit et al. (1978)
90	12 to 14	25.3d	Visser and Salamon (1974)
85 to 95	12 to 13	37.4d	Visser and Salamon (1973)
90 to 150**	12	6.1cd	Salamon (1967)
90 to 150**	12	4.2cd	Salamon (1967)
100	12 to 14	18.4a	Maxwell and Hewitt (1986)
100	55*	18.8b	Maxwell and Hewitt (1986)
120	1 to 15	18.2cd	Salamon and Lightfoot (1970)
120	15 to 25	28.3cd	Salamon and Lightfoot (1970)
150	12	12.5d	First et al.(1961)
150 to 160	12 to 14	42.9d	Salamon (1971)
160**	1 to 27	50cd	Lightfoot and Salamon (1970)
180	12 to 14	32d	Visser and Salamon (1974)
200	12 to 14	29d	Maxwell et al. (1980)
213	12	30d	First et al.(1961)
450	13	38.6d	Tervit et al. (1984)
450	50 to 60*	52d	Maxwell et al. (1980)
500	----------	17.8cd	Salamon and Lightfoot (1970)
500	12	17d	First et al.(1961)
600	55*	42.2b	Maxwell and Hewitt (1986)
1500	----------	41.3cd	Salamon and Lightfoot (1970)

(*); Hours after sponge removal, (**); No. motile sperm. Fertility was recorded by (a); ultrasound 40 days after insemination, (b); Slaughter 50 days after insemination, (c); non return 40 to 50 days after insemination, (d); lambing.

1.6.3 Intrauterine artificial insemination with frozen-thawed semen.

Because poor results have been obtained with cervical insemination, even deep in the cervix (Salamon, 1976), the need for depositing the semen into the uterus was evident. Some of the attempts have been done by Fukui and Roberts (1976, 1978) and more recently by Halbert et al. (1990) with the deposition of semen into the uterus by the forceful entrance through the cervix. Such techniques are not practical because it is time consuming to localize the cervix, and difficult to pass through it. Furthermore, the restraint needed requires experience and trained personnel and may be stressful for the ewe. More field trials are needed to prove its application in practical conditions.

Another approach to intrauterine insemination involved a full surgical laparotomy. This is also impractical because of the effect of the major surgery and the possibility of increased

embryonic loss (Salamon and Lightfoot, 1967; Mattner et al, 1969). The surgical intervention, involving opening the abdominal wall and the direct injection of semen into either the fallopian tubes or uterine horns, has been shown to give a high fertilization rate but subsequent lambing performances have been poor (Salamon and Lightfoot, 1967; Mattner et al., 1969; Killeen, 1969; Killeen and Moore, 1971). Salamon and Lightfoot (1967) and Mattner et al. (1969) did not provide any evidence of the fate of eggs fertilized by surgical insemination in their studies. Killeen and Moore (1971) provided evidence that the low lambing rates following surgical insemination were not due to fertilization failure. Their findings also suggested that the low lambing performances observed following surgical inseminations by laparotomy may be due to abnormal transport of the eggs resulting from surgical interference.

A technique for intrauterine insemination in sheep with the aid of laparoscope was first reported by Killeen and Caffery (1982), making the use of frozen-thawed semen practical, economical and efficient (Kelly and Allison, 1976). The method was faster and could reduce the risk of adhesions and infection compared with the laparotomy method (Kelly and Allison, 1976). In summary, the laparoscopic insemination consists of the insertion of two cannulas under local anesthesia into the abdominal cavity. Through one of the cannulas, the telescope with light source is introduced and in the other, the manipulating probe. The uterus is localized and then the manipulating probe is replaced with the insemination pipette which contains the diluted semen. The uterine wall is stabbed with the tip of the pipette to reach the lumen, and the semen is expelled. Both horns are inseminated with the same procedure. The method is fully described by Killeen and Caffery (1982) and Evans and Maxwell (1987).

The technique of laparoscopy has also been used to record the ovarian activity in the sheep and especially ovulation rate by observation of the ovarian structures (Roberts, 1968; Fletcher and Geytenbeek, 1970; Kelly and Allison, 1976; Oldham et al., 1976; Oldham and Lindsay, 1980; Maxwell, 1986a; Nottle et al., 1990) or for pregnancy diagnosis (Roberts, 1968; Walker et al., 1984). Before this method existed, the available methods were slaughter which in many cases was not practical (Oldham et al., 1976) or laparotomy (Lamond, 1963; Cutten, 1970; Killeen and Moore, 1971; Cumming, 1972a; Oldham et al., 1976). There is a modified method by Hulet and Foote (1968) in which a laparotomy incision is performed and a

fast and accurate examination is made *in situ* with the aid of a plastic speculum. This seems to reduce traumatization and bacterial contamination but still requires time and experience. Thus laparoscopy (or endoscopy) is the most appropriate method. There is evidence that this method does not interfere with the reproductive performance of the ewe (Kelly and Allison, 1976; Oldham et al., 1976) but it appears that the genital tract is most sensitive to manipulation between 15 and 28 days after mating, compared with untreated ewes (Oldham et al., 1976).

Since the introduction of the laparoscopic insemination method, much effort has been made to refine it, in particular in choosing the appropriate time for insemination (Killeen et al., 1982; Davis et al., 1984; Maxwell et al., 1984; Evans et al., 1986; Hunton et al., 1986; Maxwell 1986a; Hunton, 1987) and dose of inseminate (Maxwell and Butler, 1984; Salamon et al., 1985; Eppleston et al., 1986; Maxwell, 1986b; Jabbour and Evans, 1991) in order make it more efficient and obtain better results. These two factors are linked as there is evidence to suggest that relatively low doses of semen are effective if they are inseminated at a precise time in relation to ovulation, whereas larger doses are required at other times (Evans, 1988). This is probably related to the viability of the spermatozoa inside the tract and the life span of the ovulated egg (Maxwell et al., 1984; Maxwell, 1986a; Evans, 1988).

1.6.3.1 Influence of synchronization of oestrus and time of insemination on the success of laparoscopic insemination.

During the normal breeding season (generally late January to April/May in Australia) about 6-8 % of the ewes in a flock can be expected in oestrus every day. In order to detect each ewe in oestrus during a traditional AI program, drafting of the flock and subsequent insemination is carried out daily for a period of 19 days (Salamon, 1976). This practice is time consuming, does not allow the best utilization of manpower available, is not efficient and raises difficulties in the management of the flock. Thus, laparoscopic AI is almost always used in conjunction with synchronization of oestrus along with stimulation of ovulation, so that all the females can be inseminated over a short period (Maxwell, 1984; Evans, 1988).

1.6.3.1.1 Time of insemination.

A number of physiological factors have been studied in relation to this point, trying to find the best time.

Firstly, it is known that ovulation in sheep normally occurs 24 hours after the onset of oestrus (Killeen and Moore, 1970b), but ovulation is also believed to occur about 24 hours (21-26 hours) after the onset of the LH surge (Cumming et al., 1971, 1973) and the coincident surge of FSH (Salamonsen, 1973). These intervals appear to be constant in natural or controlled oestrous cycles, but evidence in cattle suggests that "stress" may extend the interval (Cumming et al., 1976). Moreover, the pre-ovulatory surge of LH does not start immediately at the onset of oestrus (Goding et al., 1969). In extreme cases it is possible for these two events to be completely dissociated, e.g. the first ovulation in the breeding season is not accompanied by overt oestrus. Nutritional, environmental and lactational factors can also dissociate the pre-ovulatory LH surge, and thus ovulation and oestrus.

Once ovulation is completed (24 to 30 hours after the onset of oestrus; Killeen and Moore, 1970c), the eggs remain highly fertile for 12 to 24 hours. On the other hand, frozen-thawed semen survives in the ewe tract maintaining its capacity to fertilize eggs for approximately 18 to 35 hours (Lightfoot and Salamon, 1970) or 48 hours according to Green (1947). Hawk (1983) reported that the retention and viability of frozen-thawed semen is less than for fresh semen. This was supported by Evans et al. (1986) who working with superovulated sheep, concluded that the time of insemination is more important for frozen-thawed semen than for fresh semen, due to the different capacity of survival. Embryo loss in sheep is relatively high even after natural mating (Edey, 1976) and the situation may be aggravated after insemination with spermatozoa subjected to ageing (Salisbury et al., 1976). In studies on the time of insemination, Maxwell et al. (1984) found that embryo mortality is increased when the semen is exposed to ageing and when the insemination is performed at an early stage (24 to 36 hours after sponge removal) before the ovulation occurs. Such effects were not observed when the semen was deposited closer to the time of ovulation (48 to 60 hours after sponge removal). This was supported by Eppleston et al. (1986) who using as low

as 5 million motile spermatozoa inseminated 60 to 66 hours after sponge removal, obtained good fertility results. McKelvey et al. (1985) reported that in their flock the peak of LH occurred about 42 hours after sponge removal for ewes which conceived after AI. Assuming ovulation occurs 21 to 26 hours after the LH surge (Cumming et al., 1971), most of the ewes were expected to have ovulated at 63 to 68 hours after sponge removal, which was later than reported by Maxwell (1986a) who found the mean time of ovulation at 56-60 hours after sponge removal. Therefore inseminations later than 55 hours after sponge removal are preferable and could theoretically still be effective between 60 and 72 hours after sponge removal.

Some authors have reported that good fertility is achieved when the insemination took place earlier in relation to the synchronization treatment. Killeen et al. (1982) and Davis et al. (1984a,b) reported that insemination 48 to 55 hours after sponge removal gave good fertility results. This was supported by Evans et al. (1986) and Jabbour and Evans (1991), who found that insemination at 44 hours after sponge removal gave higher fertilization rates than at 24 and 64 hours after sponge removal in superovulated ewes. The various recommended times for intrauterine insemination are given in Table 1.7.

Table 1.7. Recommended times for intrauterine insemination.

Time of insemination Related to sponge removal	Reference
44 hrs	Jabbour and Evans (1991) 1
44 hrs	Evans et al. (1986)1
48 hrs	Rodriguez et al. (1988)1
48 to 55 hrs	Killeen et al. (1982)2
50 hrs	Hunton (1987)2
50 ± 0.5 hrs	Armstrong and Evans (1984)1
52 to 56 hrs fixed time	Mekonnen et al. (1980)2
50 to 56 hrs	Davis et al. (1984ab)2
50 to 56 hrs	Hamilton and Killeen (1987)2
~~52 to 56 hrs fixed time~~	~~Mekonnen et al. (1980)2~~
after 55 hrs	McKelvey et al.(1985)2
59 to 63 hrs	Hunton (1987)2
59 to 63 hrs	Hunton et al. (1987)2
60 hrs	Maxwell and Hewitt (1986)2
60 to 64 hrs	Salamon et al. (1985)2
60 to 66 hrs	Eppleston et al. (1986)2
60 to 72 hrs	Maxwell (1986a)2
60 to 72 hrs	Maxwell et al.(1984)2

(1) Superovulated ewes with synchronized oestrus, (2) non-superovulated ewes with synchronized oestrus

Insemination at a fixed time of 50 to 52 hours after sponge removal has advantages in operating an AI program at the farm level, as it fits in well with the working schedule (Mekonnen et al., 1980; Evans, 1988).

1.6.3.1.2 Synchronization system.

Various methods for synchronization of oestrus have been developed based either on shortening of the luteal phase of the cycle by injections of prostaglandins (Hearnshaw, 1974; Acritopoulou et al., 1978) or the suppression of the cycle in all ewes by treatment with

progestagen-impregnated sponges for 12 to 14 days (Robinson, 1965). An additional treatment which is extensively used involves an intramuscular injection of PMSG (pregnant mare serum gonadotropin) to synchronize the oestrus and to increase the ovulation (Robinson and Smith, 1967a; Evans and Robinson, 1980; Scaramuzzi, 1984) as well as the conception rate (Robinson and Smith, 1967a). Some observations on the time of sponge insertion and the amount of PMSG are given in Table 1.8.

Table 1.8. Some observations on the time of sponge insertion and dose of PMSG.

Dose of PMSG	Length of sponge insertion	Reference
400 to 800 I.U.	-------	Hamilton and Killeen (1987).
400 I.U.	12 days	Hunton (1987); Maxwell et al. (1984); Maxwell (1986a,b);Maxwell and Hewitt (1986); Hunton et al. (1987); Davis et al. (1984a,b); Salamon et al. (1985); Rival et al. (1984).
500 I.U.	12 days	Mekonnen et al. (1980).
500 I.U.	12 to 14 days	Killeen et al. (1982); Halbert et al. (1990).
400 I.U.	15 to 16 days	Tervit et al. (1978).

It has been shown that ewes treated with progestagen sponges exhibit oestrus within 48 hours of sponge removal (Robinson and Smith, 1967b) and when also treated with PMSG ovulate with a median time of 56 hours (with 95 % fiducial limits, Maxwell, 1986a) and a range of 48 to 72 hours after sponge removal in 85 to 95 % of the treated ewes (Maxwell, 1984). Maxwell (1986a) found more variation in the time of onset of oestrus after treatment (<12 to 72 hours) compared with Evans and Robinson (1980) who reported that the mean time of the onset of oestrus was 43.2 hours (range 30-46 hours) after sponge removal and PMSG injection. With the use of sponges plus PMSG, detection of oestrus is not necessary but ewes should be joined with 10 % teaser rams at sponge removal to help stimulate ovulation, particularly when the

technique is used in the non-breeding season (Pearce, 1983). This practice is currently used by some authors in their AI programs (Maxwell et al., 1984; Maxwell, 1986a,b; Maxwell and Hewitt, 1986; Hunton et al., 1987). Maxwell (1986a) has reported in his experiments that despite the fact that the onset of oestrus varied greatly, the time of ovulation was well synchronized whether or not ewes were exposed to teasers rams. The teased ewes ovulated some 4 hours earlier than ewes kept isolated from rams. Although the trials were run in two different flocks, isolated ewes were detected in oestrus at or after 60 hours, whereas teased ewes had a median time of ovulation at 55.8 hours after sponge removal. It would be expected that the time of ovulation in relation to the onset of oestrus is shortened by PMSG treatment (Killeen and Moore, 1970a; Evans and Robinson, 1980).

Fertility in the first oestrus after sponge removal is lower than in untreated ewes (Robinson and Moore, 1967) but this is improved with the inclusion of PMSG to synchronize the time of ovulation. The same results can be achieved much more cheaply during the breeding season by avoiding the use of PMSG and inseminating the ewes in the second oestrus after sponge removal (Robinson, 1976; Salamon, 1976). The ewes in the second oestrus are still reasonable well synchronized 80-90 % in oestrus over a period of 6-7 days. In a different study, Davis et al. (1985), reported that progesterone therapy for 6 days after intrauterine AI increases lambing rate probably by a reduction in embryo mortality; a similar reaction may also occur with the mild superovulation following PMSG, possibly brought about by increase in the amount of luteal tissue.

Another way to stimulate ovulation is the treatment of ewes with GnRH (gonadotropin releasing hormone) after sponge removal. This does not necessarily increase the number of pregnant ewes, but considerably improves the synchronization of ovulation which is helpful in reducing the number of spermatozoa needed for each inseminate dose (Fukui et al, 1985; Smith et al., 1986; Walker et al., 1986). This technique probably has better application in embryo transfer programs.

1.6.3.2 Inseminate dose for intrauterine insemination.

The use of intrauterine insemination with the aid of a laparoscope has reduced considerably the amount of semen needed for good egg fertilization and lambing rates (Killeen et al., 1982; Maxwell et al., 1984; Davis et al., 1984a,b; Eppleston et al., 1986; Maxwell, 1986b) either for fresh semen (Killeen and Caffery, 1982; Rival et al., 1984; Tervit et al., 1984) or frozen-thawed semen (Killeen et al., 1982; Maxwell and Butler, 1984; Maxwell et al., 1984; Rival et al., 1984; Tervit et al., 1984; McKelvey et al., 1985; Maxwell, 1986a,b; Maxwell and Hewitt, 1986; Hunton et al., 1987).

A linear relationship has been found between the number of spermatozoa deposited by cervical insemination and lambing rates over a range of 28 to 128 x 10^6 spermatozoa (Salamon, 1962). This author predicted that within this range of sperm dose, each additional 25 x 10^6 spermatozoa would increase the lambing rate by approximately 13 %. No improvement was seen as the number of spermatozoa is increased beyond 150 x 10^6 (Salamon and Robinson, 1962). These observations suggest that the relationship is described by an asymptotic model. Such a model describes that fertility increases as the number of spermatozoa is increased from zero; but as the level of fertility increases and approaches the asymptotic or maximum value, the curve relating the fertility to the number of spermatozoa inseminated should flatten, until no further increases in fertility can be gained through increasing the number of spermatozoa. This relationship has also been reported for AI with bull semen by Salisbury and Van Demark (1961) and Saacke (1982). Furthermore, Pace et al. (1981) reported that such threshold responses in fertility can also be applied to other semen traits such as motility of sperm, proportion of intact heads, etc. The same principle may also apply to intrauterine insemination. However, to date good results have been obtained from both high and low doses of inseminate (Evans, 1988). It is necessary, therefore, to determine the appropriate dosage to make the insemination as efficient as possible. Also this variability may be related to the relative time of insemination and ovulation, though the time of ovulation is rarely documented. Some observations on the fertility obtained after using fresh or frozen semen for intrauterine insemination are given in Table 1.9.

Table 1.9. The relationship between dose of fresh and frozen ram spermatozoa and fertility determined at different times after intrauterine insemination.

Dose of spermatozoa x 10^6	Type of semen Fresh/Frozen	Pregnancy Rate %	Time of pregnancy determination	Reference
12.5	Fresh	70	70 Days	Davis et al.(1984ab)
20	"	60	70 days	Davis et al. (1985)
30	"	83	Lambing	Tervit et al. (1984)
40*	"	67	Lambing	Salamon et al (1985)
50	"	52	Non-return	Rival et al. (1984)
0.5*	Frozen	29	Lambing	Maxwell 1986b)
5*	"	50	50 Days	Eppleston et al. (1986)
10*	"	58	Lambing	Salamon etal. (1985)
12*	"	54	30 Days	Walker et al. (1984)
25*	"	56	Lambing	Maxwell (1986b)
40	"	54	Late pregnant	Maxwell et al. (1984)
40	"	52	100 Days	Maxwell and Butler(1984)
40	"	53	Lambing	Maxwell and Butler(1984)
48	"	83	88 Days	McKelvey et al.(1985)@
50	"	60	Non-return	Walker et al. (1984)
50	"	59	Non-return	Hamilton and Killeen (1987)
60	"	38	Lambing	Tervit et al. (1984)
80	"	61	Non-return	Hamilton and Killeen (1987)
100	"	56	50 Days	Maxwell and Hewitt(1986)
>100	"	64	50 Days	Maxwell and Hewitt(1986)
120	"	69	Non-return	Killeen et al. (1982)

(*) Motile spermatozoa in the dose; the rest of the values of dose are expressed as total number of spermatozoa in the inseminate. (@) very low number of ewes inseminated.

1.6.3.3 Site of insemination.

Intrauterine insemination into the lumen in the middle of the uterine horn, has been reported to give better fertility results than insemination at the tip or bottom of the horn (Maxwell,1986b). Since the middle of the horn is the part most readily visualized during insemination, it is the site of choice for deposition of semen. There is some disagreement over the advantage of depositing semen in one or two uterine horns. Evans et al. (1984) reported no differences in fertilization rates when semen was deposited either in both or just one uterine horn, indicating that spermatozoa are capable of travelling from one horn to the other. However, Killeen et al. (1982) and Maxwell (1986b) found that insemination of both horns gave better results, measured as higher non-returns and lambing rates respectively. The reasons are unclear, but there is one opinion that by inseminating two horns the amount of spermatozoa deposited into the tract is larger and so the probability of fertilization is higher. Another reason may be related to the necessity to manipulate the tract to find the ovulated ovary to perform an ipsilateral insemination which affects transport of spermatozoa and/or future embryonic survival (Maxwell, 1986b).

1.7 The purpose of this study.

The aim of this study was to examine several aspects of male fertility which have a direct influence on female fertility such as semen evaluation, processing and freezing, semen production and factors which affect it, the 'male effect' and its influence on fertility and embryo loss, the relationship between objectively measured semen characteristics and fertility, and the possibility of fertility prediction in rams.

A computerised image analysis system was used to examine methods of semen processing and freezing. Improvements of such methods have been hampered by the lack of objective and reliable semen assessment methods. A series of experiments were conducted on the processing and freezing of ram semen in straws and pellets. The cervical and intrauterine methods of artificial insemination were compared using different doses of frozen-thawed semen. Mixing the diluted semen before being drawn into straws for frozen storage was examined to see if the post-thawing semen characteristics could be improved. The ratio of two components of a commonly used diluent for freezing semen in pellets were varied in an attempt to improve the protection of ram spermatozoa and increase fertility following intrauterine insemination. The first values were obtained for fresh and frozen ram semen characteristics as measured by computerised image analysis. The variation of semen characteristics before and after freezing was estimated between rams and between samples within rams, and the minimum number of samples to be assessed to obtain an accurate semen assessment was determined.

The second aim of the study was to examine the 'male effect' on fertility and embryonic mortality in normal rams from different strains, and subfertile rams due to heat treatment or separation of the epididymis, and to study the semen production and quality in such rams.

The effect of the separation of the epididymides from the testes on semen production and quality, and fertility and embryo loss following mating was determined. The effect of

heating the scrotum with a slight increase of temperature but sustained for a long period of time on the semen production and quality and also on fertility and embryo loss was also studied. The 'male effect' was also studied by the mating of normal ewes with groups of rams from different strains. The male effect was then measured as the ram performance, ovulation rate, fertility and the embryo loss.

To examine the relationship between semen characteristics (as assessed by computerised image analysis) and fertility in normal and subfertile rams and from this to determine the most useful semen characteristics for predicting fertility in rams, data on semen characteristics obtained from the experiments conducted in the project were correlated with fertility.

CHAPTER 2. MATERIALS AND METHODS.

2.1 Location and climate.

The semen processing and freezing in experiments 1 to 3 and 6 was conducted at Austbreed Genetic Services, Mount Brian, South Australia. This is located 176 km north of Adelaide in a valley of the Mt Lofty ranges. The climate is Mediterranean with 400 mm annual rainfall of about 80 % winter incidence. Experiment 4 part I (laboratory test), was a joint project with the University of Sydney and the semen was processed and frozen from rams housed at Department of Animal Science, University of Sydney, and University of Sydney Farms, Cobbity, Camden, N.S.W. For experiments 7 and 8 the semen evaluation and/or processing and freezing were performed at the Waite Agricultural Research Institute (Animal Sciences Department), located 7 km south east of Adelaide. Experiments 5 and 10 were conducted at "Turretfield Research Centre" (South Australia Department of Agriculture), located at Rosedale 85 km north of Adelaide on the edge of the Barrosa Valley, with a similar climate and rainfall to Mount Brian. All semen evaluations were performed at the Waite Agriculture Research Institute (Animal Sciences Department).

The fertility trial for experiment 2 was conducted at "Newikia Creek", Mt Brian. The fertility trial in Experiment 4, (part II) was carried out at "Sugarloaf Hill", Robe, in the south east of South Australia, about 350 km south from Adelaide. The field trials for experiments 7 to 9 were performed at "Glenthorne", O'Halloran Hill, which is located approximately 20 km south of Adelaide. The field trials of experiment 10, were also carried out at Turretfield Research Centre.

2.2 Source of sheep.

The animals used came from different sources, but the were all medium wool Merino sheep. In experiment 2, the sheep came from the various families of the Collinsville stud of

South Australia, were 3.5 to 6 years old, and managed under grazing condition. The animals for experiment 7 to 9 were obtained from Martindale Holdings, which was established in 1950, based on Peppin bloodlines from Wanganella in New South Wales, and is now managed by the University of Adelaide. The animals used for experiment 10 came from the Turretfield Merino Resource Flock which was set up in 1988. Each year 12 sires are sampled at random from each of four South Australian studs, and mated with 40 randomly allocated ewes. This ewe flock is based on 1920 South Australian Merino sheep representative of the Collinsville and Bungaree family groups. The Collinsville family group is represented by the 'Collinsville' and 'Southrose' studs and the Bungaree group is represented by 'East Bungaree' and 'Anama' studs. The animals for experiment 2 and 4 part II were adult Merino ewes bred on the properties Newikia Creek and Sugarloaf Hill respectively.

2.3 General management of the animals.

All animals were randomly divided into groups of different sizes, according to need, and managed as homogeneously as possible to avoid variations due to environment or other conditions which could influence results. All the animals were identified by plastic ear tags, stenciled side numbers and colour spray marks.

The animals were regularly checked for condition and some were eliminated and replaced when problems were detected e.g. legs problems, weak or poor condition, pink eye etc. When the animals were kept until lambing, they were drenched and vaccinated before pregnancy detection at 65 days after insemination or natural mating.

2.4 Semen collection.

For most of the experiments, the collection of semen was performed by artificial vagina and for experiment 4, chapter 7 by electro-ejaculation.

(a) Semen collection by artificial vagina.

The method was performed as described by Frank (1950), Laing (1955) Mattner and Voglmayr (1962), Watson (1978) and Evans and Maxwell (1987). The rams were trained for about two to three weeks before the experiments started. The training consisted first of the introduction of the rams to the shed and hand feeding. Once the rams had became accustomed to the housing conditions and the presence of humans, ewes exhibiting strong oestrus behavior were introduced to the rams. Natural mounts were allowed first without the presence of humans and then in presence of an operator, so the rams were confident. After several services, the operator approached on the right side of the ram and started to use the artificial vagina for semen collection.

Two protocols were randomly used for treatment of the ewes to induce oestrus so they could be used as teasers for semen collection are:

Protocol A.
Day 0, 20 mg progesterone*
Day 2, 15 mg progesterone
Day 4, 15 mg progesterone
Day 5, 200 µg oestradiol
Day 7, ewes showing estrus.

Protocol B
Day 0, Twice daily 200 µg of oestradiol**
Day 1, Twice daily 200 µg of oestradiol
Day 3 up to day 7; ewe showing estrus.

(*) Progesterone (4-Pregneno-3, 20-dione), Sigma Chemical Company.
(**) Oestradiol Benzoate, Intervet Pty. Ltd., Australia.

b) Semen collection by electro-ejaculation.

This method was used in experiment 4 chapter 7 because of the lack of time for ram training and the number of rams used. For this method a bipolar rectal electrode was used with a power source and a variable voltage device. The procedure was very similar to the one described by Blackshaw (1954) and Evans and Maxwell (1987) which involved the restraint of

the ram on a table. The animal was first held in a vertical position by ties around the front and rear legs, then the table was moved to a horizontal position so that the animal was restrained on its side. The penis was exposed from the prepuce by straightening the sigmoid flexure, and secured by a piece of gauze behind the glans penis. The penis was introduced in to a warm clean glass or plastic tube and held with one hand. Then the rectal probe was lubricated with paraffin and inserted into the rectum. The probe was pressed towards the floor of the pelvis and several stimuli were applied, starting from very low voltage to the maximum of 12 volts, while with the other hand the penis was massaged in forward direction between the stimuli. The intervals of stimuli and increments were similar to those reported by Cameron (1977).

For both methods of collection, the semen was collected in a dry tube, and kept warm and away from light, water and other factors which may affect the semen viability (Evans, 1987; Evans and Maxwell, 1987). The semen was processed and assessed within 10 minutes of collection.

2.5 Semen processing and freezing.

Semen was processed and frozen by two methods: the pellet and straw method.

2.5.1 The pellet method.

2.5.1.1 Diluent for the pellet method.

The diluent for the pellet method was the same as first published by Salamon (1976) and consisted of a tris-glucose-egg yolk medium with glycerol as a cryoprotective agent (Diluent A). The components of Diluent A are presented in Table 2.1.

Table 2.1. The composition of the diluent for freezing ram semen by the pellet method (Diluent A).

Component	Dilution rate 1+4
Tris (hydroxymethyl) amino-methane	3.634 g
Glucose	0.500 g
Citric acid (monohydrate)	1.990 g
Egg yolk	15 ml
Glycerol	5 ml
Penicillin	100, 000 I.U.
Streptomycin	100 mg
Glass distilled water to	100 ml.

The chemicals were first dissolved in an appropriate volume of glass distilled water (65 ml) and mixed with the sodium penicillin (1000 I.U) and streptomycin (1 mg per ml of diluent). Then the glycerol and the egg yolk (previously separated and dried off on filter paper from the egg white) were added and mixed, and more water was added to make up the final volume. The diluent was then placed in a water bath at 30 to 32°C until used.

2.5.1.2 Semen dilution and freezing.

The semen was diluted (30-32°C) with Diluent A at a ratio of 1:4 (semen:diluent), so that the pellets contained reasonable numbers of spermatozoa and were easy to split in order to achieve some of the inseminate doses recommended for the intrauterine insemination with frozen-thawed semen (Maxwell et al., 1984; Eppleston et al., 1986; Evans and Maxwell, 1987). The diluent was always added to the semen and never in the opposite way, and the addition was done very slowly to avoid any thermal or osmotic shock. The diluted semen and some more diluent in a different test tube with a Pasteur pipette were placed in a beaker containing water from the water bath and cooled down to 4 to 5°C over 1.5 to 2 hours

(Salamon and Visser, 1972; Salamon, 1976; Evans and Maxwell, 1987) in a cold room or refrigerator. The diluent in the second test tube was used to rinse the pipette maintaining the same temperature as the diluted semen, to avoid cold shock.

For freezing of semen in pellets, a block of dry ice (solid carbon dioxide, -79°C) with a smooth surface was used. Several lines of holes were engraved in the surface using a stainless steel device sterilized with 70 % alcohol. The pelleting operation was done inside a cold room (4 -5°C) with iced water in which the beaker containing the diluted semen was placed to maintain the correct temperature. Then the pipette was rinsed with the cooled diluent and filled with diluted semen. Volumes of 0.1 ml (4 drops) were pipetted in quick succession into the holes on the dry ice surface, allowed to remain there for 3 to 4 minutes, and then transferred into a previously identified goblet or plastic tube containing liquid nitrogen. The pellets were stored in liquid nitrogen (-196°C) until thawing for assessment and/or insemination.

2.5.2 The straw method.

2.5.2.1 Diluent used.

The semen was frozen in straws using the method of 'one step' dilution and cooling reported by Hunton et al. (1987) and the following diluent: 360 mM tris, 33.3 mM fructose, 113.7 mM citric acid, 18 % (v/v) egg yolk and 6 % (v/v) glycerol (Diluent B).

For the dilution of semen, the same precautions were taken as for pellets, but the dilution rate changed. The semen was normally diluted to a concentration of 200 million spermatozoa per millilitre, according the following formula:

First the semen was manually evaluated for motility under a microscope and concentration of spermatozoa by photocolorimetry and the concentration value was substituted in the formula as follows:

$$\text{Diluent volume required} = \frac{\text{Semen Vol. X Concentration**}}{\text{number of spermatozoa needed per ml (200 K)*}} - \text{Semen Vol.}$$

(K)* Constant used in this case which gives 200 x 10^6 spermatozoa/ml or 50 x 10^6 spermatozoa/ straw; (**) Optical reading transformed to a concentration of spermatozoa/ml (calibrated graph).

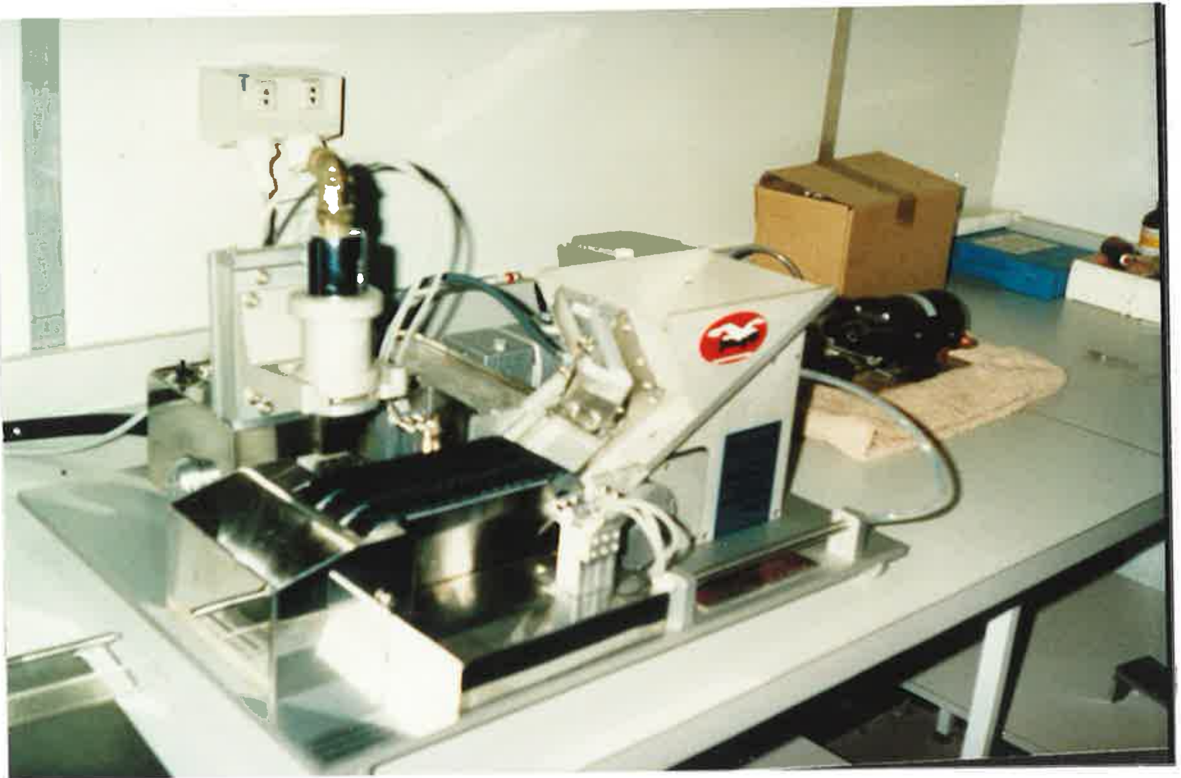
The semen was diluted as described for the pellet method (using Diluent B), and cooled down in the same way.

2.5.2.2 Freezing procedure.

While the semen was cooling down, the straws to be used were printed with the sire identification and cooled to the same temperature as the diluted semen.The diluted and cooled semen was drawn into the 0.25 or 0.50 straws (IMV, L'Aigle, France) manually with Cassou equipment (Cassou, 1984) or three straws at the time with the aid of an automatic filling and sealing machine (Plate 1) (IMV, MRS-3, Automatic Filler Sealing Machine, L'Aigle, France). A small air space was left in each straw to avoid cracking on freezing. For freezing, the straws were place horizontally on a cold rack (5°C), lowered into liquid nitrogen vapor (-120°C) for 15 minutes (Plate 2), and then plunged into liquid nitrogen. Then the straws were carefully packed into goblets and stored in liquid nitrogen.

Plate 1. The automatic filling and sealing machine (IMV, MRS-3, Automatic Filler Sealing Machine, L'Anigle, France).

Plate 2. Procedure of freezing straws in static liquid nitrogen vapor (- 120 °C).



2.6 Semen assessment.

Fresh and frozen-thawed semen was evaluated by two methods: the traditional manual assessment for fresh semen and by computerised image analysis (Hamilton Thorn Motility Analyzer, Version 6.03-7.0 Daintree Ind., Victoria; HTM).

2.6.1 Traditional manual semen analysis.

Several semen traits were evaluated as follows:

1) Volume.

Once the semen was collected the volume was recorded straight from the graduated collecting tube (Bishop et al., 1954; Evans and Maxwell, 1987).

2) Motility.

Two types of motility were assessed: a) mass motility or wave motion and b) individual motility.

a) Mass motility or wave motion.

This motility was assessed by placing a drop of fresh semen on a clean, pre-warmed (32°C) microscope slide without a coverslip, and the wave motion was observed at low power (40x) under a microscope (Bishop et al., 1954; Hulet and Ercanbrack, 1962). The slide was kept warm by a thermostatically-controlled warm stage on the microscope. The estimation was performed based on the vigour of the wave motion and the overall activity. It was scored on a scale of 1 to 4+ (very similar to the one reported by Evans and Maxwell (1987)).

b) Percentage of motile spermatozoa.

Motility was determined by placing a drop of semen on a clean, dry, pre-warmed microscope slide (normally the same as for mass motility to save time) and covered with a coverslip (Hulet and Ercanbrack, 1962; Hulet et al., 1965; Linford et al., 1976). This time a greater magnification was used (400x or 1000x) to observe individual spermatozoa. The observations were performed on several fields around the sample and the proportion of the spermatozoa moving forward was assessed as a percentage. Sometimes the sample was too dense, so the examination was repeated until a good view was achieved.

3) Concentration of spermatozoa.

The concentration of spermatozoa was measured with the aid of a haemocytometer (Improved Neubauer cell; Weber, England) and a photocolorimeter. The procedure was a modification of that originally described by Willet and Buckner (1951) then by Laing (1955) and modified by Zaneveld and Polakosky (1977). Semen was killed with an spermicidal solution of 10 % Formaldehyde (AnalaR®, BDH Chemicals, Australia Pty.Ltd., Vict.) in 0.9 % of saline solution (Travenol®, Travenol Laboratories Pty. Ltd., N. S. W.). The semen was diluted 1:500 (Semen:spermicidal solution) and a small drop was placed on both sides of the haemocytometer chamber between the counting slide and the coverslip. The sample was left for 3 minutes to settle, and then 5 large squares in a diagonal direction from both sides were counted and averaged. If the difference between the counts on each side exceeded 10 %, the estimate was repeated. The number of sperm counted in five large squares represents the number in 80/4000 cubic millimetres of diluted semen.

When the concentration of spermatozoa was measured by photocolorimetry, the method used was the same as originally described by Emik and Sidwell (1947) and explained in detail by Foote (1972), Sorensen (1986) and Evans and Maxwell (1987). The semen was collected and diluted with a spermicidal solution (at a known dilution rate 1:200) which did not interfere with operation of the apparatus. Measurements of optical density were made using a light of a certain wave length (540 nm) which were then transformed to concentration per millilitre by a calibrated

graph. The photocolorimeter readings were calibrated with both haemocytometer counts and with HTM readings. The calibration consisted of diluting semen at different rates, so a wide range of concentrations were obtained. Then, the diluted semen was measured by both the photocolorimeter and the HTM and the results were recorded. Then the results from the photocolorimeter were related to the concentration by haemocytometer and HTM by regression.

4) Live and dead ratio and morphology of sperm.

For the determination of live and dead sperm and the study of morphology, a special eosin-nigrosin stain was prepared as follows:

Eosin (water soluble).................. 1.67 g
Nigrosin (Water soluble).............. 10.00 g
Sodium citrate.2 H_2O.................. 2.90 g
Distilled water to 100 ml. (Campbell et al., 1956; Evans and Maxwell, 1987)

The stain was prepared and mixed with boiling water , then cooled down and filtered before use. The stain was warmed up to semen temperature and kept at that temperature until used within 0.5 hour. Two or three drops of semen were mixed with 0.25 ml (about 8 drops) of stain and allowed to react for 3 to 5 minutes. Then a small drop of mixture was placed on a microscope slide, and with the aid of a long coverslip spread along the slide to form a thin film. The smear was then allowed to dry and the evaluation was performed under 400x magnification (Bishop et al., 1954; Evans and Maxwell, 1987). A total of 200 spermatozoa were counted per slide as either stained or not. The criteria for live or dead sperm was determined as follows: transparent or white heads were criteria for live sperm cells and pink or coloured head were criteria for dead sperm cells (Mayer et al., 1951; Swanson and Bearden, 1951; Sorensen, 1986).

For the study of morphology, the same procedure was applied and the traits recorded were: normal sperm, headless sperm, coiled and bent tails (termed abnormal tails). A total of 200 cells were counted (slightly higher than recommended by Salisbury and Mercier (1945)), and the results were expressed as a percentage of normal and abnormal cells.

2.6.2 Computer assisted semen analysis.

A Hamilton Thorn Motility Analyzer (HTM; see section 1.2.3.6, Plate 3) was used. The machine was set up as presented on Table 2.2.

Table 2.2. Set up of the analyzer and main gates for fresh and frozen ram semen.

Main Gates	Fresh semen	Frozen semen
Analyzer set up		
Temperature	37.0 °C	37.0 °C
Chamber	Makler (10 µm)	Makler (10 µm)
Image type	Phase Contrast	Phase Contrast
Field selection	Manual selection	Manual Selection
Calculate ALH(1)	yes	yes
Main gates *		
Frames	20	20
Frames acquisition	25/second	25/second
Minimum contrast (pixels)	4	5
Minimum size (1x pixels)	7	7
Lo/Hi size gates (pixels)	0.3, 2.2	0.4, 2.2
Lo/Hi intensity gates (pixels)	0.3, 2.2	0.4, 1.2
Non motile head size (pixels)	6	6
Non motile intensity (pixels)	125	125
Medium VAP(2) value	95	95
Low VAP value	10	10
Slow cells motile	no	no
Threshold STR(3)	60	60
Dilution (Semen:Extender)	1:50	1:10 and 1:50**

(1); Lateral head displacement, (2); path velocity in microns per second, (3); straightness, (*) To calibrate the main gates, some screens were evaluated with the playback function which enables the operator to examine if all non-motile and motile cells have been identified by the machine. With the same function, debris can be avoided (Gill et al., 1988).(**) 1:10 for semen frozen in straws and 1:50 for semen frozen in pellets.

The procedure for the semen analysis depended on whether the semen was fresh or frozen.

a) Fresh semen. Fresh semen collected either by artificial vagina or electro-ejaculation was kept in a water bath at 32°C and assessed within 5 to 10 minutes after the semen collection. A 100 µl sample from the ejaculate was diluted to 1:50 with Dulbecco's Phosphate solution (Dulbecco's phosphate buffered saline solution, single strength, CSL, Victoria) at 32°C to avoid thermal shock. The machine (already switched on and with the stage warm) was set up for fresh semen and a 10 µl sample was drawn from the diluted semen and placed into a Makler counting chamber (Palte 4) (Sefi-Medical Instruments, Israel) with 10 µm depth, and covered with a coverslip. The Makler chamber was placed in the automatic temperature controlled stage of the HTM. Normally each sample was analyzed to count not less than 600 cells, which could be counted in 6 to 9 different fields selected among the ones which were clean from debris (Plate 5). The cell counting depended upon the concentration of the sample. Sometimes when the sample was not concentrated, the number of fields were increased to achieve the required number. The machine counts automatically from the first to the last chosen fields and the last one appears on the screen for the "playback" function which allows a check to be made to see if the sample was assessed correctly (Mahony et al., 1988; Hamilton, 1989). The machine marks the motile spermatozoa with a blue dot and the non motile ones with a red dot, thus when the last field is presented all cells should be selected with one of the dots. If all of the cells are dotted, the machine is selecting all cells properly and no more adjustments are needed; when some of the cells were not dotted, then a slight adjustment on the main gates was needed to select all cells in the sample and make the assessment as accurate as possible. When the sample was assessed and the required number of cells achieved, then results were saved and printed out as a summary at the end of the session. The results were presented on the screen (Plate 6) and then printed or recorded on a computer attached to the HTM.

Plate 3. The HTM machine.

Plate 4. The Makler counting chamber (Sefi-Medical Instruments, Israel).

Plate 5. A view of the screen in the HTM. The stage position (inside the machine) and temperature are presented in the upper left and right corners of the screen respectively. One of the multiple fields chosen for computerized semen analysis is shown. Some motile and non-motile sperm cells and two brighter spots of debris can be observed. In the bottom of the screen are the main functional keys for the analyzer.

Plate 6. In this Plate the first page of results from the HTM is presented. In the first section of the screen, the total and motile concentration of spermatozoa per ml, the concentration of progressively motile spermatozoa per ml are presented in the first column. In the second column, the same information is presented but per ejaculate if the volume of the ejaculate is recorded, and in the third column the actual number of cells counted for each of the above semen characteristics is presented.

In the second section of the screen, the percentage of motile spermatozoa, the percentage of progressively motile spermatozoa and the path velocity (VAP) are shown. The third section of the screen the motile concentration is divided according to certain speed thresholds in four groups of sperm cells: percentage of rapid, medium, slow and static spermatozoa.

STAGE AT 18.0 MM
37.5°C
START SCAN
ADD SCAN
SELECT 0
SHOW SETUP
DATA PAGE

DATA SUMMARY
CONC. (M/ml) SAMPLE (M) COUNT
WHO
TOTAL 1069.6 1069.6 99
MOTILE 54.0 54.0 5
PROG. 10.8 10.8 1
20M ml
MOTILITY 5 %
PROGRESSIVE 1 %
VELOCITY (VAP) 62 MIC/SEC
50%
VELOCITY
4 RAPID 1 %
3 MEDIUM 4 %
2 SLOW 1 %
0-1 STATIC 94 %
25%

b) Frozen semen: Regardless of the method of freezing, most of the procedures were the same as for fresh semen. The dilution rate was different and in some tests an incubation period for 6 hours at 37°C was used in which the assessment of the semen was performed just after thawing, and after 3 and 6 hours of incubation. When semen was frozen in pellets, one pellet was thawed in a clean and dry tube which was shaken vigorously in a water bath at 37°C (Killeen and Caffery, 1982; Maxwell et al., 1984; Maxwell, 1986) then diluted 1:50 at 32°C with the same solution as for fresh semen and assessed by the same procedure. When semen was frozen in straws, the straws were thawed by placing them straight into the water bath at 37°C for 1 minute (Colas, 1975; Watson and Martin, 1975b; Hunton et al, 1987) drying them off and cutting both ends; the semen was then placed in a dry tube and diluted 1:10 with the same solution as mentioned above, and kept at 32°C for assessment by the standard method.

The semen traits assessed were: TOTAL, MOTILE, PROGR, MOT, Mean VSL, mean VCL, mean VAP, mean ALH, mean STR Straightness (or linear Index), mean LIN %, mean LIN, RAPID, RAPID%, MEDIUM, MEDIUM %, SLOW, SLOW % , STATIC, STATIC % and the standard deviations of some of these traits in which an ending ST is added to the abbreviation (Gill et al., 1988; Hamilton Thorn Research, 1989; Pedigo et al., 1989). All abbreviations and characteristics are fully described in para 1.2.3.6.1 and Table 1.2.

2.7 Method of synchronization of oestrous.

The synchronization of oestrus for artificial insemination was accomplished by the insertion of intravaginal progestagen-impregnated sponges (30 mg Chronogest, Intervet, Aust. Pty Ltd., NSW) for 12 days and an injection of PMSG (400 i.u., Folligon, Intervet, Aust. Pty Ltd., NSW) at sponge removal. The ewes were run with 4 or 10 % of teaser rams fitted with harnesses and crayons for oestrus detection from the time of sponge removal (Radford et al., 1960; Maxwell et al., 1984; Maxwell, 1986a,b; Maxwell and Hewitt, 1986; Hunton et al., 1987).

2.8 Insemination techniques.

Intrauterine insemination with the aid of a laparoscope was used in most experiments (Plate 7), with cervical insemination also used in experiment 2 chapter 3 and natural mating in experiments 7 chapter 4, 9 chapter 6 and 4 chapter 7.

2.8.1 Intrauterine insemination with frozen-thawed semen.

This technique was performed as first described by Killeen and Caffery (1982) with slight modifications as recommended by Evans and Maxwell (1987). Semen was thawed as described in para 2.4.2. section b, and kept at 32°C for no longer than 5 to 10 minutes before insemination. Ewes allocated to intrauterine insemination were deprived of food and water for 24 to 36 hours before insemination. The place in the shed in which the inseminations were performed was cleaned and the ewes were prepared for insemination by assistants working in a different area.

The inseminations were performed 50 hours after sponge removal with a dose of 50×10^6 spermatozoa in each uterine horn or with some fixed doses of motile spermatozoa. The concentration of spermatozoa was estimated previously with the HTM and the number of spermatozoa controlled by varying the volume of the dose. The site of insemination was in the middle of the uterine horn (Maxwell, 1986b) and both uterine horns were inseminated as recommended by Killeen et al. (1982) and Maxwell (1986b) and described by Evans and Maxwell (1987).

Plate 7. Intrauterine insemination. The Plate shows the moment of the insertion of the insemination pipette into the lumen of the uterine horn. The next step is to depress the plunger of the syringe (with the aid of an assistant) to expel the semen into the uterine horn.

2.8.2 Cervical or "over the rail" insemination.

The method used was the same as reported by Lightfoot and Salamon (1970), Tervit et al. (1984), Maxwell and Hewitt (1986) and Rodriguez et al. (1988) and described by Evans and Maxwell (1987). This technique consisted of the deposition of frozen-thawed semen to a depth of up to 3 cm into the cervix. The cervical insemination was performed 55 hours after sponge removal, with ≥ 200 x 10^6 motile spermatozoa (Lightfoot and Salamon, 1970; Salamon, 1976; Tervit et al., 1978; Tervit et al., 1984; Maxwell et al., 1980; Maxwell and Hewitt 1986; Evans and Maxwell, 1987).

2.8.3 Natural Mating.

Natural mating was used in experiments 7 chapter 4, 9 chapter 6 and 4 chapter 7. For experiments 7 and 9, 50 ewes were randomly allocated in individual paddocks to each ram. Harnesses with distinctive coloured crayons (Radford et al., 1960) were placed on the rams for oestrus and mating detection, and the raddle marks were checked each day for 35 to 40 days. For experiment 4 chapter 7, 40 ewes were randomly allocated to each ram without the use of harnesses.

2.9 Ovulation rate.

Ovulation rate was calculated on a per ewe present basis in experiment 9 chapter 6. The ewes marked by the sire were recorded and drafted from the group and deprived immediately of food and water to enable observation of the ovulation rate 24 hours later. The method of laparoscopy used was as described by Oldham et al.(1976). The number of corpora lutea (CL) and follicles (FL) on each ovary were recorded.

2.10 Pregnancy diagnosis.

Pregnancy diagnosis was performed either at 17 days after insemination or natural mating by measuring the peripheral plasma level of progesterone by radioimmunoassay (RIA), or later at 50 to 65 days after insemination by ultrasound examination.

2.10.1 Pregnancy diagnosis by peripheral progesterone level assay.

Blood samples for progesterone determination as a pregnancy test, were obtained 17 days after insemination or natural mating. They were obtained via jugular venipuncture either by vacutainer (100 x 16 mm) with 19G needle (Vacutainer system, Becton Dickinson, USA.) impregnated with heparin or by using a 19G needle attached to a 10 ml syringe and heparin plastic tubes. The samples were then kept in ice, and centrifuged at 2000 rpm for twenty minutes. The plasma was pipetted off and placed in a 12 x 75 mm identified glass tubes, and stored at -20°C until required for assay. All chemicals used were analytical grade and obtained from Ajax Chemicals (Sydney, New South Wales, Australia) unless otherwise stated. This technique has been used by several workers such as Bassett et al (1969), Thorburn et al. (1969), Robertson and Sarda (1971), Robertson (1977) to study the levels of the hormones during the oestrus cycle or as an early pregnancy test and the methodology was the same as reported by D'Occhio et al. (1988).

2.10.1.1 Phosphate buffers.

A) 0.01 M phosphate-buffered saline (PBS), pH 7.5.

2.84 g Na_2HPO_4 (anhydrous)

0.74 g EDTA

2.0 g Sodium Azide (NaN_3)

16.36 g Sodium Chloride (NaCL)

pH adjusted to 7.5 with 1N HCL and made up to 2 l. with Type 1 water.

B) 0.01 M Gel PBS.

As above with gelatine 4 .0 g in 2 l buffer.

2.10.1.2 Other solutions for procedure.

A) Solvent: Heptane (extraction).

B) Dextran Coated Charcoal (DCC);

6.25 mg charcoal Norit-A

62.5 mg Dextran T 70 in 100 ml PBS.

C) Scintillation fluid;

5.0 g PPO (2,5 - Diphenyloxazode) (Scintillation grade, United Technologies Packard) in 1 lit. toluene.

