The effects of aging and various freezing methods on the survival of ram spermatozoa
by Thomas Austin Ree

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE in Animal Science
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Abstract:
To determine the effects of freezing, diluting, and aging semen at 5° C on conception rate, 22 ewes of varous breeds and ages were inseminated intrauterally or cervically with fresh, frozen or aged semen, diluted 1:4 (v/v) or 1:8 (v/v) with an egg yolk citrate diluent. Four of the 22 ewes had fertilized eggs or became pregnant, one by natural service, and three using fresh 1:8 (v/v) diluted semen inseminated intrauterally. There were no conceptions observed using aged or frozen semen. Also, no conceptions were observed following the use of cervical insemination. It was concluded that ewes inseminated intraterally had an advantage over ewes inseminated cervically as did semen diluted 1:4 (v/v) as compared to 1:8 (v/v) and fresh semen as compared to frozen or aged semen.

This phase of the study was undertaken to determine the effects of glycerol, final equilibration time, and method of freezing on ram spermatozoa. Ejaculates were collected from three trained rams, diluted, equilibrated and frozen individually in liquid air for 24 hours, thawed and scored for percent motility, progressive motility, live count, coiled tail and detached heads. Each pooled ejaculate was extended with egg yolk citrate, equilibrated for two hours, split and diluted with 3% glycerol or 12% glycerol (final concentration) and resplit and equilibrated for either 2 hours or 6 hours and frozen in either ampules or pellets. It was found that semen containing 3% glycerol, equilibrated for 6 hours and frozen in ampuled form was superior in nearly all respects to all other freezing methods. It was also found that semen frozen in pellets yielded the lowest survival rate, of the two methods tested.

It was concluded that semen could be frozen in ampules with a relatively good recovery rate and pelleted semen tended to be lower in all criteria used to measure semen survival rate. Glycerol level and final equilibration time also had a significant effect on the survival rate of the semen frozen in either pellets or ampules.

However, motility of the thawed sample may not be a good indication of the semen's fertilizing capacity.

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by

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A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree

of

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ABSTRACT

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INTRODUCTION

In the dairy and beef industry the extended use of superior sires through artificial insemination has greatly improved the quality of cattle. The advantages of artificial insemination in the sheep industry are similar to those of cattle. Limited success has been reported with insemination using frozen ram semen, and although motility of ram semen is generally unaffected by freezing, the fertilizing capacity of the frozen semen is reduced in most cases by 70 to 90 percent. It is unknown what causes this loss in fertilizing capacity. Since there are various reports of limited success using frozen ram semen, this experiment was initiated to determine the effects of various diluents and freezing techniques on semen survival and fertilizing capacity. Equilibration time, glycerol level and method of freezing were tested to determine if and how they influenced semen survival and if there was interaction between the factors being tested.

REVIEW OF LITERATURE

The most extensive application of artifical insemination in sheep has been on the large collective state farms of Russia. Since 1928, when the first experiments were conducted by Ivanov, its use had been expanded until in 1955, over 28,000,000 ewes were inseminated. Artificial insemination in sheep has been applied on a much smaller scale in other areas, such as Argentina, Australia, Brazil, England, Kenya, South Africa, and Uruguay. In the United States it is still in the experimental stage (Almquist, 1959).

The major advantages of artificial insemination in sheep are similar to those of cattle. To name a few: the extended use of superior sires, the number of rams needed, and uniformity of offspring. The major disadvantages, however, are quite different from those of the cattle industry. The external signs of estrus are weakly expressed and difficult to observe in the ewe. Therefore, detection of estrus requires the use of vasectomized rams. Obviously, under range conditions, more time and labor would be necessary during breeding season to corral, sort, and inseminate ewes than when ewes are allowed to breed naturally. Another major disadvantage to artificial insemination is the seasonal variation in semen characteristics of the rams semen. Continuous heat seems to be more detrimental to fertility than intermittent high and low temperatures. Artificial cooling of the rams will correct or prevent summer sterility and shearing of rams before hot weather was found to be helpful (Dutt, 1960).

Raising of testicular temperatures to about 36° C or higher, results in degeneration of germinal epithelium. Sperm in the seminiferous tubules and in the epididymis are also damaged. Abnormal sperm are evident within two to three weeks, and the damage is related to the duration of the high temperature (Terrill, 1968b).

Another major drawback to artificial insemination of sheep is the method of actually inseminating the ewe. The ewe is restrained in the raised breeding stall so that the vulva is at eye level with the technician, or she may be placed across a rail and her hindquarters elevated. For large scale operations, a rapid and simple insemination procedure has been used in Australia and Uruguay. Revolving platforms have also been devised, and are illustrated in several publications (Robinson, 1956). As many as 120 ewes can be inseminated per hour (Almquist, 1959). After cleansing the vulva, the lubricated speculum is inserted into the vagina, and the cervix located with the aid of a light. The inseminating pipette is introduced through the speculum, and the tip inserted into the cervix as far as possible, usually only about 0.5 (0.3-2.0) cm. In some cases, it is impossible to insert the pipette into the cervical canal, and the semen must be deposited on or near the os cervix or in the anterior vagina (Dunn, 1955). Deep cervical and uterine deposition of semen is difficult or impossible, excluding the