2.10.1.3 Stock solution and standards.

Stock solution = 1 µg progesterone/ml
80 µl in 4 ml gel PBS ....2000 pg/100 µl (Duplicate)
.......1000 pg/100 µl (Duplicate)
.... 500 pg/100 µl (Duplicate)
.... 250 pg/100 µl (Duplicate)
.... 125 pg/100 µl (Duplicate)
.... 62 pg/100 µl (Duplicate)
.... 31 pg/100 µl (Triplicate)
.... 15 pg/100 µl (Triplicate)

NSB 400 µl stock solution in 3.5 ml gel PBS (Triplicate)
Zero gel PBS (Quadruplicate)

Sample or standard: 100 µl

Solvent Heptane:	1 ml
^3H tritiated progesterone:	200 µl (10,000 cpm)
Antiserum:	200 µl.

2.10.1.4 Assay procedure.

Day 1

All the glass tubes (12 x 75 mm) for the standard curve and the samples were labelled. The plasma samples were thawed and 100 µl was drawn from each sample in duplicate; the standard curve was prepared by placing 100 µl of the different standards in tubes, the samples at this stage were kept at 4°C over night or the extractions started immediately.

Day 2

Extraction: 1 ml of heptane was added to each tube which were vortexed for 1 minute. The aqueous phase was frozen in ethanol cooled with liquid nitrogen, and the solvent was decanted into a second glass tube (12 x 75 mm). Next, the tubes in the rack were dried in an air extractor cabinet, by blowing air and heating in a water bath at 37°C (Nitrogen was sometimes used instead of air).

When the tubes were perfectly dry, 200 µl of tritiated progesterone and 200 µl of antiserum were added into each of the tubes which were then gently vortexed. The tubes were left at 4°C over night.

Day 3

Dextran-coated charcoal was added to each tube (200 µl), the tubes were vigorously shaken and left at 4°C for 15 minutes. Then, the tubes were centrifuged at 800 g for 20 minutes at 4°C.

When this was completed, the supernatant was decanted into polyethylene counting vials and 1 ml of scintillation fluid was added to each vial. Then, the vials were securely capped and shaken for 20 to 30 minutes on a mechanical shaker.

The amount of radioactivity was then measured with a beta counter (LKB-Wallac, 1215 Rackbeta II, Liquid scintillation counter).

The ewes were classified as pregnant if the progesterone concentrations were greater than 2 ng/ml which is in the mean of values reported by Bassett et al.(1969) and Thorburn et al.(1969) and the same as reported by Robertson (1977). Intra-and inter-assay coefficients of variation for all radioimmunoassays were < 10% based on duplicate samples, and the sensitivity of the assays was 0.19 ng/ ml.

2.10.2 Pregnancy diagnosis by ultrasound scanning.

The scanner used was a Toshiba Sonolayer L (Sal 32 a, Linear Electronic Scanner) with a transducer probe (PL 508 m, centre frequency) of 5 MHZ. This method was used between 50 and 80 days after insemination or mating. The embryo loss was calculated from the difference between these results and the progesterone results. The method was similar to that described by Lindahl (1969) and Fowler and Wilkins (1984) which can be summarized as follows.

The wool from the belly area and side of the hind legs was removed as close to the skin as possible using standard shearing equipment. The ewes were pulled out from the shute and restrained for scanning whilst lying on their backs in a specially designed cradle. The belly was lubricated with polyunsaturated vegetable oil to improve contact for ultrasonic transmission, and the transducer was applied to the lower part of the belly and moved from side to side directing the beam in different positions and planes so the boundaries of the uteri were defined and their contents examined for pregnancy and sometimes number of foetuses. The scanning was performed under semi-darkened conditions to aid the clarity of images of the screen. A ewe was diagnosed non-pregnant when either of the fetal heart beat or movement were not found. The

pregnant ewes were marked with a distinctive colour spray and were located in the same paddock.

2.11 Ram surgical procedures (hemicastration and epididymis separation).

2.11.1 Hemicastration.

Rams in all treatments were prepared for surgery in a similar manner. All rams were first trained to ejaculate into an artificial vagina and the semen traits were assessed by manual analysis as described in para 2.5.1. The animals were deprived of food and water for at least 24 hours before surgical intervention.

The wool from the scrotum was removed with fine clippers reaching close to the skin. Then the whole area over the wall of the scrotum and up to the escutchion was scrubbed with soap and warm water and sterilized with tincture of iodine in 70 % alcohol.

The animals were then initially anaesthetized with 10 to 20 ml of Pentobarbitone Sodium (60 mg/ml) (Nembutal®, Boehringe Ingelheim, NSW, Aust.). The animal was restrained on a surgical table, and an endotracheal tube was introduced to help breathing. The site of surgery was swabbed with tincture of iodine and 70 % alcohol, and all procedures were undertaken using sterile gloves, surgery gowns and instruments in a clean surgery, with a full complement of aseptic drapes.

An incision, large enough to expose and exteriorise one testis, was made midway down the posterior side of the scrotum, between the two testicles. Two nylon ligatures were tied, one around the spermatic cord and one separately around the cremaster, to remove the left testis and epididymis together with their investments. The hemicastration was performed on each of the assigned rams. When no further treatment was applied to a group of animals, they were termed "Hemicastrated Control" rams (HC).

2.11.2 Separation of the epididymis.

Two groups of rams were hemicastrated as above, then the right testis exposed and the peritoneal covering opened. The membrane was cut with scissors from the caudal region to the caput-corpus junction of the epididymis, as close to the epididymis as possible. The testis and epididymis were then located to allow free access to the cauda of epididymis. The epididymis was then separated from the testis up to the caput-corpus junction, also severing the epididymal-peritoneum membrane attachments. This procedure was accomplished by painstaking and delicate snipping at the connective tissue between the tail of the epididymis and the testis while gently holding the epididymis and peeling it back. All the bleeding was stopped during the process and lymphatic vessels and blood capillaries were ligated.

In one of the groups of rams, the isolated corpus and cauda epididymides were placed outside the ipsilateral peritoneal sac, but maintaining a similar orientation to their original position, within the contralateral peritoneal sac. The two sacs were sutured together proximally and distally. The epididymis and testis were separated by suturing the peritoneal membranes separately. This group was named the "Separate Epididymis" rams (SE).

The final treatment group, termed "Separate Epididymis Pouch" (SEP) rams, were surgically treated in the same manner as the SE, except a pouch of skin was constructed to hold the epididymis in the empty peritoneal sac.

All animals were cleaned up and antibiotic powder was placed liberally inside the scrotum prior to suturing. The suture was covered with antiseptic cream and antibacterial fly repellant spray. They were closely monitored during recovery, and a course of penicillin was administered up to 2 weeks post-operation.

2.12 Statistical analysis.

Because the amount and variety of experiments, several procedures were included:

* Analysis of Variance by contrasts and factorial analysis.

* Chi-square analysis.

* Correlation.

* Repeatability and Coefficients of variance.

* Bartletts test of homogeneity of variance.

The statistical analysis for each experiment is explained in detail in the following sections. The main statistical programs used were the SAS version 6.03 (Statistical Analysis System Institute, 1988) and the Genstat 5 (Reference manual, 1987) and the text by Snedecor and Cochran (1980).

CHAPTER 3. THE VIABILITY AND FERTILITY OF RAM SPERMATOZOA FOLLOWING PROCESSING AND FREEZING.

3.1 Viability of ram spermatozoa following frozen storage in maxi and mini straws at two concentrations.

3.1.1 Introduction.

Ram semen can be frozen in pellets (Salamon and Visser, 1972) or straws (Colas, 1975). There are two sizes of straws: maxi (0.50 ml) and mini (0.25 ml) straws. There has been little investigation on the effect of pre-freezing dilution rate and concentration on the survival of ram spermatozoa after frozen storage in these two sizes of straws. Hunton (1987) and Hunton et al. (1987) found no effect of concentration on post thawing motility or fertility when semen was frozen in mini straws at different concentrations produced by different dilution ratios. The same result was obtained using a constant dilution ratio by Hamilton and Killeen (1987).

Satisfactory conception rates have been obtained when ram semen frozen by the pellet method has been used for the intrauterine AI of ewes (Maxwell et al., 1984; Maxwell, 1986a,b). The use of straws for frozen storage of ram semen has been limited due to poor post-thawing motility of spermatozoa (Maxwell et al, 1980). Recently, however, no difference was reported in the post-thawing motility of spermatozoa or fertility following intrauterine insemination when semen was frozen by either method (Hamilton and Killeen, 1987; Hunton, 1987). Using the straw freezing method, "two step" dilution was originally recommended (Colas, 1975), but "one step" dilution is faster and easier to use (Hunton et al., 1987, 1988; Wilson, 1988).

The use of straws has the advantage of positive identification of each inseminate dose, accurate measurement of the semen dose delivered and efficiency of storage (Hunton et al., 1987). This technique also allows the export and international exchange of genetic material

(Hamilton and Killeen, 1987). There are also other advantages related to the freezing rate and thus survival of spermatozoa. Watson and Martin (1975b) reported that in straws the freezing rate is more uniform and the formation of ice crystals is less than in pellets, and therefore less damage is expected to the sperm cells.

Kroesch (1988) reported better fertility (60-90 days non-return rate) when bull semen was frozen in mini straws than in maxi straws. Fertility was increased when total sperm numbers per straw were reduced, and the use of mini straws increased the efficiency of storage. This size has been more widely accepted for daily use with bull semen than the maxi straws.

The present study examined the post-thawing viability of ram spermatozoa frozen in maxi or mini straws at two different concentrations.

3.1.2 Experimental procedure.

Semen from three mature Merino rams was collected by artificial vagina and samples with a mass motility of > 3 on scale of 1 to 4+ were evaluated for concentration of spermatozoa by photocolorimeter (calibrated with the HTM). The semen was split into two fractions for dilution with Diluent B to concentrations of 200 and 400 x 10^6 sperm/ml, and cooled to 5°C over 1.5 hours. Half of the cooled semen was drawn into maxi straws (0.5 ml) and the other half into mini straws (0.25 ml). The straws were frozen in liquid nitrogen vapour (15 minutes at -120°C) and then stored in liquid nitrogen.

Three weeks later, the semen was thawed at 37°C, prepared for analysis (para 3.5.2. section b) and assessed using the HTM for percentage of progressively motile cells (PROGR) and percentage of motility (MOT). These two parameters are defined in Table 1.2.

3.1.3 Statistical analysis.

The data were analyzed with the SAS program by analysis of variance of a factorial experiment, considering the following factors:

1) Rams: A vs B vs C

2) Size of straws: maxi vs mini

3) Concentrations: 200 vs 400 million sperm/ml

with n=18 (n=number of straws) per treatment.

3.1.4 Results.

Pre-freezing concentration of spermatozoa had no effect on their post-thawing viability, so the data were pooled for concentration and are presented in Table 3.1.

Table 3.1. Effect of ram and size of straw on percentage of progressively motile cells (PROGR) and percentage of motility (MOT) measured by the HTM.

Ram	PROGR		MOT	
	maxi straws	mini straws	maxi straws	mini straws
	Means ± SEM (n=72)			
A	61.79 ± 4.11	41.85±4.11	67.63 ± 4.39	45.48 ± 4.39
B	39.97 ± 4.11	39.89±4.25	43.11 ± 4.39	44.40 ± 5.54
C	63.17 ± 8.23	64.02±8.23	69.71 ± 9.79	71.30 ± 8.79

There was an interaction between ram and type of straw for both PROG and MOT ($p < 0.001$) and a clear variation due to individual rams ($p < 0.01$). Semen from ram A had better viability following freezing in maxi than in mini straws, whereas semen from ram B and C had similar viability after freezing in either type of straw.

3.1.5 Discussion.

Pre-freezing concentration of spermatozoa had no effect on post-thawing motility. This is similar to the findings of Hunton et al.(1987), who reported that the motility of spermatozoa was similar following freezing in straws at different dilution rates. The values of sperm motility for rams A and C in this experiment were higher than reported by Hunton et al.(1987).

There is a great variation in semen traits among males from the same breed (Saacke and White, 1972) and there is also a wide variation in post-thawing motility of sperm frozen from different rams and between ejaculates within rams (Eppleston et al., 1986). This was confirmed by the present results. There was an interaction between ram and type of straws on post-thawing sperm survival mainly due to a clear variation due to individual rams. A distinctive difference or advantage from one type of straw over the other on survival of spermatozoa could not be demonstrated because of the main effect of ram variation. Further experimentation is required to elucidate any consistent advantage of either type of straw on fertility as found by Kroetsch (1988). It will also be important to determine whether the variation is due to the dilution rate, the pre-freezing cell concentration, the freezing rate achieved by the type of straw *per se* or the insemination dose used. Maxi straws would be useful to test different dosages of inseminate due to greater volume and easy splitting.

3.2 The effect of dose of motile spermatozoa inseminated by intrauterine and cervical inseminations on fertility.

3.2.1 Introduction.

Although satisfactory fertility has been obtained with cervical insemination of frozen-thawed semen (. Lightfoot and Salamon, 1970; Salamon and Lightfoot, 1970; Salamon, 1971; Colas, 1975; Maxwell et al., 1980; Mekonnen, 1988), these studies involved the preselection of semen, semen re-concentration and very high inseminate doses of spermatozoa and/or double inseminations. Conception rates of 10 to 40 % are common (First et al, 1961; Salamon, 1967; Salamon and Lightfoot, 1970; Visser and Salamon, 1974; Tervit et al., 1978; Maxwell and Butler, 1984; Tervit et al., 1984; Maxwell and Hewitt, 1986) which confirms that cervical insemination with frozen semen is less efficient than intrauterine insemination.

The introduction of intrauterine insemination (Killeen and Caffery, 1982) made the use of frozen-thawed semen more efficient, since the minimum amount of spermatozoa required for good fertility was reduced to as low as 5×10^6 motile spermatozoa or 40×10^6 total spermatozoa as reported by Eppleston et al. (1986) or Maxwell and Butler (1984) respectively, compared with the minimum recommended of 125×10^6 spermatozoa for maximum fertility after cervical insemination with frozen semen (Salamon, 1962).

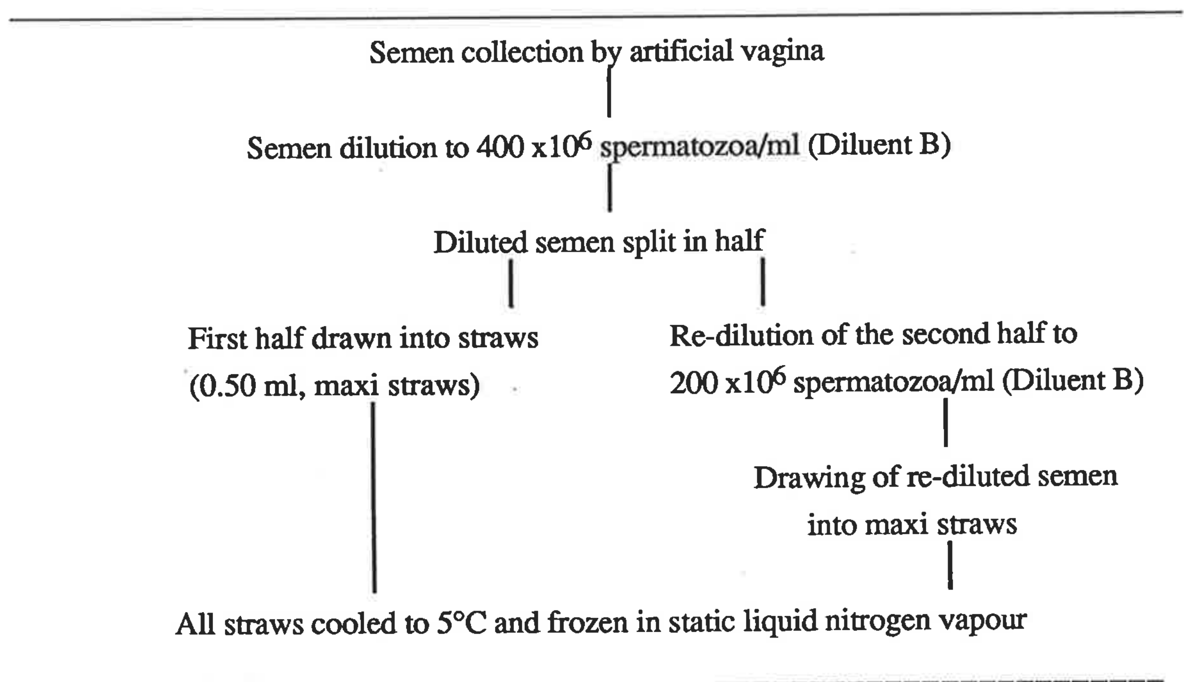
Most of the successful studies using frozen-thawed semen for intrauterine insemination have been done with semen frozen by the pellet method. Therefore it is important to determine if intrauterine insemination with semen frozen in straws will give the same rate of success. The reports to date comparing semen frozen in pellets and straws have shown no differences between the methods in survival or fertility of spermatozoa (Hamilton and Killeen, 1987; Hunton et al., 1987).

The use of low inseminate doses of sperm requires an efficient and accurate semen assessment, so that the semen can be divided to give very specific numbers of spermatozoa in each dose. The purpose of this experiment was to examine the pregnancy rate of ewes following intrauterine insemination with five different doses of frozen-thawed motile spermatozoa (MOTILE) (accurately measured by the HTM) and cervical insemination with high numbers of motile spermatozoa.

3.2.2 Experimental procedure.

Figure 3.1. shows the general procedure which has been described in detail in paragraphs 3.6.2.1. and 3.6.2.2..

Figure 3.1. General procedure for freezing semen in maxi straws (0.50 ml).



The semen was thawed (37°C) and assessed for number of motile cells using the HTM. With these results, and by varying the volume of thawed diluted semen, six treatments were determined as follows:.

Treatment 1: Intrauterine insemination with < 35 x 10^6 motile spermatozoa/ml.
Treatment 2: Intrauterine insemination with 35 to 50 x 10^6 motile spermatozoa/ml.
Treatment 3: Intrauterine insemination with 50 to 65 x 10^6 motile spermatozoa/ml.
Treatment 4: Intrauterine insemination with 65 to 85 x 10^6 motile spermatozoa/ml.
Treatment 5: Intrauterine insemination with > 85 x 10^6 motile spermatozoa/ml.
Treatment 6: Cervical insemination with 244 to 280 x 10^6 motile spermatozoa/ml.

A group of 373 ewes were treated with progestagen sponges for 12 days and received 400 I.U. PMSG at sponge removal. The ewes were inseminated 50 hours after sponge removal, and pregnancy was determined by ultrasound 50 days after insemination.

3.2.3 Statistical analysis.

The data were analyzed by analysis of contrasts (Chi-Square), following logit transformation of pregnancy rate, using the CATMOD procedures of the SAS program.

3.2.4 Results.

The results are presented in Table 3.2. and the analysis of data in Table 3.3.

Table 3.2. Pregnancy following artificial insemination with frozen-thawed semen in straws.

Treatment	Mean number motile spermatozoa inseminated x 10^6	No pregnant ewes/inseminated	(%)
1	23.3	15/37	(40.5)*
2	39.5	33/63	(52.4)
3	58.6	44/77	(57.1)
4	77.1	39/68	(57.4)
5	104.5	18/31	(58.1)
6	189.7	14/97	(14.4)**

* Treatment 1 tends to be different from treatments 3 to 5, but did not reach significance ($p=0.09$), ** Treatment 6 showed lower fertility than the rest of treatments ($p \leq 0.001$).

Table 3.3. Analysis of contrast.

Contrast	DF	Chi-Square	Probability
1 vs 6	1	9.97	0.0016
1 vs 5	1	2.05	0.1520
1 vs 4	1	2.68	0.1017
1 vs 3	1	2.72	0.0989
1 vs 2	1	1.30	0.2539
2 vs 6	1	123.90	0.0001
2 vs 5	1	0.27	0.6033
2 vs 4	1	0.33	0.5678
2 vs 3	1	0.32	0.5733
3 vs 6	1	31.31	0.0001
3 vs 5	1	0.01	0.9302
3 vs 4	1	0.00	0.9796
4 vs 6	1	30.01	0.0001
4 vs 5	1	0.001	0.9470
5 vs 6	1	20.52	0.0001

The pregnancy rate was lower after cervical insemination than intrauterine insemination, even with the lowest dose of spermatozoa.The maximum pregnancy rates of 57.4 and 58.1 % were obtained with motile sperm doses of 77.1 and 104.5 x 10^6 respectively, but they were not significantly different from the minimum pregnancy rate (40.5 %) obtained with a mean of 23.3 x 10^6 motile sperm

The pregnancy rates obtained by intrauterine insemination with the 5 different sperm doses are presented in Figure 3.2, and for intrauterine and cervical insemination Figure 3.3.

Figure 3.2.

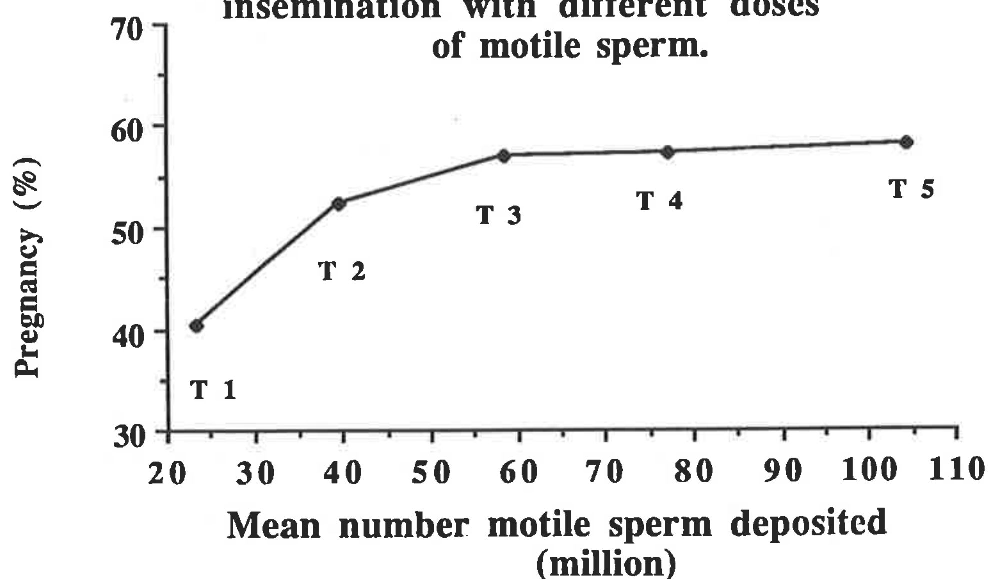
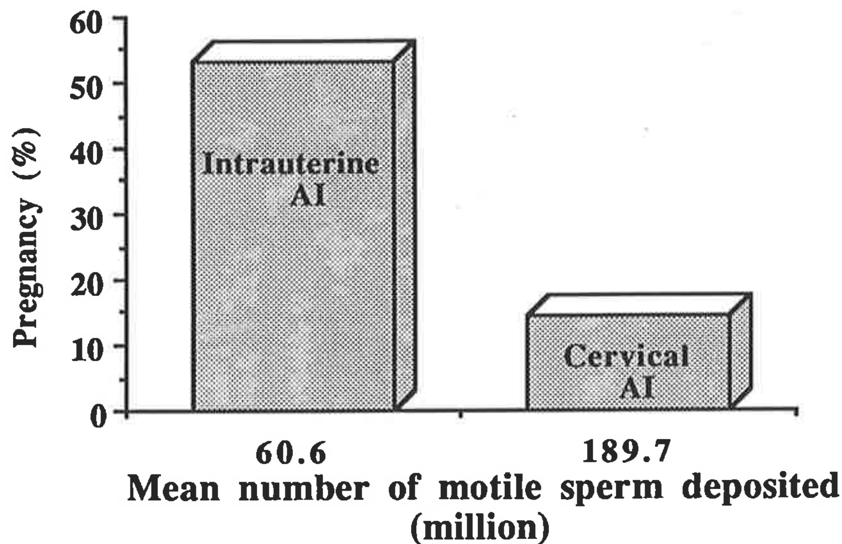


Figure 3.3. Pregnancy following intrauterine and cervical inseminations.



3.2.5 Discussion.

The fertility results obtained after intrauterine insemination with frozen-thawed semen were much better than after cervical insemination as already reported by Maxwell et al.(1984) and Maxwell and Hewitt (1986). These authors found that even with much lower doses of frozen-thawed sperm, it was possible to achieve much higher fertility with intrauterine than cervical insemination. Furthermore, intrauterine insemination is as effective as cervical insemination with fresh semen.

In the present experiment the minimum dose of 23.3 x10^6 motile sperm achieved a pregnancy rate of 40.5 %, which is lower than reported by Walker et al. (1984), Eppleston et al. (1986) and Maxwell (1986b) who obtained higher conception rates with lower doses of motile spermatozoa. In the present study there appeared to be no advantage in increasing the inseminate dose for intrauterine insemination above 23.3 x 10^6 motile spermatozoa. The overall pregnancy rate achieved was 53.5% with an average dose of 60.6 x10^6 motile spermatozoa which is higher than the threshold reported by the above authors (12 million motile sperm, 5 million motile sperm and 25 million motile sperm respectively), but lower than that found by Maxwell and

and Hewitt (1986) or Killeen et al. (1982) who reported 100 and 120 x 10^6 total sperm doses respectively achieving 69 and 56 % pregnancy at day 50 and 69 respectively.

In general terms the results obtained with the straw freezing technique were acceptable and comparable to other reports (Hunton, 1987, 1988; Hunton et al., 1987; Wilson, 1988). Most intrauterine inseminations have been done using semen frozen-thawed in pellets. From the results obtained here it appears that semen frozen in straws could be as fertile as that frozen in pellets. Straws facilitate the use of semen in breeding programs so the achievement of adequate pregnancy rates could have important commercial applications.

The results obtained by cervical insemination in this experiment are interesting, although the main aim of the study was an assessment of intrauterine insemination. Pregnancy rates were low following cervical insemination with frozen-thawed semen as reported by Salamon (1967) and Salamon and Lightfoot (1967, 1970). Some authors using frozen semen in pellet form, with lower doses of sperm and earlier times of insemination, achieved higher conception rates (Salamon and Lightfoot, 1970, 28.3 %; Salamon, 1971, 42.9 %; Visser and Salamon, 1973, 37.4 %, Visser and Salamon, 1974, 25.3 % to 32 %). In the present experiment, the pregnancy rate obtained after cervical insemination with frozen-thawed semen was lower than obtained by Maxwell et al.(1980) and Tervit et al.(1984) using similar procedures for freezing semen, synchronization of oestrus and inseminate dose. Probably for cervical insemination the skills of the operator, time of insemination and the viability of spermatozoa have a more important influence on fertility than the freezing procedure.

3.3 The effect on some semen traits of mixing diluted semen before freezing in straws.

3.3.1 Introduction.

A number of aspects of the processing of ram semen for freezing are likely to affect the viability of spermatozoa after thawing e.g. the type of vessel containing the semen (bull semen; Kroesch, 1988), the variation between rams and within rams (Eppleston et al., 1986), the diluents used (Salamon and Visser, 1972; Colas, 1975), the temperature before dilution and freezing (Salamon, 1968; Evans and Maxwell, 1987; Hunton, 1988; Wilson, 1988), and the temperature of thawing (Pace et al., 1981). Much effort has been focused on these factors, but very little has been done on small details during processing, like mixing the diluted semen before filling it into straws.

Experiments 3.1 and 3.2 revealed that semen frozen in straws had satisfactory survival of spermatozoa and fertility after intrauterine AI. Slight modifications of semen processing methods might enhance the action of the diluent protecting semen during the freezing process. Semen tends to sediment rapidly which may cause variation in post-thawing survival of spermatozoa within samples. The present experiment examined the effect of mixing diluted semen before freezing in straws on some semen characteristics measured by the HTM.

3.3.2 Experimental procedure.

Semen from 4 mature Merino rams was collected by artificial vagina and samples with a mass motility of > 3 on scale of 1 to 4+ were evaluated for concentration of spermatozoa by photocolorimeter (calibrated with the HTM). The semen was then diluted at 30°C to a concentration of 200 x 10^6 spermatozoa/ml with Diluent B.

The diluted semen was divided into two parts; one half was loaded into straws without mixing and the other was mixed continually during filling and sealing of the straws. Approximately 200 straws were loaded from each ram (100 mixed semen, 100 non-mixed semen) using an automatic filling and sealing machine (IMV, MRS-3, automatic filling and sealing straws, France). This automatic machine holds the semen in a plastic cone which is connected by rubber pipes to the vacuum pump which draws the diluted semen into the straws. This cone is easily removed, so the mixing was performed by interrupting the machine, removing the cone from its base, gently swirling it for a short time, and then replacing it and re-starting the machine. Every tenth straw (76 for the non-mixed and 79 for the mixed semen) was selected and identified for later assessment. The straws were frozen in liquid nitrogen vapor and stored in liquid nitrogen.

The straws were thawed at 37°C and prepared for semen assessment using the HTM as described in para 3.5.2 section b; the parameters measured were: TOTAL (millions/ml), MOTILE (millions/ml), PROGR (%), MOT (%), mean VSL (mic/sec) and mean LIN (%) which are described in Table 1.2.

3.3.3 Statistical analysis.

The experiment was of 4 x 2 factorial design, with the following factors:

1) Rams: 4 rams.

2) Treatments: mixed diluted semen vs non-mixed diluted semen.

The data was analyzed by the Bartletts test of homogeneity of variance.

3.3.4 Results.

Rams had no effect on the post-thawing semen characteristics. The results pooled for rams are presented in Table 3.4.

Table 3.4. The effect of mixing diluted semen during processing on some post-thawing semen characteristics measured by the HTM.

Semen Characteristic	**mixed** (n=79)	**non-mixed** (n=76)	Significance
	Mean ± SEM		
* TOTAL(x 10^6/ml)	250.3 ± 0.8	255.6 ± 0.9	N.S.
* MOTILE (x 10^6/ml)	91.3 ± 0.7	90.0 ± 0.6	P<0.05
* PROGR (%)	33.6 ± 0.2	15.7 ± 0.2	P<0.01
* MOT (%)	36.3 ± 0.2	35.3 ± 0.2	P<0.05
* VSL (mic/sec)	82.1 ± 0.2	80.8 ± 0.18	P<0.05
*LIN (%)	90.0 ± 0.04	89.6 ± 0.03	P<0.05

Mixing diluted semen before freezing improved the post-thawing MOTILE, PROGR, MOT, mean VSL and mean LIN, but had no effect on the TOTAL.

3.3.5 Discussion.

The values of motility of spermatozoa obtained in this experiment were similar to those reported by Colas (1975) and Hunton et al. (1987) confirming that the semen was successfully frozen in straws. The improvement in motility achieved by mixing the diluted semen before packing into the straws suggests that a simple constant mixing could help to improve this particular technique. Mixing probably improved the mixture between the sperm and the components of the diluent, thus the protective action was increased and so the survival of spermatozoa.

However, if the difference in post-thaw motility was due to mixing only, then one might expect a greater variability between straws in the unmixed semen, but the standard errors indicate that the between-straw, within method variability was similar. The differences reported here are only small, and in other experiments, greater between-straw variability may well mask the effect of mixing.

In section 3.5 of this chapter, the repeatability of the estimations of straw-frozen semen characteristics was very low. This was as a result of small numbers of rams and a large variability between straws. The variability between straws may be reduced by mixing the semen as in the present study, thus the accuracy and reliability of the estimations could be increased.

In chapter 7, it was found that it may be possible to improve fertility with a relatively small increase in some of the semen characteristics measured by the HTM. Thus, further work is required to determine if improved processing of semen for freezing, would not only improve the viability of spermatozoa and reduce the variation between straws, but would also increase fertility.

3.4 The effect of five different diluents varying the ratio of tris:glucose for pellet freezing on post-thawing survival and fertility of ram spermatozoa.

3.4.1 Introduction.

The development of intrauterine insemination by laparoscopy has enhanced the fertility achievable with frozen-thawed semen. Similar viability of spermatozoa frozen either in pellets or in straws has also been established. The development of diluents and processing methods for freezing semen has been restricted by the lack of an objective semen analysis system. The best diluent so far used for pellet freezing was developed by Salamon and Visser (1972). Many workers use this diluent as a control or standard in experiments designed to improve semen processing methods for freezing. The present experiment examined the effect of frozen storage of ram semen in five different diluents (including that of Salamon and Visser (1972) as the control) on semen quality measured by the HTM and fertility following intrauterine insemination.

3.4.3 Experimental procedure.

Semen was collected by artificial vagina from 3 mature Merino rams. Only ejaculates (n=9) of good initial motility (> 3, on a subjective 0-4^+ assessment scale) and a minimum concentration of 3 x 10^9 spermatozoa per ml (determined by photocolorimeter calibrated against the HTM) were used.

Semen was split for dilution (1:4; semen:diluent) at 30°C in five different diluents before freezing on dry ice in pellet form. All the diluents contained 15 % (v/v) egg yolk and 4 % (v/v) glycerol, and the pH was adjusted to 7.0 with citric acid. The five diluents varied in their tris:glucose ratios as follows: (1) 300:30 (Salamon and Visser, 1972), (2) 250:95, (3) 200:160, (4) 150:225, (5) 100:290 mM. The composition of the diluents maintained the osmolarity at 325 mosmol.

Semen was thawed at 37°C after three weeks in storage and extended 1:50 with Dulbecco's phosphate-buffered saline solution. Sperm concentration and motion characteristics were measured using the HTM immediately after thawing and after 3 and at 6 hours incubation at 37°C.

Pellets remaining from the laboratory test were thawed (37°C), pooled, and used within 15 minutes for intra-uterine insemination of 321 Merino ewes .The ewes were treated with intra-vaginal progestagen-impregnated sponges for 13-14 days. At sponge removal, the ewes received an intramuscular injection of 400 I.U. of PMSG. The ewes were inseminated 50 hours after sponge removal. For each insemination a total of 100 x 10^6 spermatozoa (50 x 10^6 spermatozoa per uterine horn) was used. Pregnancy was determined by ultrasound 48 days after insemination.

3.4.4 Statistical analyses.

For the effect of the diluents on semen characteristics, a 3x5x3 factorial design with a total of 135 observations was assessed using analysis of variance by the SAS program considering the following factors:

1) Rams: A vs B vs C
2) Diluents: 1 vs 2 vs 3 vs 4 vs 5
3) Time of assessment: 0 vs 3 vs 6 hours after incubation.

For the fertility trial, the pregnancy rate was analyzed by Chi-Square analysis. To determine the relationship between semen traits and fertility, a multiple correlation analysis was carried out.

3.4.5 Results.

1. Laboratory assessment of semen.

The three rams differed in their post-thawing semen characteristics as shown in Table 3.5. Frozen-thawed semen from ram B had better motility characteristics than the other two rams. Ram A had higher TOTAL than rams B or C ($p<0.001$). Ram C had a larger VAPsd and VSLsd than ram A or B ($p<0.001$).

Table 3.5. The effect of ram on frozen-thawed semen characteristics (means ± s.e.m).

Semen characteristic	Ram A	Ram B	Ram C
TOTAL (x 10^6/ml	4102.5 ± 59.19**	3155.0 ± 68.35	3354.5 ± 83.71
PROGR	38.0 ± 1.33	49.0 ± 1.54**	38.4 ± 1.88
MOT (%)	43.0 ± 1.53	54.0 ± 1.77**	44.0 ± 2.16
MOTsd	127.5 ± 3.33**	112.8 ± 3.84	107.1 ± 4.71
VSL(mic/sec)	62.2 ± 1.06	73.3 ± 1.22*	69.0 ± 1.50
LIN (%)	87.0 ± 0.30	89.5 ± 0.35*	88.2 ± 0.42
STR	76.7 ± 0.92	82.4 ± 1.07**	80.1 ± 1.31
VAP(mic/sec)	66.8 ± 1.16	77.6 ± 1.34*	74.0 ± 1.64
VAPsd	24.9 ± 0.25	23.8 ± 0.29	26.1 ± 0.35**
VSLsd	25.1 ± 0.25	24.3 ± 0.29	26.5 ± 0.35*
RAPID (No)	67.1 ± 3.74	106.7 ± 4.32**	80.7 ± 5.29
RAPID %	15.6 ± 0.88	25.8 ± 1.02**	19.0 ± 1.25
SLOW (No)	84.7 ± 3.14**	71.4 ± 3.62	80.4 ± 4.44
SLOW %	19.7 ± 0.69*	17.0 ± 0.79	17.7 ± 0.97
STATIC %	37.3 ± 1.70*	29.1 ± 1.97**	37.5 ± 2.41

(*) $p<0.01$, (**) $p < 0.001$

Ram semen characteristics were affected to varying degrees by the five diluents (Table 3.6). Diluents 1 and 2 were similar in the way they preserved the semen, achieving significantly better results for MOTILE, PROGR, ALH, RAPID No., RAPID % and STATIC % than the other diluents (at levels of $p< 0.01$, < 0.001). Diluent 2 achieved the lowest number of STATIC, whereas diluent 5 had the lowest SLOW % of motile cells.

Table 3.6. The effect of diluent on frozen-thawed semen characteristics of motility (means ± s.e.m.).

Semen characteristic	DILUENTS 1	2	3	4	5
MOTILE(x 10^6/ml)	1822.4 ± 110.5*	1759.8 ± 110.5*	1661.9 ± 110.5	1592.6 ± 110.51	1405.3 ± 110.50
PROGR %	44.9 ± 2.06*	46.2 ± 2.06*	41.2 ± 2.00	39.8 ± 2.06	37.4 ± 2.06
MOT. (%)	50 0 ± 2.37**	52.2 ± 2.37*	45.8 ± 2.37	45.2 ± 2.37	41.3 ± 2.37
ALH (mic)	4.4 ± 0.08*	4.4 ± 0.0	4.1 ± 0.08	4.2 ± 0.08	4.2 ± 0.08
RAPID (No)	95.1 ± 5.0**	99.4 ± 5.0**	77.9 ± 5.00	80.4 ± 5.0	71.5 ± 5.00
RAPID %	22.4 ± 1.37*	23.7 ± 1.37*	18.3 ± 1.37	19.2 ± 1.37	16.9 ± 1.37
SLOW %	20.0 ± 1.07	18.5 ± 1.07	19.2 ± 1.07	18.9 ± 1.07	15.6 ± 1.07*
STATIC(No)	129.2 ± 12.87	111.8 ± 12.87**	151.8 ± 12.87	149.2 ± 12.87	164.5 ± 12.87
STATIC %	29.4 ± 2.64*	29.2 ± 2.64*	34.9 ± 2.64	36.4 ± 2.64	43.1 ± 2.64

(*) $p<0.01$, (**) $p<0.001$.Diluents 1 and 2 compared with diluents 3, 4 and 5.

Incubation time had a direct influence on all the semen characteristics assessed ($p < 0.01$, and $p< 0.001$) except for the TOTAL concentration which remained constant. Some of the data are presented in Table 3.7 The motility parameters PROGR and MOT together with RAPID, MEDIUM and SLOW, decreased during incubation, while STATIC increased. However, the velocity parameters VSL and VAP increased during the first 3 hours of incubation and then decreased to levels similar to recently thawed spermatozoa. No significant differences were found when velocities were compared at thawing or after 6 hours incubation at 37°C. LIN increased during incubation due to a disproportional increase and decrease in VCL and VSL during the first 3 hours and at 6 hours incubation respectively (Table 3.7.).

Table 3.7. The effect of time on frozen-thawed semen characteristics (means ± s.e.m.).

Semen characteristic	INCUBATION TIME 0	3 hours	6 hours
MOTILE (x 10^6/ml)	1898.8 ± 85.61	1636.5 ± 85.61	1410.4 ± 85.61*
PROGR %	48.3 ± 1.60	41.3 ± 1.60	36.2 ± 1.60*
MOT (%)	55.1 ± 1.84	45.9 ± 1.84	40.0 ± 1.84*
VSL (mic/sec)	64.0 ± 1.28	73.4 ± 1.28**	66.9 ± 1.28
VAP (mic/sec)	69.8 ± 1.39	77.6 ± 1.39**	71.0 ± 1.39
VCL (mic/sec)	0.7 ± 0.01	0.8 ± 0.01	0.7 ± 0.01
LIN (%)	85.6 ± 0.36	88.8 ± 0.36	90.1 ± 0.36*
ALH (mic)	4.5 ± 0.07	4.3 ± 0.06	3.9 ± 0.06**
RAPID (No)	92.6 ± 4.49	93.0 ± 4.29	69.0 ± 4.29**
MEDIUM(No)	147.8 ± 5.20	98.0 ± 5.20	97.0 ± 5.20**
SLOW (No)	87.3 ± 3.77	77.5 ± 3.77	71.7 ± 3.77*
STATIC (No)	112.1 ± 9.96	149.4 ± 9.96	162.4 ± 9.96**

(*) $p < 0.01$; (**) $p < 0.001$. Comparing 6 hours vs 3 hours vs 0 hours. When 3 hours was compared with 0 hours, there were no significant differences in any characteristic.

Rams were involved in a first order interaction with diluent on PROGR, MOT, VSL, VAP and RAPID % (Table 3.8). Type of diluent had no effect on the semen characteristics of rams A and C. However, for ram B most of the semen characteristics were better following freezing with diluents 1 and 2 than 3, 4 and 5.

Table 3.8. The effect of rams and diluents on frozen-thawed semen characteristics (means ± s.e.m.).

Ram	Diluent.	PROGR	MOT	VSL	VAP	RAPID
A	1	37.2 ± 2.9	42.6 ± 3.4	64.0 ± 2.4	69.0 ± 2.6	74 1 ± 8.4
A	2	40.9 ± 2.9	46.8 ± 3.4	62.4 ± 2.4	67.6 ± 2.6	75.1 ± 8.4
A	3	40.7 ± 2.9	45.2 ± 3.4	58.1 ± 2.4	62.1 ± 2.6	63.3 ± 8.4
A	4	33.3 ± 2.9	38.0 ± 3.4	62.3 ± 2.4	66.7 ± 2.6	55.2 ± 8.4
A	5	38.0 ± 2.94	2.4 ± 3.4	64.2 ± 2.4	68.6 ± 2.6	64.3 ± 8.4
B	1	54.7 ± 3.4*	59.8 ± 3.9*	76.4 ± 2.7**	80.3 ± 3.0*	120.0 ± 9.7*
B	2	56.9 ± 3.4**	63.2 ± 3.9**	76.3 ± 2.7**	81.0 ± 3.0*	127.6 ± 9.7**
B	3	46.1 ± 3.4*	50.8 ± 3.9	72.6 ± 2.7*	76.6 ± 3.0	101.5 ± 9.7
B	4	47.4 ± 3.4*	52.7 ± 3.9	72.4 ± 2.7	77.4 ± 3.0	108.7 ± 9.7
B	5	39.7 ± 3.4	43.3 ± 3.9	68.5 ± 2.7	72.5 ± 3.0	75.7 ± 9.7
C	1	42.8 ± 4.2	49.2 ± 4.8	67.7 ± 3.4	72.6 ± 3.7	87.6 ± 11.8
C	2	41.6 ± 4.2	46.8 ± 4.8	70.2 ± 3.4	78.4 ± 3.7	95.5 ± 11.8
C	3	36.6 ± 4.2	41.3 ± 4.8	63.6 ± 3.4	68.0 ± 3.7	69.0 ± 11.8
C	4	38.8 ± 4.2	4.8 ± 4.8	67.3 ± 3.4	71.4 ± 3.7	77.3 ± 11.8
C	5	34.7 ± 4.2	38.4 ± 4.8	75.2 ± 3.4	79.6 ± 3.7	74.3 ± 11.8

(*) $p< 0.01$, (**) $p< 0.001$.

There was no interaction between time of assessment and any of the diluents. The diluents were equally effective in preserving semen characteristics during incubation at 37°C.

2. Field fertility test.

Table 3.9 shows the results of the field fertility test. There were no significant differences between diluents in either the percentage of ewes pregnant or in the numbers of foetuses per ewe.

Table 3.9. Pregnancy and number of foetuses after intrauterine insemination with semen frozen-stored in different diluents.

Diluent	No. Preg. ewes	(%)	Number of/fetuses/ewe 1	2	3	4	Total foetuses(%)
1	28/63	(44.4)	12	15	1	0	45 (71.4)
2	26/65	(40.0)	16	7	2	1	40 (61.5)
3	36/64	(56.2)	17	18	1	0	56 (87.5)
4	30/64	(46.9)	10	18	1	0	52 (81.3)
5	26/65	(40.0)	15	10	1	0	36 (55.4)
Total	146/321	(45.48)	70	68	6	1	229 (71.34)

3. Relationship between semen characteristics and fertility.

There was no significant correlation between post-thawing semen characteristics and fertility. The low number of observations and the unexpected high fertility achieved by diluent 3 made it difficult to detect any correlation. The relationship between some semen characteristics and fertility are shown in Figures 3.4, 3.5 and 3.6.

Figure 3.4 RELATIONSHIP BETWEEN MOT % AND FERTILITY

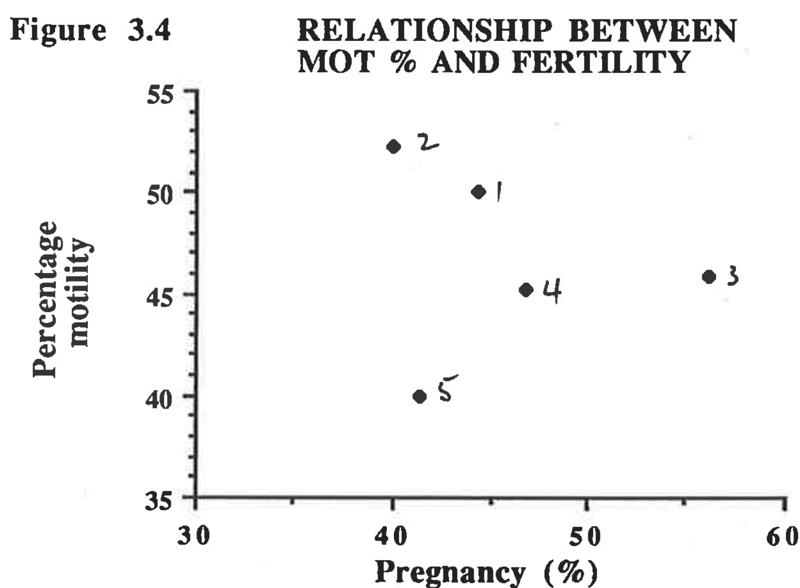


Figure 3.5 RELATIONSHIP BETWEEN PROGR % AND FERTILITY

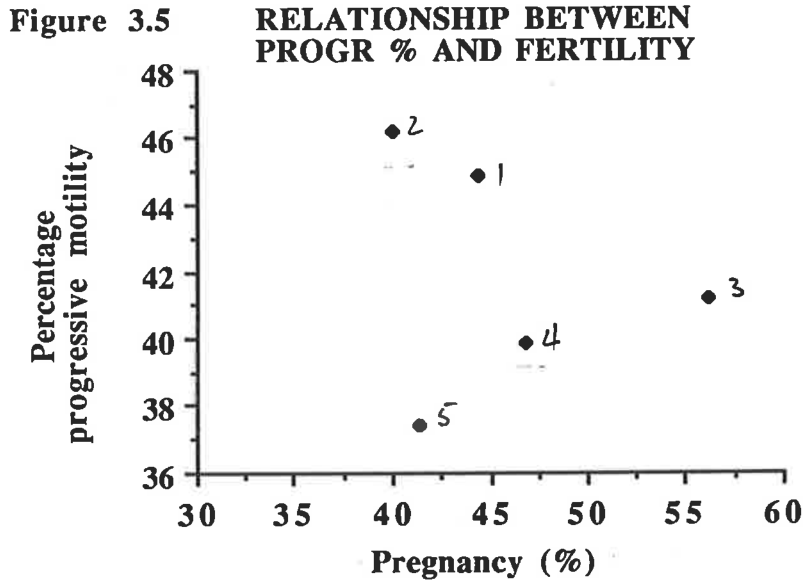
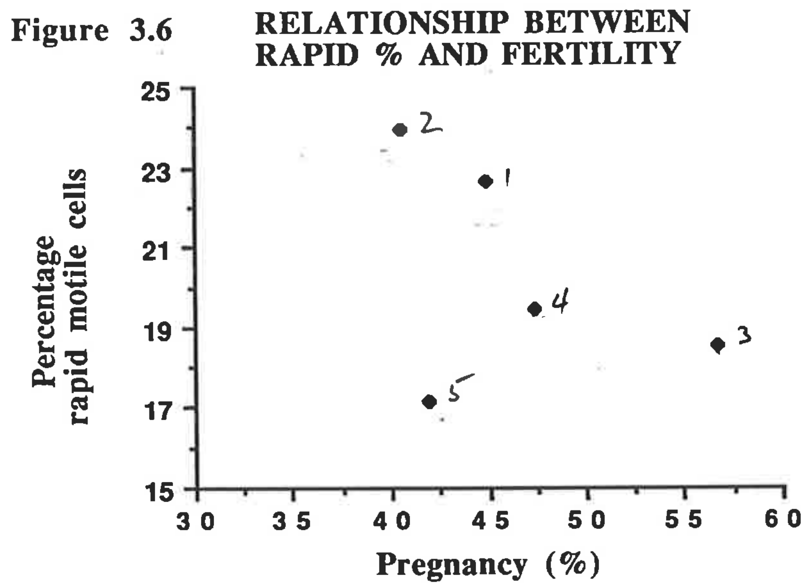


Figure 3.6 RELATIONSHIP BETWEEN RAPID % AND FERTILITY



3.4.6 Discussion.

When semen was extended with different diluents varying the ratio of tris:glucose, the standard or control diluent (1) obtained even better motility than originally reported by Salamon and Visser (1972). As mentioned by Eppleston et al. (1986), there was a significant variation between rams. One of the rams (B) was better than the other two in the survival of spermatozoa using the different diluents, although ram (A) had a greater density.

It was interesting to note that during post-thawing incubation at 37°C, parameters measuring aspects of motility decreased, whereas those measuring aspects of velocity increased for a period of time after thawing, and then decreased (Table 3.7). For example, mean LIN (the ratio of VSL/VCL) increased during incubation, partly due to a disproportional increase in VSL during the first 3 hours of incubation and a decrease in the second 3 hours of incubation. This may also signify that the spermatozoa were moving more slowly along the track,but with more rapid circular movements. These increases in velocity (Table 3.7) (sometimes confused with progressive motility) have been observed elsewhere, and may be due to a stimulatory effect of thawing temperature (Salamon, 1968), or the post-thawing medium. It is not clear whether such changes in sperm velocity and motion characteristics are important for their passage through the female reproductive tract, or whether they have any effect on the fertilizing capacity of the spermatozoa.

Two of the diluents were better in preserving semen characteristics measured by the HTM. It was concluded that increasing the concentration of sugar in the diluent did not increase the level of cryoprotection because sugars are probably poor penetrants of ram sperm membranes. Despite these differences in post-thawing semen characteristics, there were no differences in fertility when semen frozen in the different diluents was used for artificial insemination. The only conclusion which can be reached is that adequate sperm protection during pellet freezing can be achieved with diluents containing between 250 to 300 mM of tris and 30 to 95 mM glucose.

In the present experiment, a first attempt to correlate post-thawing ram semen characteristics with fertility was performed, but it was a poor estimation because of the unexpected good results in conception rate with diluent 3 (56.2 %) despite the poor semen quality found using the HTM compared with the quality achieved by diluents (1) and (2). One of the reasons may be the small number of observations of semen quality (1 sample per treatment before insemination) from which the correlation was estimated as mentioned by Foote et al. (1977), O'Connor et al. (1981) and Saacke (1982). On the other hand, the inseminate dose used (100 x 10^6 spermatozoa/ml) was perhaps greater than the minimum required for

maximum fertility. Saacke (1982) suggested that when a large dose of sperm is used in a fertility trial, the ~~estimation of~~ magnitude of the correlation ~~is difficult~~ coefficient may be reduced and the value of semen characteristics for fertility prediction tends to be poor.

Using the best combination of tris and glucose (between 250 and 300 mM tris and 30 to 95 mM glucose), with a larger number of samples it may be possible to demonstrate a difference between the two best diluents and find significant differences which could represent a step towards the improvement of semen processing in pellets. To be able to calculate the correlation of semen traits with fertility, it is necessary to have large numbers of observations of semen samples, a considerable range of semen quality, and a large number of ewes inseminated.

3.5 The variability of semen characteristics in semen frozen in straws.

3.5.1 Introduction.

It is known that there is a variation in semen characteristics after semen has been frozen in pellets and straws due to the different freezing rates. In pellet freezing, about 50 % of the pellet is in contact with the dry ice during freezing (Lightfoot and Salamon, 1969b), but straws are generally frozen in liquid nitrogen vapour and the rate of freezing is more precisely controlled (Watson and Martin, 1975b). A variation in post-thawing motility of sperm has been observed between both rams and between ejaculates within rams (Eppleston et al., 1986). The variation between straws may be because the freezing curves produced by static liquid nitrogen vapour vary greatly from sample to sample within freeze and especially from freeze to freeze (Fiser et al., 1987).

There is no information on the partitioning of variance components for post-thawing semen characteristics between rams, ejaculates (often termed batches) within rams or between straws within batches. There is a need for more objective semen analysis which gives more reliable data on semen quality (Salamon, 1987; Evans, 1988). Thus the aims of this experiment were to provide some values of ram semen characteristics as assessed by the HTM and to estimate the variance components and repeatabilities of measurements of straw-frozen ram semen characteristics.

3.5.2 Experimental procedure.

Semen from 4 mature Merino rams was collected on different dates by artificial vagina and samples with a mass motility of > 3 on a scale of 1 to 4+ were evaluated for concentration of spermatozoa by photocolorimeter (calibrated with the HTM). Semen was diluted with Diluent B to a concentration of 200 x 10^6 sperm/ml, and cooled to 5°C in 1.5 hours. The

cooled semen was drawn into maxi straws (0.5 ml), frozen in liquid nitrogen vapor (15 minutes at -120°C), and stored in liquid nitrogen.

Three weeks later, the semen was thawed at 37°C, prepared for analysis, and assessed using the HTM for concentration and sperm motility characteristics.

3.5.3 Statistical analysis.

The data had a hierarchical structure. The experimental design considered 4 rams, 3 batches within each ram and 10 to 54 straws within each batch. Variance components were estimated by fitting a model which included the effects of rams, batches within ram, and straws within batch. All effects were treated as random and the last mentioned term was the error.

The mathematical model fitted was $Y_{ijk} = \mu + R_i + B_{ij} + e_{ijk}$

where Y_{ijk} is the kth straw within the jth batch within the ith ram, μ is the overall mean, R_i is the effect of the ith ram, B_{ij} is the effect of the jth batch within the ith ram, and e_{ijk} is the error term. Ram and batch within ram were treated as random effects and the coefficients of expected mean squares were as follows:

Source of variation	df	Expected Mean square value
Ram	3	$VAR + nVAR_B + bnVAR_W$
Batch within ram	8	$VAR + nVAR_B$
Error	335	VAR

When the total variance was separated into its components, termed as Ram, Batch and Error, they were expressed as actual values and as percentages. These percentages show the proportion of the total variance of the estimated variables coming from each of the components; i.e. how much of the variance found in each of the variables came from rams, batches within rams or straws within batches within rams. Repeatabilities were estimated from the intra-class correlation, combining the appropriate variance components. The equations used were:

VAR_R= Variance component between rams

VAR_B= Variance component between batches

VAR_W= Error variance component,

so total variance = $VAR_R + VAR_B + VAR_W$ and within a ram the variation is

$VAR_{WithinRam} = VAR_B + VAR_W$

Thus, repeatability of straws from the same batch was calculated from the variance components as:

$$r_B = \frac{VAR_R + VAR_B}{VAR_R + VAR_B + VAR_W}$$

and repeatability of batches from same ram

$$r_R = \frac{VAR_R}{VAR_R + VAR_B + VAR_W}$$

3.5.4 Results.

The mean, standard deviation and the minimum and maximum values for the frozen-thawed ram semen characteristics estimated by the HTM are presented in Table 3.10.

Table 3.10. Mean, standard deviation and range of frozen-thawed semen characteristics.

Semen characteristics	Mean	Standard Deviation	Range	
			Minimum	Maximum
TOTAL	262.60	44.93	128.2	397.3
MOTILE	119.94	47.14	11.9	280.7
PROGR	31.20	12.33	4.9	63.7
MOT	45.33	13.77	13.7	77.3
VSL	117.51	16.69	52.2	158.6
LIN %	89.51	3.30	74.7	98.9
ALH	5.23	0.84	2.4	7.2
LIN	81.75	4.33	66.7	89.5
VAP	126.93	16.52	57.6	170.3
RAPID %	32.46	12.95	4.9	66.9
MEDIUM %	12.83	5.07	3.5	37.3
SLOW %	2.10	9.81	0.0	108.0
STATIC %	53.18	14.07	17.3	85.7

The values of the mean squares of the sources of variation for each of the semen characteristics and their statistical significance are shown in Table 3.11. There was a significant

variation due to the ram in only three of the variables assessed (LIN %, LIN and MEDIUM %). There was a significant difference in most of the variables assessed due to the batch from which the straws were analyzed within the same ram .

The variance components for the post-thawing semen characteristics are shown in Table 3.12. For most of the semen characteristics, the greatest variation was found first between straws within batches within ram, and second between batches within ram.

Table 3.11. Mean squares and their significance from the sources of variation for each of the semen frozen-thawed characteristics.

	SOURCE OF VARIATION		
Semen characteristics	Ram	Batch	Error
TOTAL	11139.73	7556.01***	1809.40
MOTILE	28014.30	10354.25***	1796.99
PROGR	2989.670	803.45***	110.97
MOT	1937.06	1332.23***	146.65
VSL	3655.92	2188.19***	202.71
LIN %	236.61**	21.68*	8.61
ALH	8.12	13.07***	0.34
LIN	566.46**	67.09***	12.73
VAP	3519.74	2115.95***	199.99
RAPID %	3063.05	882.26***	124.78
MEDIUM %	471.43*	109.84***	19.74
SLOW %	27.41	157.94	95.34
STATIC %	1807.77	1397.60***	154.99

(*) $p < 0.05$; (**) $p < 0.01$; (***) $p < 0.001$

Table 3.12. Values and percentages of the variance components for each of the frozen-thawed semen characteristics.

Semen characteristic	Variance components (value) Ram	Batch	Error	Variance components (percentage) Ram	Batch	Error
TOTAL	38.25	200.04	1809.40	1.87	9.77	88.36
MOTILE	199.30	308.61	1796.99	8.65	13.39	77.96
PROGR	25.11	24.97	110.97	15.59	15.51	68.90
MOT	5.75	42.76	146.65	2.95	21.91	75.14
VSL	15.03	71.61	202.72	5.20	24.75	70.05
LIN %	2.53	0.47	8.61	21.82	4.06	74.12
ALH	-0.07	0.46	0.34	0.00	57.18	42.82
LIN	5.86	1.96	12.74	28.51	9.53	61.96
VAP	14.36	69.10	200.00	5.07	24.38	70.56
RAPID %	24.97	27.32	124.78	14.10	15.43	70.47
MEDIUM %	4.18	3.25	19.74	15.39	11.96	72.65
SLOW %	-1.62	2.26	95.34	0.00	2.31	97.69
STATIC %	3.38	44.81	155.00	1.66	22.05	76.28

The values of repeatability for the semen characteristics measured for straws from the same batch and of batches from the same ram are presented in Table 3.13. In general terms the values of both estimations were very low, but the lowest values of repeatability were recorded for ALH and SLOW % for batches from same ram.

Table 3.13. The repeatability of the measurements of frozen-thawed semen characteristics for straws from the same batch and batches from the same ram.

Semen characteristic	REPEATABILITY r_B= straws from the same batch	r_R= batches from same ram
TOTAL	0.12	0.02
MOTILE	0.22	0.09
PROGR	0.31	0.16
MOT	0.25	0.03
VSL	0.30	0.05
LIN %	0.26	0.22
ALH	0.48	0.00
LIN	0.38	0.29
VAP	0.29	0.05
RAPID %	0.30	0.14
MEDIUM %	0.27	0.15
SLOW %	0.02	0.00
STATIC %	0.24	0.02

3.5.5 Discussion.

Major limitations of this experiment were that the number of rams used was too small for a reliable estimation of the variance components between rams, and the similarity of the rams which were selected for their semen production and quality. Thus much more accurate results would be possible with a larger number of unselected rams. One of the purposes of the present study was to provide some values of semen characteristics as a reference for future research in the field.

In studies by Eppleston et al. (1986), and in section 3.1 chapter 3 of this study, a variation was found in the freezability of semen from different rams. In the present experiment, however, the variation between straws was significantly larger than the variation between batches from the same ram or the variation between rams. Some of the variation in fertility following AI may be explained by this variation between batches and particularly between straws. The variation between straws within batches and between batches within ram was probably due to sampling, variation in semen processing and freezing, and to a lesser extent the protocol for semen assessment which may cause a slight variation between the successive estimations. The small variation between rams observed was due to the use of a small number of rams which had been pre-selected for production of similar semen quality. These variations then affect the repeatability of the estimations, but there is potential for them to be reduced.

The repeatability refers to the correlation between successive measurements of a semen characteristic. In this experiment, the repeatability was very low when it was measured for straws from the same batch and of batches from the same ram, due to the variation between straws. If the variation between straws could be reduced, then the repeatability would be increased, and so the correlation between measurements of the same characteristics would be greater. These low values of repeatability indicate that more than one straw must be assessed to obtain reliable information from a batch of frozen semen, and several batches must be assessed from each ram. Further studies are required using larger numbers of unselected rams.

3.6 The variability of fresh and pellet-frozen semen characteristics.

3.6.1 Introduction.

In experiment 3.5, a large variation and low repeatability between straws was found, but also ~~particularly due to~~ the small number of rams which was considered as a limitation in the experiment. The variation between rams was also small because the rams were pre-selected for their similar production and quality of semen. This experiment was designed to study the variation between semen characteristics from fresh and frozen-thawed samples collected from a large number of unselected rams, and to estimate the repeatability of these characteristics.

3.6.2 Experimental procedure.

3.6.2.1 Semen collection, processing and freezing.

Semen was collected by electro-ejaculation from sixty-five 16-month old rams from four South Australian studs. Only one ejaculate per ram was obtained. Each ejaculate was divided into two parts. Four sub-samples of fresh semen (100 µl each) were then taken from one of the parts. The other part was diluted 1:4 (semen:diluent) with Diluent A, cooled to 5°C, frozen on dry ice in pellet form and stored in liquid nitrogen (-196°C).

3.6.2.2 Semen assessment.

Fresh and frozen-thawed semen was assessed with the HTM. The four sub-samples (100 µl) from each ejaculate were assessed within 10 minutes of collection. Three weeks later, 8 to 9 pellets per ram were thawed at 37°C and assessed. Two hundred and fifty nine fresh and 509 frozen-thawed semen samples were assessed from the 65 rams.

3.6.3 Statistical analyses.

3.6.3.1 Analysis of variance for fresh and frozen-thawed semen characteristics.

The data was analyzed by analysis of variance. Type of semen was treated as a fixed effect and ram as a random effect. The following mathematical fitted model was used:

$$Y_{ijk} = \mu + T_i + R_j + (T^*R)_{ij} + e_{ijk}$$

where Y_{ijk} is the kth observation within the ij^{th} sample of the ith type of semen within the jth ram, and μ is the overall mean. T_i is the effect of the ith type of semen (fresh or frozen), R_j is the effect of the jth ram, $(T^*R)_{ij}$ is the ij^{th} sample of the ith type of semen of the jth ram and e_{ijk} is the error term.

3.6.3.2 The estimation of the variance components of fresh and frozen-thawed semen characteristics and the repeatability of the estimations.

For the fresh samples there was a mean of 3.98 samples from 65 rams, and for frozen-thawed semen 8.095 samples from 63 rams. Variance components were estimated by analysis of variance. The fresh semen data and frozen semen data were analyzed separately, fitting a model which included the effects of rams and samples within ram.

The general mathematical fitted model = $Y_{ij} = \mu + R_i + e_{ij}$

where Y_{ij} is j^{th} sample (fresh sample or thawed pellet) within i^{th} ram, μ is the overall mean. R_i is the effect of the ith ram and e_{ij} is the error term. Ram and sample within ram were treated as random effects and the coefficients of expected mean squares were as follows:

Fresh semen:

Source of variation	df	Expected Mean square value
Between Rams (R)	64	VAR + $nVAR_R$
Samples within ram (error)(S)	194	VAR
Total	252	

Frozen-thawed semen:

Source of variation	df	Expected Mean square value
Between Rams (R)	62	VAR + $nVAR_R$
Pellets within ram (error)(S)	447	VAR
Total	509	

The repeatability analysis was calculated from the intra-class correlation, combining the appropriate variance components:

VAR_B= Variation between rams.
VAR_W= Variation within rams.

so Total VAR = $VAR_B + VAR_W$ and within a ram the variation is
VAR_W= MS between samples or pellets.

Thus, repeatability of the semen characteristic is calculated...

$$r = \frac{VAR_B}{VAR_B + VAR_W}$$

3.6.4 Results.

3.6.4.1 Analysis of variance for fresh and frozen-thawed semen characteristics.

The results of the analysis of variance are presented in Table 3.14.

Table 3.14. Mean square values for semen characteristics as affected by the type of semen (T), rams (R) and sample within type of semen within ram (STR).

Semen characteristic	MEAN SQUARES VALUES T	R	STR
TOTAL	483.04	4.70	1.12
MOTILE	1719.56	15.65	3.07
PROGR	371.24	7.39	1.67
MOT	348.86	4.90	1.43
VSL	28.69	4.22	1.23
LIN %	8.48	2.30	0.97
ALH	32.54	0.67	0.27
LIN	5.54	2.44	0.93
VAP	36.41	3.97	1.28
RAPID %	402.70	87.36	1.61
MEDIUM %	163.93	3.75	1.91
SLOW %	3.00	0.68	0.48
STATIC %	121.47	0.74	0.67

All values significant at $p < 0.001$.

All fresh semen characteristics were significantly different to frozen-thawed semen characteristics ($p < 0.001$, Table 3.15 vs 3.17). All semen characteristics varied significantly between rams within type of semen ($p < 0.001$). Rams produced a wide range of fresh semen quality, with low and high values in the different semen characteristics and these were affected differently by processing and freezing. Some rams produced semen of good freezability (more resistant), whereas semen characteristics from other rams were drastically affected by freezing producing significantly lower post-thawing semen quality.

3.6.4.2 Variance components of fresh semen characteristics and the repeatability of the estimations.

The mean, standard deviation and the range for fresh semen characteristics are presented in Table 3.15..

Table 3.15. Mean, standard deviation and range of fresh semen characteristics.

Semen characteristic	Mean	Standard Deviation	Range	
			Minimum	Maximum
TOTAL	2517.0	1708.64	99.0	9926.0
MOTILE	1552.0	1308.64	22.0	8306.0
PROGR	37.0	20.19	0.0	80.1
MOT	59.0	22.38	5.5	94.2
VSL	115.0	32.77	41.0	196.8
LIN %	86.7	6.91	60.4	97.7
ALH	6.4	1.17	3.2	10.0
LIN	75.7	10.74	44.6	93.4
VAP	128.3	32.41	48.0	211.0
RAPID %	39.9	21.51	0.0	83.4
MEDIUM %	19.1	15.00	0.0	73.1
SLOW %	0.4	0.53	0.0	4.5
STATIC %	40.4	22.26	5.8	93.1

A large range of values were observed for most of the semen characteristics, due to the large number of unselected rams. The SLOW % recorded the lowest value and had a standard deviation bigger than the mean. In this particular set of data, this characteristic may not have been measured very accurately and would have low repeatability. The variance components and repeatabilities for fresh semen characteristics are presented in Table 3.16.

Table 3.16. Variance components expressed as actual values and percentages and repeatabilities for fresh semen characteristics.

Semen characteristic	Variance components (value) Between rams	Within ram	Variance components (percentage) Between rams	Within ram	Repeatability
TOTAL	2255201.00	684300.00	76.72	23.28	0.77
MOTILE	1225351.70	51100.00	70.57	29.43	0.71
PROGR	312.56	97.86	76.16	23.84	0.76
MOT	368.29	135.90	73.05	26.95	0.73
VSL	829.49	251.20	76.76	23.24	0.77
LIN %	34.95	13.12	72.71	27.29	0.73
ALH	0.95	0.43	68.97	31.03	0.69
LIN	84.16	32.98	71.85	28.15	0.72
VAP	831.41	227.30	78.53	21.47	0.79
RAPID %	352.34	113.70	75.60	24.40	0.76
MEDIUM %	183.81	43.04	81.03	18.97	0.81
SLOW %	0.04	0.24	14.13	85.87	0.14
STATIC %	358.24	140.40	71.84	28.16	0.72

Except for the SLOW %, which had the lowest variance and repeatability, the fresh semen characteristics varied to a greater extent due to the rams (between rams) than between samples within ram and had a high repeatability.

3.6.4.3 Variance components of frozen-thawed semen characteristics and the repeatability of the estimations

The mean, standard deviation and the range for frozen-thawed semen characteristics are presented in Table 3.17.

Table 3.17. Mean, standard deviation and range of frozen-thawed semen characteristics.

Semen characteristic	Mean	Standard Deviation	Range Minimum	Range Maximum
TOTAL	478.4	504.04	36.0	3322.2
MOTILE	117.0	251.30	0.0	2053.0
PROGR	10.2	9.78	0.0	43.1
MOT	17.6	13.60	0.0	69.7
VSL	98.5	48.01	0.0	253.6
LIN %	84.5	22.40	0.0	99.9
ALH	4.2	1.98	0.0	11.6
LIN	76.4	21.69	0.0	99.8
VAP	104.6	48.45	0.0	256.6
RAPID %	10.5	10.01	0.0	44.7
MEDIUM %	7.1	6.37	0.0	38.2
SLOW %	0.9	2.09	0.0	21.9
STATIC %	81.5	13.83	26.9	100.0

There was a larger range in the values of frozen-thawed semen characteristics than found in fresh semen. The values of the semen characteristics were lower for frozen-thawed semen.

The variance components and repeatabilities for frozen-thawed semen characteristics are presented in Table 3.18.

Table 3.18. Variance components expressed as actual values and percentages and the repeatability for frozen thawed characteristics.

Semen characteristic	**Variance components** (value)		**Variance components** (percentage)		**Repeatability**
	Between rams	Within ram	Between rams	Within ram	
TOTAL	224920.32	32270.00	87.45	12.55	0.87
MOTILE	54758.62	9154.00	85.68	14.32	0.86
PROGR	71.53	25.28	79.89	26.11	0.80
MOT	141.10	44.59	75.99	24.01	0.76
VSL	1090.55	1229.00	47.02	52.98	0.47
LIN %	146.42	357.20	29.07	70.93	0.29
ALH	1.25	2.70	31.75	68.25	0.32
LIN	156.74	315.00	33.23	66.77	0.33
VAP	1098.33	1264.00	46.49	53.51	0.46
RAPID %	74.89	26.33	73.99	26.01	0.74
MEDIUM %	20.69	20.16	50.65	49.35	0.50
SLOW %	1.22	3.18	27.73	72.27	0.27
STATIC %	140.19	52.90	72.60	27.40	0.73

The variance components and repeatabilities for frozen-thawed semen characteristics were different to those for fresh semen due to the semen processing and freezing. With frozen-thawed samples the variation in TOTAL, MOTILE, PROGR, MOT, RAPID % and STATIC % was larger between rams than between samples within ram, and the variation in VSL, LIN %, ALH, LIN, VAP, MEDIUM % and SLOW % varied more between samples than between rams. The variation in semen characteristics between samples within ram is inversely proportional to the repeatability of their estimation.

3.6.5 Discussion.

The previous experiment was similar to this but using semen collected by artificial vagina and frozen in straws. The limitation of the previous experiment was the small number of selected rams used. In the present experiment, the main limitation was the small number of samples per ram. More meaningful results would probably be obtained if the larger number of rams was combined with greater numbers of samples per ram. It was not practical to collect more ejaculates per ram in this experiment because of the use of electro-ejaculation and the large number of rams involved.

Data from fresh and frozen-thawed ram semen collected by electro-ejaculation and assessed by computerised image analysis are presented for first time in this experiment and can be used as a reference for future research and comparisons. The collection of semen by electro-ejaculation may have had an adverse effect on some semen characteristics. Unfortunately it was not possible to compare methods of collection in this experiment, but it is expected that electro-ejaculated semen compared with semen collected by artificial vagina would have a greater volume due to the amount of plasma produced, less wave motion, with no differences in other semen characteristics (Borisov, 1947; Mattner and Voglmayr, 1962). Therefore the findings of this experiment should be largely applicable to semen collected by artificial vagina.

Fresh semen characteristics were changed significantly by processing and freezing in pellet form. Despite a careful protocol of processing and the appropriate diluent for freezing,

ram semen was drastically affected by cryopreservation. In this population of rams, a wide range of fresh semen quality was observed. This variation apparently increases when the population of rams is large and particularly when they are randomly selected. When an artificial breeding program is undertaken, it may be possible to reduce this variation by a pre-selection of sires with high semen quality.

The interaction between type of semen (fresh and frozen) and ram found in this study means it may be possible to identify sires which produce fresh semen of high quality, and also sires which produce semen with higher or lower resistance to processing and freezing. Such sires are more likely to be of use in AI programs with frozen-thawed semen. If such rams can be identified, it will be interesting to determine if freezability of semen is heritable. Thus, it might be possible to breed stock which produce semen ~~of high resistance to cryopreservation~~. which shows good motility after freezing and thawing.

When fresh and frozen-thawed semen characteristics were analyzed independently, most of the variation in fresh semen was due to the rams; in other words, the variation in semen characteristics between rams was larger than the variation in semen characteristics between samples within ram. This variation was larger than in the previous experiment due to the larger randomly selected population of rams. A wide range in semen quality was found between the rams and most of the semen characteristics had high repeatability. The small variation between pellets within ram had a direct effect on repeatabilities which were high. The analysis indicated that all fresh semen characteristics were estimated accurately by computerised image analysis.

For half of the frozen-thawed semen characteristics the variation was larger between pellets within ram, and for the other half the variation was larger between rams. This suggests that semen characteristics are affected to different extents between pellets by processing and freezing. Semen characteristics related to the velocity (VSL, VAP) and direction (LIN %, LIN, ALH) were more susceptible to processing and freezing then motility characteristics, as the major variation was found between pellets within ram. Thus, in these frozen-thawed semen characteristics the variation caused between rams is not as important as the variation produced by processing and freezing. The repeatability of these semen characteristics was also lower,

suggesting that these semen characteristics are measured less accurately than those with higher repeatability.

However, variance components and repeatability estimates from fresh and frozen semen were in general of similar magnitude, and higher than semen frozen in straws in the previous experiment. Such repeatabilities suggest that reliable estimates of semen characteristics could be obtained by computerised image analysis of fresh and pellet-frozen semen. The difference in repeatability between fresh and frozen-thawed semen characteristics may have an effect in terms of the number of samples to be assessed. Those with low repeatability (particularly frozen-thawed characteristics) may require a larger number of samples to be assessed in order to increase the accuracy of their estimation.

CHAPTER 4. THE EFFECT OF SEPARATION OF THE EPIDIDYMIS FROM THE TESTIS AND HEMICASTRATION ON SEMEN QUALITY AND FERTILITY.

4.1 Introduction.

The structure and function of the epididymis have been extensively studied, although some aspects are still not well understood. The importance of the relationship between the testis and the epididymis is not known although these two organs are physically very close. In most mammals, extensive and strong fibrous connective tissues hold the epididymis in place. The influence of the epididymis on spermatozoa is thought to be mediated by the circulatory blood, lymphatic and the luminal fluid (Jones and Glover, 1975; Cooper, 1986). The composition of the fluid varies along the length of the duct as a consequence of modification of the prevailing distal current of testicular exocrine secretion, by the processes of secretion into, and resorption from the lumen. These processes must occur across the epithelium and are mediated by the epithelial cells (Mann and Lutwak-Mann, 1981). Fluid in the tail of the epididymis (see Jones and Glover, 1975) is more likely to influence the maintenance of sperm fertilizing capacity (sperm storage) than sperm maturation, which occurs more proximally (Mann and Lutwak-Mann, 1981).

The importance of the relationship between the testis and epididymis for semen quality and fertility is not well known. In an attempt to determine the effect of separation and heating of epididymis on semen quality during storage in the epididymis, Bedford (1978) studied rats and rabbits in which the epididymides but not the testes were reflected into the abdomen; the continuity of the excurrent duct with the functional testes was maintained. Under these circumstances the spermatozoa underwent maturation but the storage in the epididymis was compromised and sperm motility was adversely affected. In this work, the effect of increased temperature was confounded with the effect of the separation of epididymides. Increased testicular temperature is known to affect semen quality, fertilizing capacity (Dutt and Simpson,

1957; Fowler and Dun, 1966; Fowler, 1968; Howarth, 1969; Rathore, 1970b), and to increase embryo mortality (Dutt et al., 1959, Alliston and Ulberg, 1961; Howarth, 1969; Rathore, 1970b; Setchell et al., 1988), but it is usually thought that sperm in the epididymis are unaffected.

In a study of the lymphatic connections between the testis and epididymis, Jones (1984) supported the theory of a direct transfer of materials from the testis to the ipsilateral epididymis in rats and rams. Specifically, radioactive iodine labelled human serum albumin (I^{125} HSA) with a molecular weight of about 60,000 was transferred from testicular lymph to the ipsilateral epididymis. Albumin is about the same size as the androgen binding protein found in testicular lymph, and much larger than steroids. Jones's experiment suggests the lymphatics of the ram testis may play some part in the fluid exchanges which occur during maturation, storage and subsequent release of sperm. The epididymis functions through its luminal fluid which surround the sperm cells. Thus, the exchange of fluids from the testis by the lymphatic connections to the epididymis could have more influence on the spermatozoa than previously thought, and the lymphatic connections may contain the essential component which produces the maturation of the sperm cells and confers the motility needed for the sperm to reach the fertilization site.

To be able to perform a separation of the epididymis from the testis, it was necessary to perform a hemicastration, so the control rams were also hemicastrated. This requirement for hemicastration must be considered because hemicastration itself can cause some changes in the remaining testis.

It is known that a compensatory hypertrophy of the Sertoli cells occurs as a result of hemicastration. Waites et al. (1983) reported an increment of Sertoli cell mass in hemicastrated ram lambs, but it was not determined whether the change was due to hypertrophy or hyperplasia. Mirando et al. (1989) in similar studies, reported that 150 days after hemicastration or unilateral castration (UC) the mean testis weight was increased by 56 % and the mean epididymal weight by 15 %; moreover, the observed difference in mean volume was 146 % greater for UC rams than the control rams. The hemicastration resulted in a significant

hypertrophy and this was consistent with the findings of other workers (Voglmayr and Mattner, 1968; Walton et al., 1978). Epididymal weight also increases with hemicastration (Brown et al., 1987; Mirando et al., 1989) and thus, a larger epididymis is associated with an increase of sperm production.

For the purposes of the present study such changes were not considered and the hemicastrated control animals were used as a reference point. One intact ram was also studied to give some assessment of the effect of hemicastration.

The present experiment was designed to determine the effect of separation of the epididymis from the testis (without any change in temperature) on semen quality, fertility and embryo mortality.

4.2 Experimental procedure.

This experiment commenced in September 1988 with the semen collection, assessment and selection of the rams to be used. Operations were done in November 1988. Then the collections were continued during 1989 until November when the rams were involved in a mating program for approximately 45 days and then kept for further studies.

4.2.1 Semen collection and assessment.

Twelve rams were selected with the following characteristics: a) ability to be trained for collection by artificial vagina, b) semen of good motility and concentration, c) good general health.

Semen was collected twice a week by artificial vagina at least one or two months before surgery and re-started 6 to 19 days after surgery according to recovery which varied between rams. Freshly collected semen was held at 32°C, and manually assessed for mass motility, individual motility or motility %, volume, ratio of live and dead in 200 cells and concentration

of spermatozoa. During 1989, semen was regularly collected and assessed to detect any changes in quality over time until November 1989, when the rams were randomly assigned to groups of mature ewes to mate and thus to assess the effect of the treatments on fertility.

When the SE+SEP group of rams was formed (see below), two characteristics of semen quality which seemed most affected by the treatments were chosen: motility and percentage of live spermatozoa. These two characteristics were compared on the basis of their frequency below certain pre-set standards which were considered to be lower than in a normal semen sample. These two standards were set at 60 % of motility and 50 % of live cells.

4.2.2 Surgical procedure.

Rams were divided into three groups each of 4 rams for the following treatments: 1) Hemicastrated control (HC), 2) Separation of epididymis (SE) 3) Separation of epididymis and location in a separate pouch (SEP). The surgical procedures were performed through November-December, 1988 and are described in paragraphs 2.11.3.1 and 2.11.3.2. Unfortunately one control, two separated epididymis and one separated epididymis pouch rams died during the experiment, so the data from two groups of rams with the separated epididymis were pooled, and the groups were called SE+SEP (separated epididymis), and HC (hemicastrated). An intact ram was included in order to compare the performance of hemicastrated control rams as complementary data.

4.2.3 Mating program.

The mating program was conducted at Glenthorne farm in November 1989. Before the joining of the rams with the ewes, semen was collected and assessed in order to compare semen quality with fertility. Then, each ram was fitted with a raddle harness (Radford et al., 1960) and run in a separate paddock with 50 randomly selected ewes. The raddled ewes were noted each day and separated from the ram. Rams remained with the ewes for approximately 45 days and

were then withdrawn from each of the groups. The ewes were bled 17 days after mating for progesterone assay as an early pregnancy test, and examined a second time by ultrasound for pregnancy 50 to 65 days after mating. The embryo loss was determined by the difference between the pregnancy rates at 17 and 65 days after mating.

4.3 Statistical analysis.

The data was analyzed by Chi-square analysis of contingency tables between treatments.

4.4 Results.

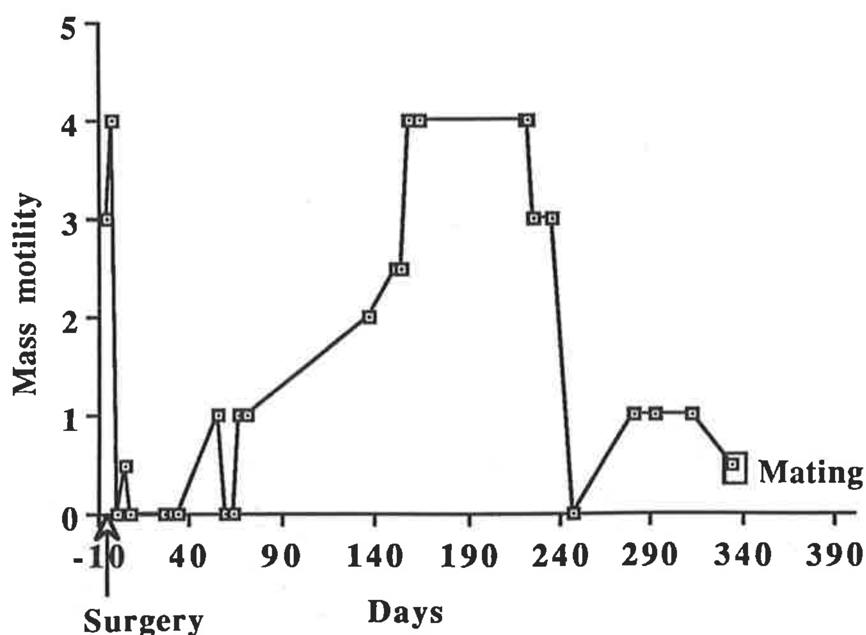
4.4.1 Variation in semen characteristics up to the mating program.

1. Semen characteristics from SE, SEP, HC and the intact ram.

The semen characteristics of rams evaluated through time after treatments are presented on a per ram basis within group in Figures 4.1 to 4.16.

MASS MOTILITY OF SEMEN FROM INDIVIDUAL RAMS THROUGH TIME AFTER TREATMENT: GROUPS SE, SEP, HC AND INTACT RAM.

Figure 4.1. SE Rams.



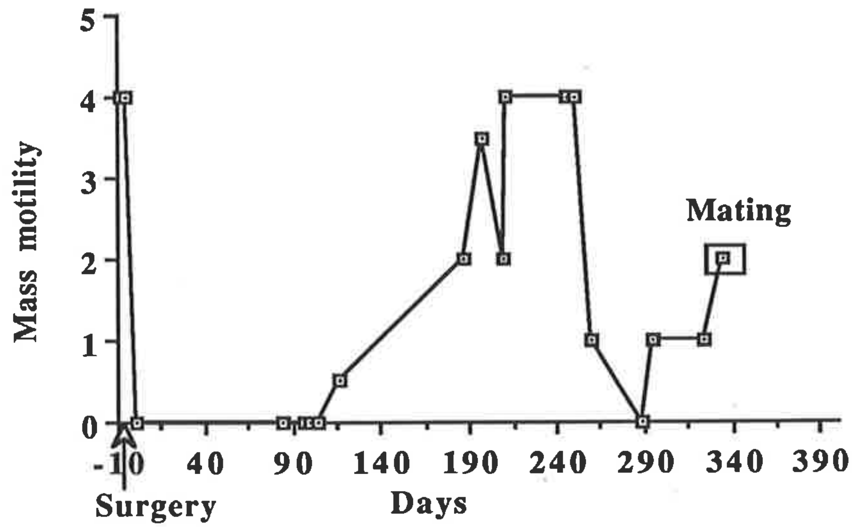
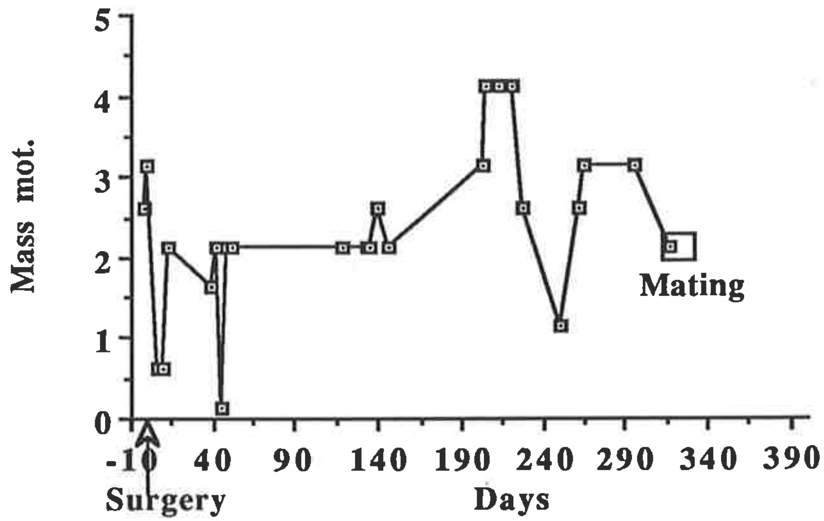
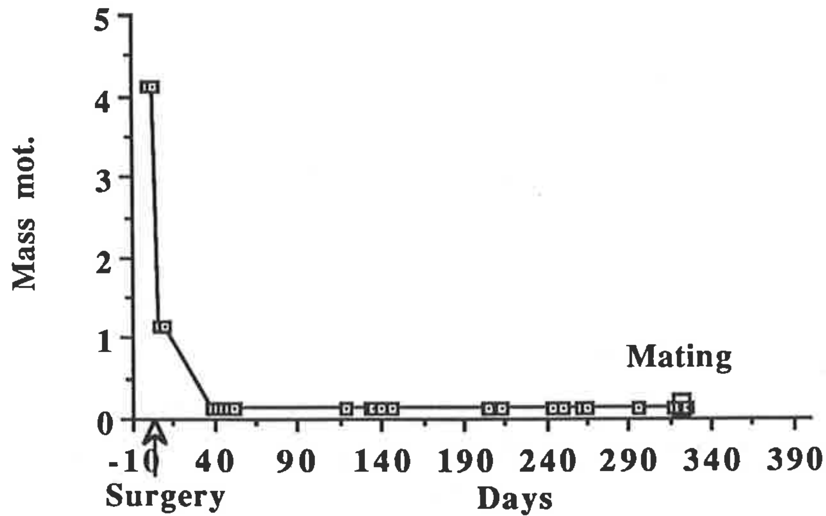


Figure 4.2. SEP Rams.





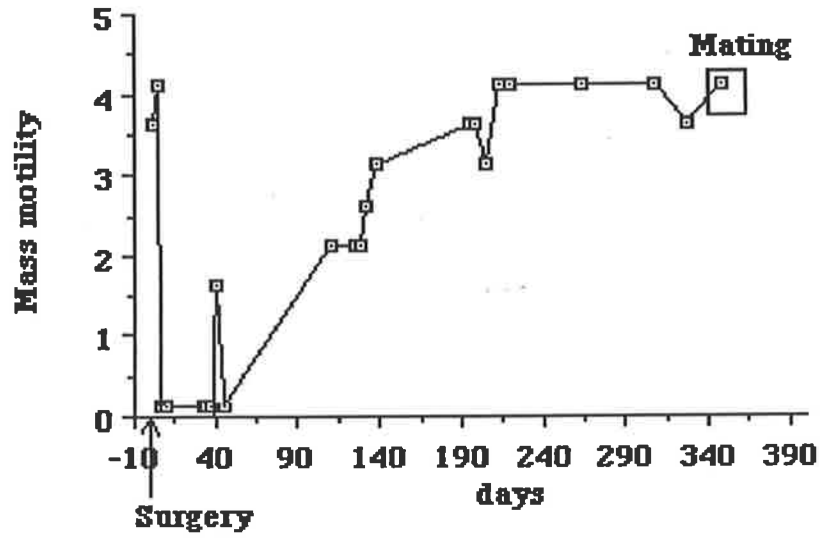
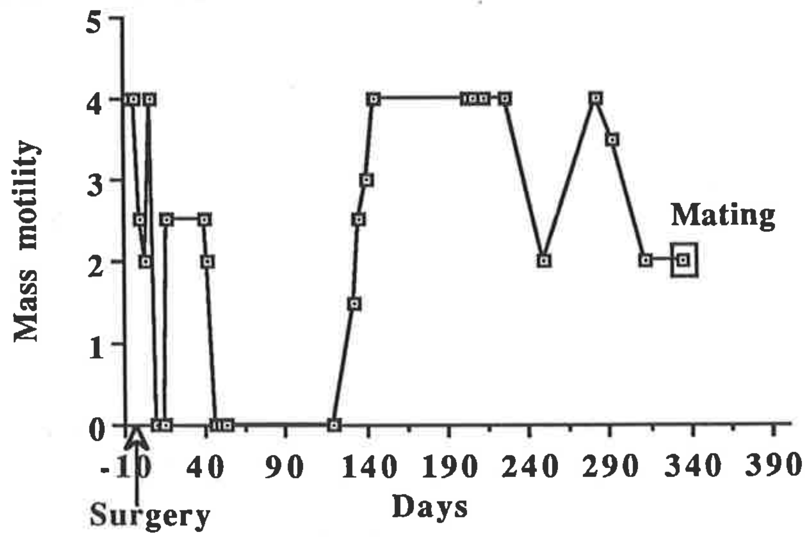
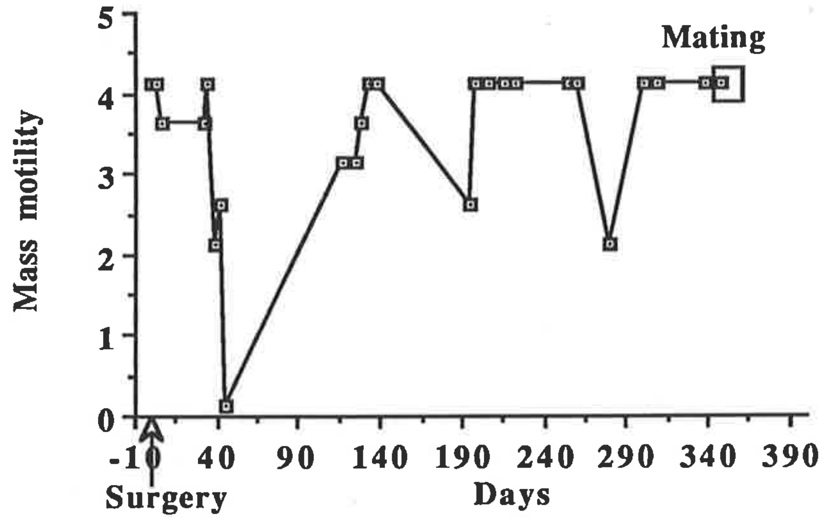


Figure 4.3. HC Rams.



Ram n. 480



Ram n. 498

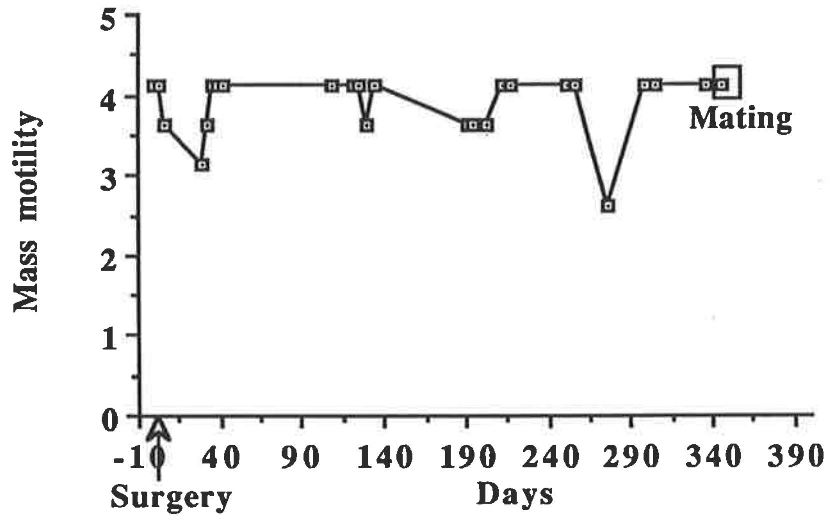
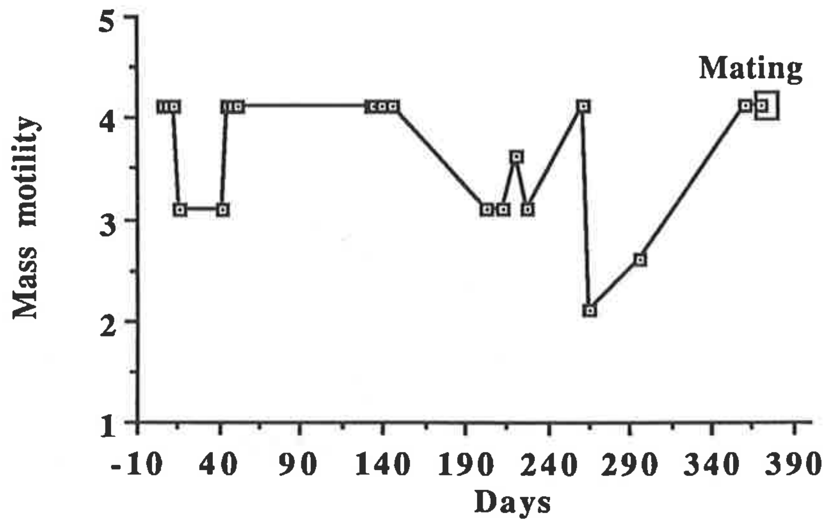


Figure 4.4. Intact ram.

Ram n. 138



(a) Mass motility.

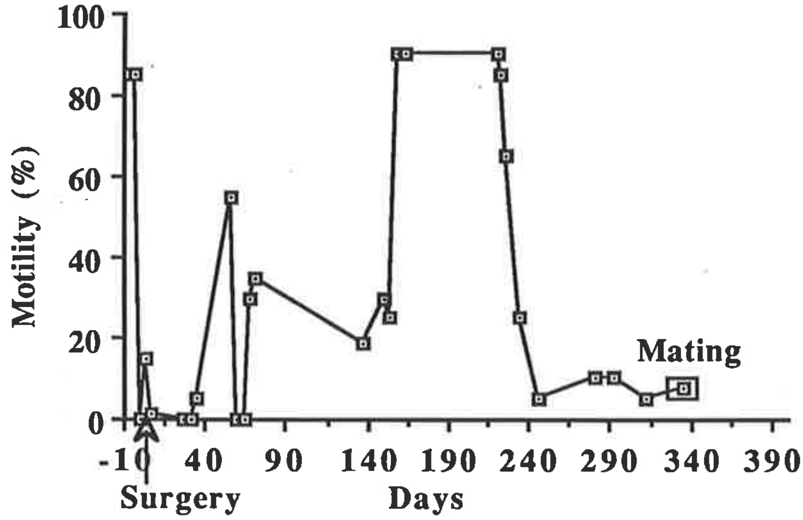
There was a large variation between rams within groups. It was not possible to determine in detail how many days after surgery it was possible to collect and assess the first semen sample from each ram. Ram 48 and 483 gave the first ejaculate at 8 and 19 days after surgery respectively; rams 465, 467 and 478 gave the first ejaculate 15 days after surgery; and rams 53, 480 and 498 gave the first ejaculate at 11, 11 and 10 days after surgery respectively. Rams from SE and SEP groups recovered and started working later than the HC rams. In the SE group, Ram 48 recovered the original mass motility about 150 days after the surgery while ram 483 did not do so until about 215 days after surgery (Figure 4.1). Ram 483 did not work at all for some 90 days after the surgery (Figure 4.1). SEP rams 465 and 478 recovered the original or even better mass motility about 215 days after surgery, whereas ram 467 did not recover original mass motility at all; this ram maintained very low semen quality after surgery (Figure 4.2). It was also noticed that SEP rams 483 and 478 took longer to recover than SEP ram 48, and about the same time as ram 483 from the SE group. Rams from the SE and SEP groups responded in a similar manner to treatments, showing more variation through time than the HC group or the intact ram. All HC rams had a lower mass motility after surgery probably due to the intervention and stress caused, but they recovered much faster than the SE and SEP group about 140 days after surgery (Figure 4.3). Ram 498 had a very fast recovery and maintained a better mass motility than the rest. The effect of hemicastration showed less diminution on the mass motility than the separation of epididymides. The intact ram was very constant, having a low mass motility on day 265 similar to the rest of the rams, which was probably due to the collection technique and the season.

(b) Motility (%).

As for mass motility, motility was affected differently by the different treatments. In group SE, rams recovered sperm motility at about the same time (Figure 4.5), but ram 483 started to work a little later than ram 48. As for mass motility, rams 465 and 478 recovered their original sperm motility at about the same time; some recovery was noted around day 40 after surgery reaching maximum values around 190 days after surgery while ram 467

MOTILITY (%) OF SEMEN FROM INDIVIDUAL RAMS THROUGH TIME AFTER TREATMENT: GROUPS SE, SEP, HC AND INTACT RAM.

Figure 4.5. SE Rams.



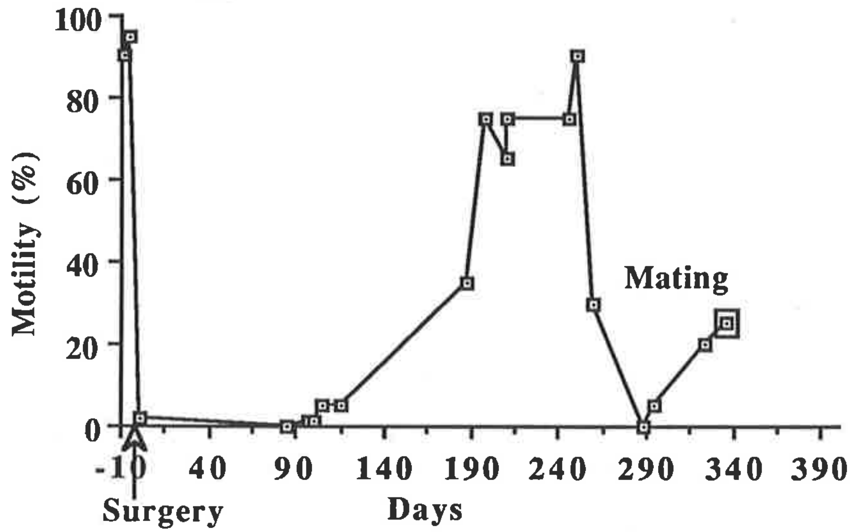
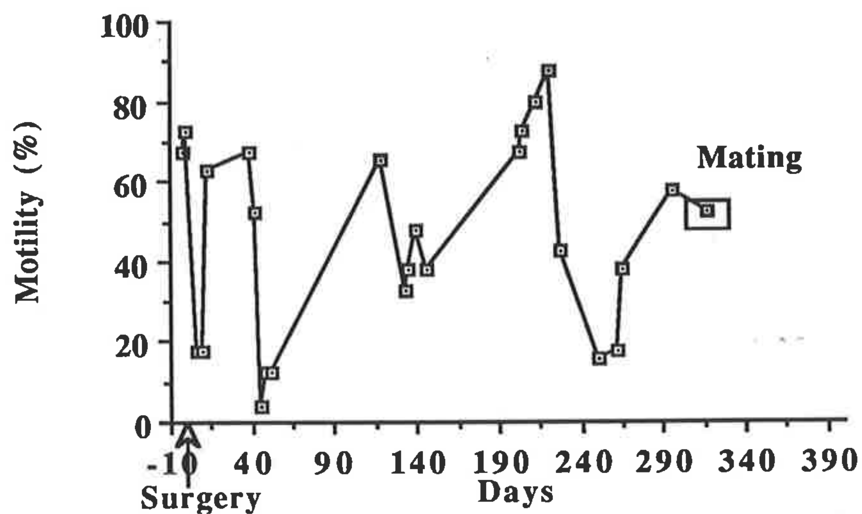
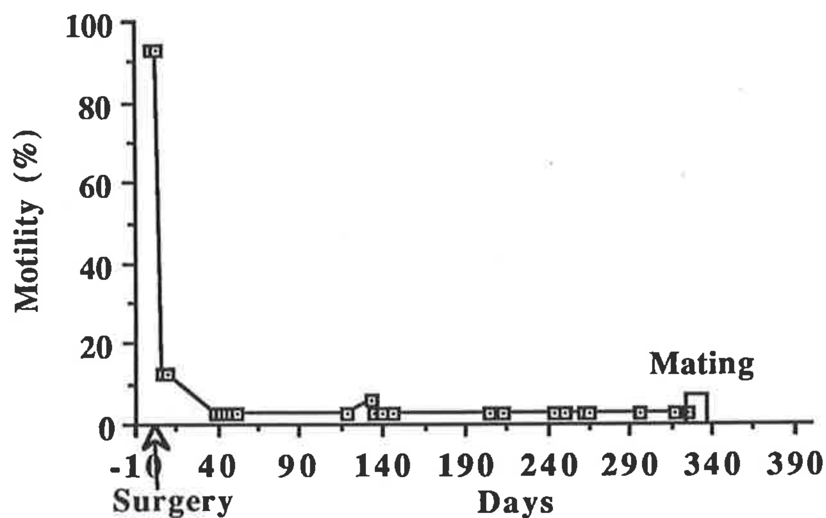


Figure 4.6. SEP Rams.

Ram n. 465



Ram n. 467



Ram n. 478

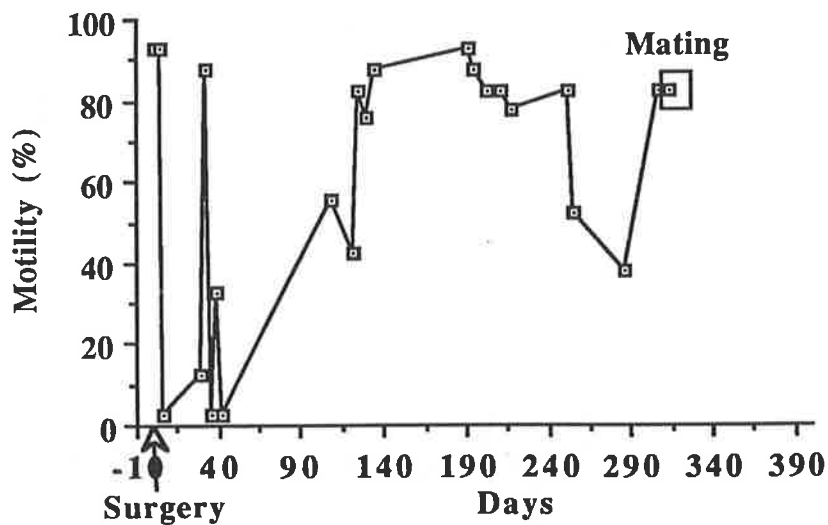
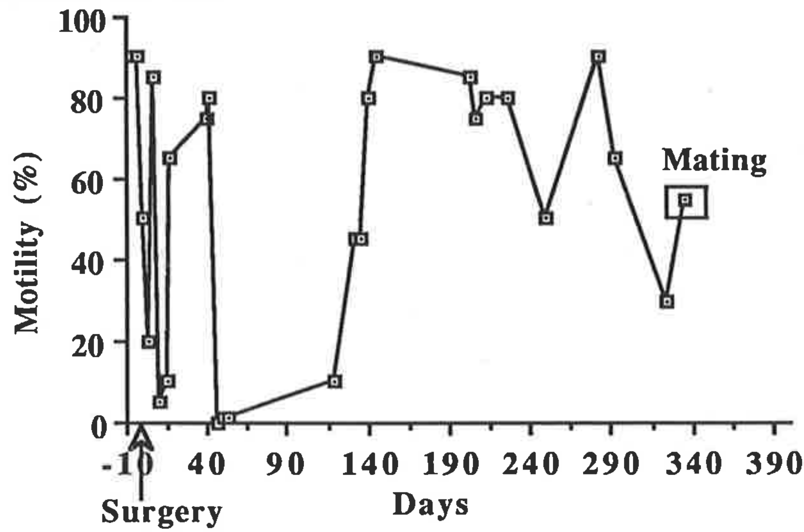
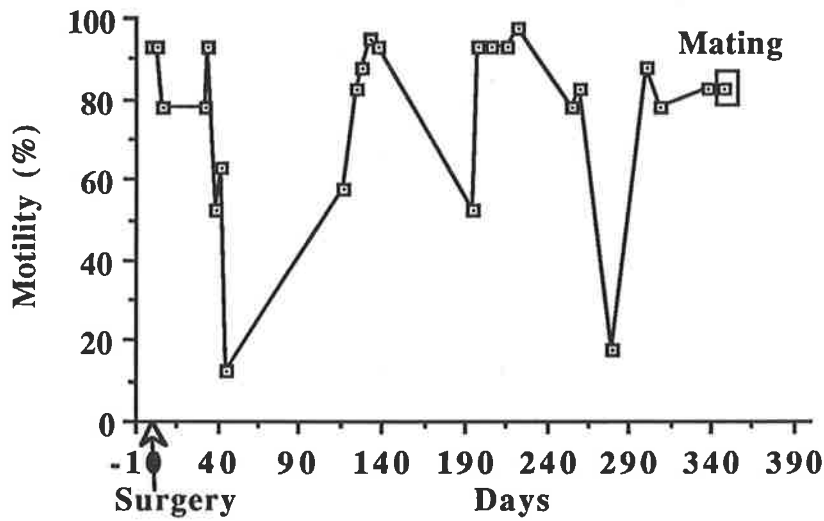


Figure 4.7. HC Rams.





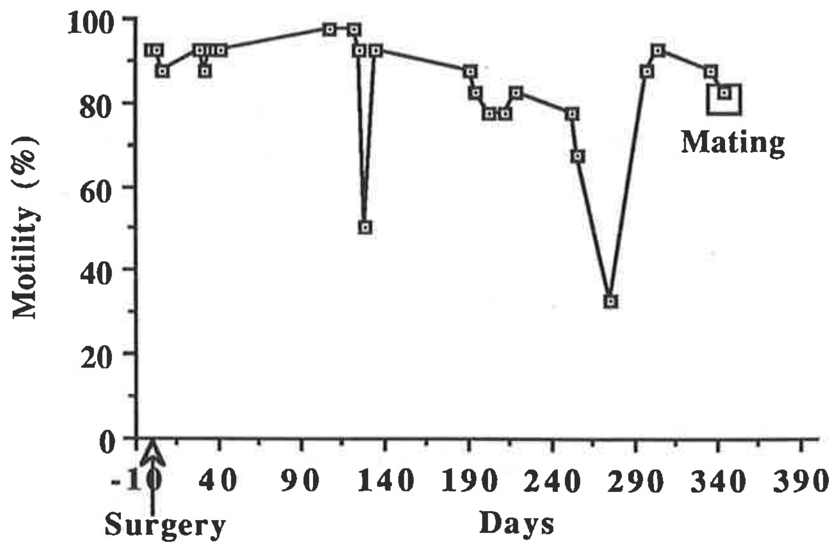
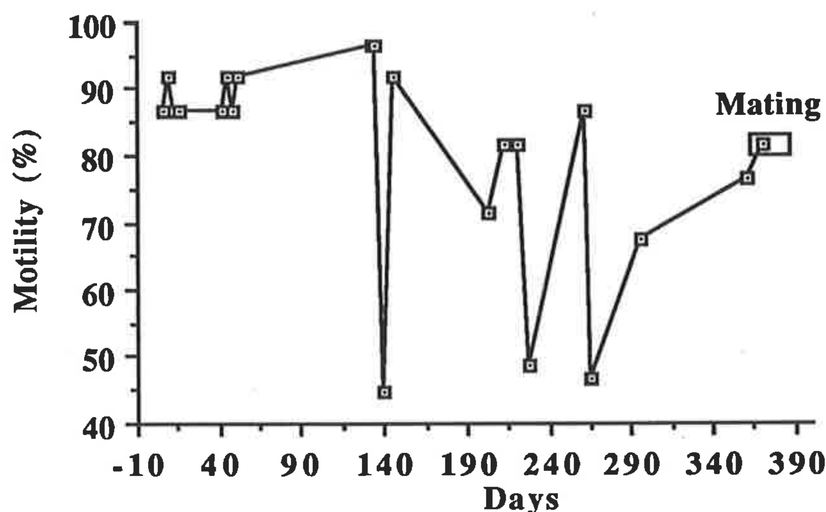


Figure 4.8. Intact ram.



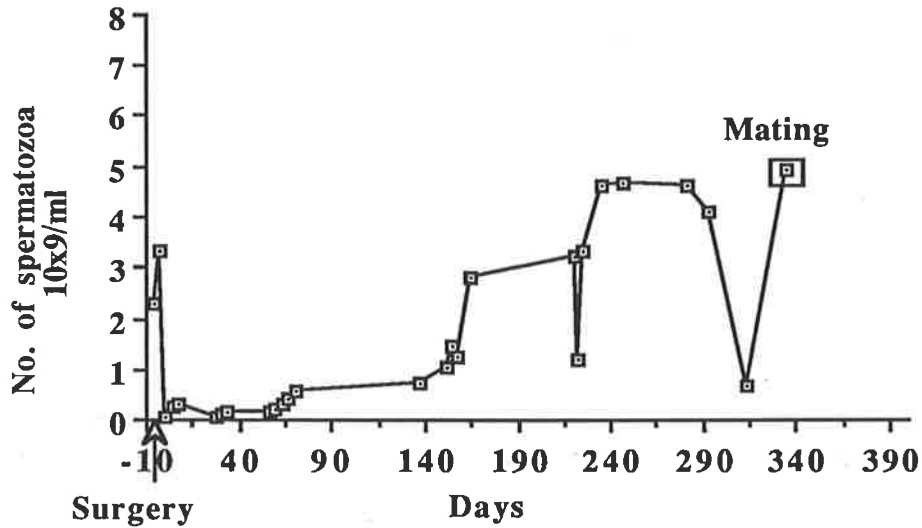
never recovered its original sperm motility (Figure 4.6). HC rams 53 and 480 had a very similar recovery of motility; motility peaked at around day 40 after surgery, then fell and recovered slowly, reaching the maximum values at about 100 days after surgery (Figure 4.7). Ram 498 had the fastest recovery of spermatozoa. This ram had no appreciable fall in sperm motility after surgery and maintained quite normal semen quality until mating (Figure 4.7). The intact ram showed a greater variation through time compared with the control rams from experiment 5.

(c) Concentration of spermatozoa.

Concentration of spermatozoa was also affected by the different treatments. In the SE group, ram 48 had better recovery than ram 483, reaching higher values than before surgery. In group SEP, rams 465 and 478 recovered similarly, but ram 467 as for mass motility and sperm motility never recovered the original concentration of spermatozoa; it remained with the lowest values recorded throughout the observation time. Group HC was very homogeneous, with all rams recovering at a similar rate (Figures 4.9, 4.10 and 4.11). The intact ram showed variation in concentration of spermatozoa through the observation time, probably due to the collection and assessment technique or seasonal effects (Figure 4.12).

CONCENTRATION OF SPERMATOZOA IN SEMEN FROM INDIVIDUAL RAMS THROUGH TIME AFTER TREATMENT: GROUPS SE, SEP, HC AND INTACT RAM.

Figure 4.9. SE Rams.



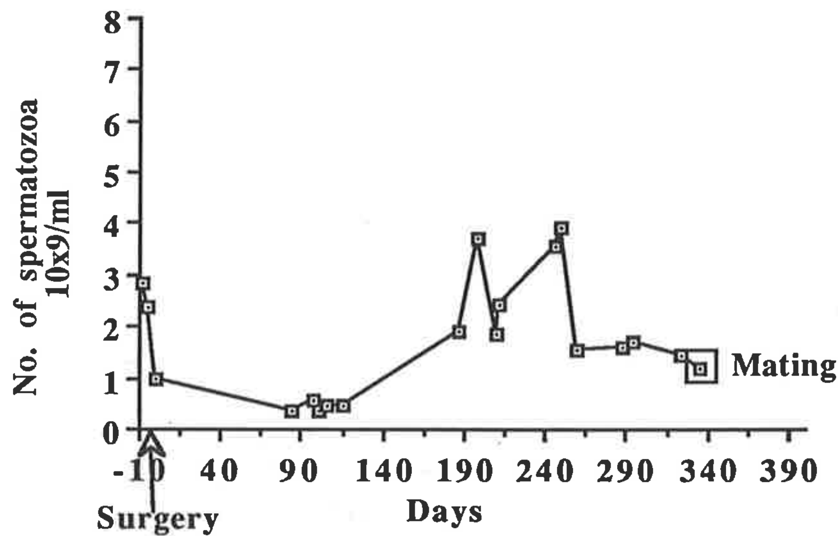
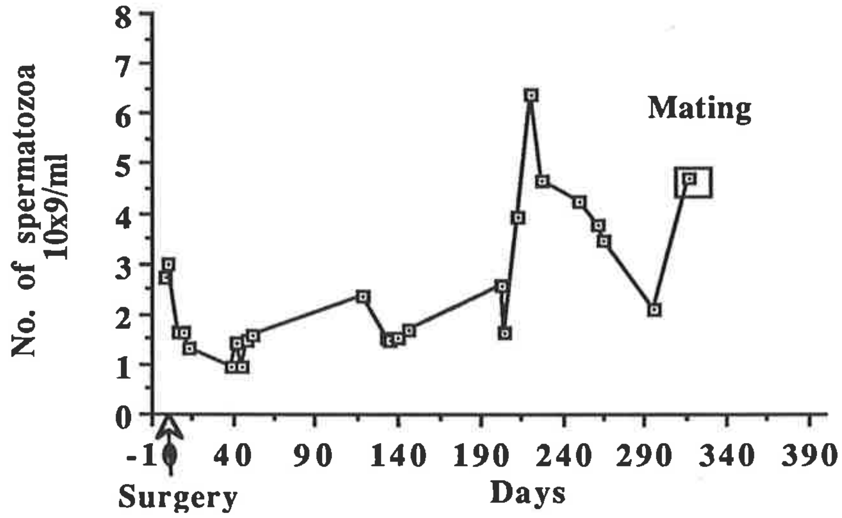
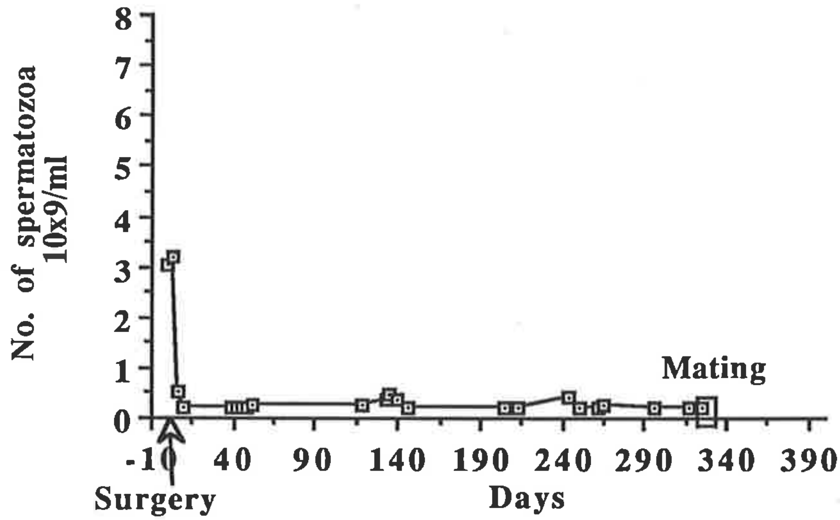


Figure 4.10. SEP Rams.





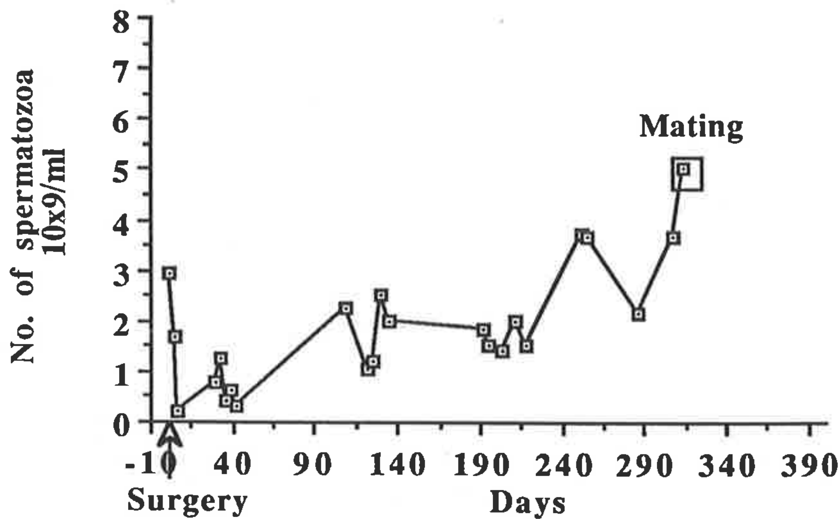
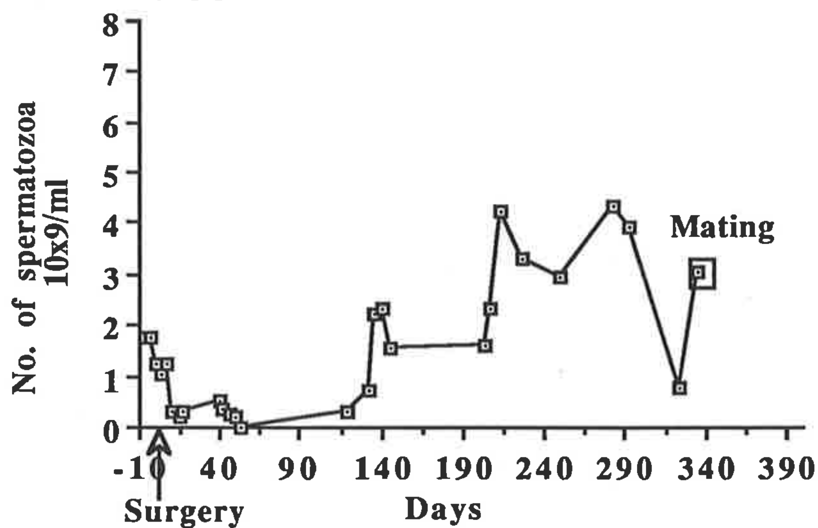
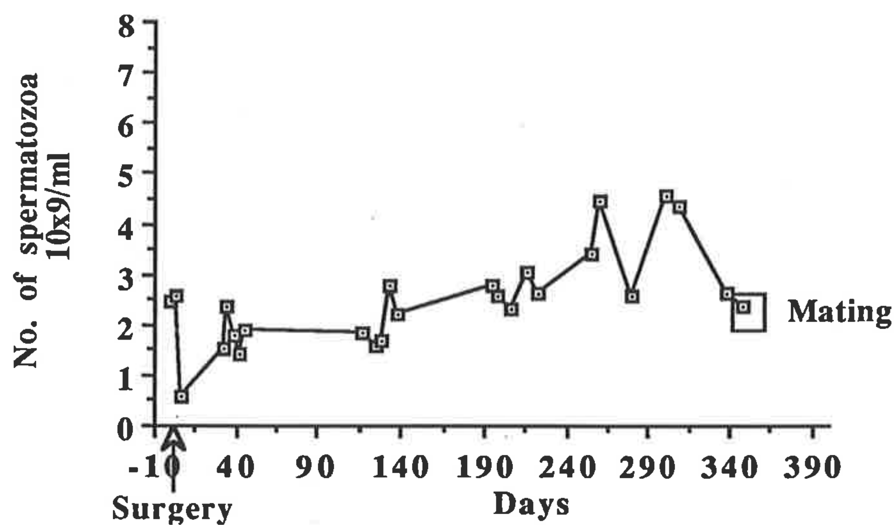


Figure 4.11. HC Rams.





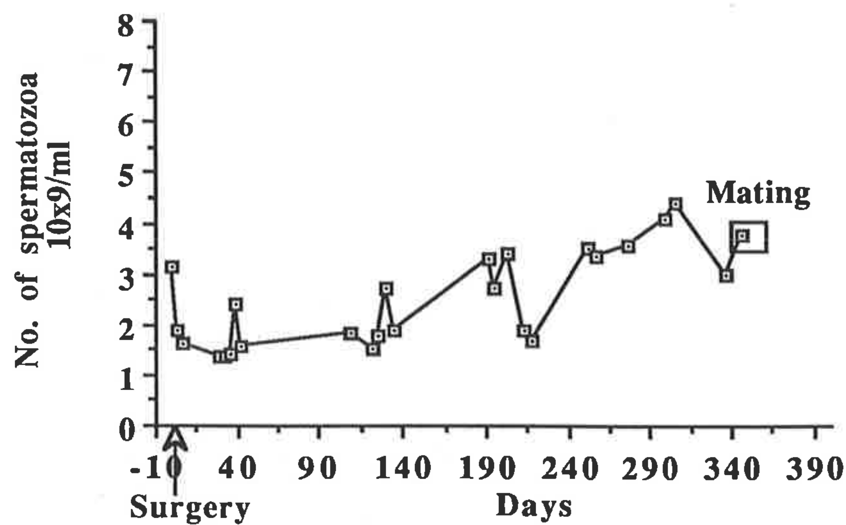
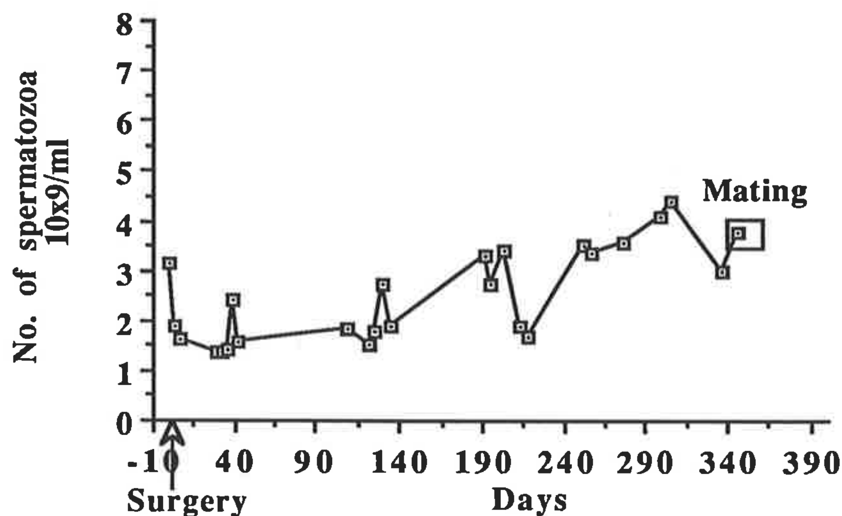


Figure 4.12. Intact ram.

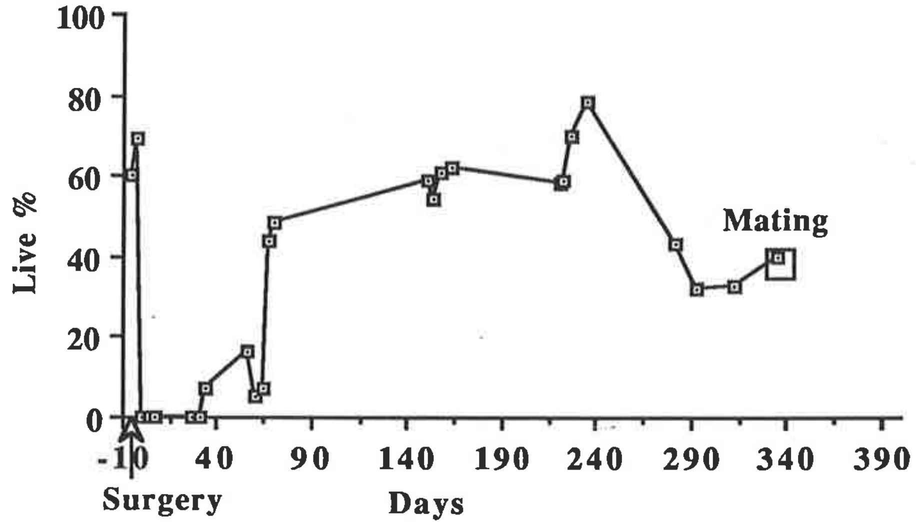


In the SE group, the rams recovered the original percentage of live sperm about 235 days after surgery (Figure 4.13). In group SEP, ram 478 had the best recovery followed by ram 465, while ram 467 never recovered to a normal level of live sperm (Figure 4.14). Rams from group HC recovered between 140 and 240 days after treatment (Figure 4.15).

There were large variations between rams within the various groups, so it was difficult to characterize the response in each particular group by using such information. The small number of animals and the loss of some rams made it even more difficult.

PERCENTAGE OF LIVE SPERMATOZOA IN SEMEN FROM INDIVIDUAL RAMS THROUGH TIME AFTER TREATMENT; GROUPS SE, SEP, HC AND INTACT RAM.

Figure 4.13. SE Rams.



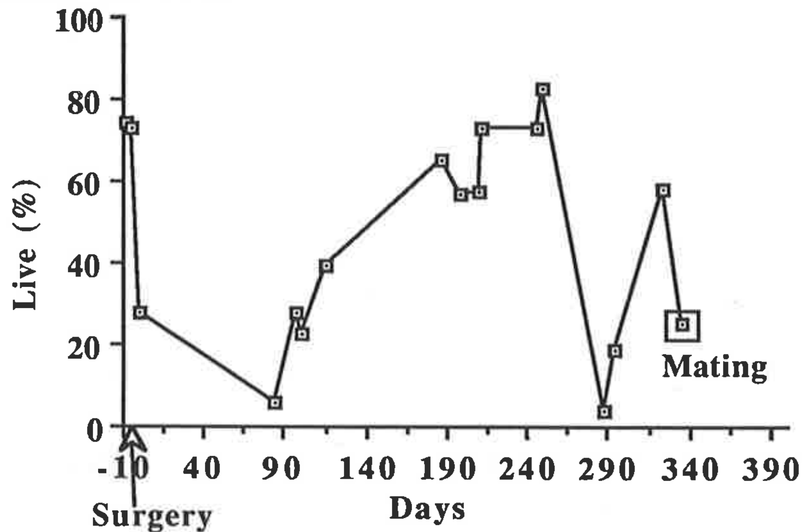
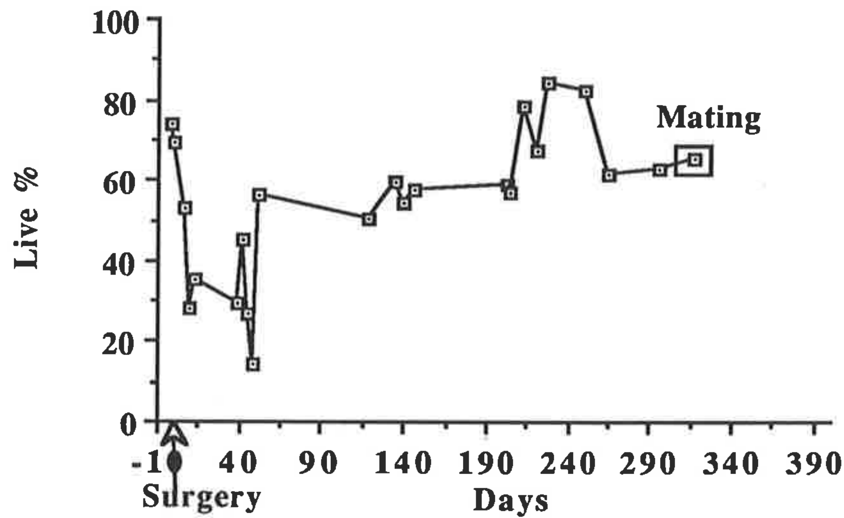
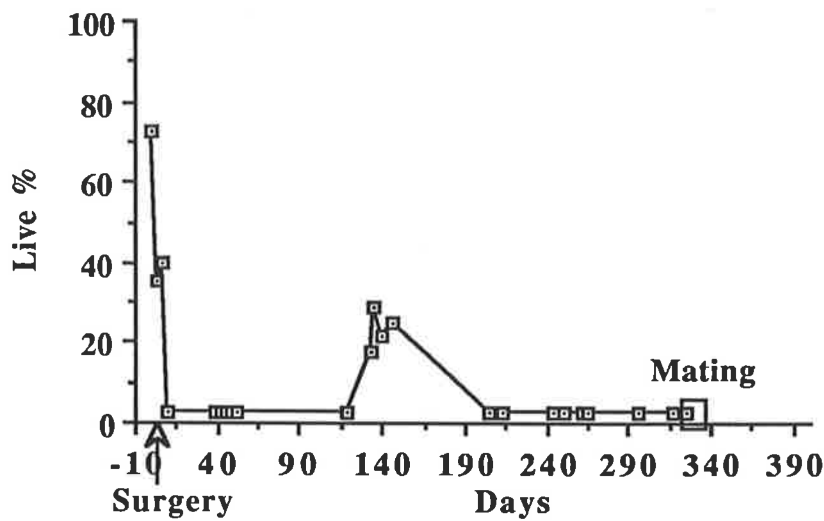


Figure 4.14. SEP Rams





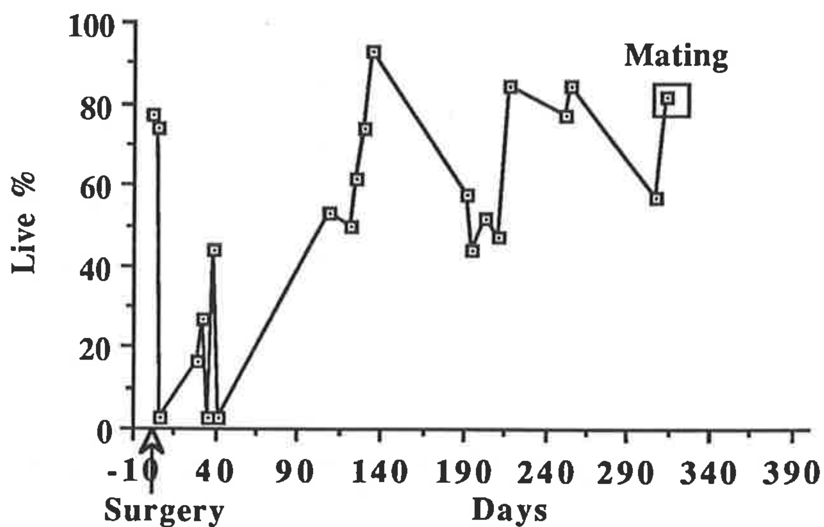
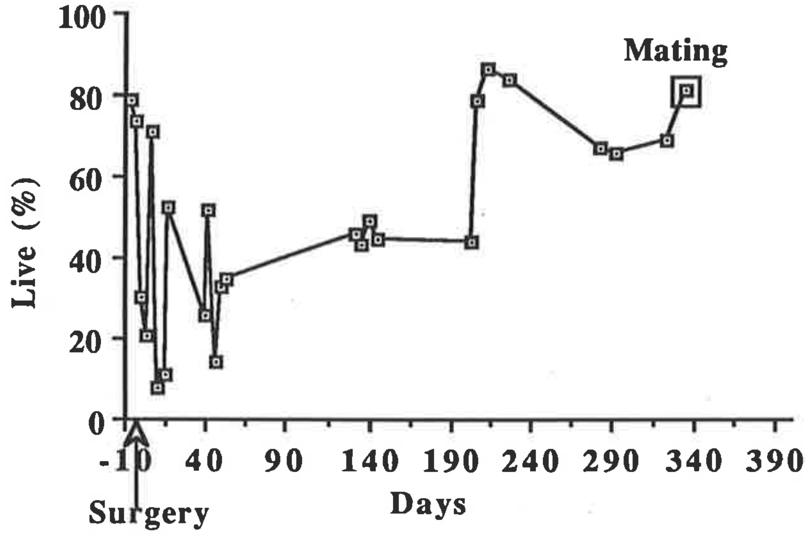
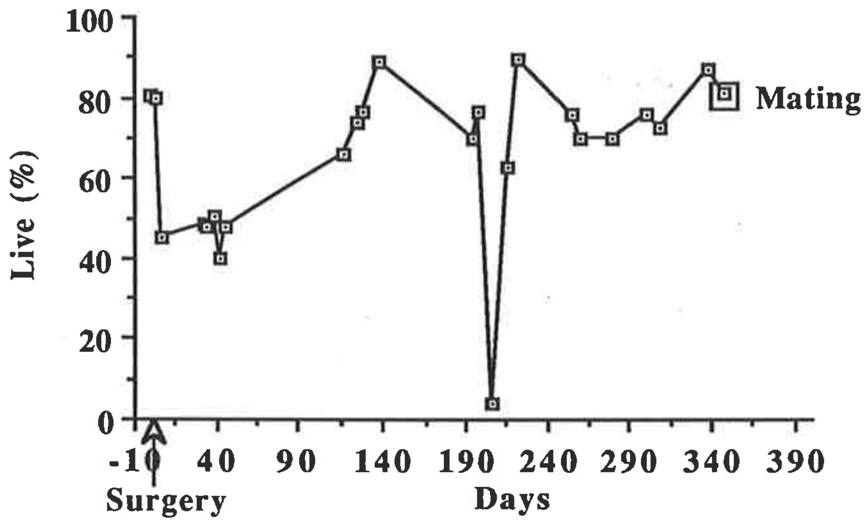


Figure 4.15. HC Rams.





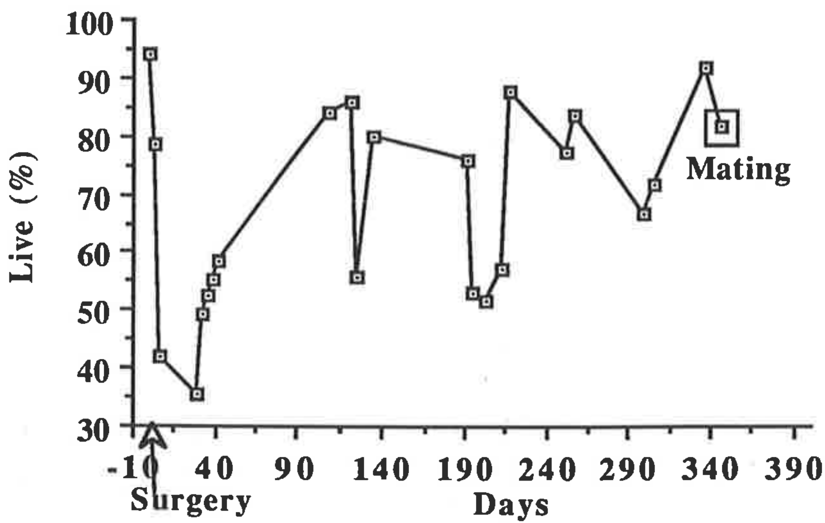
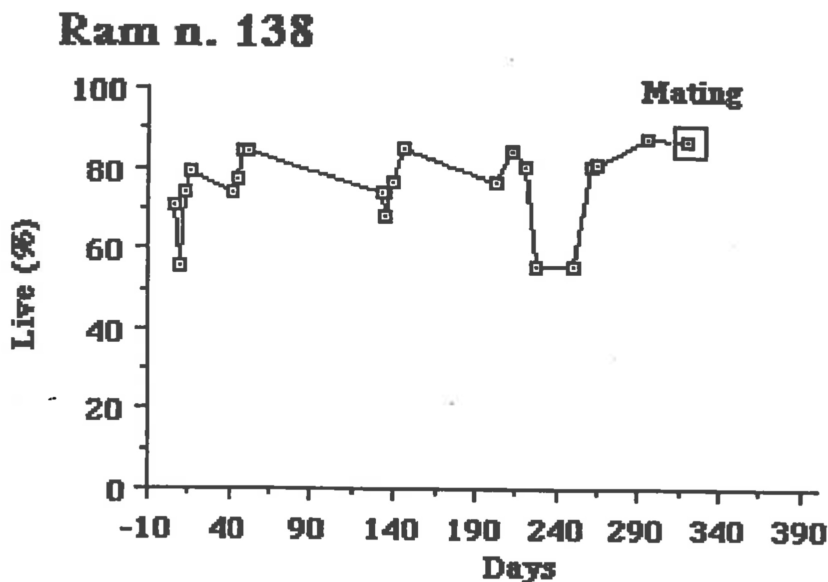


Figure 4.16. Intact ram.



2. Motility and percentage of live sperm from pooled results of SE+SEP, HC and the intact ram.

The frequency with which motility and percentage of live sperm fell below the arbitrary standards set for a normal semen sample is shown for each ram in Table 4.1. The following section of results correspond to the pooled group SE+SEP, HC and the intact ram. No attempt was made to account for seasonal variation.

Table 4.1. The frequency with which motility and percentage of live sperm were below normal standards for individual rams within groups of treatment.

Ram	Treatment	Frequency (%) motility		% live sperm	
48	SE	23/27	(85.2)	17/25	(68.0)
483	SE	14/18	(77.8)	7/15	(46.7)
465	SEP	17/27	(63.0)	7/25	(28.0)
467	SEP	22/24	(92.0)	23/24	(95.8)
478	SEP	11/24	(45.8)	10/23	(43.5)
53	HC	14/27	(51.85)	14/25	(56.0)
480	HC	7/23	(30.4)	6/22	(27.3)
498	HC	3/22	(12.6)	4/22	(18.2)
138	Intact ram	3/19	(17.8)	0/19	(0.0)

The frequency with which motility was below 60 % during the observation period is shown in Figure 4.17. The separation of the epididymis from the testis increased the frequency with which motility was below 60 % compared with the HC group ($p < 0.001$). Both SE+SEP and HC rams had a higher frequency than the intact ram ($p < 0.01$). The results for the intact ram are similar to the control rams used in experiment 5, where none of the control rams had a motility which was below 60 %.

The frequency of samples in which more than 50 % of the cells were dead is presented in Figure 4.18. As for motility, frequency was greater in SE+SEP rams than in the HC group ($p < 0.001$). Both of the latter groups had a greater frequency when more than 50 % of the cells were dead than the control ram ($p < 0.001$). The results from the intact ram were similar to the results from the control rams in experiment 5. None of the latter rams had a percentage of live sperm below 50 %, and they also showed similar variations through the observation time as the intact ram in this experiment.

Figure 4.17. Effect of separation of epididymis from the testis (SE+SEP), hemicastration (HC) and control (intact ram) on the frequency with which motility was below 60 % .

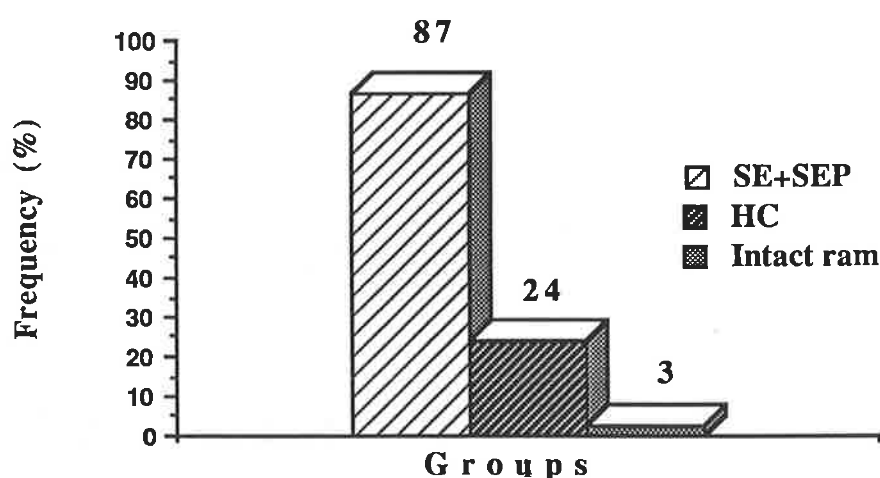
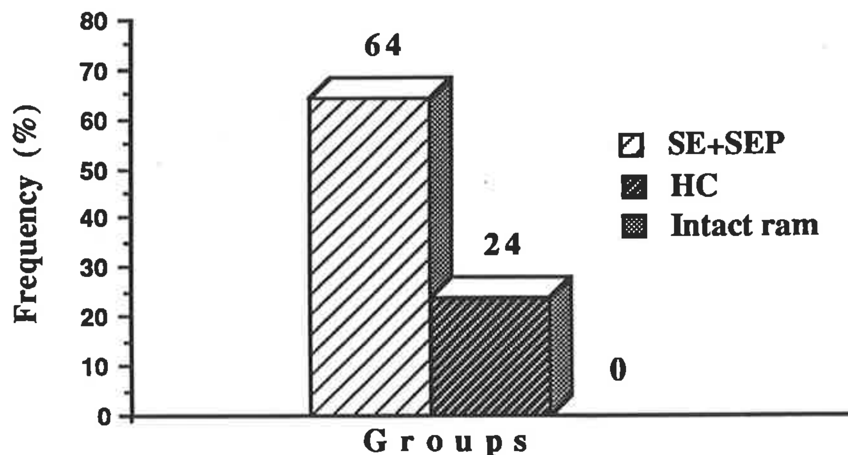


Figure 4.18. Effect of separation of epididymis from the testis (SE+SEP), hemicastration (HC) and control (intact ram) on the frequency with which percentage of live sperm was below 50 %.



4.4.2 Fertility results.

All groups of rams raddled similar proportions of ewes (Figure 4.19). The results from the semen assessment prior to the mating program and the pregnancy rates 17 and 65 days after mating are presented for each ram in Table 4.2. In the SE+SEP group, the three rams with poor quality semen characteristics (48, 483 and 467) also had low fertility. The two SE+SEP rams with similar semen characteristics to rams in the HC group (465 and 478), also had similar fertility to the HC rams. The pregnancy rates from rams 465 and 478 were high and not significantly different from the HC rams or the intact ram. Unfortunately, due to the individual ram variation found in this experiment, it was not possible to be conclusive about the effect of each of the treatments on either semen characteristics or fertility. However, a positive correlation was found between the semen characteristics assessed and fertility. Figure 4.20 shows the correlation between pregnancy rate at 65 days after mating and motility ($r = 0.968$, $p < 0.001$) and percent live sperm ($r = 0.945$, $p < 0.001$). Similar values were found for the rest of semen characteristics assessed.

Figure 4.19. Percentage of ewes raddled by rams with the epididymis separated from the testis (SE+SEP), hemicastrated rams (HC) and the control (intact).

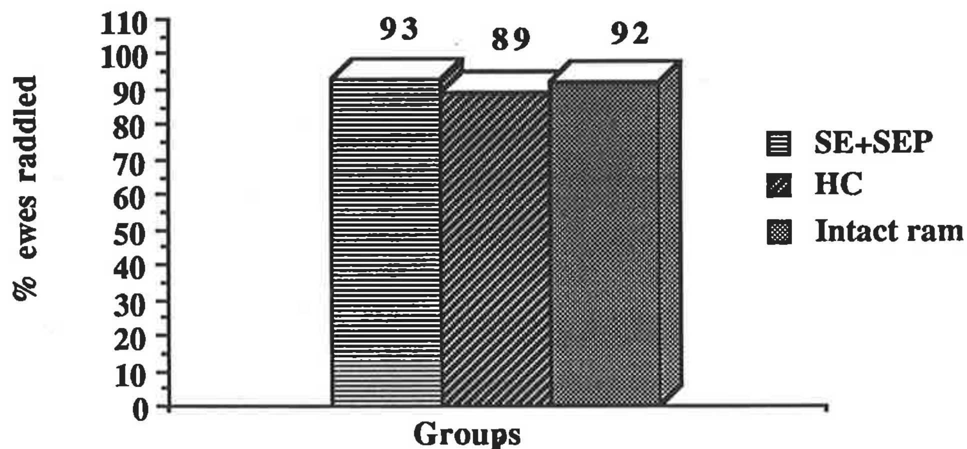


Table 4.2. Semen characteristics prior to the mating program and pregnancy 17 and 65 days after mating for each group and ram.

Group	Ram	Mass motility	Individual motility (%)	Concentration of spermatozoa x10^9/ml	% live	Ewes pregnant/ ewes mated(%) 17 days after mating	65 days after mating
SE	48	0.5	8	4.95	40	10/46 (21.8)	0/46 (0.0)
	483	0	5	0.2	21.5	12/45 (26.7)	1/45 (2.2)
SEP	465	2	50	4.5	72.5	38/46 (82.6)	30/46 (65.2)
	467	0	0	0	0	15/48 (31.3)	0/48 (0.0)
	478	4	78	3.05	87.0	45/49 (91.8)	38/49 (77.6)
	53	2	55	3.03	81.0	39/45 (86.7)	35/45 (77.8)
HC	480	4	80	2.15	78.5	38/44 (86.4)	35/44 (79.5)
	498	4	80	3.55	80.0	38/44 (86.4)	35/44 (79.5)
Intact	**138**	4	80	6.25	82.0	39/46 (84.4)	36/46 (78.3)

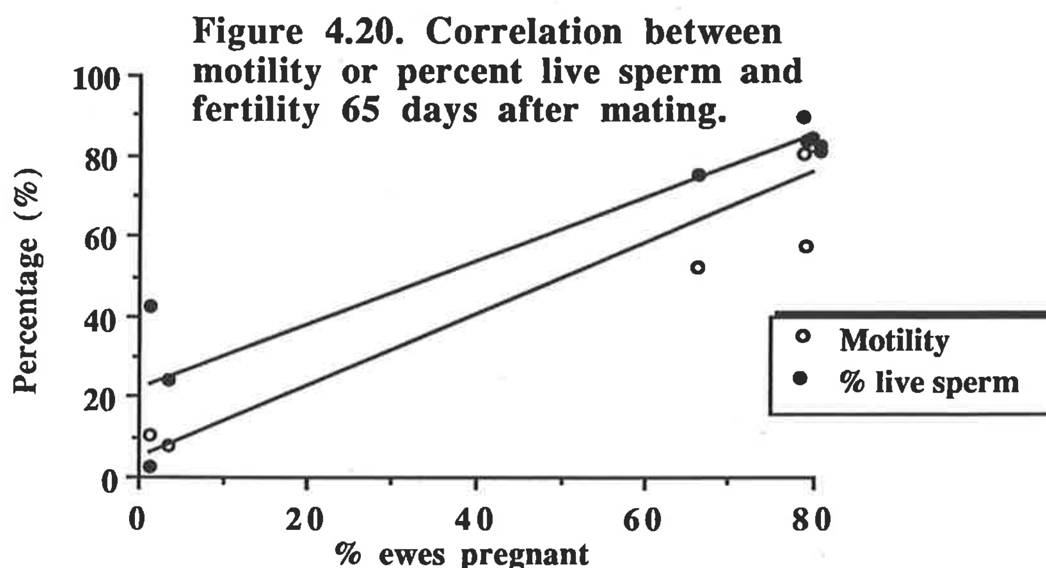


Figure 4.20. Correlation between motility or percent live sperm and fertility 65 days after mating.

The number of ewes mated, ewes pregnant and the estimated number of embryos lost are summarized in Table 4.3.

Table 4.3. Number of rams, ewes mated, pregnancy at 17 and 65 days after mating and number of embryos lost by the SE+SEP, HC and intact rams.

Group	No. of rams	No. ewes mated	No. ewes pregnant (%) 17 days after mating	No. ewes pregnant (%) 65 days after mating	~~No. embryo lost (%)~~ No. ewes pregnant at 17d but not at 65d.
SE+SEP	5	234	120 (51.3)	69** (29.5)	51 (42.5)
HC	3	133	115 (86.5)	105 (79.0)	10 (8.7)
Intact ram	1	46	39 (84.8)	36 (78.0)	3 (7.6)

(**) $p < 0.001$.

A comparison of pregnancy at 17 and 65 days after mating is shown in Figure 4.21 The pregnancy rates both at 17 and 65 days achieved by the SE+SEP rams was significantly lower than the HC group and the intact ram ($p < 0.001$). However, when the HC group was compared with the intact ram, and there was no significant difference. There was a significant

reduction in the pregnancy rate at 17 days compared with 65 days in the SE+SEP group ($p < 0.001$), whereas in the HC rams and the intact ram no significant difference was found between the two pregnancy tests. Thus, the SE+SEP group had higher embryo mortality than the HC group and intact ram (Figure 4.22.).

Figure 4.21. Comparison of pregnancy at 17 and 65 days following mating of ewes to rams with the epididymis separated from the testis (SE+SEP), hemicastrated rams (HC) and the control ram (intact).

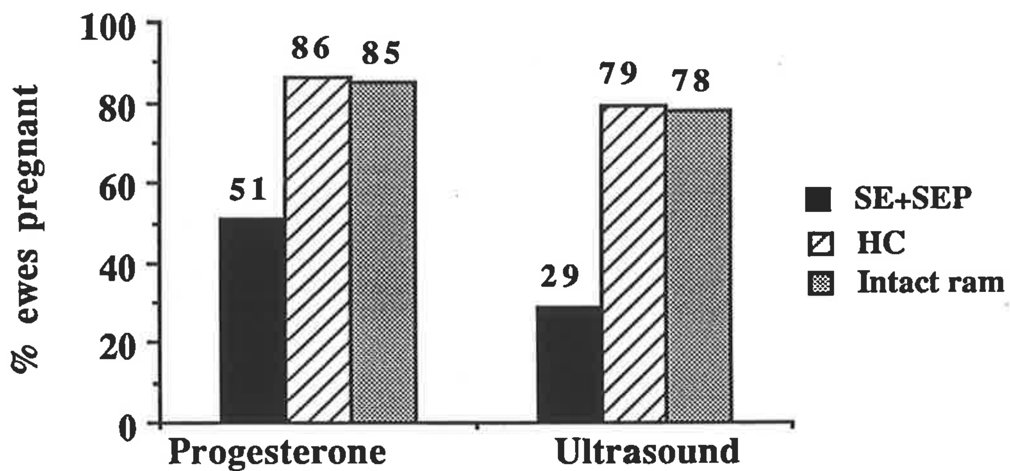
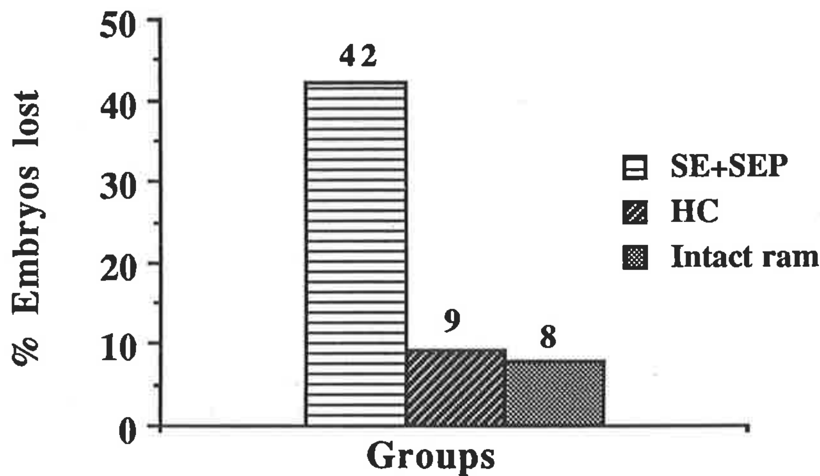


Figure 4.22. Percentage of embryos lost following mating of ewes to rams with the epididymis separated from the testis (SE+SEP), hemicastrated rams (HC) and the control ram (intact).



4.5 Discussion.

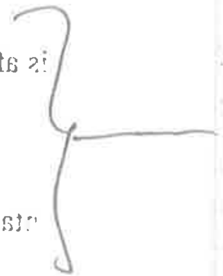
In the present experiment, a large variation was observed between rams within treatments which confused the effect of each of the treatments on semen quality and fertility. Two of the rams from the SE+SEP group responded similarly to rams from the HC group, and some rams were lost during the experiment making the results difficult to interpret. Nevertheless, in the rams from the SE+SEP group which presented an effect on semen quality through time, the recovery was slower, and the mass motility, individual motility, % live sperm and concentration of spermatozoa were reduced in comparison with unaffected rams. In general terms semen from such rams presented more variability through time than the HC group and the intact ram. There was an effect of hemicastration over a short period of time, which was related to the stress and effects of the surgery. This effect was manifested by an increased frequency of motility below 60 % and percentage of live spermatozoa below 50 % compared with the intact ram.

When the semen quality from the intact ram was also compared with the semen of control rams from experiment 5, the number of times in which motility was below 60 % and percentage of live spermatozoa below 50 % were similar.

These results are similar to those reported by Bedford (1978) who performed a similar experiment, but also exposed the epididymides to abdominal temperatures. The number of immature spermatozoa was increased and a large proportion of spermatozoa with low motility were present, which he suggested was due to the effect of temperature on the epididymis. He attributed such effects to an increase in the rate of passage of the spermatozoa caused by a reduction in the storage capacity of the epididymis. He also concluded that the abdominal temperature apparently does not alter the environment of the epididymis required for maturation. Unfortunately, Bedford (1978) made no attempt to assess fertility.

In subsequent work by Jones (1984), the effect of similar separation of the epididymides from the testis was established. He reported a larger diminution of sperm production in SEP rams than HC rams. The SEP rams reached a much lower production in the

Unfortunately, the design of this experiment, as was also the case with the experiment described in the next chapter, did not allow determination of the stage at which embryo loss occurred, nor did it allow for the assessment of embryonic loss before 17 days of pregnancy. There are also inbuilt erors in the use of blood progesterone levels to diagnose pregnancy at 17 days after insemination, and no estimates were made of fetal loss after 65 days of pregnancy.



short term and a recovery of about 20 % of normal production was observed by around 80 days after surgery. The motility, on the other hand, was reported unchanged. In the present experiment, the production of viable sperm was decreased soon after the operation, and the recuperation was different in each of the groups but later than reported by Jones (1984). In three of the rams from the SE+SEP group semen production never recovered. However, the motility was also decreased independently of sperm production, and was affected to a greater extent in the SE+SEP group of rams. The lymphatics of the testis may play an important role during fluid exchanges which occurs during maturation, storage and subsequent release of sperm, thus fertility may also be affected.

A fertility test was performed in the present experiment, and it was observed that semen characteristics were correlated with fertility; thus rams with low values for semen characteristics had low fertility after mating. The separation of epididymis from the testis affected fertility by dramatically reducing the percentage of pregnant ewes at an early stage (17 days) after mating, with an even greater effect at 65 days of pregnancy. Thus, it appears that not only is the fertilizing ability of the sperm affected by the separation of the epididymides from the testes, expressed by the lower proportion of pregnant ewes, but also the viability of the fertilized eggs is affected, reflected in an increased embryo wastage by 65 days after mating.

Although the HC group had greater frequency of low motility and dead cells than the intact ram or the control rams from experiment 5, the fertility and the embryo survival achieved was the same as the intact ram, although individual variation was also observed and one of the hemicastrated rams had lower semen quality than the other two. Thus, although hemicastration may temporarily change semen quality, a full recovery is achieved in a few months, and fertility and embryo viability can be normal.

Failure of fertilization could be because of reduced sperm quality (particularly motility) and fertilizing capacity which are acquired during passage through different parts of the epididymis (Mann and Lutwak-Mann, 1981; Amann, 1987). This is reflected by the reduction of pregnancies up to day 18 of the cycle (Edey, 1966, 1967; Cumming, 1972a). Embryo mortality can occur even during implantation or later, but also it may be a continuation of a

process which began earlier (Robinson, 1951). In the fertility trial in this experiment, it was observed that motility % was reduced in SE+SEP rams and this was reflected in low fertilizing capacity of the spermatozoa indicated by reduced fertility at 17 days after mating.

There are some observations about embryo mortality caused by immature spermatozoa. Embryo loss is increased when eggs are fertilized by sperm from the caput or the distal corpus of the epididymis (Amann, 1987). The fertilization of eggs by immature sperm may cause delayed early cleavage divisions which lead to improper synchrony between embryo development and the uterine environment (Fournier-Delpech et al., 1981). Amann (1987) attributed the maturation of sperm to exchanges between the caput and the cauda epididymis during the sperm passage along a normally-attached epididymis with the complement of lymphatic drainage from the testis. Final maturation, motility and fertilizing capacity are achieved during this passage, and one of these must be affected when the epididymis is separated from the testis. Fournier-Delpech et al.(1981) showed that when the epididymis is ligated, the spermatozoa from different parts presented unequal degrees of maturation, and when the semen was used for AI, the amount of embryo mortality was increased with the number of immature spermatozoa inseminated. This supports the findings of the current fertility trial, where apart from reducing the fertilizing capacity of the spermatozoa, the separation of epididymides from the testis may have influenced the function of the epididymides resulting in the production of more immature spermatozoa capable of fertilizing the eggs but not of producing viable embryos.

Bedford (1978) reported that the number of immature spermatozoa was increased by an increase in epididymal temperature. It was suggested by Jones (1984) that a similar effect might occur when the epididymis was separated from the testis. The lymphatic connections between the testis and epididymis may be of considerable importance and have a direct influence on maturation and fertilizing capacity of the spermatozoa, and therefore with the viability of the fertilized egg. Unfortunately, due to the large variation of responses it was not possible to reach a definitive conclusion on this aspect in the current experiment.

CHAPTER 5. THE EFFECTS OF HEATING THE TESTES AND EPIDYDIMIDES OF RAMS ON FERTILITY AND EMBRYO MORTALITY IN EWES INSEMINATED WITH FROZEN SEMEN.

5.1 Introduction.

Spermatogenesis in mammals with scrotal testes is susceptible to damage if the testicular temperature is raised above normal levels (Phillips and MacKenzie, 1934; MacKenzie and Berliner, 1937; Bogart and Mayer, 1946; Waites and Setchell, 1990). Morphological changes to spermatozoa, such as an increase in the amount of abnormal cells, often occur after exposure to high temperatures (Dutt and Hamm, 1957; Dutt and Simpson, 1957; Moule and Waites, 1963; Rathore, 1968; Howarth, 1969; Smith, 1971; Bornman et al., 1989), but this effect shows considerable variation between individuals (Dun, 1955) and breeds (Lindsay, 1969). These abnormalities are more often present in the sperm head, in the form of increased numbers of pyriform heads and acrosome damage (Rathore, 1968, 1970a, 1970b; Colas, 1983). The damage depends on the length of exposure to heat and its intensity (Moule and Waites, 1963; Waites and Setchell, 1964; Fowler, 1968).

Other semen characteristics affected by heat stress include lower motility of sperm (Dutt and Hamm, 1957; Dutt and Simpson, 1957; Lindsay, 1969; Smith, 1971) and decreased concentration of spermatozoa (Dutt and Simpson, 1957; Moule and Waites, 1963; Waites and Setchell, 1964; Rathore, 1968; Howarth, 1969; Braden and Mattner, 1970; Rathore, 1970; Smith, 1971)

Fertility and embryo survival are also affected. The fertilizing capacity of sperm is severely reduced after a heat exposure (Dutt and Simpson, 1957; Fowler and Dun, 1966; Fowler, 1968; Howarth, 1969; Braden and Mattner, 1970; Rathore, 1970 b). This is apparent in ewes mated with heat stressed males as early as 3 or 4 days after treatment, and the duration of this failure depends on the duration and intensity of the treatment (Rathore, 1968; Colas,

1983). In infertile men, Mieusset et al.(1987) reported that 1 to 3°C increase above normal testicular temperature produced a marked depression in spermatogenesis.

Several reports have shown that high temperatures can lead to increased embryo mortality (Howarth, 1969; Rathore, 1970 b). Dutt and Simpson (1957) reported higher embryo loss from ewes mated to heat stressed rams, but this was not significantly greater than control rams. Rathore (1968) confirmed this finding of higher embryo mortality in similar experiments and suggested that high temperatures affect embryo survival due to damage to cell morphology. Thus the sperm are capable of fertilizing an egg but a pregnancy may not be maintained.

The effect of scrotal heating on fertility has been reported in a number of species. Wettemann et al.(1976) reported a decreased foetus to corpus luteum ratio when normal gilts were joined to heat stressed boars. Setchell et al. (1988) found that the number of foetuses per corpus luteum was reduced due to heat stress in rats, suggesting that embryo mortality was increased. Recently, an increase in the number of miscarriages was reported in women whose partners had a high frequency of hot baths (Spira, 1990). In some instances there is normal fertilization but increased embryo death (mice: Bellvé, 1972; rabbit: Howarth et al., 1965, Burfening and Ulberg, 1968); in others, there is both a failure of fertilization and an increase in embryo death (ram: Rathore, 1970 b; Wettemann and Bazer, 1985; mice: Burfening et al., 1970; rat: Setchell et al., 1988). However, it is difficult to estimate the relevance of many of these reports since in most of them body temperature was increased due to the elevated ambient temperature but testicular temperature was not measured, and in the studies using local heating, testicular temperature was raised to unphysiological levels (40 to 43°C).

The purpose of the present experiment was to assess the fertilization rate and embryo mortality in normal female sheep inseminated with frozen-thawed semen from rams submitted to moderate but sustained elevations in their scrotal temperature.

5.2 Experimental procedure.

5.2.1 Animals.

Eight mature Merino rams aged between 3 and 5 years and weighing between 65 to 75 kg were kept in individual pens in a controlled temperature and light room, at temperatures ranging between 19 and 23°C under a 16 hour-light and 8 hour-dark regimen. Four animals were treated and 4 used as a control. They were fed ad libitum with standard rations twice a day with free access to water.

5.2.2 Treatments.

The testes and scrotum were insulated for 16 hours/day, beginning 4 hours before the end of the light period, for 21 successive days. Scrotal insulation was achieved by means of a bag consisting of one layer of aluminium foil inserted between two layers of cotton cloth, and on the outside, one layer of water-proof cotton cloth (Plate 8, Appendix D of the thesis) (Malmgren and Larsson, 1989). The bag surrounded the scrotum without inducing any compression and was maintained in this position by four strings tied over the back of the ram.

Preliminary assessment of the increase in scrotal temperature was performed on 5 other similar rams. Under ~~local anaesthesia~~ sedation induced with 0.4 ml of xylazine i.v. (Rompun; Bayer, Australia), a probe for recording temperature was placed into the space between the tunica vaginalis and the scrotal skin through a small incision in the skin about 10 cm above the testis. The probe was maintained in position by suturing the scrotal skin and temperatures were recorded every 5 minutes for 2 to 6 hours from the first to the fifth day of surgery, with the scrotum insulated as above. In all cases the mean subcutaneous scrotal temperature was between 1.4 and 2.2°C above control values, with a mean (± s.e.m.) increase of 1.7±0.3°C.

5.2.3 Semen assessment, processing and freezing.

Semen was collected twice weekly by artificial vagina and analyzed manually for sperm concentration and the proportion of live and dead spermatozoa in 200 cells. Motility characteristics were assessed by the HTM. On days -49 before treatment, and 4, 15 and 21 during treatment, semen was diluted five-fold with Diluent A at 32°C. Then the diluted semen was frozen in pellet form on dry ice (-79°C) and stored in liquid nitrogen (-196°C).

The pellets were kept frozen in liquid nitrogen until thawed at 37°C in clean, dry tubes by shaking in a water bath, and the semen was used for intrauterine AI by laparoscopy within 10 minutes of thawing. Semen was assessed fresh and after thawing for motility and concentration characteristics with the HTM, so the concentration and quality was estimated and a fixed inseminate dose was established (total 100×10^6 spermatozoa per ewe divided into two halves, one for each uterine horn) which was achieved by varying the dose volume from 0.01 to 0.1 ml.

5.2.4 Insemination program.

A group of mature Merino ewes were randomly distributed into 8 groups of 80 ewes and their oestrous cycles were synchronized with intravaginal sponges and an intramuscular injection of 400 I.U. PMSG at sponge removal. Each day, 80 ewes were inseminated 50 hours after sponge removal, 40 with semen from heated rams and 40 with semen from control rams, with the date of collection selected at random. On three occasions, twice in the control and once in the heated group, no semen could be collected from one ram. Therefore, 324 ewes were inseminated with semen from heated rams and 312 with semen from control rams.

5.2.5 Pregnancy diagnosis and embryo mortality.

Two successive determinations of pregnancy were performed on each ewe at 17 and 65 days after insemination. The 17 days diagnosis was performed by the determination of plasma progesterone level (assessed in duplicate by RIA). The second determination was performed at 65 days after insemination by ultrasound. The embryo mortality was expressed as a percentage of the number of females pregnant at 17 days after insemination minus the number of females pregnant at 65 days after insemination divided by the number of females pregnant at 17 days after insemination.

5.3.5 Statistical analyses.

The proportion of pregnant females and embryo loss were compared by a Chi-square test. Between group comparisons of the semen parameters were done by an unpaired Student's t-test, and within ram comparisons at different times by a paired Student's t-test.

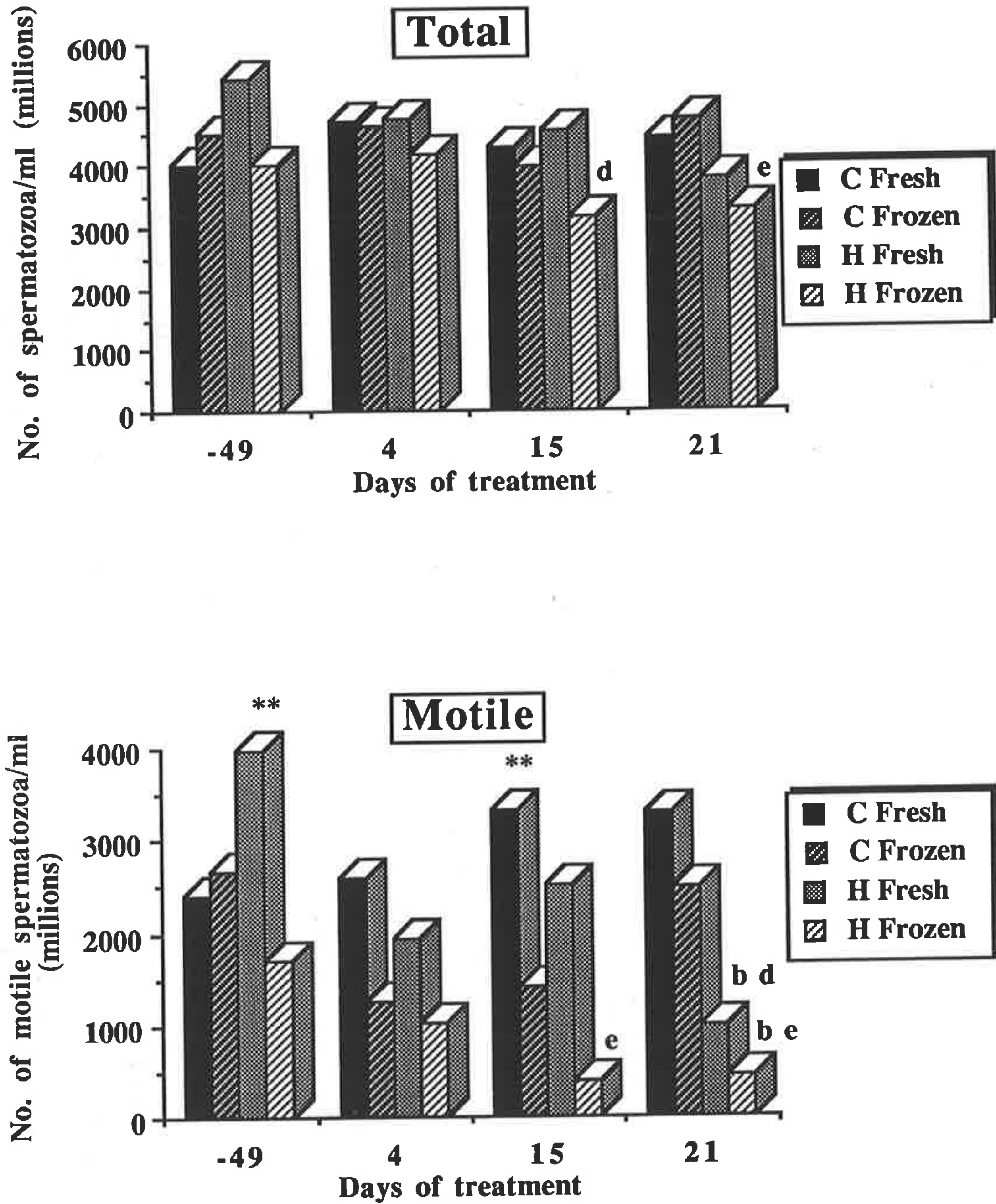
5.4 Results.

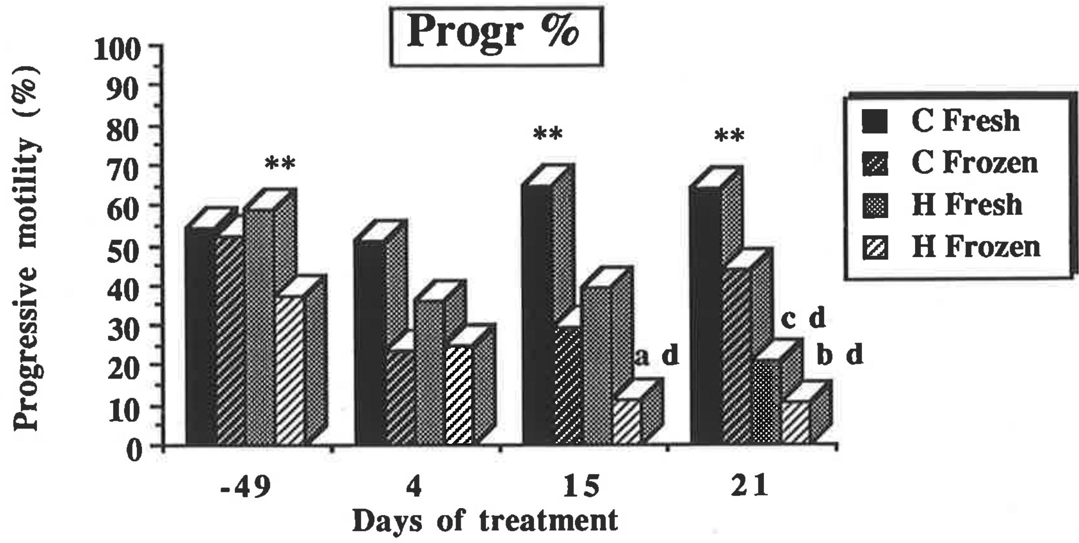
5.4.1 Semen characteristics.

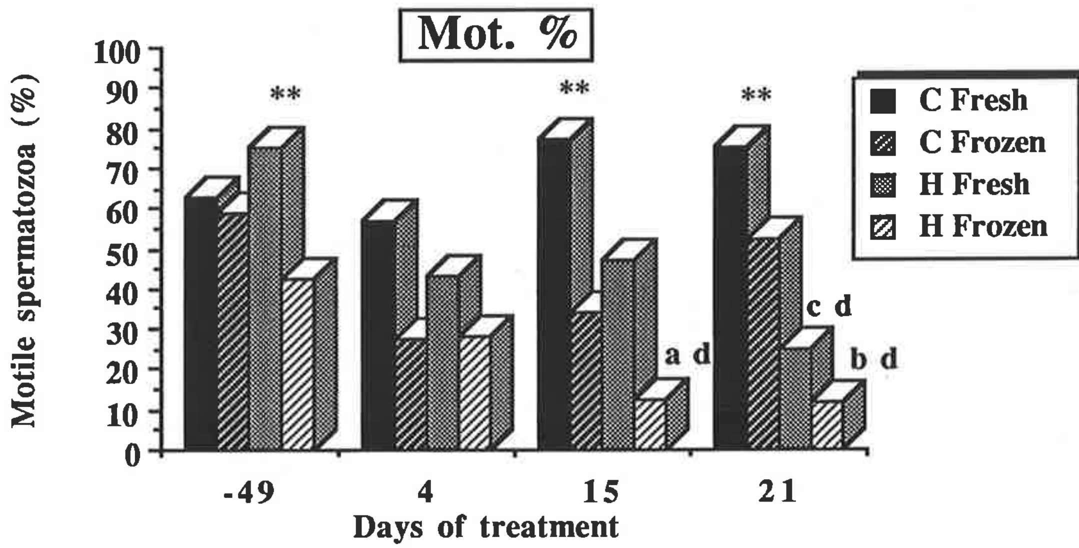
Semen characteristics determined by computerised image analysis and manual assessment of fresh and frozen-thawed semen from individual rams within treatment groups at days -49, 4, 15 and 21 of treatment are presented in Tables 5.1, 5.2 and 5.3. in Appendix A of the thesis.

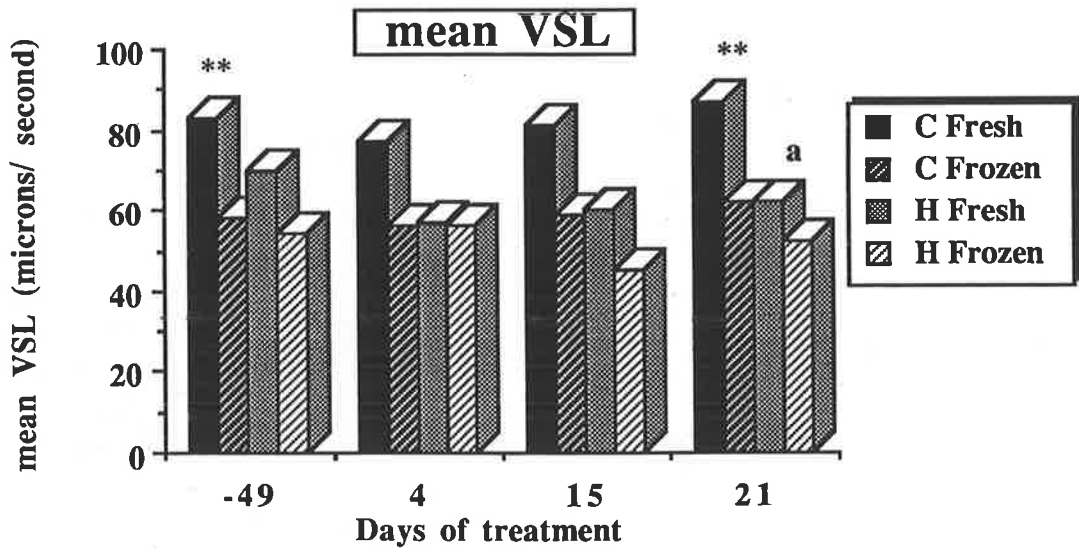
Using the information from Appendix A, the values with their respective significant differences for all fresh semen characteristics from rams in each group are presented in Figure 5.1.

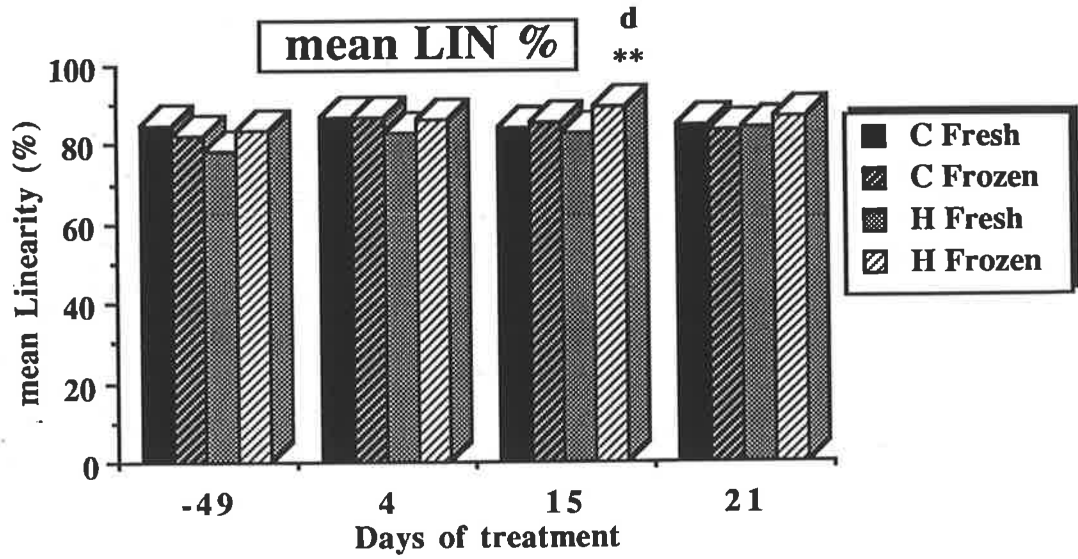
Figure 5.1 Fresh semen characteristics assessed by computerised image analysis from the control (C) and heated (H) rams.

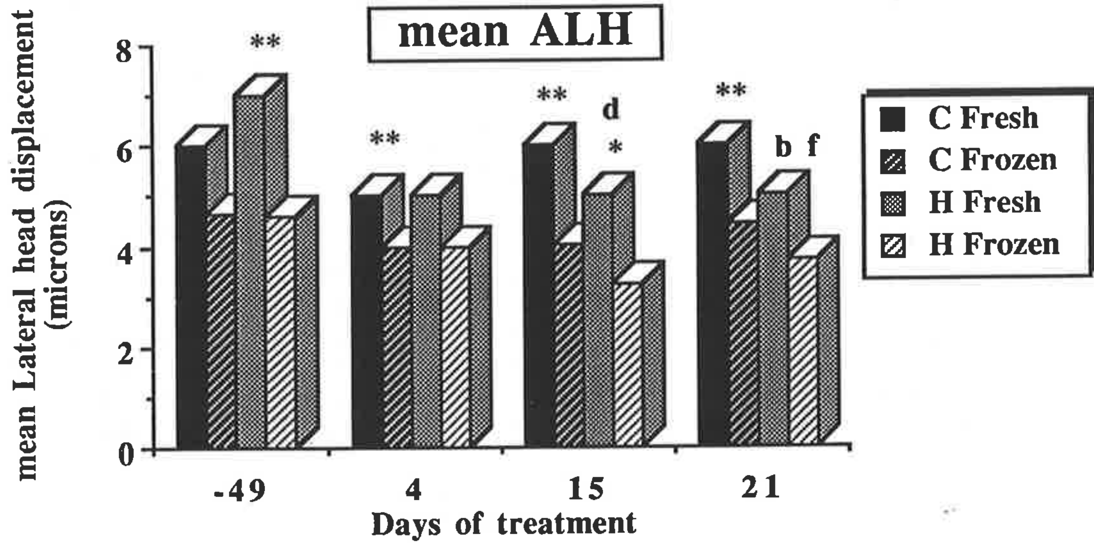


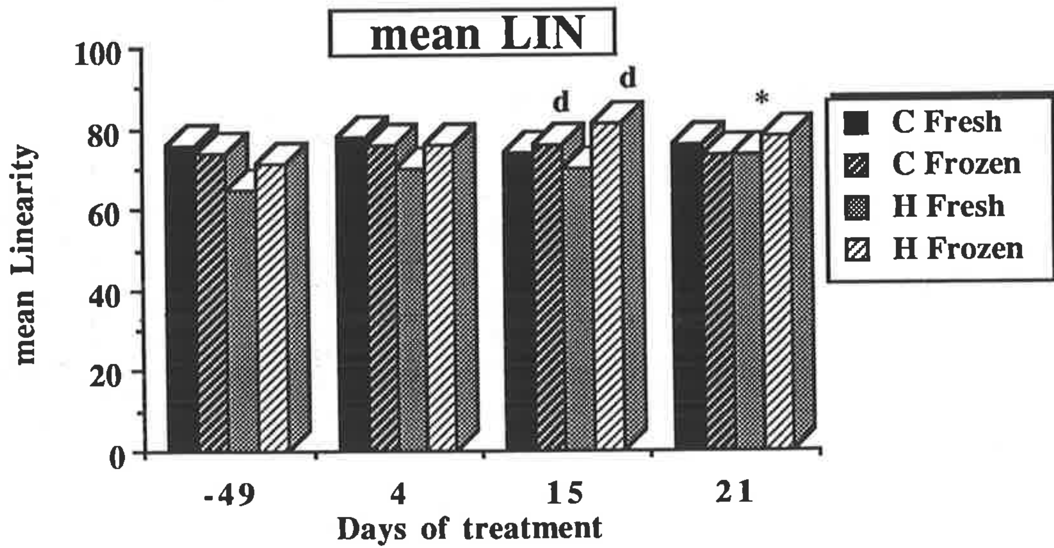

Progr %
Progressive motility (%)
100
90
80
70
60
50
40
30
20
10
0
**
**
**
a d
c d
b d
-49
4
15
21
Days of treatment
C Fresh
C Frozen
H Fresh
H Frozen

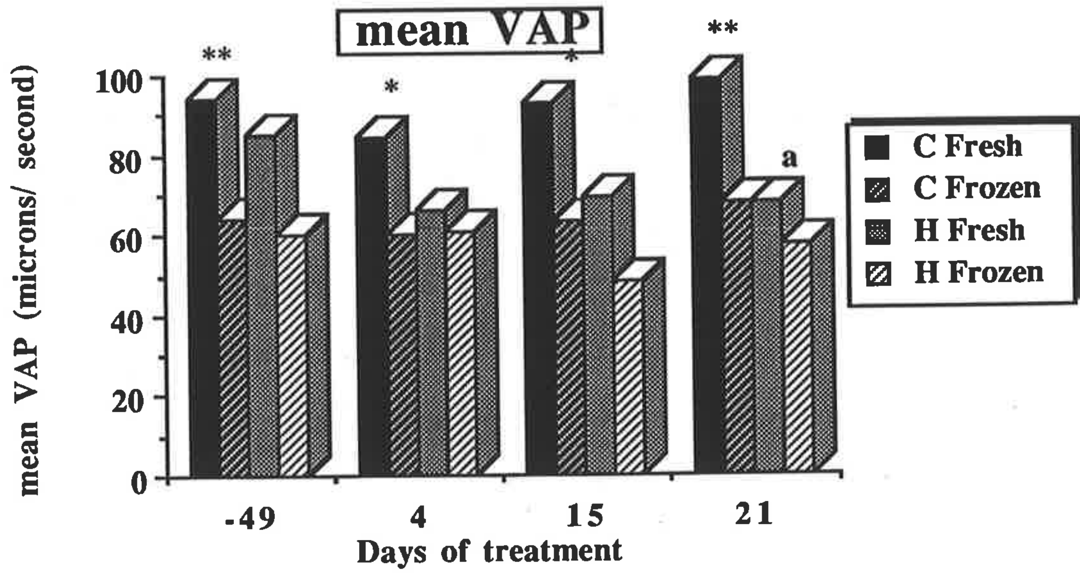

Mot. %
Motile spermatozoa (%)
100
90
80
70
60
50
40
30
20
10
0
**
**
**
a d
c d
b d
-49
4
15
21
Days of treatment
C Fresh
C Frozen
H Fresh
H Frozen

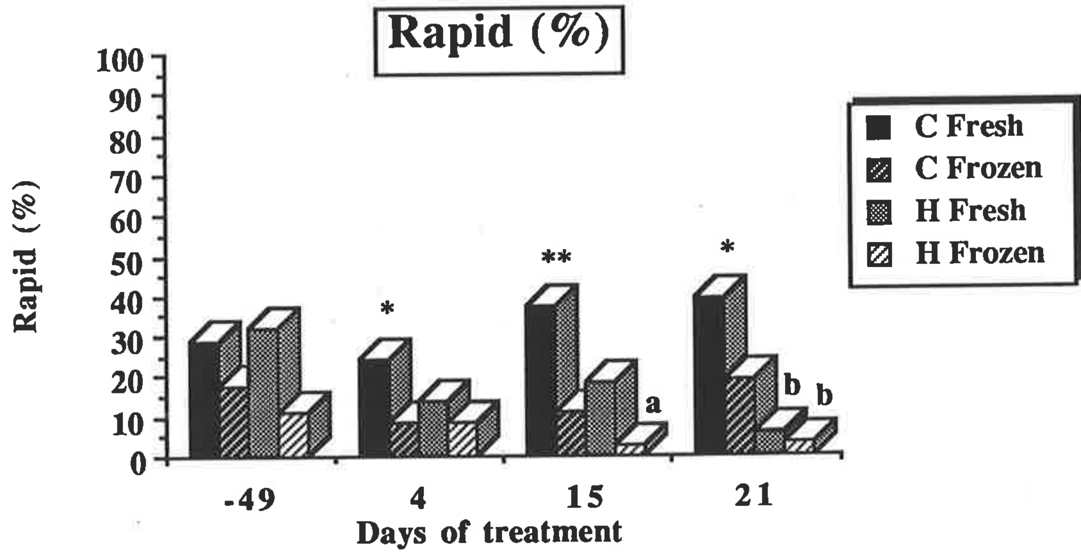

mean VSL
mean VSL (microns/ second)
100
80
60
40
20
0
**
**
a
-49
4
15
21
Days of treatment
C Fresh
C Frozen
H Fresh
H Frozen

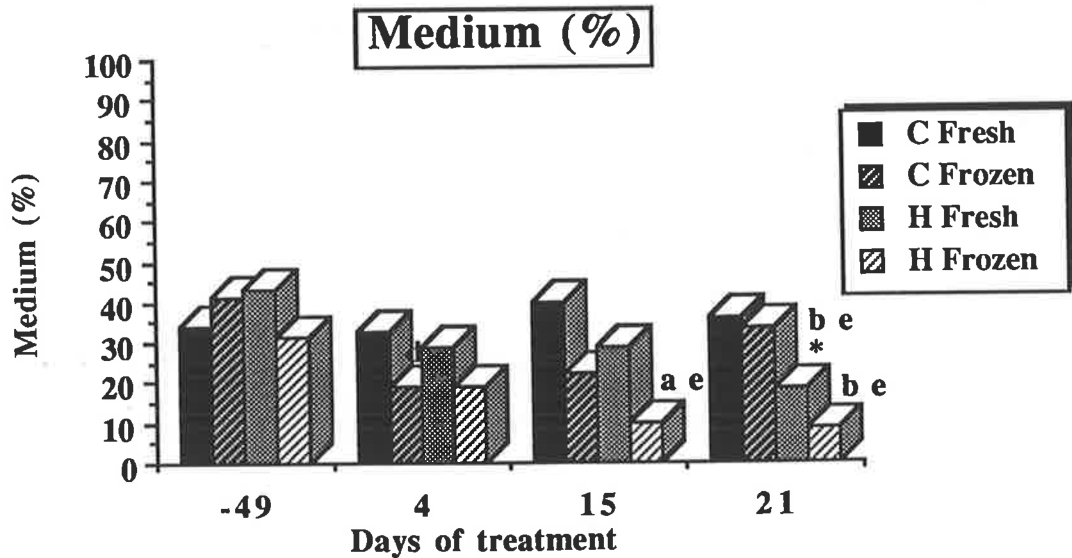

mean LIN %
d
**
100
80
60
40
20
0
mean Linearity (%)
-49
4
15
21
Days of treatment
C Fresh
C Frozen
H Fresh
H Frozen

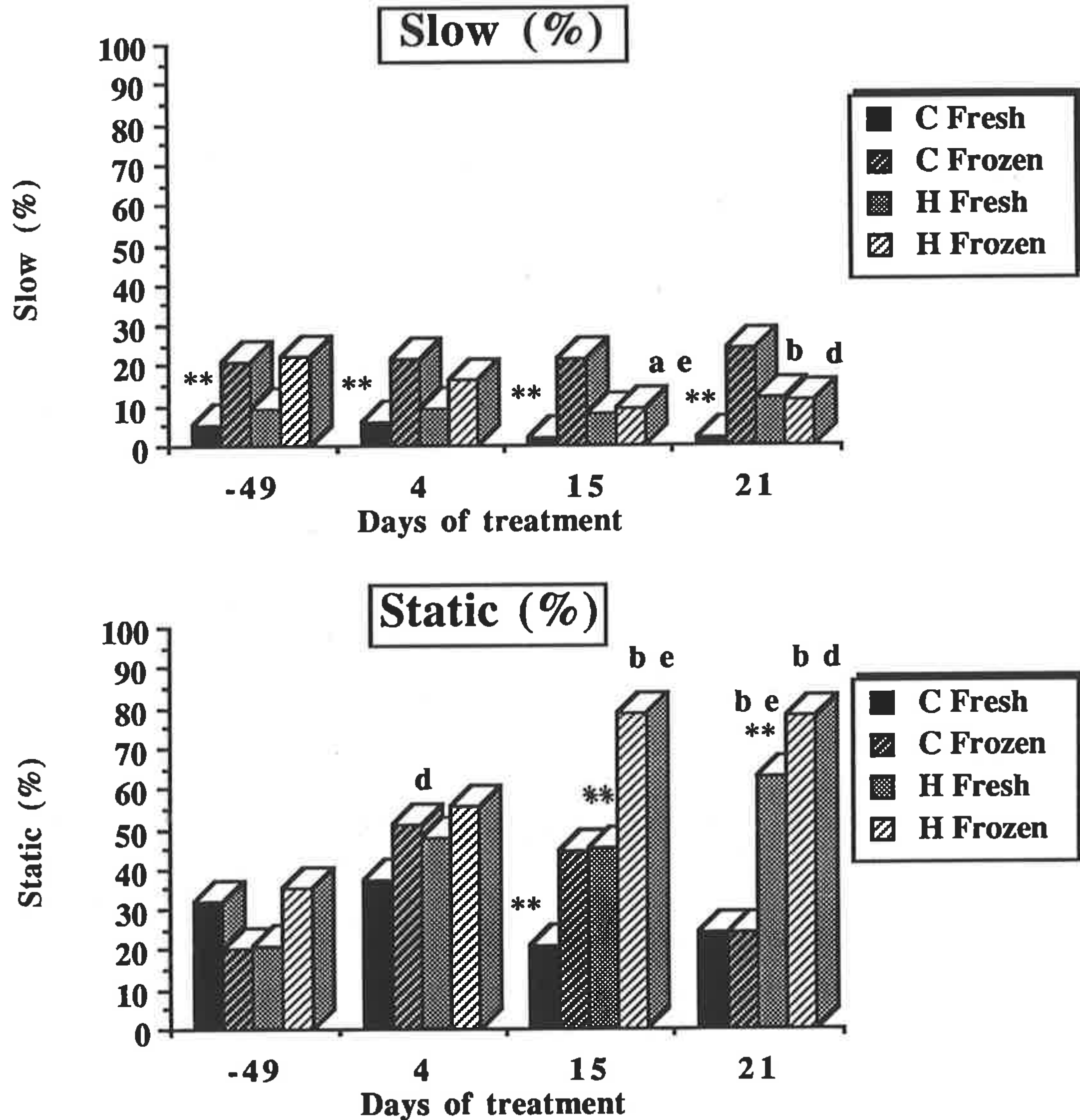

mean ALH
8
6
4
2
0
mean Lateral head displacement (microns)
**
**
**
d
*
**
b f
-49
4
15
21
Days of treatment
C Fresh
C Frozen
H Fresh
H Frozen


mean LIN
100
80
60
40
20
0
mean Linearity
d
d
*
-49
4
15
21
Days of treatment
C Fresh
C Frozen
H Fresh
H Frozen


mean VAP
mean VAP (microns/ second)
100
80
60
40
20
0
**
*
*
**
a
-49
4
15
21
Days of treatment
C Fresh
C Frozen
H Fresh
H Frozen


Rapid (%)
Rapid (%)
100
90
80
70
60
50
40
30
20
10
0
*
**
*
a
b
b
-49
4
15
21
Days of treatment
C Fresh
C Frozen
H Fresh
H Frozen


Medium (%)
Medium (%)
100
90
80
70
60
50
40
30
20
10
0
a e
b e
*
b e
-49
4
15
21
Days of treatment
C Fresh
C Frozen
H Fresh
H Frozen



(a); $p < 0.05$, (b); $p < 0.01$, (c) $p < 0.001$ fresh or frozen semen characteristic compared with control group on same day, (d); $p < 0.05$, (e); $p < 0.01$, (f); $p < 0.001$ fresh or frozen semen characteristic compared with values on day -49 (within group), (*); $p < 0.05$, (**); $p < 0.01$ fresh semen characteristic compared with frozen-thawed semen characteristic on same day within group.

When fresh semen from the rams was compared before the scrotal insulation started, no difference was detected between the two groups for any of the semen characteristics measured (Figure 5.1). No significant differences were detected in the semen characteristics assessed by computerised image analysis on days 4 and 15 of treatment. On day 21 of treatment, a reduction was observed in VSL ($p < 0.05$), VAP ($p < 0.05$), MOTILE ($p < 0.01$) and ALH ($p < 0.01$) in the heated rams compared with the control rams. RAPID % and MEDIUM % cells was lower in the heated rams whilst SLOW % and STATIC % cells was higher ($p < 0.01$). PROGR and MOT were much lower in heated rams than in control rams (p

< 0.001). Probably these two semen characteristics were affected by heating to a larger extent than the rest. TOTAL, LIN and LIN % remained unchanged when compared with control group (Figure 5.1). TOTAL and MOTILE, PROGR and MOT % were significantly different when the values from day 21 were compared with the values before treatment ($p < 0.05$); likewise MEDIUM % and STATIC % ($p< 0.01$) and ALH ($p < 0.001$) (Figure 5.1).

Fresh semen characteristics were compared with frozen-thawed semen characteristics within group on the same day. In control rams, except for TOTAL, LIN%, LIN and MEDIUM %, the rest of the semen characteristics were significantly different in some of the days during the experiment ($p < 0.05$). In the heated rams, the following semen characteristics were significantly different in fresh and frozen-thawed semen: MOTILE, PROGR., MOT, LIN%, ALH, LIN, MEDIUM %, and STATIC %. In this ram group semen characteristics were already affected by the heating treatment and they were particularly low in fresh semen.

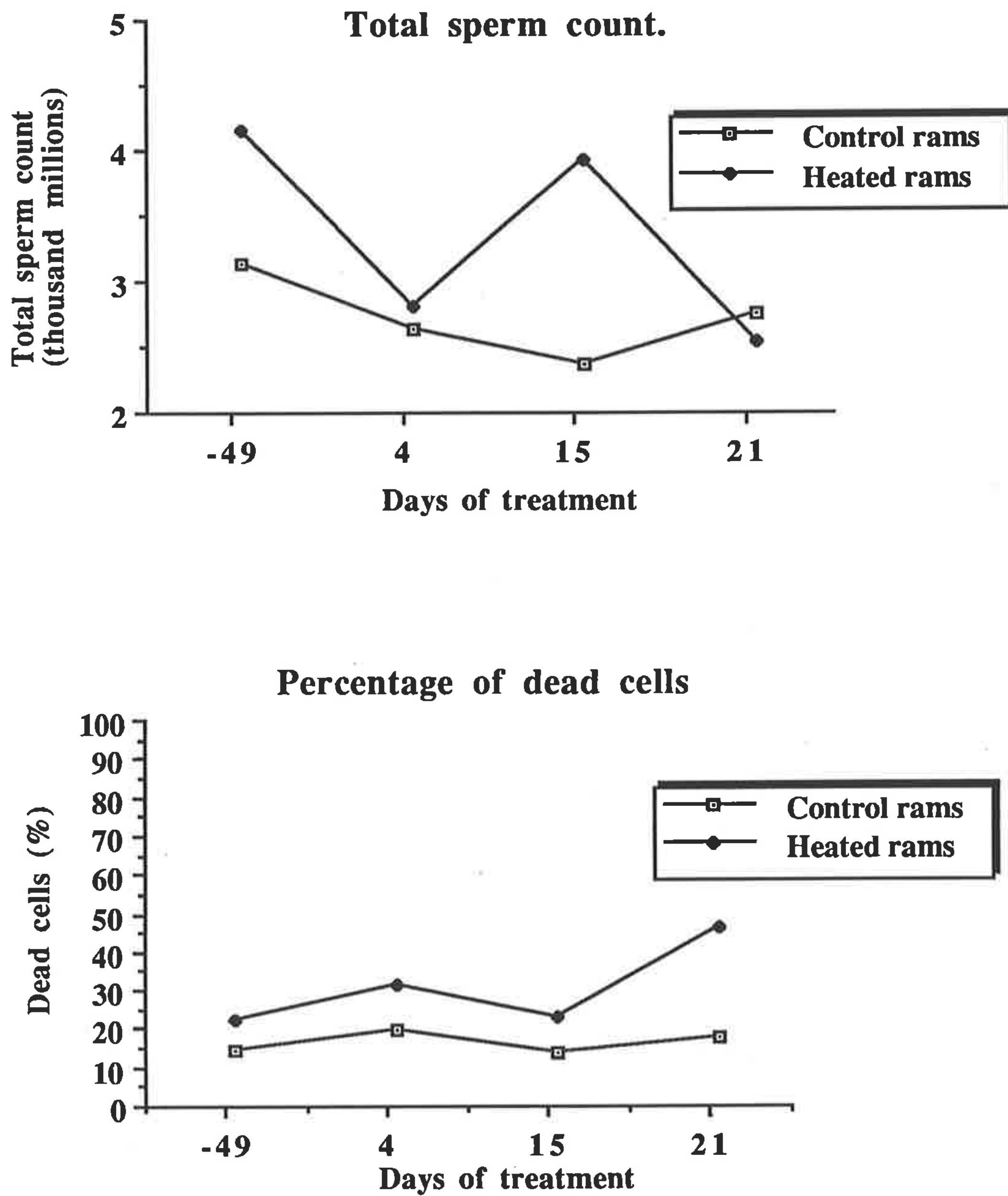
When frozen-thawed semen characteristics from treated rams were compared with those from control rams, it was found that PROGR, MOT, RAPID %, MEDIUM %, SLOW % ($p < 0.05$) and STATIC % ($p < 0.01$) spermatozoa were significantly different on day 15. On day 21, it was also found that MOTILE, PROGR, MOT, RAPID %, MEDIUM % and STATIC % spermatozoa were significantly different in treated and control rams ($p < 0.01$).

When frozen-thawed characteristics were compared to values on day -49 within groups, most of the differences observed were in semen from the heated rams. On day 15 the following semen characteristics were significantly different to day -49 in heated rams: TOTAL, PROGR, MOT, LIN %, LIN ($p < 0.05$), MOTILE, MEDIUM %, SLOW % and STATIC % ($p < 0.01$). On day 21, TOTAL, MOTILE, MEDIUM % ($p < 0.01$), PROGR, MOT, SLOW %, and STATIC % ($p < 0.05$) were significantly different to day -49. The control rams showed significant differences on day 4 in MEDIUM % and STATIC % when compared with day -49 ($p < 0.05$).

The manually assessed semen characteristics of heated and control rams are presented in Figure 5.2. The total count remained unchanged while percentage of dead cells showed a significant increase on day 21 of treatment in the heated group compared with the controls (p <

0.01). There was no significant difference between the semen characteristics on the days of treatment and before treatment for either of the ram groups.

Figure 5.2. Total sperm count and percentage of dead cells in fresh semen manually assessed from control and heated rams.



5.4.2 Pregnancy 17 and 65 days after insemination and embryo mortality.

The fertility after AI with frozen-thawed semen per sire per day by group are summarized in Table 5.4 in Appendix B of the thesis. The pregnancy and embryo mortality per day of treatment by ram group is shown in Table 5.5, and Figures 5.3, 5.4, and 5.5. Rams within groups responded in a similar way throughout the experiment (Table 5.4., Appendix B).

No statistical difference was found in the pregnancy rate at day 17 after insemination between females inseminated with frozen-thawed semen from the control rams compared with the heated rams before treatment (56 % vs 60.2 %); at day 4, control rams obtained a slightly lower (non significant) pregnancy rate than heated rams (65.2 % vs 76.2 %); at day 15 no difference was observed (66.7 % vs 63.6 %) and at day 21, ewes inseminated with semen from control rams had a slightly greater pregnancy rate (60.3 % vs 48.2 %, Table 5.5.and Figure 5.3).

Percentages of ewes pregnant 65 days after insemination did not differ when semen collected before treatment was used (22.7 % for the control group and 25.2 % for the heated group). However, the percentage of ewes pregnant at 65 days was lower with semen from heated rams at day 4 (14.3 % vs 29.3 %, $p < 0.05$), 15 (13.6 % vs 27.3 %, $p < 0.05$) and 21 (3.5 % vs 20.7 %, $p < 0.001$) of treatment (Table 5.5, Figure 5.4).

Table 5.5. Pregnancy and embryo mortality in heated and control rams.

DAYS	No. ewes pregnant/inseminated (%) DAY 17	DAY 65)	EMBRYO MORTALITY[1] (%)
HEATED RAMS			
-49	53/88 (60.2)	19/88 (21.6)	34/53 (64.1)
4	48/63 (76.2)	9/63 (14.3)*	39/48 (81.2)*
15	56/88 (63.6)	12/88 (13.6)*	44/56 (78.6)*
21	41/85 (48.2)	3/85 (3.5)**	38/41 (92.7)**
CONTROL RAMS			
-49	51/91 (56.0)	23/91 (25.3)	28/51 (54.9)
4	60/92 (65.2)	27/92 (29.3)	33/60 (55.0)
15	44/66 (66.7)	18/66 (27.3)	26/44 (59.1)
21	38/63 (60.3)	13/63 (20.7)	25/38 (65.8)

(*) $p < 0.05$; (**) $p < 0.001$.

1 $\frac{\text{No. ewes pregnant at 17 days — No. ewes pregnant at 65 days}}{\text{No. ewes pregnant at 17 days}}$

Before treatment, no difference was observed between the groups in embryo mortality (54.9 % in the controls and 64.1 % in the heated group). However, embryo mortality was significantly increased at days 4 (39/48, 81.2 %; $p < 0.001$), 15 (44/56, 78.6 %; $p < 0.05$) and 21 (38/41, 92.7 %; $p < 0.001$) of treatment in heated rams compared with control rams (33/60, 55.0 %; 26/44, 59.1 %; 25/38, 65.8 %, respectively). In control rams there was a slight increase in embryo mortality at the end of the program, but this did not affect the results (Table 5.5, Figure 5.5).

Figure 5.3. Pregnancy rate 17 days after insemination with semen collected from heated and control groups on days -49, 4, 15 and 21 of treatment.

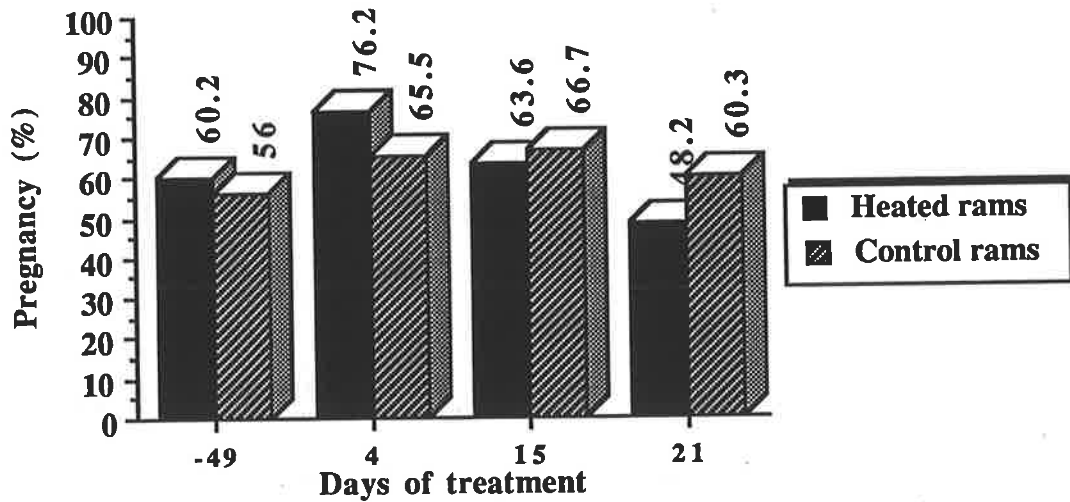


Figure 5.4 .Pregnancy rate 65 days after insemination with semen collected from heated and control groups on days -49, 4, 15 and 21 of treatment.

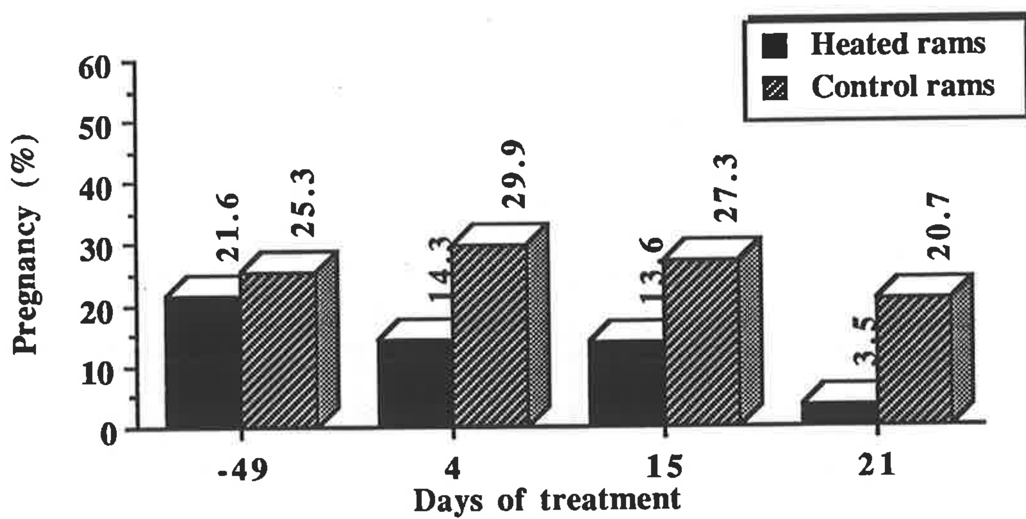
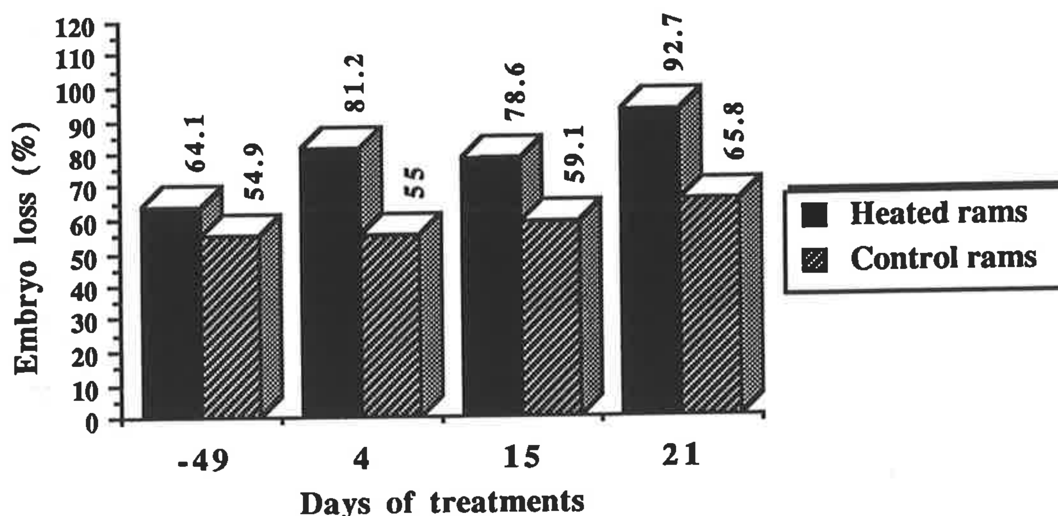


Figure 5.5. Percentage of embryo mortality at 65 days after insemination with semen collected from heated and control groups on days -49, 4, 15 and 21of treatment.



5.5 Discussion.

There was no difference in the early pregnancy rate of females inseminated with frozen-thawed semen from control and heated rams. From this it can be concluded that an increase of about 2°C in subcutaneous scrotal temperature for 16 hours per day does not reduce the fertilizing capacity of the spermatozoa after 4, 15 or 21 days of treatment. These results are apparently in contradiction with those reported in previous studies dealing with the effects of heat on fertilization rate in rams (Dutt and Simpson, 1957; Fowler and Dun, 1966; Rathore, 1968, 1970; Howarth, 1969; Braden and Mattner, 1970), boars (Wettemann and Bazer, 1985) and rats (Setchell et al., 1988). The differences between the present study and the previous studies may be due to the heating procedure, as earlier authors elevated testicular temperature to 40 or 43°C (Braden and Mattner, 1970; Setchell et al., 1988), or used treatments which caused increases in whole body temperature (Dutt and Simpson, 1957; Fowler and Dun, 1966; Rathore, 1968, 1970; Howarth, 1969; Wettemann and Bazer, 1985).

More important, the reduction in the fertilization rate reported in some of these studies was obtained from animals in which semen characteristics were drastically altered by a long exposure to heat, ranging from 45 to 80 days (Dutt and Simpson, 1957; Wettemann and Bazer, 1985).

In the present study embryo mortality was also calculated as the numeric difference between the number of ewes pregnant at 17 and 65 days after insemination. Such estimation may underestimate the rate of embryo death, as a ewe can be pregnant with two embryos at 17 days and with only one foetus at 65 days. There are two main opinions on this matter; Geisler et al. (1977) and White et al (1981) reported that embryo survival for a single ova is higher than for a twin ova and is inversely proportional to ovulation rate, as more ova are shed. On the other hand, and supporting our point of view, despite this relationship, the pregnancy rate from twin ovulations has been found greater than for single ovulations since embryo loss generally occurs at random (Restall et al., 1976). Moreover, Wilkins (1989) found in numerous observations that pregnancy failed in 10 % of single ovulating ewes versus 4 % in flock of females with twin ovulations.

The high embryo mortality rate (55 to 66 %) in the ewes inseminated with frozen-thawed semen from control rams may be due to a partial interference by the surgical technique of insemination as suggested by Maxwell et al. (1984), but this is not a main factor because when insemination is performed by laparotomy, the embryo loss is much higher as reported by Salamon and Lightfoot (1967), Mattner (1969), Killeen (1969) and Killeen and Moore (1971). These latter authors concluded that the high embryo loss with this technique is due to abnormal transport of the egg resulting from surgical interference.

There are probably other factors which influenced the embryo mortality in this experiment as the normal basal embryo mortality due to genetic and environmental factors can be as high as 20 to 30 % (Bishop, 1964; Edey, 1969). The environmental temperature during AI may affect embryo mortality (Thwaites, 1969, 1971), and environmental temperatures towards the end of the present experiment reached 39 to 41°C. In early reports, Dutt et al. (1959) and Alliston and Ulberg (1961) concluded that continued high temperatures caused

death of a high proportion of embryos in early pregnancy and the peak incidence was found to be during the first week after mating. Sheep handling may also affect embryo mortality by inducing tension or stress which increases adreno-cortical activity (Reid and Mills, 1962) in the female at the time of drafting prior to insemination and during insemination. The use of frozen-thawed semen has also been reported to increase the embryo mortality (Salamon and Lightfoot, 1967), and this is increased by ageing spermatozoa when the time between the thawing and the actual insemination increases as reported by Maxwell (1978).

In the present experiment embryo mortality was significantly increased in ewes inseminated with semen from the heated rams as early as day 4 of treatment. The absence of modification in the fertilization rate associated with an increase in embryo mortality is in agreement with results reported in mice (Bellvé, 1972; Burfening et al., 1970) and rats (Setchell et al., 1988) when females were mated with males immediately after heat treatment, and in the rabbit when spermatozoa were heated before insemination (Howarth et al., 1965; Burfening and Ulberg, 1968). However, as implantation of the embryo takes place in the ewe between 15 to 24 days after fertilization (Robertson, 1977), and as the first diagnosis of pregnancy was performed at 17 days after insemination, it cannot be decided from the present study whether the embryo loss occurred before or after implantation. In this respect, most of the literature suggests that embryo mortality occurs mostly between day 12 and day 18 of the cycle and this is reflected by the number of ewes returning to service at day 19 to 24 after first insemination or mating (Edey, 1966, 1967; Cumming, 1972a; MacKenzie and Edey, 1975a, 1975b; Sawyer and Knight, 1975). This is different to foetal loss which occurs at later stages of pregnancy (Morley, 1954). Moor and Rowson (1964) working with embryo transfer concluded that in the presence of the embryo, the signal for maintenance of the corpus luteum (CL) appears to be given on day 12 to 13 of the cycle, since removal of embryos up to, but not later than this time, results in cycles of normal length. Thus, when deaths occur during this time (first 12 to 13 days), the ewes return to oestrus at the normal time, whereas when the embryo loss occurs after this time, a delay in ewes returning to oestrus is observed (Edey, 1966). Embryo loss may also occur after the implantation as a continuation of a process which began earlier (fertilization with abnormal spermatozoa or an abnormal egg) (Robinson, 1951).

Unfortunately, none of these suggestions could be examined in this experiment due to the lack of oestrus detection after and up to 45 days after insemination. If a ewe was detected pregnant at day 17, but then lost the embryo(s) after this day, it would be marked by the ram about 17 days later or 35 days after insemination, whereas the non pregnant ewes would be marked by the ram at 17 days after insemination.

The epididymis is currently thought to be resistant to heat due to the lack of abnormal spermatozoa or modification in the motility of spermatozoa in the ejaculates of rams or boars until about 14 days after heating (Glover, 1955; Moule and Waites, 1963; Waites and Setchell, 1964; Wettemann and Bazer, 1985). This apparent resistance was also confirmed in rams by the absence of a change in fertilization rate within the first week after heating (Braden and Mattner, 1970; Howarth, 1969). Nevertheless, in the rat, mouse and bull, the lower temperature of the cauda epididymis has been recently shown to facilitate the storage of sperm by enhancing oxygen availability (Djakiew and Cardullo, 1986). As in rams, passage of spermatozoa through the epididymis requires a period of about 11 to 14 days (Ortavant, 1954), the increase in embryo death at day 4 of heating in the present study indicates a positive effect of heat on the epididymal spermatozoa. Although this effect has been observed by other authors who worked with mice mated during the first week after heating, no comment was made about the possible effect on the epididymis (Bellvé, 1972; Burfening et al., 1970).

Different mechanisms can be cited for the decreased capacity of the spermatozoa to produce a viable embryo after scrotal heating. These include a developmental retardation of the embryo before implantation (Bellvé, 1972), a reduced capacity to implant, either as a direct effect of the heat, or as a result of the slower division rate of the embryo through unsynchronization with the uterine environment. Such effects could also be involved in post-implantation embryo death. Fournier-Delpech et al. (1979, 1981) reported that desynchronization of embryo development and the uterine environment which causes embryo loss is observed when eggs are fertilized with immature spermatozoa. It may be that the increased temperature affects spermatozoal maturation; however, this cannot explain the early embryo mortality observed in this particular study after only 4 days of heating, probably the effect in maturation would be expected at later times after heating. Regardless of the

mechanism, the alterations induced in the spermatozoa by the heating method used in the present study must manifest themselves at later stages than fertilization which proceeds normally.

The differences between fresh and frozen-thawed semen characteristics may be due to the processing and freezing procedures. Moreover, prior effects on fresh semen due to the heating treatment may reduce the subsequent reduction rate in semen characteristics due to processing and freezing. Thus, control rams produced a similar quality of fresh semen throughout the experiment which responded similarly to processing and freezing. However the fresh semen produced by the heated rams changed in quality and production towards the end of the experiment, which may have changed the survival of sperm following processing and freezing.

In the present experiment the number of females inseminated from each semen sample were enough to give a good estimate of fertility, but the number of treated rams should be increased to make the results more meaningful and to determine the effect of variation between rams.

The results of the present experiment show that increasing the subcutaneous scrotal temperature by about 2°C for 16 hours per day in rams induced, as early as day 4 of treatment, an increase in embryo mortality without any modification in the fertilization rate at least until day 21 of heating, at which time the quality and to a lesser extent the quantity of the spermatozoa in the ejaculates was also affected. Thus, the earliest effects of heat seem to be on the epididymal spermatozoa. From these results and from those already reported in the literature, it is suggested that the sequence of heat effects on spermatogenesis could be firstly a reduction in the capacity of the spermatozoa to produce viable embryos, secondly a decrease in the capacity of the spermatozoa to fertilize the ovum, and finally a decrease in the number of spermatozoa produced. More detailed analyses of the characteristics of the spermatozoa are required to establish a relationship between the semen characteristics and this sequence.

CHAPTER 6. OVULATION RATE, FERTILITY AND EMBRYO MORTALITY IN EWES MATED TO RAMS FROM TWO DIFFERENT STRAINS (SOURCES).

6.1 Introduction.

Embryo mortality during the early part of pregnancy is an important contributor to reproductive failure, but it is usually attributed to physiological or environmental factors affecting the female on or about the time of implantation (Kelly, 1984). However, Courot and Colas (1986) suggested that the male side may also contribute to the reproductive failure by affecting embryo survival. When semen quality is low, fertilization with abnormal sperm may occur resulting in degeneration of the embryo and subsequent embryo mortality.

The role of the male as a factor in embryo mortality has been controversial. Fournier-Delpech et al. (1979, 1981) showed a clear influence of the male in an experiment in which eggs were fertilized by ram spermatozoa from different parts of the epididymis and the fertility was evaluated at different stages. These authors concluded that in eggs fertilized by immature spermatozoa, the fertilization may be normal but the embryo often dies. Some authors have reported that embryo mortality increased when normal ewes were mated with subfertile rams (Rathore, 1968, 1970b; Howarth, 1969; Braden and Mattner, 1970). In chapter 5 of this thesis, when rams were treated with a small but sustained increase in scrotal temperature, embryo mortality was increased in ewes mated to such rams as early as 4 days after treatment commenced, indicating a direct male influence on embryo mortality.

Another factor which may affect embryo survival is the genetic influence of the male. Such effects might be manifested between individual rams, or between strains or ram sources. Bradford (1972) suggested that rams may contribute to variation in the litter size of their mates through differences in the fertilizing capacity of their semen or in the pre-natal survival of their offspring. Burfening et al. (1977) reported obtaining embryo mortality of 11 % and 19 % respectively from ewes mated to rams born from dams selected for high and low prolificacy.

Similar findings have also been reported by Moore (1981), but no differences were found by Baker and Land (1970).

Jefferies et al. (1988) mated Merino rams from 3 different sources to randomly allocated ewes during two mating seasons (1986 and 1987) and found no significant differences in number of pregnant ewes. However, the rams from different sources caused differences in the number of foetuses present at 12 weeks of pregnancy, and more surprisingly in the number of lambs surviving at marking.

The aim of the present study was to examine such a ram source effect on ovulation rate, fertility and embryo mortality in ewes mated to rams from different sources.

6.2 Experimental procedure.

The study was performed from March to August, 1989 at "Glenthorne".

6.2.1 Animals.

Groups of 6 mature Merino rams were randomly selected from average grade rams from two South Australian Merino studs (sources A and B). Each ram group was run with 50 randomly allocated mature ewes (from one, different source) in separate but adjacent paddocks of comparable pasture. The ewes remained in these paddocks until they were mated. The sheep were supplemented at the same rate with hay and grain for maintenance with free access to water.

The 600 ewes were drafted and identified with plastic ear tags and two side numbers for easy identification and a distinctive colour/mark on the head and rump. Sires were equipped with raddle harnesses with a distinctive colour which were checked frequently.

6.2.2 Recording of raddles, ovulation rate and pregnancy.

The mated (raddled) ewes were recorded and drafted off each day. They were then deprived of food and water for 24 hours and the ovulation rate determined by laparoscopy. The ewes were then put in homogeneous groups and run together until the completion of the experiment. Pregnancy was diagnosed in the ewes by progesterone assay (day 17) and ultrasound (day 65). The difference between pregnancy at days 17 and 65 was used to calculate the percentage of embryo mortality.

6.3 Statistical analyses.

The number of raddled and pregnant ewes and the incidence of single and twin foetuses were analyzed by Fisher's Exact test of 2x2 tables. The comparison of pregnancies at 17 and 65 days and the embryo loss was performed by Chi-square analysis of contingency tables.

6.4 Results.

Rams from source A raddled significantly more ewes ($p < 0.001$) than rams from source B (Table 6.1). The ovulation rates of the ewes mated to both strains of rams were similar (Table 6.1).

Table 6.1. Ewes raddled, number of corpora lutea (CL) and ovulation rate in ewes mated to two sources of rams.

Source of rams	No. ewes mated	No. ewes raddled (%)	No.CL	Ovulation rate
A	300	253 (84.33)**	366	1.45
B	300	234 (78.0)	333	1.43
Total	600	487 (81.17)	699	1.44

(**) $p < 0.001$.

Rams from the two sources produced similar proportions of ewes pregnant at 17 days after mating. However, rams from source B produced significantly more pregnant ewes 65 days after mating than rams from source A ($p < 0.001$, Table 6.2).

Table 6.2. Ewes pregnant at 17 and 65 days after mating, the incidence of single and twin pregnancies at 65 days after mating, and the rate of embryo loss in ewes mated to two sources of rams.

Source of rams	Ewes pregnant/ mated (%) 17 days	Ewes pregnant/ mated (%) 65 days	Single preg- nancies	Twin preg- nancies	No.foetuses (% of ewes pregnant)	Ewes pregnant at 17 but not at 65 days (%)
A	197/253 (77.7)	64/253 (25.30)	38	26	90 (140.6)	133/197(67.5)*
B	181/234 (77.3)	82/234 (35.0)**	56	26	108 (131.7)	99/181(54.7)

(*) $p < 0.05$; (**) $p < 0.001$.

Rams from source A produced significantly higher embryo mortality than rams from source B ($p < 0.05$). There was no was significant difference in the incidence of single and twin pregnancies between ram sources nor the number of foetuses present 65 days after mating (Table 6.2).

6.5 Discussion.

Although Jefferies et al. (1988) reported no differences in the fertility between rams from different sources, the present study found an increase in the number of ewes raddled by rams from one source (A). These rams also produced a significantly lower proportion of pregnant ewes and higher embryo loss than the rams from source B. The embryo mortality for both ram groups was very high, but this may have been due to the frequency of handling and a period of extremely hot weather during early pregnancy.

Previous studies (Burfening et al., 1977; Moore, 1981) have suggested that rams from different strains have different abilities to fertilize single and multiple ovulating ewes. Some rams were less able to fertilize more than one ovum, while rams from other strains were able to fertilize two or more ova. The progesterone assay we used to detect pregnancy 17 days after mating could not differentiate between single and multiple pregnancies, so our embryo loss figures are likely to be underestimated, and we cannot confirm these earlier studies.

The observation that rams from the two sources had similar fertilizing capacity but different capacities of producing embryos which survive to become viable offspring confirms the findings reported by Courot and Colas (1986), that rams may contribute to embryo mortality by transmitting lethal genes (Bishop, 1964) and that there is a basal embryo mortality in sheep of 20 to 30 % which eliminates the less adequate genotypes (Edey, 1966). This basal embryo mortality can be increased by interaction with the environment (Edey, 1979). This may have been important in the present experiment because of the abnormal management of the ewes and the heat wave conditions during and after the mating program which probably increased the rate of embryo mortality. Thus, we conclude that the difference between ram sources in pregnancy and rate of embryo mortality probably had a genetic and reproductive basis inherent to the strains of rams, and was enhanced by an interaction with the environment.

CHAPTER 7. CORRELATIONS BETWEEN SEMEN CHARACTERISTICS WITH FERTILITY AND FERTILITY PREDICTION.

7.1 Introduction.

Fertility has been correlated with some semen characteristics of fresh and frozen-thawed semen by subjective assessment (Hulet and Ercanbrack, 1962; Hulet et al., 1965; Wiggins et al., 1953; Linford et al., 1976) or fresh and frozen-thawed semen using video tapes and computer-assisted semen analysis (Budworth et al., 1988).

The accuracy of the estimation of correlation is influenced by the population of males from which semen is collected, the number of ewes which are mated or inseminated with a particular semen, the variability of the semen quality and the threshold of inseminate dose (Foote et al., 1977; O'Connor et al., 1981; Saacke, 1982). It is not possible to generalize from the results of individual experiments to the whole male population, but estimations of correlation between semen characteristics and fertility could be valuable for prediction of fertility.

This chapter aimed to use all the information generated from fresh and frozen-thawed semen assessment from normal and subfertile rams and the fertility achieved in each case, to estimate the correlation of all semen characteristics with fertility. It was also proposed to study the relationships between the measured semen characteristics to find if they are correlated, to estimate the confidence limits within which an increase in the value of semen characteristics would predict an improvement in fertility, and to determine the minimum sample size required to enable such predictions. An attempt was made to estimate an equation of fertility prediction using all the semen characteristics measured (for each particular set of data from the previous experiments). The standard regression coefficients were also estimated and used to determine which of the correlated semen characteristics had the most impact in fertility.

7.2 Experimental procedure.

7.2.1 Experiment 1. This experiment was conducted to find the effect of different doses of post-thawing motile sperm inseminated by laparoscopy on fertility and to compare the latter insemination technique with the cervical insemination technique (experiment 2, chapter 3). Thirty observations of frozen-thawed semen quality were used to estimate the correlation with pregnancy rate at 65 days after intrauterine insemination. With the estimated correlation coefficients, the confidence limits for semen characteristics assessed by HTM were estimated within which increments of 5, 10 and 15 % in fertility could be predicted, and the minimum sample size required to enable such predictions was also estimated. For the latter estimation, the sample size was calculated from the variance components obtained in experiment 5 and the mean, standard deviation and coefficient of regression of the Y (pregnancy) and Xs (correlated semen characteristics) variables were determined. All semen characteristics were used to estimate regression coefficients and a prediction equation of fertility, and the best model with the semen characteristics is presented. The standard regression coefficients were estimated to determine which of the correlated semen characteristics had the greatest impact on fertility.

7.2.2 Experiment 2. This experiment was conducted to examine the effect of separation of the epididymis from the testis on semen quality, fertility and embryo mortality (experiment 7, chapter 4). The major limitation of this experiment was the number of rams (n = 9) and the wide range of semen quality from normal and subfertile rams, which made the estimation of correlation unreliable. In this experiment, fresh semen characteristics assessed by both subjective and objective analysis (HTM) were correlated with 17 and 65 day pregnancy rates. There were 9 observations of semen quality; one for each of the rams before the mating program. It was not possible to perform an analysis of regression because of the low number of observations.

7.2.3 Experiment 3. This experiment was conducted to examine the effect of slightly increased but sustained scrotal temperature on semen quality, fertility and embryo mortality (experiment 8, chapter 5). In this experiment the number of rams was limited (n = 9), but the number of observations of semen quality was adequate for the estimation of correlation and the conduct of a regression analyses. From this experiment, 29 observations of semen quality of frozen-thawed semen samples assessed by HTM were correlated with pregnancy 17 and 65 days after intrauterine insemination. The standard regression coefficients were estimated to determine the rank of importance of the semen characteristics on fertility.

7.2.4 Experiment 4. This experiment was conducted at "Turretfield Research Centre". Sixty five rams of 16 months of age from the Collinsville and Bungaree families were involved for the study of repeatability of fresh and pellet-frozen semen (experiment 6, chapter 3) and from this group, 46 rams were each joined with 40 randomly allocated ewes in adjacent and similar paddocks for the breeding season (November-December, 1990). The data on semen characteristics and fertility from this experiment were used for the correlation and regression analysis.

Semen was collected by electro-ejaculation from each of the rams (only one ejaculate per ram was obtained, because of the number of animals and lack of training). The semen was kept at 32°C in a water bath and assessed within 5 to 10 minutes of collection. Semen was assessed in two ways: morphology was estimated manually and semen characteristics measured by computerised image analysis. The morphology of spermatozoa was assessed for proportion of live and dead sperm and percentages of normal, headless, coiled and bent tail cells (termed abnormal tail). The semen assessment by the HTM was performed on 4 sub-samples (100 μl) from the electro-ejaculated semen. Pregnancy was determined at 42 and 70 days after mating by ultrasound and at lambing.

All fresh semen characteristics assessed by both of the analyses (subjective: one observation of each morphological characteristic; and objective: mean of the 4 observations of

semen characteristics from the 4 sub-samples) were correlated with pregnancy rate at 70 days after mating and at lambing. The confidence limits, sample size and standard regression coefficients were estimated as in experiment 1. For the sample size, the variance components estimated in experiment 6 (chapter 3) were used because they were estimated from the same population of rams under the same conditions. An equation for fertility prediction was calculated using the regression coefficients.

7.2.5 Abbreviations used:

Pregnancy 17 days = Pregnancy rate at 17 days after insemination or mating determined by RIA of progesterone.

Pregnancy 65 days = Pregnancy rate at 65 days after insemination or mating determined by ultrasound.

Pregnancy 70 days = Pregnancy rate at 70 days after mating determined by ultrasound.

Lambing = Proportion of ewes lambing.

The abbreviations for the semen characteristics assessed by the HTM are presented in Section 1.2 para 1.2.3.6.1.to 1.2.3.6.3. of the literature review.

The characteristics from manual assessment are presented in paragraph 1.2.2. of the literature review and section 2.6. paragraph 2.6.1. of the Materials and Methods and the abbreviations used are follows:

Mass motility: Mass mot.
Percentage of motile spermatozoa: Mot (%).
Percentage of live cells : Live (%)
Percentage of dead cells: Dead (%)
Proportion of live cells over 200: Live
Proportion of dead cells over 200: Dead
Concentration of spermatozoa x 10^6/ ml: Density
Proportion of normal cells: Normal
Proportion of cells without head: Headless

Porportion of cells with coiled tail: Coiled
Porportion of cells with bent tail: Bent
Porportion of cells with coiled + bent tail termed Abnormal tail: Abnormal tail.

7.3 Estimation of correlations, multiple regressions, confidence limits, minimum sample size and standard regression coefficients for the fresh and frozen-thawed semen characteristics and fertility.

7.3.1 Correlation between semen characteristics and fertility.

With data of each experiment in which semen assessment and fertility trials were involved (AI or mating programs), simple correlations were estimated between all the semen characteristics and fertility, and between the semen characteristics assessed.

7.3.2 Coefficients of regression, confidence limits of the correlated semen characteristics and minimum sample size.

Definitions:
Y = Mean of fertility or dependent variable
b = Coefficient of regression
b' = Standard regression coefficient
a = Constant of the regression equation which is the interception point when X= 0
X = Mean of semen characteristics or independent variable
r = Coefficient of correlation
L = Confidence limit for X in which an increment in fertility is found
Xi = Value of semen characteristics which increments the required value of fertility
Y= a + b X equation of simple linear regression
VAR wr = Variance component (value) within ram (from the repeatability studies sections 3.5 for semen frozen in straws and 3.6 of chapter 3 for fresh and frozen semen in pellets)
n = minimum sample size = 4 (variance within rams)/ L^2

Using the correlation coefficients of the semen characteristics and fertility, it was possible to obtain a coefficient of regression which was also used to calculate confidence limits

for each of the correlated semen characteristics. It was thereby possible to determine which values of the semen characteristics would produce differences in fertility of 5, 10 and 15 %. The minimum sample size required to predict these increments in fertility was also calculated.

The following equations were used in the calculation of the regression coefficients, the intercept, and the minimum sample size;

$r = Cov\ XY / STD\ x\ STD\ y$

$b = (r) \times STD\ Y / STD\ X$

$a = Y - b * X$

$L = Xi - X$

$n = 4\ (VARwr)/ L^2$

For example, the pregnancy rate or fertility is 55 % with mean motility of 57.5 % from a large number of samples from one ram. If it is required to predict an increase of 10 % in fertility, it is necessary to know the confidence limit for motility within which an in crease in motility would predict this improvement in fertility. If the correlation coefficient between motility and fertility is r = 0.5163, the STD Y = 18.890 and the STD X = 7.412 then:

$b = r * STD\ Y/ STD\ X = 0.5163 * 18.890/7.412 = 1.31$

$a = Y - b\ X = 55.0 - (1.31)(57.5) = -20.32$

If a 10 % increment in fertility is required, then the estimation of the increase in motility needed, and the confidence limits of the motility increase are as follows:

$Y = a + b\ X$ thus

$X = Y - a / b$

$X = 65 - (-20.32)/ 1.31$

$X = 65.13$ % motility is required, and

$L = Xi - X$

$L = 65.13 - 57.5$

$L = 5.63$ % motility.

To obtain these confidence limits the minimum number of samples to be assessed is also estimated as follows:

Assuming that the variance component within ram for motility is VAR wr = 189 (from experiment 5) then,

using the equation $n = 4\ (VARwr)/\ L^2$

$n = 4\ (189)/\ (5.63)^2$

$n = 23.85$ or about 24 samples.

Thus in this example, it was necessary to assess at least 24 samples of the semen in order to estimate a confidence limit of 5.63 % of motility, corresponding to predicted fertility differences of 10 %.

7.3.3 Multiple linear regression and the standard regression coefficients.

In sets of data with more than 25 observations, it was possible to estimate the regression equation or prediction equation of fertility and the impact of each of the semen characteristics on fertility. For the analysis, the program used considered as a limit the significance level of 0.5 for entry into the model.

The general model for multiple linear regression is given by:

$Y = a + biXi + bjXj + e$

$e= N(0, VAR)$ and for a given sample of 2 independent variables (X1 and X2) then

$Y = a + b1X1 + b2X2$

In this equation b estimates the average change in Y associated with unit increase in X. But in multiple regression analysis it is important to know which of the X variables are most important in determining Y. One way is by estimating the so-called 'standard regression coefficients' which have been used as measures of relative importance, so that Xs may be

coefficients' which have been used as measures of relative importance, so that Xs may be ranked in order of the sizes of these coefficients (ignoring sign, Snedecor and Cochran, 1980). When the Xs are correlated, however, the contribution of an X to the Y variation depends on the order in which this X is introduced to the model. Sometimes one X will contribute more or less to the change of Y in the presence of a second X, therefore it is important to select the Xs in order of importance or change produced in Y. To do this it is necessary to eliminate the units of each X because it may affect the coefficient value or magnitude, and this is done by standardizing the units by the standard regression coefficients which are estimated as follows:

$$b'i = bi\ (\sqrt{VARXi}/\ VARY) \text{ or } bi\ (STDXi/\ STDY) \qquad (7.5)$$

In this way the Xs can be rated in the order of the sizes of standard regression coefficients in absolute terms.

All statistical procedures described above and used in this chapter were performed using the SAS program.

The regression equations estimated in the present study were applied for the particular experiments in which they were estimated. Until more information is available, the findings presented are not conclusive. Nevertheless, the information can be used as a reference.

7.4 Results.

7.4.1 Estimation of correlations and regressions.

Experiment 1.

<u>1.Correlation analysis, estimation of confidence limits and minimum sample size.</u>

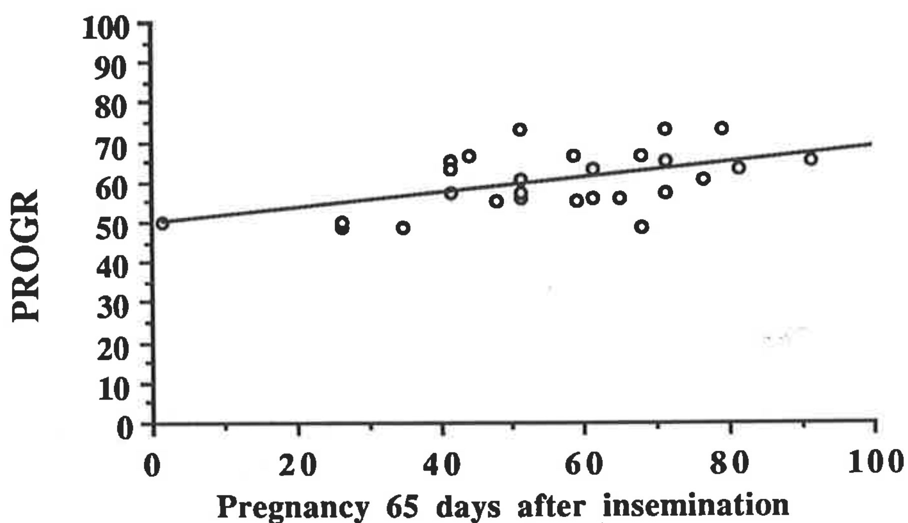
The correlation between 30 observations of frozen-thawed semen characteristics and pregnancy rate 65 days after insemination is presented in Table 7.1.

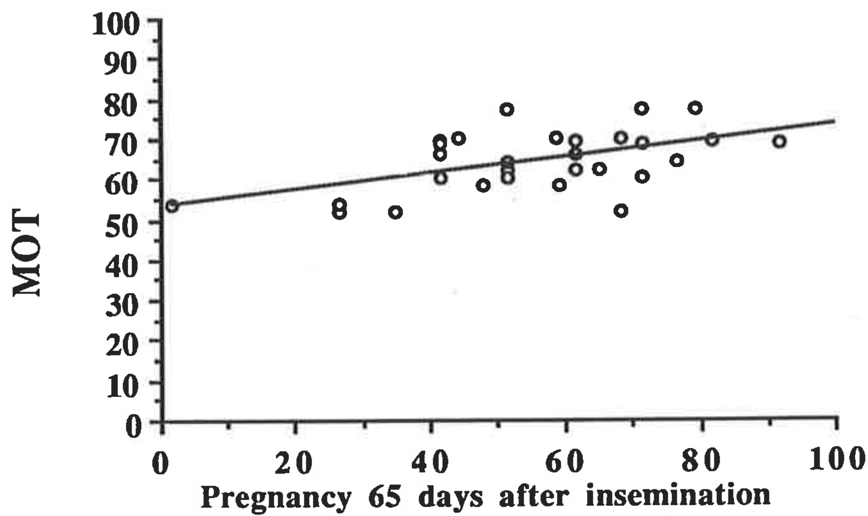
Table 7.1. Correlation matrix of post-thawing semen characteristics and pregnancy: Pearson correlation coefficients/ Prob > R. n = 30.

	MOTILE	PROGR	MOT	VSL	LIN%
PROGR	0.31241 0.0928				
MOT	0.34175 0.0645	0.98402 0.0001			
VSL	-0.06884 0.7185	-0.20374 0.2802	-0.26973 0.1495		
LIN %	-0.5711 0.7644	0.07827 0.6810	-0.04360 0.8190	0.28314 0.1295	
Pregnancy 65 days	0.20746 0.2713	0.50860 0.0041	0.51631 0.0035	0.08542 0.6536	0.0959 0.6340

In this experiment, fertility was positively correlated with progressive motility and motility percentage. The correlations with the rest of the semen characteristics were not significant. The correlations between PROGR, MOT and pregnancy are presented in Figure 7.1.

Figure 7.1. Relationship between PROGR, MOT and pregnancy.





The mean, standard deviation and correlation coefficients for progressive motility and motility percentages with fertility are presented in Table 7.2; and the confidence limits for these characteristics, and the minimum sample size to be assessed to predict differences in fertility are presented in Table 7.3.

Table 7.2. Mean and standard deviation of the correlated semen characteristics (PROGR and MOT) and the coefficients of correlation with pregnancy (r).

Characteristic	Mean	Standard dev.	r
PROGR	57.59	7.11	0.51
MOT	61.61	7.41	0.52
Pregnancy 65 days	53.57	18.89	

Table 7.3. The values of progressive motility and motility (X), the confidence limits for these characteristics (L), and the minimum sample size to be assessed (n) to predict differences of 5, 10 and 15 % in fertility.

Correlated semen characteristic	Increment in fertility required (%)			
	0 X	5 X(L, n)	10 X(L, n)	15 X(L, n)
PROGR	57.6	61.3 (3.7, 40)	65.0 (7.4, 10)	68.7 (11.1, 5)
MOT	61.6	65.4 (3.8, 52)	69.2 (7.6, 13)	73.1 (11.4, 6)

Thus increments of 3.7, 7.4 and 11.1 % in progressive motility or 3.8, 7.6 and 11.4 % in motility, would be predicted to result in 5, 10 and 15 % differences in pregnancy rate. Sample sizes required to enable such predictions are 40, 10 and 5 straws respectively for progressive motility and 52, 13 and 6 straws respectively for motility.

2. Analysis of regression: The estimation of the regression coefficients and the standard regression coefficients.

The regression coefficients (b_n) and standard regression coefficients (b'_n) for the semen characteristics included in the best two variables regression model (MOT and VSL) are presented in Table 7.4.

Table 7.4. The regression coefficients (b_n) and standard regression coefficients (b'_n) estimated for the semen characteristics included in the best two variables regression model.

Semen characteristics	Parameter estimated	
	Regression coefficient (b_n)	Standard regression coefficient (b'_n)
MOT	1.48	0.5815
VSL	0.69	0.2420

With the above data the regression equation was:

$Y = -98.79 + 1.482\ (MOT) + 0.696\ (VSL)$

if MOT = 61.61, and VSL = 87.2 from data set then

$Y = -98.79 + 1.482\ (61.61) + 0.696\ (87.2) = 53.2$ which is approximately the pregnancy rate obtained in this experiment.

The standard regression coefficients reflect the absolute impact or change that MOT and VSL semen characteristics produces on fertility. MOT had more importance in fertility than VSL.

Experiment 2.

Correlation analysis.

The correlation between fresh semen characteristics assessed subjectively and objectively (HTM) and fertility are presented in Tables 7.5. and 7.6. respectively.

Table 7.5. Correlation matrix of fresh semen characteristics assessed manually and pregnancy: Pearson correlation coefficients/ Prob > R. n = 9

	Mass mot.	Mot. (%)	Live (%)	Dead (%)	Density	Normal	Head-less	Coiled	Bent	Preg-nancy 17day
Mot. (%)	0.986 0.0001									
Live (%)	0.8857 0.0015	0.9318 0.0003								
Dead (%)	-0.4920 0.1785	-0.4940 0.1764	-0.3208 0.399							
Density	0.4885 0.1823	0.4916 0.1789	0.6391 0.0639	-0.0698 0.8585						
Normal	0.7973 0.0101	0.8097 0.0082	0.8413 0.0045	-0.0355 0.9277	0.5999 0.0877					
Headless	-0.6077 0.0828	-0.6164 0.0771	-0.4757 0.1955	0.9714 0.0001	-02501 0.5148	-0.2068 0.5934				
Coiled	-0.4038 0.2811	-0.4038 0.2811	-0.4054 0.2790	-0.1783 0.6462	0.8130 0.0077	0.0612 0.8757	-0.1320 0.7349			
Bent	0.4199 0.2606	0.4985 0.1719	0.7063 0.0334	0.0454 0.9077	0.4792 0.1918	0.3706 0.3262	-0.0884 0.8211	0.2568 0.5048		
Pregnan-cy 17 day	0.8917 0.0012	0.9478 0.0001	0.9195 0.0005	-0.5879 0.0959	0.4202 0.2601	0.7355 0.0239	-0.6968 0.0370	-0.5027 0.1678	0.5186 0.1526	
Pregnan-cy 65 day	0.9147 0.0005	0.9678 0.0001	0.9450 0.0001	-0.5331 0.1394	0.4651 0.2071	0.7584 0.0179	-0.6537 0.0561	-0.4321 0.2455	0.5753 0.1051	0.9922 0.0001

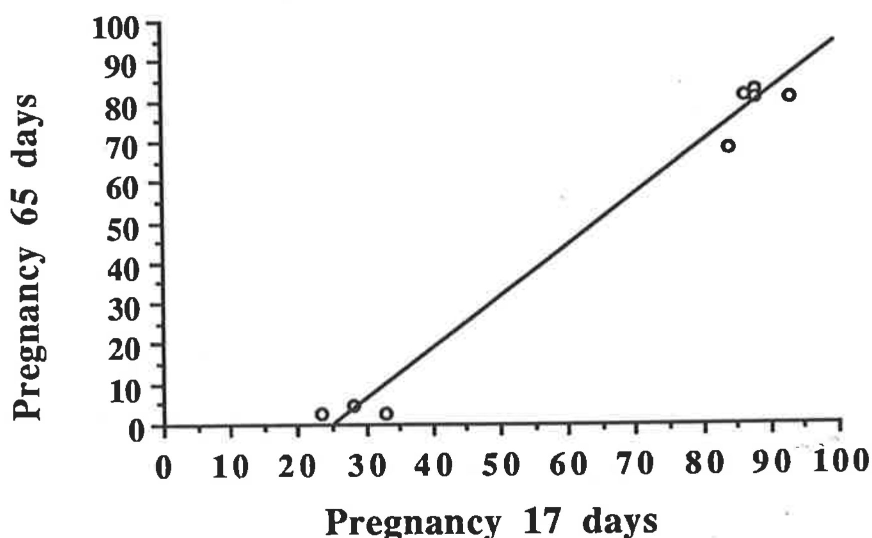
Table 7.6. Correlation matrix of fresh semen characteristics assessed by the HTM and pregnancy: Pearson correlation coefficients/ Prob > R. n = 9

	TOTAL	MOTILE	PROGR	MOTILE sd	MOT	VSL	LIN (%)	ALH	LIN	VAP	VAPsd	VSL sd	RAPID (%)	MEDIUM (%)	SLOW (%)	STATIC (%)	Pregnancy 17 days
MOTILE	0.9786 0.001																
PROGR	0.9204 0.0004	0.9515 0.0001															
MOTsd	0.9862 0.0001	0.9932 0.0001	0.9659 0.0001														
MOT	0.9544 0.0001	0.9809 0.0001	0.9892 0.0001	0.9888 0.0001													
VSL	0.1830 0.6374	0.1044 0.7893	0.1717 0.6587	0.1341 0.7310	0.1599 0.6811												
LIN (%)	0.5711 0.1082	0.4897 0.1808	0.5575 0.1188	0.5286 0.1434	0.5407 0.1328	0.8931 0.0012											
ALH	0.4609 0.2119	0.4363 0.2403	0.4130 0.2693	0.4376 0.2388	0.4397 0.2363	0.7432 0.0217	0.7546 0.0188										
LIN	0.52240.1 490	0.4377 0.2387	0.5203 0.1510	0.4796 0.1914	0.4955 0.1750	0.9028 0.0009	0.9958 0.0001	0.7065 0.0334									
VAP	0.2849 0.4574	0.2103 0.5871	0.2523 0.5126	0.2350 0.5427	0.2546 0.5079	0.9903 0.0001	0.9147 0.0005	0.8065 0.0086	0.9134 0.0006								
VAPsd	-0.0909 0.8166	-0.1523 0.6956	-0.1599 0.6812	-0.1406 0.7183	-0.1407 0.7181	0.7819 0.0128	0.5517 0.1235	0.8133 0.0077	0.5352 0.1376	0.7860 0.0120							
VSLsd	-0.0131 0.9723	-0.0668 0.8644	-0.0782 0.8415	-0.0581 0.8819	-0.0560 0.8863	0.7931 0.0108	0.5882 0.0957	0.8591 0.0030	0.5670 0.1114	0.8070 0.0086	0.9952 0.0001						
RAPID (%)	0.8710 0.0022	0.9130 0.0006	0.9218 0.0004	0.9186 0.0005	0.9482 0.0001	0.2110 0.5858	0.5110 0.1597	0.4415 0.2330	0.4682 0.2037	0.3067 0.4221	-0.0849 0.8282	0.0045 0.9908					
MEDIUM (%)	0.7132 0.0310	0.6932 0.0384	0.6420 0.0623	0.7028 0.0347	0.6665 0.0499	0.2967 0.4382	0.5214 0.1500	0.7863 0.0120	0.4452 0.2182	0.3832 0.3086	0.4057 0.2786	0.4667 0.2054	0.5828 0.0996				
SLOW (%)	0.0438 0.9110	-0.0464 0.9055	-0.0095 0.9806	0.0035 0.9928	-0.0318 0.9352	0.4277 0.2507	0.3856 0.3054	0.5721 0.1075	0.3763 0.3182	0.4165 0.2648	0.6764 0.0454	0.6751 0.0460	-0.0724 0.8531	0.6020 0.0863			
STATIC (%)	-0.9572 0.0001	-0.9776 0.0001	-0.9883 0.0001	-0.9889 0.0001	-0.9977 0.0001	-0.1889 0.6263	-0.5667 0.1116	0.4785 0.1925	-0.5208 0.1505	-0.2833 0.4601	0.097 0.8086	0.0100 0.9796	-0.9435 0.0001	-0.7072 0.0331	-0.0358 0.9272		
PREGNANCY 17 DAYS	0.9637 0.0001	0.9743 0.0001	0.9664 0.0001	0.9889 0.0001	0.9813 0.0001	0.0462 0.9061	0.4578 0.2153	0.3579 0.3443	0.4094 0.2739	0.1418 0.7158	-0.2237 0.5629	-0.1449 0.7099	0.9055 0.0008	0.6828 0.0427	-0.0147 0.9701	-0.9801 0.0001	
PREGNANCY 65 DAYS	0.9717 0.0001	0.9817 0.0001	0.9747 0.0001	0.9938 0.0001	0.9883 0.0001	0.1073 0.7836	0.5132 0.1576	0.4239 0.2556	0.4642 0.2081	0.2051 0.5965	-0.1557 0.6891	-0.0764 0.8450	0.9001 0.0009	0.7027 0.0348	0.0080 0.9837	-0.9886 0.0001	0.992 0.0001

When correlations were estimated with the data on manually assessed semen characteristics, mass mot., mot. (%), live (%), normal cells and headless cells were correlated with both of the pregnancy estimates (Table 7.5). Except for the headless cells, the characteristics were correlated positively with fertility. When correlations were estimated for objectively measured semen characteristics (Table 7.6), TOTAL, MOTILE, PROGR, MOTILEsd, MOT, RAPID %, MEDIUM % and STATIC % (negatively correlated) were significantly correlated with either 17 or 65 day pregnancy rate ($p < 0.001$). Semen characteristics had slightly better correlations with pregnancy rate at 65 days than at 17 days after mating. Despite these good correlations, because of the source of the semen (normal and subfertile rams), the range in semen quality was very large resulting in good correlation coefficients from a small number of observations.

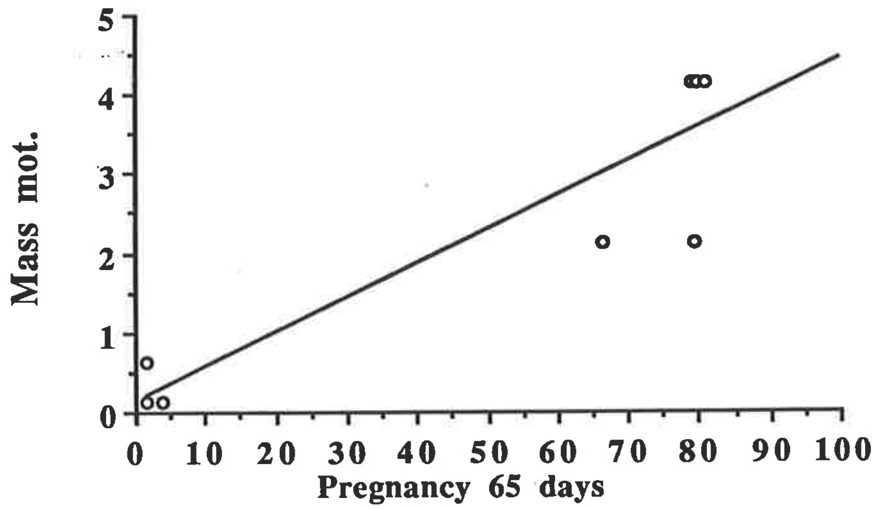
The relationship between pregnancy 17 and 65 days after mating is presented in Figure 7.2.

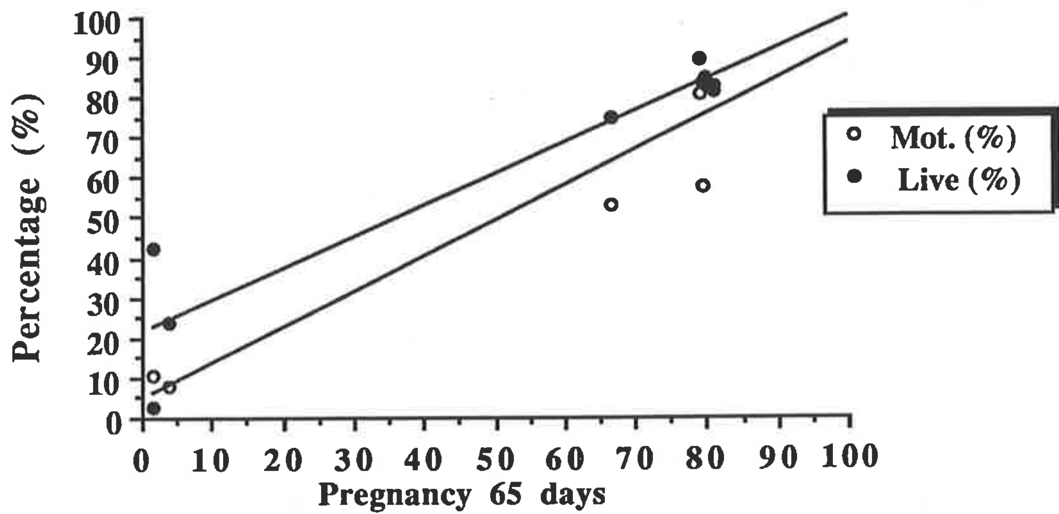
Figure 7.2. Relationship between pregnancy at 17 and 65 days.

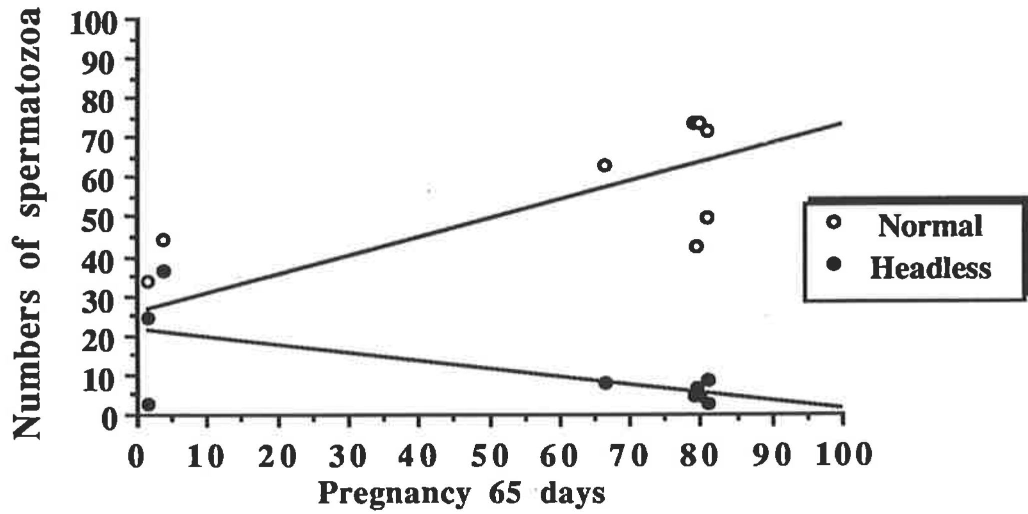


The relationship between the correlated semen characteristics subjectively measured and pregnancy 65 days after mating are presented in Figure 7.3.

Figure 7.3. Relationship between the correlated semen characteristics subjectively measured and pregnancy 65 days.

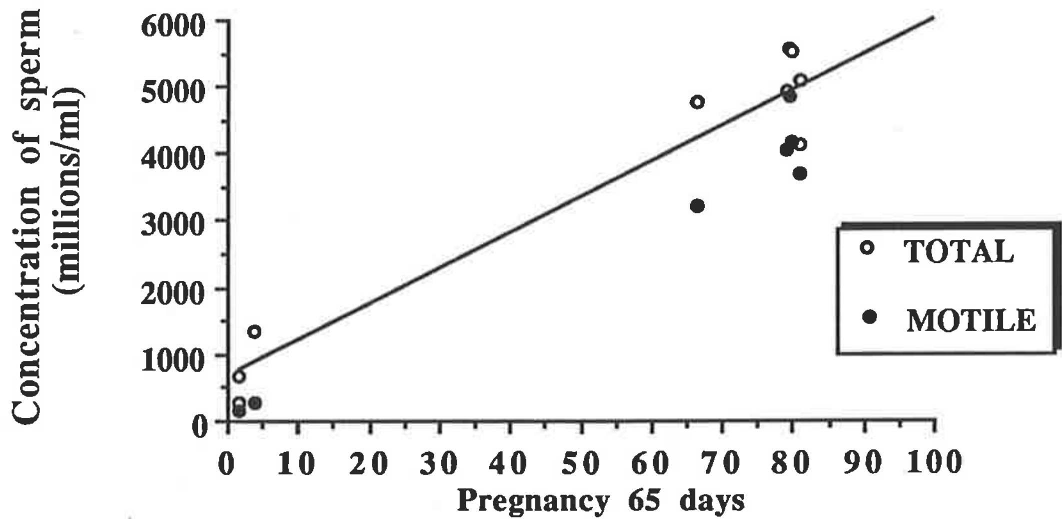


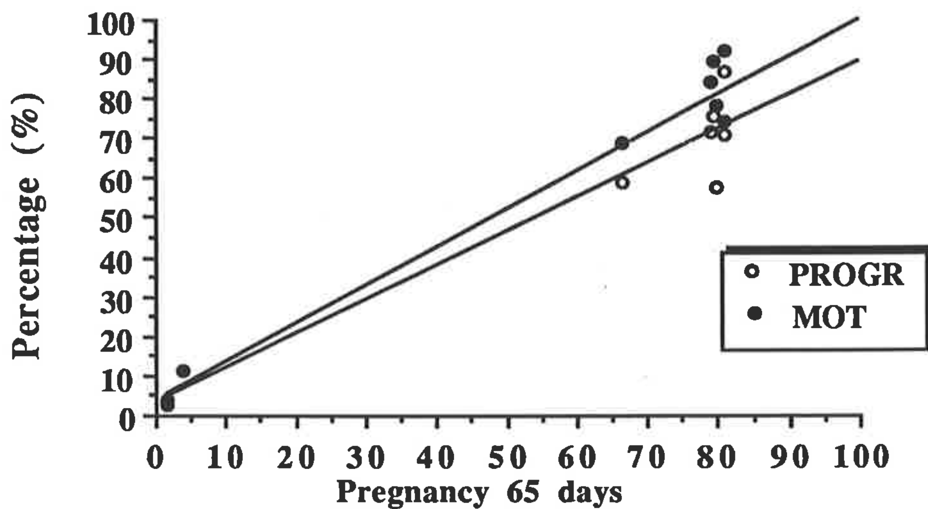


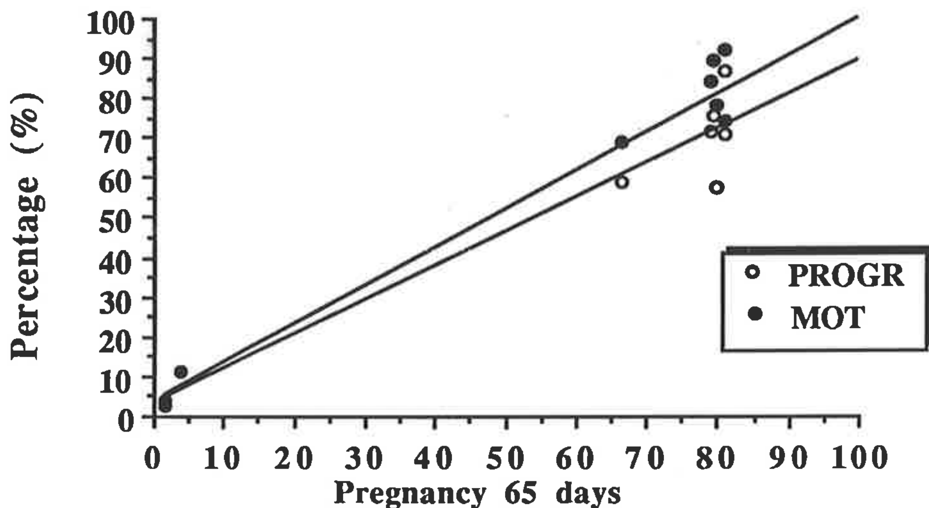


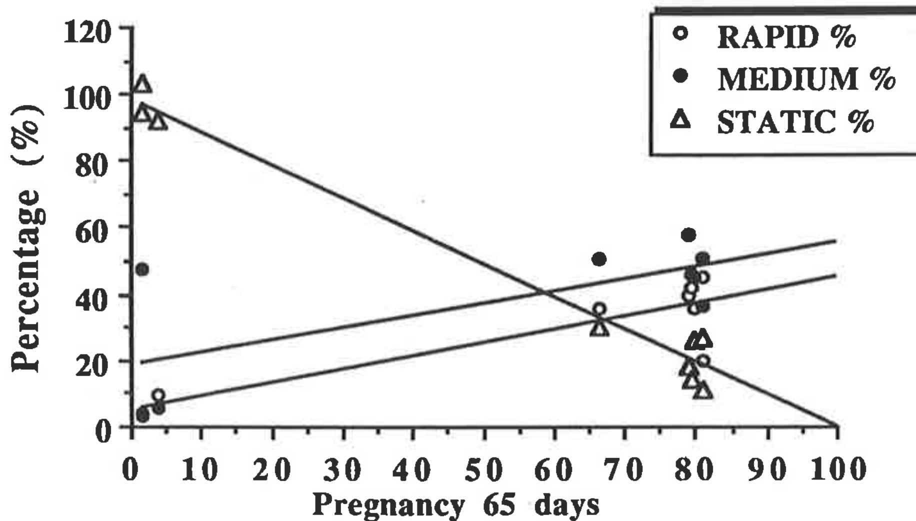
The relationship between the correlated semen characteristics objectively measured and pregnancy 65 days are presented in Figure 7.4.

Figure 7.4. Relationship between the correlated semen characteristics objectively measured and pregnancy 65 days.




Percentage (%)
100
90
80
70
60
50
40
30
20
10
0
PROGR
MOT
0 10 20 30 40 50 60 70 80 90 100
Pregnancy 65 days


Percentage (%)
100
90
80
70
60
50
40
30
20
10
0
PROGR
MOT
0 10 20 30 40 50 60 70 80 90 100
Pregnancy 65 days


Percentage (%)
120
100
80
60
40
20
0
RAPID %
MEDIUM %
STATIC %
0 10 20 30 40 50 60 70 80 90 100
Pregnancy 65 days

Experiment 3.

1. Correlation analysis.

The correlation of 29 observations of semen quality of frozen-thawed semen samples assessed by the HTM with pregnancy at 17 and 65 days after intrauterine insemination are presented in Table 7.7.

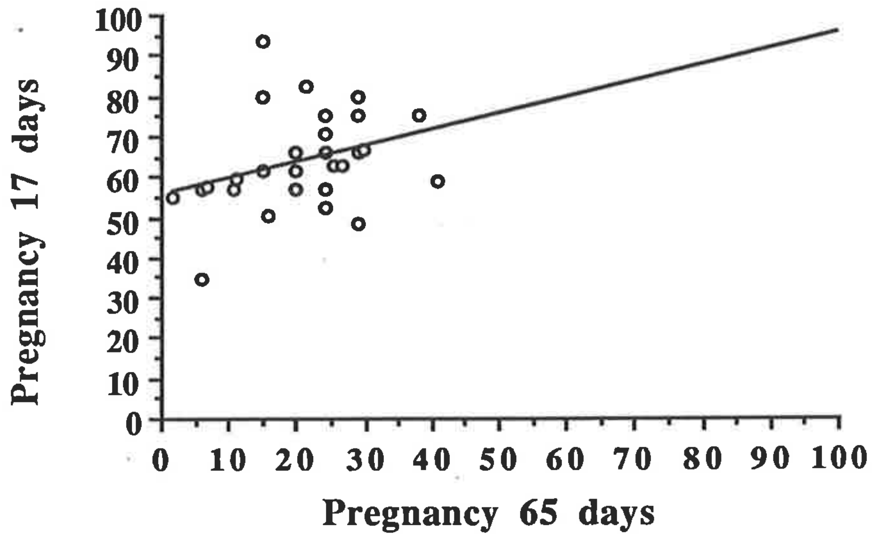
As in the experiment 2, the fertility was affected by the treatments applied to the rams. Thus semen came from normal and subfertile rams and the range of semen quality was very large. In this experiment (experiment 3), the pregnancy at 17 days did not vary significantly between control and treated rams and pregnancy at 65 days was very low compared with pregnancy at 17 days even in the normal rams. This was attributed to large embryo mortality, and consequently the pregnancy rate at 17 days was not correlated with pregnancy rate at 65 days after insemination. Semen characteristics were better correlated between each other than the fresh semen analyzed in experiment 2. Only PROGR, MOT, MEDIUM %, SLOW % were significantly correlated, and STATIC % negatively correlated, with pregnancy rate 65 days after insemination. In general the correlation coefficients were smaller than in previous experiments.

Table 7.7. Correlation matrix of post-thawing semen characteristics assessed by the HTM and pregnancy: Pearson correlation coefficients/ Prob > R. n = 29

	PROGR	MOT	VSL	LIN (%)	ALH	LIN	VAP	RAPID (%)	MEDIUM (%)	SLOW (%)	STATIC (%)	Pregnancy 17 days
MOT	0.9978 0.0001											
VSL	0.4804 0.0083	0.4693 0.0102										
LIN (%)	-0.4945 0.0064	-.05212 0.0037	-0.2611 0.1713									
ALH	0.7142 0.0001	0.7108 0.0001	0.7361 0.0001	-0.6064 0.0005								
LIN	-0.4900 0.0070	-0.5191 0.0039	-0.1919 0.3187	0.8186 0.0001	-0.6614 0.0001							
VAP	0.5601 0.0016	0.5506 0.0020	0.9001 0.0001	-0.3570 0.0573	0.8621 0.0001	-0.2819 0.1385						
RAPID (%)	0.9401 0.0001	0.9383 0.0001	0.5905 0.0007	-0.4351 0.0183	0.6985 0.0001	-0.3515 0.0615	0.6777 0.0001					
MEDIUM (%)	0.9769 0.0001	0.9812 0.0001	0.3770 0.0438	-0.5409 0.0024	0.6801 0.0001	-0.5852 0.0009	0.4507 0.0141	0.8540 0.0001				
SLOW (%)	0.5468 0.0021	0.5647 0.0014	0.2664 0.1625	-0.5346 0.0028	0.5097 0.0047	-0.6677 0.0001	0.2618 0.1701	0.4585 0.0124	0.5939 0.0007			
STATIC (%)	-0.9582 0.0001	-0.9655 0.0001	-0.4537 0.0134	0.5786 0.0010	-0.7208 0.0001	0.6197 0.0003	-0.5163 0.0041	-0.8834 0.0001	-0.9598 0.0001	-0.7601 0.0001		
Pregnancy 17 days	-0.1312 0.4974	-0.1372 0.4779	-0.0752 0.6981	0.0028 0.9886	-0.2003 0.2975	0.0019 0.9921	-0.1806 0.3486	-0.1757 0.3620	-0.1086 0.5748	-0.0231 0.9055	0.1152 0.5519	
Pregnancy 65 days	0.4313 0.0195	0.4307 0.0197	0.0717 0.7118	-0.1922 0.3179	0.1183 0.5410	-0.2101 0.2740	0.0122 0.9501	0.3326 0.0780	0.4623 0.0116	0.5425 0.0024	-0.5103 0.0047	0.2591 0.1747

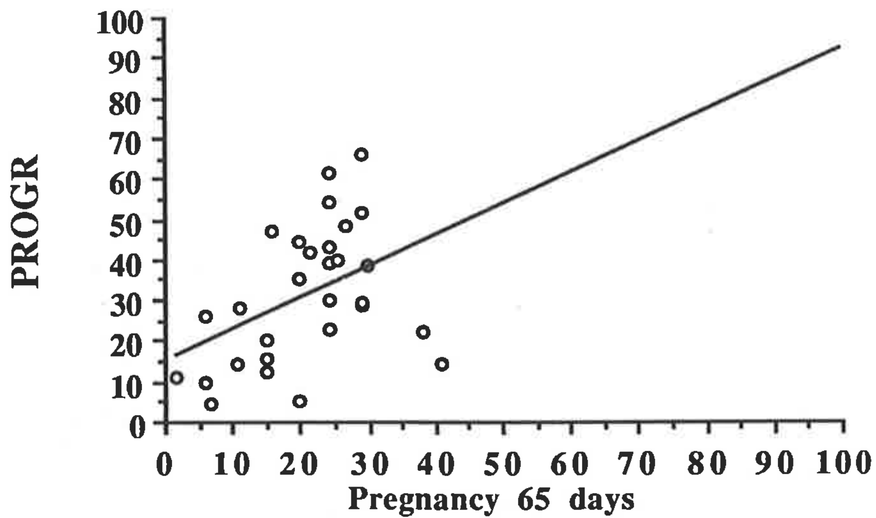
The relationship between pregnancy at 17 days and 65 days after insemination is shown in Figure 7.5.

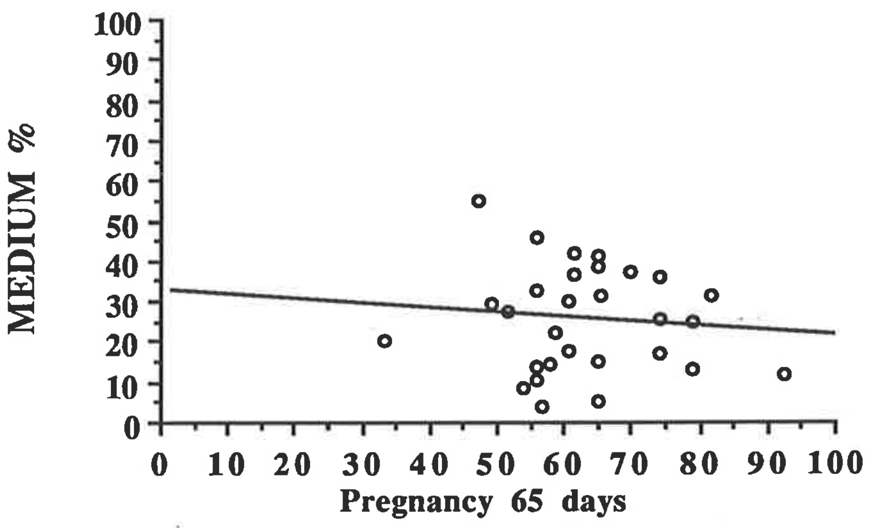
Figure 7.5. Relationship between the pregnancy 17 and 65 days after insemination.

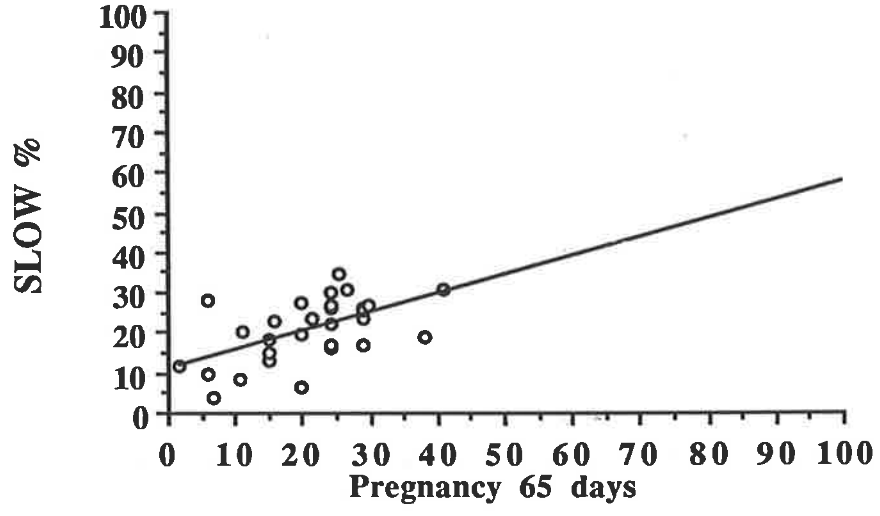


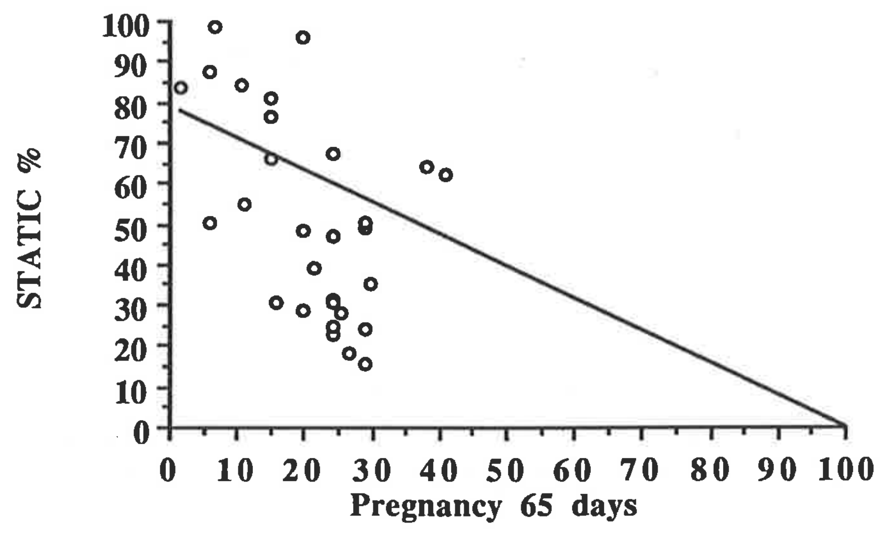
The relationships between the correlated semen characteristics measured objectively and pregnancy 65 days after insemination are shown in Figure 7.6.

Figure 7.6. Relationship between the correlated semen characteristics measured objectively and pregnancy 65 days after insemination.




MEDIUM %
100
90
80
70
60
50
40
30
20
10
0
0 10 20 30 40 50 60 70 80 90 100
Pregnancy 65 days


SLOW %
100
90
80
70
60
50
40
30
20
10
0
0 10 20 30 40 50 60 70 80 90 100
Pregnancy 65 days


STATIC %
100
90
80
70
60
50
40
30
20
10
0
0 10 20 30 40 50 60 70 80 90 100
Pregnancy 65 days

2. Analysis of regression: The estimation of the regression coefficients and the standard regression coefficients.

The regression coefficients and standard regression coefficients of the four semen characteristics included in the best four variables regression model (PROGR, MOT, ALH AND SLOW %) are presented in Table 7.8.

Table 7.8. The regression coefficients (b_n) and standard regression coefficients (b'_n) estimated for the semen characteristics included in the regression equation.

Semen characteristics	Parameter estimated	
	Regression coefficient (b_n)	Standard regression coefficient (b'_n)
PROGR	1.950	3.34
MOT	-1.436	-2.86
ALH	-6.768	-0.54
SLOW %	0.765	0.61

The regression equation was:
Y = a + b1X1 + b2X2 + b3X3 + b4X4 thus,

Y = 24.08+ 1.95 (PROGR) + (-1.436) (MOT) + (-6.768) (ALH) + 0.765 (SLOW %)

using the mean values from data set then

Y = 24.08 + 1.95 (28.68) + (-1.436) (32.85) + (-6.768) (4.06) + 0.765 (18.11) = 19.16 which is the pregnancy rate obtained in this experiment.

In this experiment, the standard regression coefficients indicated that PROGR had more importance than the rest of the semen characteristics analyzed.

Experiment 4.

The means for the fresh semen characteristics per sire are presented in Table 7.9. in Appendix C of the thesis. The mean, standard deviation and range of values for fresh semen characteristics assessed both by subjective and objective semen assessment are presented in Table 7.10.

Table 7.10. Mean, standard deviation and range of fresh semen characteristics assessed both by subjective and objective semen assessment.

Semen characteristics	Mean	Standard Deviation	Range	
			Minimum	Maximum
TOTAL	2367.00	1362.00	331.98	4876.00
MOTILE	1379.00	941.87	30.45	3887.00
PROGR	33.99	17.96	6.60	66.30
MOT	55.73	20.03	9.40	87.83
VSL	109.96	27.00	49.13	162.80
LIN %	86.50	5.35	71.73	95.98
ALH	6.43	0.99	3.83	8.80
LIN	74.56	9.07	55.50	91.15
VAP	123.04	27.87	55.80	184.20
RAPID %	35.79	19.33	2.20	76.53
MEDIUM %	19.81	14.52	1.78	70.50
SLOW %	0.40	0.32	0.00	1.38
STATIC %	42.97	20.62	2.07	90.05
Live	159.15	14.66	125.00	181.00
Dead	41.06	14.90	19.00	75.00
Normal	85.24	8.57	59.00	95.00
Headless	4.46	6.05	0.00	22.00
Abnormal tail	10.35	5.47	0.00	28.00

1.Correlation analysis, estimation of confidence limits and minimum sample size.

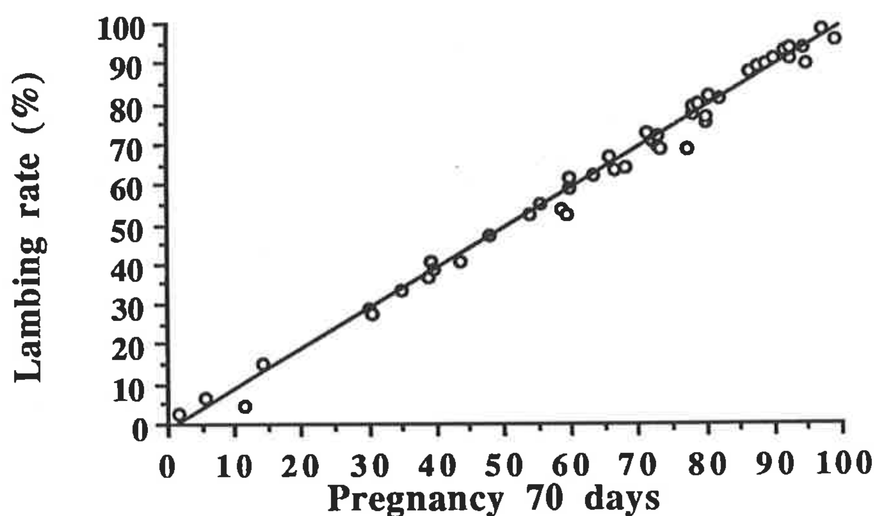
The correlation matrix for fresh semen characteristics assessed both subjectively and objectively and fertility (pregnancy at 70 days and lambing rate) is presented in Table 7.11.

Table 7.11. Correlation matrix of fresh semen characteristics assessed both subjectively and objectively and fertility (pregnancy 70 days and lambing): Pearson correlation coefficients/ Prob > R, n = 46.

	TOTAL	MOTILE	PROGR	MOT	VSL	LIN %	ALH	LIN	VAP	RAPID %	MEDIUM %	SLOW %	STATIC %	Live	Dead	Normal	Headless	Abnormal tail.	Pregnancy 70 days
MOTILE	0.8236 0.0001																		
PROGR	0.1244 0.4101	0.4927 0.0005																	
MOT	0.1220 0.4194	0.5796 0.0001	0.7683 0.0001																
VSL	0.1336 0.3759	0.2644 0.0737	0.6907 0.0001	0.1851 0.2181															
LIN %	-0.3622 0.0134	-0.1092 0.4699	0.4545 0.0015	0.2791 0.0603	0.4366 0.0024														
ALH	0.3683 0.0118	0.2488 0.0954	-0.047 0.7549	-0.0826 0.5853	0.0644 0.6708	-0.5400 0.0001													
LIN	-0.2043 0.1732	0.0095 0.9499	0.6028 0.0001	0.2196 0.1425	0.7431 0.0001	0.8419 0.0001	-0.5400 0.0001												
VAP	0.2216 0.1388	0.3244 0.0279	0.6638 0.0001	0.1616 0.2833	0.9833 0.0001	0.2728 0.0666	0.1907 0.2043	0.6273 0.0001											
RAPID %	0.1425 0.3448	0.4928 0.0005	0.9745 0.0001	0.7302 0.0001	0.7257 0.0001	0.3875 0.0078	0.0114 0.9400	0.5820 0.0001	0.7144 0.0001										
MEDIUM %	-0.0322 0.8315	0.1438 0.3402	-0.2334 0.1185	0.4172 0.0039	-0.7149 0.0001	-0.1342 0.3739	-0.1132 0.4535	-0.4798 0.0007	-0.7306 0.0001	-0.3142 0.0334									
SLOW %	0.1920 0.2011	0.0389 0.7973	-0.0857 0.5710	-0.1431 0.3428	-0.0406 0.7889	-0.1949 0.1942	-0.0831 0.5828	-0.0589 0.6973	-0.0169 0.9113	-0.0947 0.5312	-0.0974 0.5198								
STATIC %	-0.1277 0.3976	-0.5597 0.0001	-0.7033 0.0001	-0.9797 0.0001	-0.0999 0.5087	-0.2428 0.1040	0.0712 0.6382	-0.1343 0.3735	-0.0767 0.6122	-0.6448 0.0001	-0.5037 0.0004	0.1373 0.3628							
Live	-0.0435 0.7741	0.3441 0.0192	0.6099 0.0001	0.6762 0.0001	0.2931 0.0481	0.4639 0.0012	-0.1172 0.4379	0.3735 0.0106	0.2384 0.1106	0.5954 0.0001	0.1535 0.3084	-0.1769 0.2396	-0.6256 0.0001						
Dead	0.0245 0.8715	-0.3564 0.0150	-0.6152 0.0001	-0.6802 0.0001	-0.3051 0.0392	-0.4717 0.0009	0.1506 0.3176	-0.3935 0.0068	-0.2489 0.0953	-0.5998 0.0001	-0.1529 0.3104	0.1685 0.2630	0.6307 0.0001	-0.9952 0.0001					
Normal	0.0962 0.5246	0.3435 0.0194	0.5600 0.0001	0.5222 0.0002	0.4153 0.0041	0.4191 0.0037	0.1839 0.2210	0.3440 0.0192	0.3797 0.0093	0.5517 0.0001	0.0102 0.9463	-0.3325 0.0240	-0.4783 0.0008	0.7308 0.0001	-0.7316 0.0001				
Headless	0.0536 0.7148	-0.2515 0.0917	-0.4689 0.0010	-0.5531 0.0001	-0.1947 0.1948	-0.2456 0.1000	-0.2164 0.1487	-0.1401 0.3530	-0.1777 0.2373	-0.4785 0.0008	-0.1550 0.3038	0.3974 0.0062	0.5143 0.0003	-0.5803 0.0001	0.5648 0.0001	-0.7829 0.0001			
Abnormal tail	-0.2279 0.1277	-0.2709 0.0686	-0.3873 0.0078	-0.1934 0.1979	-0.5055 0.0003	-0.3913 0.0072	-0.0606 0.6890	-0.4354 0.0025	-0.4721 0.0009	-0.3837 0.0085	0.2380 0.1112	0.0729 0.6302	0.1437 0.3407	-0.5087 0.0003	0.5268 0.0002	-0.7109 0.0001	0.1302 0.3883		
Pregnancy 70 days	-0.0981 0.5165	0.0488 0.7472	0.3706 0.0112	0.3926 0.0070	0.0438 0.7724	0.2444 0.1016	-0.1372 0.3632	0.1332 0.3775	0.0081 0.9572	0.3503 0.0170	0.09332 0.5378	0.0964 0.5239	-0.3684 0.0118	0.3878 0.0078	-0.3961 0.0064	0.3747 0.0103	-0.1983 0.1864	-0.3655 0.0125	
Lambing (%)	-0.0936 0.5361	0.0489 0.7468	0.3651 0.0126	0.3739 0.0105	0.0476 0.7535	0.2367 0.1130	-0.1382 0.3598	0.1332 0.3775	0.0137 0.9280	0.3451 0.0188	0.0731 0.6290	0.1149 0.4471	-0.3490 0.0174	0.3722 0.0109	-0.3793 0.0093	0.3397 0.0209	-0.1599 0.2884	-0.3549 0.0155	0.9948 0.0001

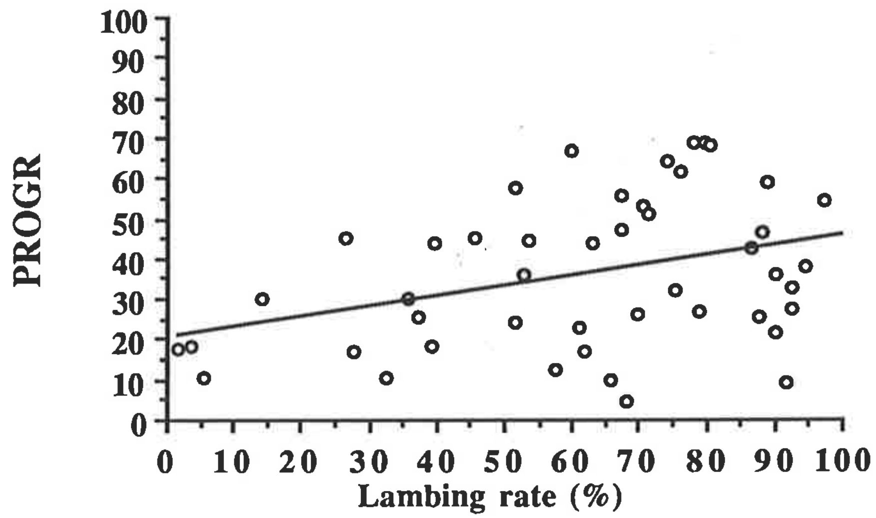
When the fresh semen characteristics were assessed by correlation analysis, PROGR, MOT, RAPID % and STATIC % (assessed by computerised image analysis) and Live, Dead, Normal and Abnormal tail sperm (assessed subjectively) were significantly correlated with both fertility parameters. All these semen characteristics showed a slightly better correlation with lambing rate than pregnancy at 70 days. However, both fertility parameters were highly correlated ($p < 0.001$), the pregnancy test at 70 days provided an accurate estimate of the lambing rate. The relationship between pregnancy at 70 days and lambing rate is shown in Figure 7.7.

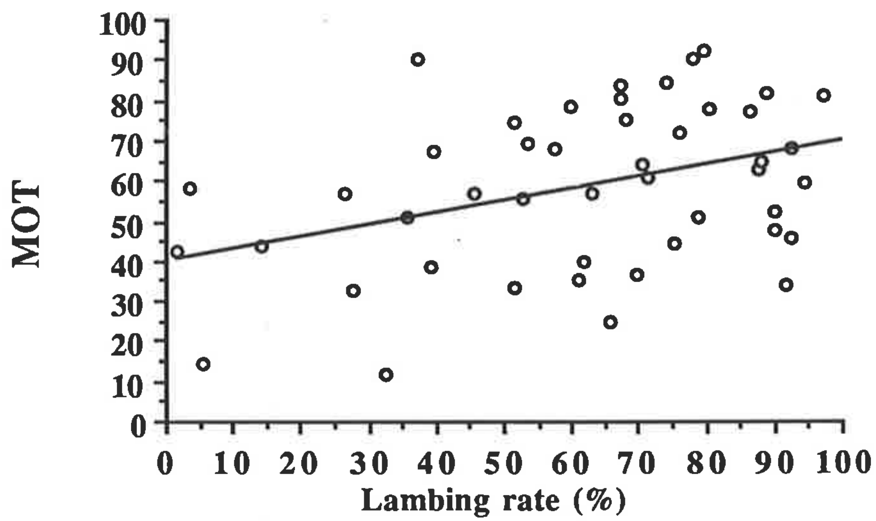
Figure 7.7. Relationship between pregnancy 70 days and lambing rate.

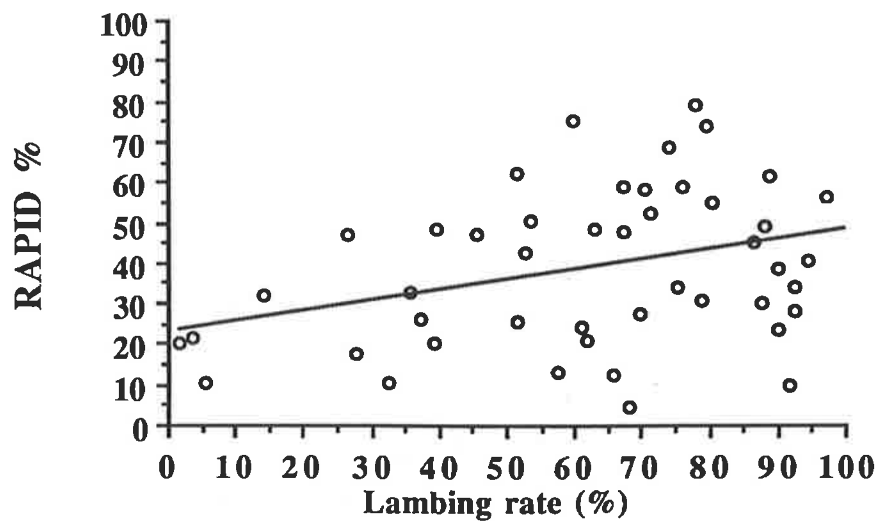


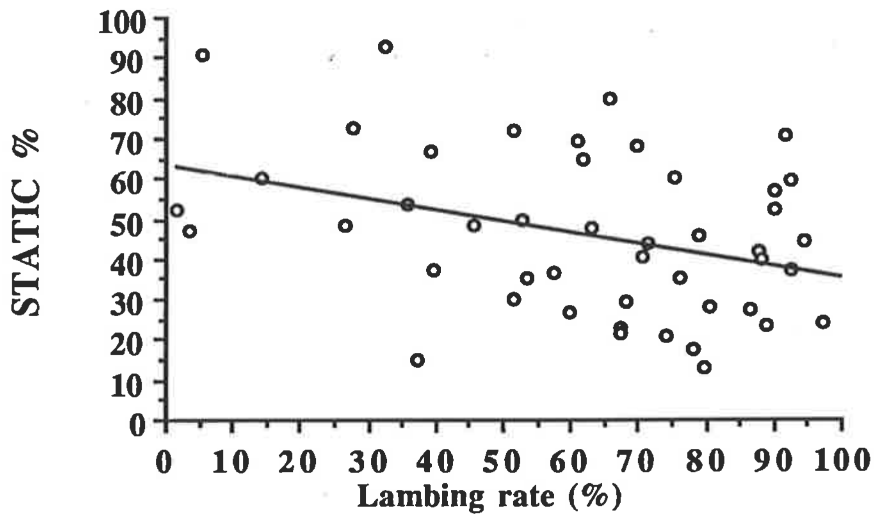
The relationships between the correlated semen characteristics measured both subjectively and objectively and lambing rate are presented in Figure 7.8.

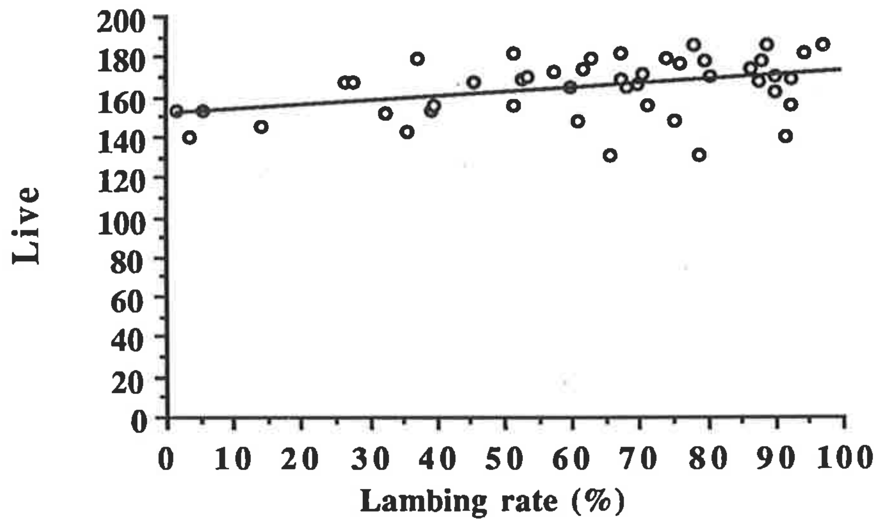
Figure 7.8. Relationships between the correlated semen characteristics measured both subjectively and objectively and lambing rate.

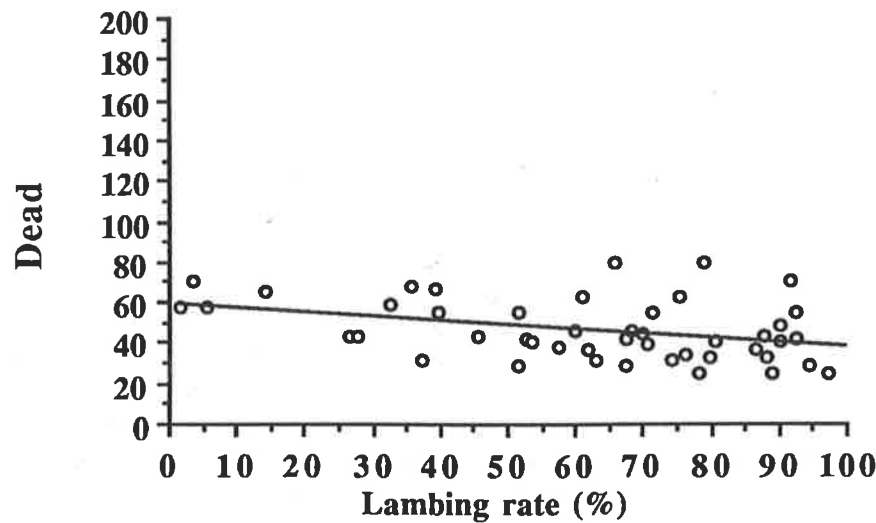


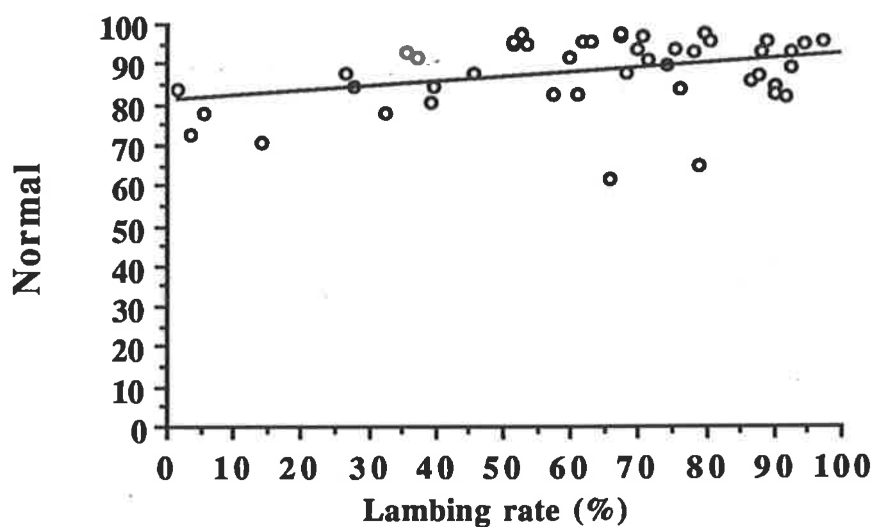


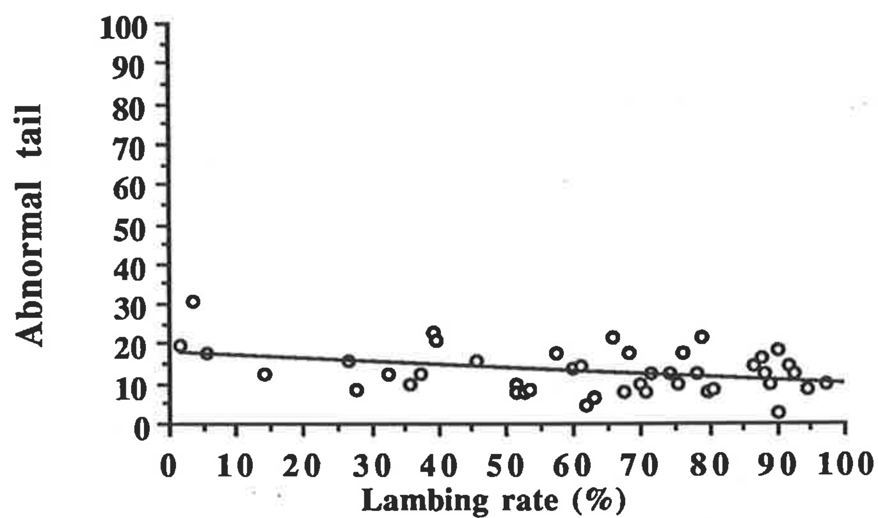



STATIC %
100
90
80
70
60
50
40
30
20
10
0
0 10 20 30 40 50 60 70 80 90 100
Lambing rate (%)


Live
200
180
160
140
120
100
80
60
40
20
0
0 10 20 30 40 50 60 70 80 90 100
Lambing rate (%)


Dead
200
180
160
140
120
100
80
60
40
20
0
0 10 20 30 40 50 60 70 80 90 100
Lambing rate (%)





The mean, standard deviation and the correlation coefficient (r) with both pregnancy at 70 days after mating and at lambing for the correlated fresh semen characteristics are presented in Table 7.12.

Table 7.12. Mean, standard deviation and correlation coefficient (r) with pregnancy at 70 days and at lambing for the correlated fresh semen characteristics.

Semen characteristic	Mean	Standard dev.	r pregnancy (%)	r lambing (%)
PROGR	33.99	17.96	0.37	0.36
MOT	55.73	20.03	0.39	0.37
RAPID (%)	35.78	19.33	0.35	0.34
STATIC (%)	42.97	20.62	-0.37	-0.35
Live	159.15	14.66	0.39	0.37
Dead	41.06	14.90	-0.40	-0.38
Normal	85.24	8.57	0.37	0.34
Abnormal tail	10.35	5.46	-0.36	-0.35
Pregnancy 70 days (%)	62.85	25.67		
Lambing(%)	60.46	25.83		

The confidence limits for the correlated semen characteristics assessed by the HTM (PROGR, MOT, RAPID % and STATIC %), and the minimum sample size to be assessed to predict differences of 5, 10 and 15 % of pregnancy 70 days after mating and at lambing are presented in Tables 7.13. and 7.14.

Table 7.13. The values of PROGR, MOT, RAPID %, STATIC % (X), the confidence limits for these characteristics (L), and the minimum sample size to be assessed (n) to predict differences of 5, 10 and 15 % in fertility as pregnancy 70 days after mating.

Correlated semen characteristic	Increment in fertility required (%)			
	0 X	5 X(L, n)	10 X(L, n)	15 X(L, n)
PROGR	33.99	43.43 (9.44, 5)	52.86 (18.87,1)	62.30 (28.31, 1)
MOT	55.73	65.74 (10.01, 6)	75.74 (20.01, 2)	85.74 (30.01, 1)
RAPID	35.79	46.65 (10.87, 4)	57.52 (21.24, 1)	68.39 (32.61, 1)
STATIC	42.97	32.08 (-10.89, 5)*	21.21 (-21.76, 2)*	10.34 (-32.63, 1)*

Table 7.14. The values of PROGR, MOT, RAPID %, STATIC % (X), the confidence limits for these characteristics (L), and the minimum sample size to be assessed (n) to predict differences of 5, 10 and 15 % in fertility at lambing.

Correlated semen characteristic	Increment in fertility required (%)			
	0 X	5 X(L, n)	10 X(L, n)	15 X(L, n)
PROGR	33.99	43.80 (9.81, 4)	53.61 (19.62, 1)	63.41 (29.42, 1)
MOT	55.73	66.36 (10.63, 5)	77.0 (21.27, 2)	87.64 (31.91, 1)
RAPID	35.79	46.89 (11.11, 4)	58.0 (22.22, 1)	69.11 (33.33, 1)
STATIC	42.97	31.61 (-11.36, 5)*	20.25 (-22.72, 1)*	8.88 (-34.09, 1)*

2. Analysis of regression: the estimation of the regression coefficients and the standard regression coefficients.

The regression coefficients and the standard regression coefficients for the fresh semen characteristics included in the best 9 variables regression model with pregnancy 70 days after mating and lambing rate are presented in Tables 7.15. and 7.16.

Table 7.15. The regression coefficients (b_n) and the standard regression coefficients (b'_n) estimated for fresh semen characteristics included in the regression model with pregnancy 70 days after mating.

Semen characteristic	PARAMETER ESTIMATED		
	Regression coefficient(b_n)	Standard regression coefficient (b_n)	Rank of importance in pregnancy 70 days after mating
MOTILE	-0.009	0.05	8
PROGR	0.810	0.37	4
MOT	-4.804	0.39	3
VSL	-0.646	0.04	9
RAPID %	5.218	0.42	1
MEDIUM %	4.851	0.09	7
SLOW %	20.545	0.10	6
Dead	0.137	-0.40	2
Abnormal tail	-2.392	-0.36	5

Table 7.16. The regression coefficients (b_n) and standard regression coefficients (b'_n) estimated for fresh semen characteristics included in the regression model with lambing rate.

Semen characteristic	**PARAMETER ESTIMATED**		
	Regression coefficient (b_n)	Standard regression coefficient (b'_n)	Rank of importance in lambing rate
MOTILE	-0.008	0.05	7
PROGR	0.871	0.35	3
MOT	-4.718	0.36	2
VSL	-0.670	0.05	7
RAPID %	5.071	0.34	4
MEDIUM %	4.684	0.07	6
SLOW %	21.422	0.11	5
Dead	0.130	-0.38	1
Abnormal tail	-2.340	-0.35	3

The regression equation for pregnancy 70 days after mating was:

$$Y = a + b1X1 + b2X2 + b3X3 + b4X4 + b5X5 + b6X6 + b7X7 + b8X8 + b9X9 \text{ thus,}$$

Y = 114.53 + (-0.009) (MOTILE) + 0.810 (PROGR) + (-4.808) (MOT) + (-0.646) (VSL) + 5.218 (RAPID %) + 4.851 (MEDIUM %) + 20.545 (SLOW %) + 0.137 (Dead) + (-2.392) (Abnormal tail)

using the mean values for these semen characteristics from the data set then

Y = 114.53 + (-0.009) (1379) + 0.810 (33.99) + (-4.808) (55.73) + (-0.646) (109.96) + 5.218 (35.79) + 4.851 (19.81) + 20.545 (0.40) + 0.137 (41.06) + (-2.392) (10.35) = 62.61

which is the pregnancy obtained 17 days after mating.

RAPID % had more importance for pregnancy 70 days after mating than the rest of the semen characteristics in this experiment.

The regression equation for lambing rate was:

Y = a + b1X1 + b2X2 + b3X3 + b4X4 + b5X5 + b6X6 + b7X7 + b8X8 + b9X9 thus,

Y = 115.17 + (-0.008) (MOTILE) + 0.871 (PROGR) + (-4.718) (MOT) + (-0.670) (VSL) + 5.071 (RAPID %) + 4.684 (MEDIUM %) + 21.422 (SLOW %) + 0.130 (Dead) + (-2.340) (Abnormal tail)

using the mean values for these semen characteristics from the data set then

Y = 115.17 + (-0.008) (1379) + 0.871 (33.99) + (-4.718) (55.73) + (-0.670) (109.96) + 5.071 (35.79) + 4.684 (19.81) + 21.422 (0.40) + 0.130 (41.06) + (-2.340) (10.35) = 61.11

which is very similar to the lambing rate obtained in this experiment.

The largest standard regression coefficient for lambing rate was with the proportion of dead spermatozoa.

7.5 Discussion.

When frozen-thawed semen characteristics from normal rams run under commercial conditions were assessed (experiment 1), progressive motility and motility were correlated with fertility, but not the progressive velocity and path velocity as reported for bull semen by Budworth et al. (1988). In the present study, the coefficients of correlation for ram semen were higher than has been reported for bulls (Budworth et al., 1988). The relationship between the semen characteristics themselves were not clear, and further research is required to give reliable information.

In experiment 3 in which a wide range of semen quality was obtained, the same semen characteristics as experiment 1 (PROGR and MOT), together with the percentage of medium, slow and static cells were correlated with fertility. However the coefficients of correlation were lower than in experiment 1, as the semen characteristics and fertility were affected either by rams, treatments or by environmental conditions.

When the data on fresh semen characteristics from normal and subfertile rams assessed manually or by computerised image analysis (experiment 2) were correlated with fertility, the number of observations was low (n=9) and semen was severely affected by some treatments. Therefore the quality of semen varied over a large range of values, and the correlation coefficients were very high, but not ~~reliable.~~ very precise

Most of the semen characteristics correlated with fertility in this experiment were also reported previously by Hulet et al. (1965) and some of them by Linford et al. (1976). In both these cases, the coefficient of correlation and the significance were smaller than the present study, and Hulet et al.(1965) reported mass motility was negatively correlated with fertility. Wiggins et al. (1953) obtained much lower estimations of correlation between proportion of normal and live sperm and fertility as extrapolated from the breeding records of rams. Percentage of motile sperm estimated subjectively by microscope was also found to be correlated with fertility by O'Connor et al. (1981) for fresh semen. The differences in correlation coefficients obtained in different studies are probably due to the different ways used to define fertility. As mentioned by O'Connor et al. (1981), some authors have measured ewe fertility at lambing, return to oestrus or as in the present experiments ewes pregnant at 17 and 65 days after mating.

The number of observations and accuracy of the estimations and the range of semen quality also varies between studies (Foote et al., 1977; O'Connor et al., 1981; Saacke, 1982). When low values of motility and percentage of live sperm were obtained in the present study, the fertility was also low, confirming that such semen characteristics are related to fertility. It was also found that the percentage of live cells was correlated with percentage of normal cells (Table 7.5), suggesting that the latter is also correlated with fertility. Percentage of headless cells was negatively correlated with fertility (Table 7.5), which would be expected. Headless, bent and coiled tail sperm would be unlikely to fertilize an egg.

Semen characteristics generally correlated better with pregnancy at 65 days than at 17 days after mating. In some of the experiments (experiment 2 and 3) early pregnancy rate (17 days) was larger than the later pregnancy rate (65 days) because pregnancy rate at 17 days after

mating or insemination did not include all embryo mortality. In some of the experiments in this study (experiment 8 and 9) semen quality did not affect the fertilization of ova (as assessed by pregnancy at 17 days), but the viability of embryos was affected (as assessed by pregnancy 65 days). Furthermore, in extreme situations such as in experiment 3, pregnancy at 17 days was not well correlated with pregnancy at 65 days (Table 7.7 and Figure 7.5), which was attributed to a high level of embryo mortality. In experiment 3, semen characteristics were correlated with pregnancy at 65 days but not 17 days.

When fresh semen characteristics in experiment 2 were assessed by computerised image analysis, concentration of spermatozoa, percentage of motile spermatozoa and progressively motile spermatozoa, and the proportion of rapid and medium sperm were positively correlated with fertility, and as expected the percentage of static sperm was negatively correlated with fertility. There have been no other studies on the relationship between these objectively assessed semen characteristics and fertility in sheep. The use of the data in the present study as a reference point needs to be considered in the context of the limited number of observations and the fact that normal conditions would probably not offer so much variation in semen quality. In this case the coefficients of correlation may be much lower and such semen characteristics will not be so strongly related to fertility. Nevertheless, it is encouraging that these semen characteristics which are normally assessed in semen for commercial purposes are correlated with fertility and may be used for fertility prediction in artificial breeding programs or for sire selection.

In experiment 4, fresh semen characteristics assessed manually were also correlated with fertility and corresponded to those previously reported (Wiggins et al., 1953; Hulet et al., 1965; Linford et al., 1976; O'Connor et al., 1981). In this experiment, the number of observations were high (n = 46) and the results much more reliable than in experiment 2. Furthermore, in this case the pregnancy at 70 days after mating and at lambing were almost perfectly correlated (Figure 7.7), thus the semen characteristics were as well correlated with either of the fertility parameters. A slight difference was observed in the correlation coefficients and their significance between pregnancy at 70 days and at lambing, but this was probably because some lambs and ewes died or were lost. In a practical situation, probably it would be

preferable to consider the values at lambing because it is more realistic and closer to the reality of the field. As for the semen characteristics assessed manually, semen characteristics objectively assessed were also correlated with fertility. Only progressively motile spermatozoa, motile spermatozoa , percentage of rapid spermatozoa and percentage of static spermatozoa (negatively) were correlated with fertility. As mentioned before, with semen in normal conditions, less semen characteristics were correlated with fertility and the correlation coefficients were much lower than in experiment 2. This is important because although less semen characteristics were correlated, the information is much more reliable. It was also observed that the correlated semen characteristics measured manually were more strongly related to fertility than those objectively assessed (Table 7.11, Figure 7.8). However, the accuracy and repeatability of the measurements by objective analysis studied were confirmed to be reliable, whereas in the manual assessment reliability was not studied. .

In experiments 1 and 3 changes in progressive motility had more effect on fertility than the rest of the semen characteristics. In experiment 1 progressive velocity was also important, and in experiment 3 percentage of motile spermatozoa, percentage of slow sperm and mean lateral head displacement were important. In experiment 4, nine semen characteristics were included in the regression model, and percentage of rapid spermatozoa and number of dead spermatozoa (in 200 cells) were the most important semen characteristics affecting pregnancy at 70 days and at lambing respectively.

It seems that although a number of semen characteristics may be correlated with fertility, not all of them had an important impact on fertility when they are included in the regression analysis. This may be because the influence of some semen characteristics on fertility depends on the levels of other semen characteristics. It is probably important to select the semen characteristics which even in presence of others still have a significant effect on fertility and make the prediction of fertility more reliable. Although the regression equations formulated in experiment 4 are more reliable than those estimated for experiments 1 and 3, there is still not enough information to generalize them to all situations.

The results of experiment 4 reliably showed that motility characteristics assessed objectively are better correlated with fertility than velocity or directional semen characteristics. However, for fertility prediction concentration, motility and velocity characteristics were considered. Although most of the manually assessed semen characteristics related to the sperm morphology were correlated with fertility, only number of dead sperm and number of sperm with abnormal tails were considered for fertility prediction. This was not surprising, since both these semen characteristics are related to the number of spermatozoa able to fertilize and their capacity of motility. Thus, semen characteristics from either of the methods of assessment are useful for the fertility prediction. From these results, much more research with the kind of analysis presented in this study is required in order to achieve the goal of general fertility prediction in rams.

The limits of confidence estimated (experiment 1) within which 5, 10 or 15 % differences in fertility are predicted were relatively low, as were the sample sizes required to predict such differences in fertility. It seems that in normal conditions it may be possible to improve fertility with a small increase in some of the post-thawing semen characteristics as in experiment 3, chapter 3, or by selecting rams with higher values for such semen characteristics (good freezability). Although the fertility in experiments 1 and 4 was similar, confidence limits presented in Table 7.14 (experiment 4) for fresh semen were larger than those presented in Table 7.3. (experiment 1) for semen frozen in straws, and the sample size was much lower for fresh semen than for frozen-thawed semen. This was because the estimations of fresh semen characteristics by HTM are more accurate and reliable than those for straw-frozen-thawed semen. On the other hand, as the semen in experiment 1 was collected from a small number of pre-selected rams, higher values of semen characteristics were observed and therefore fertility may be improved with a small increase in those values. In experiment 4, a wide range of semen quality was observed from a larger population of unselected rams. Thus values for semen characteristics were low, and therefore large increases in those semen characteristics were required to improve fertility.

CHAPTER 8. GENERAL DISCUSSION.

Since the development of methods for frozen preservation of ram semen (pellets, by Salamon, 1967 and straws, by Colas, 1975), further achievements have been hampered by the lack of an objective method for semen assessment (Evans, 1988). With the advent of more objective semen assessment methods such as computerised image analysis, the preservation of ram semen may be improved through a better understanding of the changes which occur in the spermatozoa during the freeze-thawing process. Some of the studies undertaken in this project examined aspects of ram semen processing with the aid of computerised image analysis. It was observed that semen can be frozen successfully in either of the types of straws (0.25 ml or 0.5 ml) and that pre-freezing concentration of spermatozoa has no effect on their survival. It was also found that one-step dilution of semen for freezing in straws is an easier method than the more complex two-step process reported by Colas (1975).

From several experiments it was demonstrated that a major contributor to variability in the results of semen preservation was due to rams. Semen characteristics vary considerably between rams and even between samples within ejaculates which was earlier reported by Eppleston et al. (1986). Individual rams as a source of variation also interacted with type of diluent and even type of straw. Rams are a difficult source of variation to control, and important to consider for further research.

Through the assessment of many frozen-thawed samples, a significant amount of variation between samples within ram was also found. This variation may have been due to sampling, the protocol for semen assessment, or the processing and freezing itself. On the latter point, it was suggested that during processing, semen settles down very quickly and often does not mix properly with all the components of the diluent which are required to preserve and provide cryoprotection to the spermatozoa. Thus by mixing semen at 5°C just before drawing into straws for freezing, the post-thawing characteristics and the viability of ram spermatozoa were improved (Chapter 3, experiment 3.3). Unfortunately it was not

possible to determine whether the improvement in post-thawing semen characteristics due to pre-freezing mixing of semen was associated with a reduction in variability between the straws, or if it may also have resulted in improved fertility.

Maxwell et al. (1984), Maxwell and Hewitt (1986) and Maxwell (1986a,b) among others, have shown that intrauterine insemination is more efficient than cervical insemination in sheep, especially when frozen-thawed semen is used. Our results comparing the intrauterine and cervical insemination with frozen-thawed semen were similar to those reported previously. We concluded that intrauterine insemination with frozen semen gave much higher conception rates than cervical insemination even when a low number of motile spermatozoa were inseminated.

In some of the experiments conducted in this project (Chapter 3, experiments 4 and 6 and chapter 5, experiment 8), semen was frozen in pellet form using the tris-glucose-egg yolk-glycerol diluent (Diluent A) of Salamon and Visser (1972), and its variants reported by Salamon (1976) and Evans and Maxwell (1987). In experiment 4 the ratio of tris to glucose was varied in this diluent in an attempt to improve the cryoprotection of spermatozoa. However, neither increasing the amount of sugar nor decreasing the amount of tris, had any effect on post-thawing characteristics of spermatozoa. An increase in the concentration of sugar used in the diluent (glucose) may not have increased the protection of spermatozoa because of its poor extracellular protection (Salamon, 1968). We concluded that the best media for protection of semen during pelleting are those which contain between 250 and 300 mM of tris and between 30 and 95 mM of glucose, and that tris:glucose levels can be varied as long as osmolarity is maintained within an optimum range.

The viability and fertility of spermatozoa frozen-thawed in either pellets or straws were similar to previous reports (Salamon and Visser, 1972; Maxwell, et al., 1984; Hunton, 1987; Eppleston et al., 1986; Maxwell, 1986; Maxwell and Hewitt, 1986; Hunton et al., 1987). The freezing of ram semen in straws provided the advantages of easier handling, positive identification and more accurate delivery of the amount of spermatozoa required per insemination (Hunton et al., 1987).

The use of computerised image analysis of semen has been used extensively in species other than sheep. There are reports on the precision of the commercially available systems: Cellsoft (Knuth et al., 1987; Ginsburg and Abel (1987); Budworth et al., 1988; Vantman et al., 1988; Blach et al., 1989) and Hamilton Thorn Motility Analyzer (HTM) (Gill et al., 1988; Pedigo et al., 1989). However, little is known about either the fresh and frozen-thawed ram semen characteristics measured by these systems, or the variability in estimations of these semen characteristics between rams and between samples within ram. Therefore, experiments 5 and 6 (Chapter 3) were undertaken to estimate such variations and the repeatability of estimations of ram semen characteristics using computerised image analysis.

In experiment 5(chapter 3), semen was frozen in straws from 4 rams with a large number of straws per ram. The variation observed in semen characteristics between rams was very small while the variation between straws within ram was very large. This variation was attributed to the sampling of this particular population of rams, the semen assessment protocol, and mainly to the effects of processing and freezing of the semen. The latter appears to have a major effect on the viability of the spermatozoa between individual straws, hence a low repeatability between straws was found for most semen characteristics (Table 3.13). We suggested that with a large variation between samples, there was also expected to be a wide range of semen quality throughout the batch or ejaculate, and thus a large variation in fertility. Thus, we also suggested that in general terms, when the repeatability of the estimation is low, a larger number of samples is required to have more reliable information about the ejaculated semen.

Following the aims of experiment 5(chapter 3) a second experiment (experiment 6) was conducted to study the variation in the estimation of semen characteristics. A larger number of rams was involved in this experiment although lower numbers of samples were measured per ram. Semen was analyzed from 65 rams in fresh and 63 rams in frozen-thawed samples and the variance components and repeatabilities of the semen characteristics were estimated. The results obtained were different for fresh semen compared with frozen-thawed semen. In fresh semen most of the variation in semen characteristics was between rams and to a lesser extent

between samples within ram. Conversely, in frozen-thawed semen most of the variation in semen characteristics was between samples within ram. This supported in part the findings of experiment 1 chapter 3 with semen frozen in straws, in which most of the variation was between samples within ram. Thus sampling, processing, freezing and assessing semen may contribute to variation between samples. The variation between rams in fresh semen was attributed to the sampling of unselected rams from a large population which produced a wide range of semen quality inherent to the rams.

For frozen-thawed semen, it was observed that the semen characteristics related to the velocity and direction of spermatozoa had the higher variation between pellets, hence the lower repeatability. Such semen characteristics may be more susceptible to processing and freezing than others, and because they are more variable a larger number of samples need to be assessed to increase the accuracy of their estimation. Despite the variation between samples in frozen-thawed semen, the repeatability was much higher than in experiment 5 (chapter 3). These findings suggested that the estimation of semen characteristics using computerised image analysis is reliable, and that the estimates in fresh semen are more repeatable than in frozen-thawed semen. Thus a lower number of samples is probably required for analysis of fresh than frozen-thawed semen characteristics. The variation between rams is difficult to control while the variation between samples could be reduced by further research.

Measuring variation in semen characteristics is not always an indicator of variation in fertility. This thesis studied the influence of male reproduction on female fertility. The main way in which the male can affect female reproductive performance is through the semen. Therefore, some factors were examined which might affect this aspect of male reproduction: scrotal temperature and the degree of association between the epididymis and testis.

Increased temperature applied to the scrotum reduces the production of spermatozoa and increases the number of immature spermatozoa due to a more rapid passage of the spermatozoa through the epididymis (Bedford, 1978). The epididymal environment can also be altered so that the normal maturation and storage of spermatozoa is disrupted. Such effects have been reported to produce a failure of fertilization in sheep soon after mating (Dutt and

Simpson, 1957; Fowler and Dun, 1966; Howarth, 1969), or a good fertilization rate but later embryo mortality (Bellvé, 1972; Burfening et al., 1970).

The effect of scrotal temperature on the fertilizing capacity and fertility of ejaculated spermatozoa was studied in experiment 8 (Chapter 5). Temperature was applied directly to the scrotum, so that any side effects related to the heating of the whole body were avoided. An increase in subcutaneous scrotal temperature of 2°C for 16 hours per day did not induce modification in the fertilizing capacity of the spermatozoa after 4, 15 or 21 days of treatment. These results were contrary to the findings of Dutt and Simpson (1957), Fowler and Dun (1966), Rathore (1968, 1970), Howarth (1969) and Braden and Mattner (1970) who found that increased scrotal temperature reduced the fertilizing capacity of ram spermatozoa. However, our finding agreed with those of Bellvé (1972) and Burfening et al. (1970) working with mice and Setchell et al.(1988) with rats. The different findings were probably due to differences in the heating procedure, the modification of the whole body temperature by some workers, and because some authors treated animals with much higher temperatures (Moule and Waites, 1963; Waites and Setchell, 1964; Braden and Mattner, 1970; Smith, 1971; Setchell et al., 1988) and during a longer exposure (Dutt and Simpson, 1957; Smith, 1971; Wettemann and Bazer, 1985). In our study, a difference in the pregnancy rate detected at 17 days and 65 days after insemination was observed in ewes inseminated with semen from rams with elevated scrotal temperature. When embryo loss was calculated as the difference between these two pregnancy rates, it was found that as little as 4 days of heating the scrotum increased embryo loss. This reduction in embryo survival occured without any modification to the fertilization rate for up to 21 days of heating. When scrotal heating was applied for more than 21 days, the quality and to a lesser extent the quantity of the spermatozoa in the ejaculates of rams were beginning to decline. This suggests that the earliest effect of scrotal heating is on the epididymal spermatozoa, as the spermatozoa take 14 days to pass through the epididymis (Ortavant, 1959). We concluded that the sequence of heat effects on spermatogenesis could be firstly a reduction in the capacity of the spermatozoa to produce a viable embryo, secondly a decrease in the capacity of the spermatozoa to fertilize the ovum, and finally a decrease in the number of spermatozoa produced.

The lymphatic connections between the testis and epididymis may have an important role in the maturation, acquisition of fertilizing capacity and storage of spermatozoa. This is mediated by some substances which are exchanged between the testis and epididymis during the passage of the spermatozoa through the epididymis (Jones, 1984). Thus the epididymis has an influence on the fertilizing capacity of spermatozoa and beyond, on the viability of the embryo.

In experiment 7 chapter 4, the effect of the separation of the epididymis from the testis on semen characteristics and fertility was studied. The number of dead cells and motility were decreased, therefore it was assumed that the capacity of maturation and storage of the epididymides was reduced as reported by Jones (1984). The movement of substances from the testes to the epididymides may influence the functions of the epididymides. Thus removal of the connections between these two organs may reduce semen maturation, fertilizing capacity of spermatozoa and storage capacity of the epididymis. When rams with separated epididymides were mated to normal ewes, the fertilization rate and the early pregnancy rate (17 days after mating) was lower than in rams with their epididymides attached to the testes. The embryo mortality was also increased judged by the difference in pregnancy rates at 17 days and 65 days after mating. Thus the viability of the embryo may be affected by the quality of semen produced when the epididymides are separated from the testes. When eggs are fertilized by immature spermatozoa due to a failure of the epididymides to produce normal spermatozoa, embryo mortality is also increased (Fournier-Delpech et al., 1981; Amann, 1987).

There is another way in which normal rams can affect female fertility by increasing embryo mortality due to a differential ability to produce viable embryos. This so called "male effect" has been reported in the literature as an effect of strain of ram or genotype on semen characteristics reflected in differences in the fertility, fecundity and embryo survival in ewes mated to the rams (Burfening et al., 1977; Moore, 1981; Jefferies et al., 1988). Jefferies et al. (1988) reported no differences in fertility between strains of rams. However the authors reported an increase in the numbers of foetuses in pregnant ewes and lambs at marking produced by one strain of rams. Burfening et al. (1977) and Moore (1981) concluded that

certain strains of rams are able to fertilize more eggs and to produce more viable embryos than others. In the present study two strains of rams had spermatozoa of similar fertilizing capacity (measured by pregnancy rate 17 days after mating), but the numbers of foetuses present 65 days after mating and the number of lambs at marking were different (experiment 9). Thus it was concluded that the differences in embryo loss must be inherent to the strains of rams. It was also observed that the environmental conditions, the intensity of sheep handling and the method of insemination also contributed to the level of embryo mortality. Thus it is important to avoid all sources of stress which may cause low fertility in sheep (Reid and Millis, 1962).

We concluded that in either normal or subfertile conditions, males can affect the production of viable offspring. This may be a result of increased numbers of abnormal spermatozoa which cause embryo mortality, or by genetic factors inherent to the rams. Such studies may help the understanding of conditions in which unexpected low fertility may occur without a clear reason. The application of these findings in different species, particularly in man, may be useful for the study of infertility. Some simple environmental effects on human males (e.g. hot baths, type of clothes) may interfere with the normal function of the testis or epididymis, thus reducing fertility. On the other hand, such experimental modifications might be used to establish new contraception methods without disturbing other body functions.

To further understand the usefulness of subjective and objective assessment of semen, the relationship between the quality of semen and fertility was examined. For this purpose, several statistical procedures were applied. First, the correlation with fertility of fresh and frozen-thawed semen characteristics assessed subjectively or by computerised image analysis was estimated. Second, the confidence limits were estimated within which increases in the value of semen characteristics would predict increases in fertility, and the minimum sample size required to predict such differences in fertility was determined. Third, a regression analysis was performed with all the semen characteristics measured, and used to formulate equations for fertility prediction in experiments with reasonable numbers of observations. Finally the standard regression coefficients were estimated and used to determine which of the semen characteristics had the greatest impact on fertility.

The semen characteristics of several species have been measured both subjectively (manual assessment) and objectively using computerised image analysis. Several authors have reported that percentage of motile spermatozoa (motility) is positively correlated with fertility (Hulet et al., 1965; Linford et al., 1976; Budworth et al., 1988), and the correlation was better following semen incubation for 1 to 3 hours (Pickett et al., 1961; Saacke and White, 1972). The variability between reports made the correlations unclear, and normally the predictive value has been poor. Morphology of spermatozoa and the ratio of live to dead cells have also been correlated with fertility (McKenzie and Phillips, 1934; Gunn et al., 1942; Carroll et al., 1963; Cupps et al., 1952; Mercier and Salisbury, 1946; Wiggins et al., 1953; Bishop et al., 1954; Hulet et al., 1965; Linford et al., 1976). It was not until 1987-1988 that Budworth and collaborators, in a series of experiments with bull semen, successfully correlated many of the semen characteristics as measured by computerised image analysis with fertility.

In experiment 7 chapter 4 of the present study, with fresh semen from a population of rams with their epididymides separated from their testes, most of the semen characteristics assessed manually were highly correlated with fertility. This was surprising as the number of observations was low (n=9), but may have been due to the large between-ram variation in semen quality observed in this particular population of rams. Despite this situation, the semen characteristics which were correlated with fertility were similar to those reported by Hulet et al. (1965), Linford et al. (1976), and to a lesser extent by Wiggins et al. (1953) and O'Connor et al. (1981). These semen characteristics were mass motility or score of the wave motion, percentage of motile spermatozoa, percentage of live sperm, percentage of normal sperm and the percentage of cells without heads (negatively correlated). The differences in the values of the correlation coefficients reported here and by other authors were probably due to factors such as the number of observations used for the estimation of the correlation, the range in semen quality in the particular population of males and the size of the population, the way in which fertility was determined, and the accuracy of estimation of semen characteristics or the data from fertility as reported by Foote et al. (1977), O'Connor et al. (1981) and Saacke (1982).

The following fresh semen characteristics measured in experiment 7 chapter 4 were correlated with fertility: total concentration of spermatozoa per ml, percentage of progressively motile sperm, percentage of motile sperm, percentage of rapidly motile sperm, percentage of spermatozoa with medium motility and percentage of static spermatozoa (negatively correlated). This represents the first data available on ram semen characteristics assessed by computerised image analysis.

When frozen-thawed semen characteristics from normal rams under commercial conditions were measured (experiment 2, chapter 3), only percentage of progressively motile sperm and percentage of motile sperm were significantly correlated with fertility, which agrees with the findings of Budworth et al. (1988) in bull semen. The same authors also reported that progressive velocity, path velocity, linearity, and other semen characteristics were correlated with fertility. These characteristics did not influence fertility in experiment 2.

In experiment 8 chapter 5 of the present study, a wide range of semen quality was observed from normal and subfertile rams. In this case, the values of the semen characteristics measured were low, and the fertility obtained in this experiment for both groups of rams (normal and subfertile) was also low. In this experiment fertility at 17 days was not correlated with fertility at 65 days, probably because there was significant embryo mortality which occurred between these two assessments of pregnancy. Therefore, the correlations were estimated between the semen characteristics and pregnancy 65 days after AI. The percentages of motile and progressively motile spermatozoa were correlated with fertility as in experiment 2, but in addition the percentages of rapid, medium, slow and static cells were also correlated with fertility.

Two regression analyses were performed using the data from experiment 2 chapter 3 and experiment 8 chapter 5 to obtain fertility prediction equations. In experiment 2, the prediction equation was formulated upon two semen characteristics: percentage of motile spermatozoa and progressive velocity. In experiment 8, the prediction equation was based on four semen characteristics: percentage of motile sperm, percentage of progressively motile sperm, percentage of slow cells and mean lateral head displacement. These equations have to

be estimated for each particular case until enough information is available from normal and abnormal situations in which a wide range of semen quality and fertilities are generated.

The impact of semen characteristics on fertility as indicated by the standard regression coefficients was estimated in each particular case. In experiments 2 chapter 3 and experiment 7 chapter 5, changes in progressive motility had more impact on fertility than the rest of the semen characteristics. In experiment 2 chapter 3 progressive velocity had second importance, and in experiment 7 chapter 5 percentage of motile sperm, percentage of slow cells and mean lateral head displacement had the second, third and fourth rank of importance on fertility. It was observed that although some semen characteristics were correlated with fertility, when all semen characteristics were included in the regression model, their importance or prediction value was reduced.

When the limits of confidence were estimated, it was observed that when semen came from normal fertile rams with high values for semen characteristics and fertility, the limits of confidence estimated in which differences of 5, 10 and 15 % fertility could be predicted and the number of samples required to enable such predictions were relatively small. Thus, we suggested that in normal conditions it is possible to improve fertility with a slight increase in some of the semen characteristics, particularly the ones with more impact on fertility.

In experiment 4 chapter 7, fresh semen characteristics measured both subjectively and objectively were correlated with fertility at 70 days after mating and at lambing. It was observed that both fertility parameters were highly correlated and many of the semen characteristics were also correlated between each other. The correlations coefficients were slightly higher with fertility at 70 days than at lambing, probably because there were a few losses of lambs and ewes. In this experiment, nine semen characteristics were correlated with fertility: percentage of progressively motile sperm, percentage of motile sperm, percentage of rapid sperm, percentage of static sperm (negatively correlated), proportion of live sperm, proportion of normal sperm, proportion of dead sperm and sperm with abnormal tails (negatively correlated). In general the correlation coefficients were lower than in experiment 2 chapter 7 because this experiment was performed with a large number of unselected normal

rams, and the number of observations was much larger, and there was a limited range of semen quality. The objective assessments of semen characteristics were more accurate than the subjective assessments. However, the subjectively measured semen characteristics had higher correlation coefficients and closer relationships with fertility (experiment 4 chapter 7). In the latter experiment, nine semen characteristics were included in the regression model compared with two in experiment 1 chapter 7 and four in experiment 3 chapter 7.

Despite significant correlations between semen characteristics and fertility, not all were useful for fertility prediction. Furthermore, when the standard regression coefficients were estimated (experiments 1, 3 and 4 chapter 7), different semen characteristics had the greatest impact on fertility: progressive motility for experiments 1 and 3 and percentages of rapid and dead sperm for experiment 4.

The confidence limits to predict differences in fertility for fresh semen characteristics assessed with the HTM in experiment 4 chapter 7, were larger than those estimated for experiment 1 chapter 7, but the minimum sample sizes required for such predictions were much smaller. These results suggest that the estimations of fresh semen characteristics are more accurate than the estimations in straw-frozen semen. Thus lower numbers of fresh samples are required; first to obtain reliable information about semen quality, and second to enable prediction of increments in fertility.

Despite the accuracy of estimation of semen characteristics and reasonable numbers of observations of these characteristics in the last experiment of this study, there was still not enough information about the relationship between semen characteristics and fertility to generalize a fertility prediction equation for ram semen. Further reseach in required to generate data on the relationship between semen characteristics and fertility which may be analyzed as in the present study.

CHAPTER 9. BIBLIOGRAPHY

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APPENDICES

Introduction

Four Appendices are included in this thesis. These Appendices contain supplementary information from three of the chapters (3, 5 and 7).

Appendix A, contains Tables 5.1, 5.2 and 5.3 from chapter 5. Tables 5.1 and 5.2 show the semen characteristics determined by computerised image analysis of fresh and frozen-thawed semen from individual rams within group of treatment at days -49, 4, 15 and 21 of treatment. Table 5.3 shows the semen characteristics assessed manually per ram, per group at days -49, 4, 15 and 21 of treatment.

Appendix B, contains Table 5.4 and from chapter 5. This Table shows the results of fertility and embryo loss per ram per day by group.

Appendix C, contains Table 7.9 from experiment 4 chapter 7. This Table shows the mean values of the different fresh semen characteristics assessed by computerised image analysis per sire.

Appendix D, contains Plate 8, which shows the insulating bag used for the heat treatment in experiment 8, chapter 5.

A P P E N D I X A

Table 5.1. Fresh semen characteristics as measured by the HTM of individual ram per group by day during treatment.

Control.		Days	Total	Motile	Progr	Mot%	Mvsl	Mlin %	Malh	Mlin	Mvap	Rap.%	Med. %	Slow %	Stat. %
20		-49	3594.5	2900.1	62.1	80.7	85.9	77.9	6.8	68.7	104.7	44.3	36.4	2.3	17
21			6535.5	4411.5	55	67.5	76.2	81.1	6.3	70.7	89.7	28.7	38.7	3.8	28.7
2			5105.9	1748.3	29.4	34.2	77.4	84.2	5.8	73.1	87.5	15	19.2	9.4	56.3
10			837.6	588.7	67.9	70.3	91.8	96	4.4	92.7	94.5	27.7	42.6	2.8	26.9
	Mean		4018	2412	54	63	83	85	6	76	94	29	34	5	32
	Stdev		2437	1633	17	20	7	8	1	11	8	12	10	3	17
	SEM		1218	817	8	10	4	4	1	6	4	6	5	2	8
20		4	4438.7	3104	62.3	69.9	80	88.3	5.4	81.4	87.5	28.2	41.7	3.4	26.7
21			5405.4	1484	24.4	27.5	73.5	85.7	5.2	76.9	79.8	12.6	14.9	10.3	62.2
2			4280.8	3186	64.7	74.4	76.3	84.4	6	74.7	87.1	30.2	44.3	3.1	22.5
10			4666.8	2584	51.4	55.4	76.3	88.5	5.2	78.7	82.8	24.9	30.4	8.1	36.5
	Mean		4698	2590	51	57	77	87	5	78	84	24	33	6	37
	Stdev		498	784	18	21	3	2	0	3	4	8	13	4	18
	SEM		249	392	9	11	1	1	0	1	2	4	7	2	9
20		15	4220	3172	64.2	75.2	85.1	85.7	6	77.7	96.3	37.1	38.1	0.6	24.2
21			3907.7	2873	62.4	73.5	77	84.6	5.9	74	87	32.1	41.5	3.5	23
10			4717.8	3880	68.8	82.3	81.9	81.7	6.8	71.6	96.9	41.6	40.7	3	14.7
	Mean		4282	3308	65	77	81	84	6	74	93	37	40	2	21
	Stdev		409	517	3	5	4	2	0	3	6	5	2	2	5
	SEM		236	299	2	3	2	1	0	2	3	3	1	1	3
20		21	5769.7	4218	64	73.1	79	85	6	76	89.9	27.7	45.4	1.3	25.6
21			3819.2	2910	64.7	76.2	98.5	84.8	6.9	76.6	111.4	51.5	24.7	1.6	22.2
10			3753.4	2791	62.9	74.4	84.2	84.3	6.5	74	95.7	37	37.4	1.6	24.1
	Mean		4447	3306	64	75	87	85	6	76	99	39	36	2	24
	Stdev		1146	792	1	2	10	0	0	1	11	12	10	0	2
	SEM		661	457	1	1	6	0	0	1	6	7	6	0	1

Insulated		Day	Total	Motile	Progr	Mot.	Mvsl	Mlin %	Malh	Mlin	Mvap	Rap.%	Med. %	Slow %	Stat. %
28		-49	4932.3	3084	48.4	62.5	51.4	77.3	6.4	61.2	64.7	8.9	53.6	3.7	33.7
5			7167.7	4186.8	43.6	58.4	65.1	75.4	6.9	61	81.8	22.5	35.9	2.8	38.7
26			5534.8	5105.9	75.3	92.3	86	81	7	70.5	101.3	50.6	41.7	23	4.8
27			4105.1	3553.7	67.2	86.6	78.8	80.2	6.7	67.4	92	44.3	42.3	5	8.5
	Mean		5435	3983	59	75	70	78	7	65	85	32	43	9	21
	Stdev		1295	875	15	17	15	3	0	5	16	19	7	10	17
	SEM		648	437	8	8	8	1	0	2	8	10	4	5	9
5		4	6208.8	1430	20.2	23	38.8	82.1	4.2	68.5	45.3	2.6	20.4	16.7	60.3
26			4302.6	3077	60.1	71.5	79.7	83.7	6.1	74.2	92.4	30.4	41.1	0.9	27.5
27			3774.3	1274	29	33.8	53.3	81.7	5.2	65.9	60.2	8	25.8	10.2	56.1
	Mean		4762	1927	36	43	57	83	5	70	66	14	29	9	48
	Stdev		1281	999	21	25	21	1	1	4	24	15	11	8	18
	SEM		739	577	12	15	12	1	1	2	14	9	6	5	10
28		15	1908.9	46.3	2.4	2.4	49.7	89.4	4.1	77.3	53.7	0.4	2	1.7	95.9
5			6065.8	4044	53.2	66.7	73.3	78.8	6.5	65.8	86.7	31.6	35	2.4	31
26			6188.3	3064	40.9	49.5	45.8	80.1	4.4	66.1	53.5	8.6	40.9	21.8	28.7
27			4125.6	2886	59.4	70	72.3	82.9	5.6	72.1	83.4	30.7	39.3	5	25.1
	Mean		4572	2510	39	47	60	83	5	70	69	18	29	8	45
	Stdev		2011	1720	26	31	15	5	1	5	18	16	18	9	34
	SEM		1006	860	13	16	7	2	1	3	9	8	9	5	17
28		21	4207.3	1457	28.8	34.6	42.5	82	3.9	69.6	49	4.2	30.4	17.5	47.9
5			4103.3	1155	25.1	28.1	76.6	86.9	5.1	76.7	82.3	10.9	17.3	12.4	59.5
26			3013.3	289.1	8	9.6	71.1	84.3	4.9	74.3	77.4	3.5	6	7.5	82.9
27			3821.5	1039	23.5	27.2	56	83	4.6	70.8	62.7	7.2	20	12	60.8
	Mean		3786	985	21	25	62	84	5	73	68	6	18	12	63
	Stdev		541	496	9	11	15	2	1	3	15	3	10	4	15
	SEM		270	248	5	5	8	1	0	2	8	2	5	2	7

Table 5.2. Frozen-thawed semen characteristics as measured by the HTM of individual rams per group by day during treatment.

Control.		Days	Total	Motile	Prgr	Mot	Mvsl	Mlin %	Malh	Mlin	Mvap	Rap. %	Med %	Slow %	Stat. %
20		-49	3485.6	1933	48.7	55.5	62	74.2	4.6	74	67.7	19.7	35.7	22.9	21.7
21			3903.2	2546	59	65.2	65	86.8	5.1	75	71.3	22.2	43	14.4	20.3
2			5136.5	2196	37.3	42.7	46	84	4.2	71	50.7	8.7	34	31.8	25.4
10			5509.8	4021	63.3	73	60	85	4.6	74	66.2	20.7	52.3	14.3	12.8
	Mean		4508.8	2674	52.1	59.1	58	82.5	4.63	74	64	17.8	41.3	20.9	20.1
	Stdev		967.76	932.4	11.6	13.1	8.7	5.65	0.37	2.1	9.11	6.17	8.34	8.34	5.29
	SEM		483.88	466.2	5.8	6.53	4.3	2.83	0.18	1	4.55	3.09	4.17	4.17	2.65
20		4	2369.1	507.2	20.3	21.4	71	90.7	4	84	73.7	8.9	12.5	13.9	64.7
21			5931	1858	26.7	31.3	57	83.1	4.4	71	62.6	9.2	22.1	21.1	47.5
2			4426.8	1915	36.2	43.3	61	83.7	4.3	74	66.8	14.8	28.5	24.2	32.5
10			5861.6	755.7	11.8	12.9	35	88.7	3.1	77	38.1	1.4	11.5	27.9	59.2
	Mean		4647.1	1259	23.8	27.2	56	86.6	3.95	76	60.3	8.58	18.7	21.8	51
	Stdev		1669.5	732	10.3	13.1	15	3.74	0.59	5.3	15.5	5.5	8.12	5.94	14.3
	SEM		834.73	366	5.15	6.55	7.7	1.87	0.3	2.7	7.75	2.75	4.06	2.97	7.13
20		15	3152.7	954.3	26.4	30.3	50	86.2	3.7	76	53.7	7.7	22.6	23.3	46.4
21			3757.9	822	19.8	21.9	63	86.1	4.1	77	66.9	7.5	14.4	16.6	61.5
10			4978.8	2442	41.8	49	64	83.6	4.3	74	69.6	19.1	29.9	24.9	26.1
	Mean		3963.1	1406	29.3	33.7	59	85.3	4.03	76	63.4	11.4	22.3	21.6	44.7
	Stdev		930.19	899.6	11.3	13.9	8	1.47	0.31	1.7	8.51	6.64	7.75	4.4	17.8
	SEM		537.04	519.4	6.52	8.01	4.6	0.85	0.18	1	4.91	3.83	4.48	2.54	10.3
20		21	5142.7	2479	40.8	48.2	57	83.6	4	74	63.2	14.7	33.5	23.5	28.3
21			3721.2	1916	44.7	51.5	75	86.1	4.8	77	79.8	24.6	26.9	20.5	28
10			5397.7	3040	45.9	56.3	55	81	4.6	69	61.7	16.9	39.5	27.8	15.9
	Mean		4753.9	2478	43.8	52	62	83.6	4.47	73	68.2	18.7	33.3	23.9	24.1
	Stdev		903.36	562	2.67	4.07	11	2.55	0.42	3.8	10	5.2	6.3	3.67	7.07
	SEM		521.55	324.5	1.54	2.35	6.1	1.47	0.24	2.2	5.8	3	3.64	2.12	4.08

Insulated		Days	Total	Motile	Prgr	Mot	Mvsl	Mlin %	Malh	Mlin	Mvap	Rap. %	Med %	Slow %	Stat. %
28		-49	3672.5	1352	32.9	36.8	61	86.9	4.6	76	66.4	9.4	27.4	17.2	46
5			3880	1238	27.4	31.7	45	81.7	4.5	67	52.5	6.7	25	23.9	44.3
26			3539.4	2074	51.5	58.6	62	85.3	4.8	74	68.3	20.1	38.5	19.4	22
27			4907.5	2147	36.5	43.8	46	81.1	4.4	68	52.4	9.4	34.4	27.3	28.9
	Mean		3999.9	1703	37.1	42.7	54	83.8	4.58	71	59.9	11.4	31.3	22	35.3
	Stdev		621.12	474.1	10.3	11.7	9.4	2.8	0.17	4.6	8.64	5.94	6.23	4.53	11.7
	SEM		310.56	237	5.16	5.84	4.7	1.4	0.09	2.3	4.32	2.97	3.11	2.26	5.87
5		4	5518.4	600.5	9.8	10.9	43	85.4	3.2	75	46.3	1.9	9	10.6	78.5
26			2669.9	1133	39.5	42.4	63	89	4.3	80	66.9	13.6	28.8	21	36.6
27			4375.2	1312	25.5	30	62	84.8	4.4	74	68.4	10.2	19.8	17.6	52.4
	Mean		4187.8	1015	24.9	27.8	56	86.4	3.97	76	60.5	8.57	19.2	16.4	55.8
	Stdev		1433.5	370.1	14.9	15.9	12	2.27	0.67	3.3	12.3	6.02	9.91	5.3	21.2
	SEM		827.61	213.7	8.58	9.16	6.7	1.31	0.38	1.9	7.13	3.47	5.72	3.06	12.2
28		15	2988.1	84.8	2.8	2.8	17	97.1	1	91	17.2	0.1	2.7	3.7	93.5
5			3537.4	723	17.9	20.4	62	86.1	4.3	76	66.9	5.7	14.8	15.8	63.7
26			2350.6	297.1	11.5	12.6	50	87.3	3.7	79	55.2	1.7	10.9	5.7	81.7
27			3703.5	503.8	13	13.6	51	88	4	76	54.6	3.3	10.3	12.5	73.9
	Mean		3144.9	402.2	11.3	12.4	45	89.6	3.25	81	48.5	2.7	9.68	9.43	78.2
	Stdev		611.44	273.9	6.29	7.25	20	5.04	1.52	7.2	21.6	2.39	5.06	5.68	12.6
	SEM		305.72	136.9	3.15	3.62	9.8	2.52	0.76	3.6	10.8	1.19	2.53	2.84	6.29
28		21	2528.4	204.2	6.9	8.1	34	84.2	3.1	76	38.7	0.5	7.6	7.3	84.7
5			3243.9	313.2	8.5	9.7	69	86.7	4.3	78	74	3.8	5.9	9.1	81.2
26			2970.7	66.8	2.1	2.2	45	88.9	3	81	49.3	0.6	1.6	1.4	96.4
27			4309.4	1150	23.5	26.7	62	86.6	4.5	76	66.8	8.8	17.9	25.6	47.7
	Mean		3263.1	433.6	10.3	11.7	52	86.6	3.73	78	57.2	3.43	8.25	10.9	77.5
	Stdev		757.27	488.2	9.24	10.5	16	1.92	0.78	2.5	16.1	3.9	6.91	10.4	20.9
	SEM		378.64	244.1	4.62	5.26	7.8	0.96	0.39	1.3	8.06	1.95	3.46	5.18	10.5

Table 5.3. Values and averages of fresh semen characteristics assessed manually per ram by group.

Day/Treat.	Ram No.	Volume	Concen./ml	Total count	Dead cells No.	Dead cells %
-49						
Control	20	1.20	1.49	1.79	30.00	15.00
	21	1.10	4.06	4.47	33.00	16.50
	2	1.10	3.68	4.05	14.00	7.00
	10	1.00	1.93	1.93	19.00	9.50
	Mean	1.10	2.79	3.06	24.00	12.00
Heated	28	1.10	2.45	2.70	23.00	11.50
	5	1.50	3.39	5.09	45.00	22.50
	26	1.50	2.76	4.14	21.00	10.50
	27	1.60	2.73	4.37	66.00	33.00
	Mean	1.43	2.83	4.07	38.75	19.38
4						
Control	20	1.30	1.15	1.50	11.00	5.50
	21	1.00	3.38	3.38	82.00	41.00
	2	1.20	2.93	3.51	5.00	2.50
	10	1.00	1.85	1.85	37.00	18.50
	Mean	1.13	2.33	2.56	33.75	16.88
Heated	28	1.50	2.05	3.08	48.00	24.00
	5	1.30	1.70	2.21	114.00	57.00
	26	1.10	1.88	2.06	15.00	7.50
	27	1.50	2.38	3.56	51.00	25.50
	Mean	1.35	2.00	2.73	57.00	28.50

Day/Treat.	Ram No.	Volume	Concen./ml	Total count	Dead cells No.	Dead cells %
Control						
15	20	1.30	2.68	3.48	34.00	17.00
	21	0.80	2.70	2.16	22.00	11.00
	10	0.40	2.98	1.19	9.00	4.50
	Mean	0.83	2.78	2.28	21.67	10.83
Heated						
15	28	1.40	3.33	4.66	49.00	24.50
	5	1.00	3.78	3.78	46.00	23.00
	26	1.10	3.33	3.66	15.00	7.50
	27	1.40	2.33	3.26	52.00	26.00
	Mean	1.23	3.19	3.84	40.50	20.25
21						
Control	20	1.70	2.38	4.04	13.00	6.50
	21	0.80	3.33	2.66	34.00	17.00
	2	0.90	2.85	2.57	24.00	12.00
	10	0.50	2.80	1.40	48.00	24.00
	Mean	0.98	2.84	2.67	29.75	14.88
21						
Heated	28	1.10	1.13	1.24	126.00	63.00
	5	1.70	2.15	3.66	71.00	35.50
	26	1.20	1.48	1.77	97.00	48.50
	27	1.30	2.48	3.22	58.00	29.00
	Mean	1.33	1.81	2.47	88.00	44.00

A P P E N D I X B

Table 5.4. Pregnancy at 17 and 65 days following insemination with frozen-thawed semen from days -49, 4, 15 and 21 of treatment and embryo loss per ram by group.

HEATED RAMS			EWES PREGNANT		
RAM	DAYS	No.EWES INSEMINATED	17 days after insemination	65 days after insemination	EMBRYO WASTAGE (%)
26	-49	22	14	5	9 (64.28)
	4	20	16	4	12 (75.0)
	15	22	12	2	10 (83.3)
	21	20	11	1	10 (90.9)
		84	53 (63.10)	12 (14.29)	41 (77.35)
27	-49	22	15	5	10 (66.7)
	4	21	12	2	10 (83.3)
	15	22	17	3	14 (82.35)
	21	22	7	1	6 (85.7)
		87	51 (58.62)	11 (12.64)	40 (78.43)
5	-49	22	11	5	6 (54.5)
	4	22	20	3	17 (85.0)
	15	22	13	3	10 (76.9)
	21	21	11	0	11 (100.0)
		87	55 (63.22)	11 (12.64)	44 (80.0)
28	-49	22	13	4	9 (69.2)
	15	22	14	4	10 (71.4)
	21	22	12	1	11 (91.7)
		66	39 (59.09)	9 (13.64)	30 (76.92
TOTAL		324	198 (61.11)	43 (13.27)	155 (78.28)
CONTROL RAMS					
20	-49	22	14	6	8 (57.1)
	4	22	14	5	9 (64.3)
	15	22	16	6	10 (62.5)
	21	22	16	5	11 (68.7)
		88	60 (68.18)	22 (25.0)	38 (63.33)
2	-49	25	15	6	9 (60.0)
	4	25	16	7	9 (56.2)
		50	31(62.00)	13 (26.0)	18 (58.06)
10	-49	22	10	6	4 (40.0)
	4	23	13	9	4 (30.8)
	15	22	12	4	8 (66.7)
	21	20	12	5	7 (58.3)
		87	47 (54.02)	24 (27.59)	23 (48.93)
21	-49	22	12	5	7 (58.3)
	4	22	17	6	11 (64.7)
	15	22	16	8	8 (50.0)
	21	21	10	3	7 (70.0)
		87	55 (63.22)	22 (25.29)	33 (60.0)
TOTAL		312	193 (61.8)	81 (25.96)	112 (58.03)

APPENDIX C

Table 7.9. Mean values of the fresh semen characteristics assessed by computerised image analysis and manual analysis per ram included in experiment 4.

Ram #	TOTAL	MOTILE	PROGR	MOT	VSL	Lin %	ALH	Lin	VAP	RAPID %	MEDIUM %	SLOW %
569	4026.98	3554.00	23.00	87.83	76.73	89.68	5.98	71.93	85.05	23.55	64.30	0.00
578	3247.03	1051.83	20.50	32.97	89.57	85.57	7.87	65.33	103.00	21.63	11.30	0.63
570	1737.43	1390.55	51.40	78.53	112.80	92.35	6.18	79.63	121.00	53.45	25.10	0.23
579	598.10	282.18	19.00	45.38	84.13	85.93	5.93	70.48	96.15	20.65	24.80	0.23
564	3545.78	1097.73	6.60	31.33	59.90	85.63	5.10	67.68	68.13	7.40	23.90	0.48
567	2866.28	1881.50	9.98	65.40	59.20	80.05	7.10	55.50	72.23	10.65	54.80	0.38
577	3910.05	2496.83	35.60	56.63	115.10	90.85	5.55	82.28	124.10	37.68	18.90	1.38
565	1387.83	1073.65	44.00	62.30	105.70	92.25	6.73	75.50	114.20	46.63	15.70	0.15
573	1504.20	1096.83	1.93	72.80	49.13	86.70	6.30	56.20	55.80	2.20	70.50	0.20
572	331.98	203.13	22.70	59.98	82.08	79.18	6.75	62.68	100.40	27.58	32.40	1.13
576	989.08	653.15	25.00	65.28	76.68	89.43	6.08	70.83	83.73	25.30	40.00	0.43
566	1404.90	1100.33	44.20	77.70	97.48	94.70	4.75	85.50	102.70	45.03	32.70	0.00
685	4275.70	1766.50	27.70	41.25	121.20	86.95	5.58	79.58	133.40	29.50	11.80	1.05
696	446.53	313.28	58.70	69.20	129.90	95.98	3.83	91.15	130.20	56.03	10.80	0.60
594	1789.18	998.28	42.20	54.03	130.90	90.48	6.60	82.33	141.50	44.33	9.73	0.48
690	2517.08	1811.75	54.90	71.98	142.60	89.48	6.63	81.48	155.90	59.18	12.80	0.66
697	1101.05	892.13	39.80	74.35	100.20	91.35	4.58	84.20	108.90	42.58	31.80	0.48
691	2278.13	1859.50	61.20	81.40	129.80	88.48	5.70	81.13	144.00	65.90	15.50	0.38
688	555.23	161.80	23.60	34.10	104.30	86.25	7.28	72.88	117.50	24.88	9.25	0.23
692	4441.33	3887.25	66.30	87.70	136.70	84.38	6.40	77.05	157.30	76.53	11.20	0.03
689	2295.05	2059.00	65.80	89.25	124.80	89.93	6.33	81.70	136.90	70.95	18.30	0.15
687	1022.30	798.83	56.00	79.03	114.20	90.93	5.83	80.88	124.00	58.75	20.30	0.08
699	854.38	30.45	7.63	9.40	120.30	89.53	5.85	83.13	133.10	7.63	1.78	0.55
686	698.88	248.98	15.70	35.65	79.63	81.00	8.80	58.75	96.23	17.60	18.00	0.28
593	4355.83	2890.00	41.70	66.48	121.60	81.13	7.55	69.78	141.30	47.50	19.00	0.68
589	2023.68	1194.68	48.40	58.35	137.40	91.70	6.53	84.03	147.20	49.83	8.55	0.30
587	1379.58	896.90	15.80	55.30	68.48	74.33	6.50	60.80	89.00	18.70	36.60	0.13
596	3269.00	993.95	21.50	30.58	130.40	88.23	6.85	78.50	142.80	23.05	7.55	0.18
595	2613.50	1393.50	33.50	52.90	133.30	80.88	7.93	69.28	154.70	39.95	13.00	0.20

Ram #	TOTAL	MOTILE	PROGR	MOT	VSL	Lin %	ALH	Lin	VAP	RAPID %	MEDIUM %	SLOW %
594	1789.18	998.28	42.20	54.03	130.90	90.48	6.60	82.33	141.50	44.33	9.73	0.48
588	2397.98	1933.75	53.00	80.83	127.30	88.45	6.65	79.08	139.50	56.43	24.40	0.30
585	4829.38	1928.75	15.20	39.75	85.48	79.03	7.48	62.35	103.50	17.63	22.10	0.00
590	604.23	70.50	7.73	11.65	111.30	92.45	4.80	83.85	117.30	7.93	3.75	0.13
591	2683.23	1893.53	41.30	64.83	120.00	85.95	6.05	77.10	134.20	45.58	19.30	0.68
596	2385.68	1195.58	33.60	49.90	125.30	88.18	6.70	77.98	137.90	35.93	14.00	0.30
593	4489.00	3366.75	65.10	75.00	124.20	85.30	6.80	79.00	143.00	52.00	22.00	0.63
584	2693.33	1717.73	50.60	61.75	162.80	89.33	7.15	81.85	178.60	55.75	6.00	0.30
595	2027.43	979.30	27.60	48.55	109.20	84.80	7.33	72.25	123.90	29.98	18.60	0.33
598	3904.28	2971.50	64.10	75.53	162.80	85.85	8.00	77.13	184.20	72.40	3.15	0.13
586	3447.78	1470.75	29.10	42.05	136.10	85.83	7.40	75.08	151.20	31.50	10.50	0.20
594	4876.28	1854.20	14.60	37.28	84.80	75.03	7.50	58.78	105.70	18.25	19.00	0.53
590	3514.00	1684.08	24.30	48.30	95.45	78.30	6.33	66.18	113.40	28.15	20.10	1.23
589	646.45	312.45	30.30	43.38	128.60	90.33	5.43	82.85	137.20	31.10	12.30	0.00
597	536.30	176.30	14.50	29.75	111.30	88.53	4.90	80.20	119.90	15.08	14.70	0.33
599	3814.65	2199.00	41.20	54.43	137.90	86.30	7.15	77.23	154.50	45.98	8.48	0.45
587	2784.28	627.43	7.43	22.43	70.78	71.53	6.53	56.55	94.08	9.55	12.90	0.63
mean	2367.16	1379.53	33.61	55.79	109.97	86.50	6.43	74.56	123.04	35.92	19.81	0.40
std.deviation	1362.10	941.74	18.45	20.14	27.01	5.36	1.00	9.07	27.88	19.26	14.52	0.32
variance	1855329.37	886871.98	340.34	405.43	729.32	28.76	1.00	82.25	777.09	371.08	210.81	0.10
maximum	4876.28	3887.25	66.30	89.25	162.80	95.98	8.80	91.15	184.20	76.53	70.50	1.38
minimum	331.98	30.45	1.93	9.40	49.13	71.53	3.83	55.50	55.80	2.20	1.78	0.00

Ram #	Stat %	Live	Dead	Normal	Headless	Abn. T.	Preg.%	Concep.%
569	12.18	174.00	26.00	89.00	1.00	10.00	38.10	35.70
578	66.37	142.00	58.00	80.00	8.00	12.00	61.90	59.50
570	21.25	181.00	19.00	93.00	0.00	7.00	95.45	95.45
579	54.38	165.00	35.00	82.00	2.00	16.00	90.69	88.37
564	68.23	135.00	65.00	79.00	9.00	12.00	90.00	90.00
567	34.23	167.00	33.00	80.00	5.00	15.00	58.50	56.09

Ram #	Stat %	Live	Dead	Normal	Headless	Abn. T.	Preg.%	Concep.%
577	42.03	177.00	23.00	92.00	2.00	6.00	97.67	93.02
565	37.53	173.00	27.00	90.00	0.00	10.00	93.30	86.66
573	27.00	160.00	40.00	85.00	5.00	15.00	71.42	66.66
572	38.90	162.00	38.00	84.00	2.00	14.00	86.04	86.04
576	34.33	163.00	37.00	90.00	0.00	10.00	93.02	90.69
566	20.53	164.00	36.00	94.00	1.00	5.00	75.60	65.85
685	57.70	140.00	60.00	68.00	22.00	10.00	12.50	12.50
696	32.58	171.00	29.00	81.00	4.00	15.00	76.74	74.74
594	45.50	162.00	38.00	85.00	2.00	13.00	46.51	44.18
690	27.35	176.00	24.00	93.00	2.00	5.00	52.38	50.00
697	25.15	168.00	32.00	83.00	5.00	12.00	85.00	85.00
691	18.23	174.00	26.00	87.00	3.00	10.00	78.50	72.50
688	65.68	161.00	39.00	91.00	2.00	7.00	70.45	68.18
692	14.78	180.00	20.00	90.00	0.00	10.00	76.74	76.74
689	10.60	173.00	27.00	95.00	0.00	5.00	80.43	78.26
687	20.90	180.00	20.00	93.00	0.00	7.00	87.17	87.17
699	90.05	146.00	54.00	75.00	15.00	10.00	33.33	30.95
686	64.05	148.00	62.00	78.00	2.00	20.00	37.71	37.71
593	32.88	165.00	35.00	92.00	2.00	6.00	54.16	52.10
589	41.40	150.00	50.00	88.00	2.00	10.00	70.00	70.00
587	44.58	135.00	65.00	70.00	2.00	28.00	9.80	1.96
596	69.20	150.00	50.00	92.00	1.00	7.00	58.00	50.00
595	46.93	163.00	37.00	95.00	0.00	5.00	57.14	51.00
594	45.50	162.00	38.00	85.00	2.00	13.00	29.16	25.00
588	18.90	177.00	23.00	95.00	0.00	5.00	72.00	66.00
585	49.88	148.00	52.00	81.00	2.00	17.00	0.00	0.00
590	88.23	148.00	52.00	75.00	10.00	15.00	4.16	4.16
591	34.50	150.00	50.00	82.00	0.00	18.00	42.00	38.00
596	49.80	157.00	43.00	80.00	20.00	0.00	88.37	88.37
593	25.37	165.00	35.00	93.00	1.00	6.00	79.06	79.06
584	37.93	166.00	34.00	94.00	1.00	5.00	71.42	69.00

Ram #	Stat %	Live	Dead	Normal	Headless	Abn. T.	Preg.%	Concep.%
595	51.10	137.00	63.00	90.00	3.00	7.00	37.20	34.09
598	24.35	159.00	41.00	89.00	0.00	11.00	58.53	58.53
586	57.75	142.00	58.00	91.00	2.00	7.00	78.57	73.80
594	62.15	168.00	32.00	93.00	5.00	2.00	65.11	60.50
590	42.98	125.00	75.00	62.00	19.00	19.00	77.27	77.27
597	69.90	162.00	38.00	82.00	12.00	6.00	28.57	26.20
599	45.10	174.00	26.00	93.00	3.00	4.00	66.66	61.50
587	76.95	126.00	74.00	59.00	22.00	19.00	64.28	64.28
mean	43.51	159.15	41.07	85.30	4.46	10.35	62.85	60.29
std.deviation	19.84	14.66	14.90	8.63	6.05	5.47	25.67	25.87
variance	393.47	214.98	222.11	74.44	36.56	29.88	659.16	669.32
maximum	90.05	181.00	75.00	95.00	22.00	28.00	97.67	95.45
minimum	10.60	125.00	19.00	59.00	0.00	0.00	0.00	0.00

APPENDIX D

Plate 8. The insulating bag used in experiment 8 chapter 5. With this kind of bag it was possible to increase subcutaneous scrotal temperature between 1.4 and 2.2 °C above control values, with a mean and s.e.m. increase of 1.7 ± 0.3 °C.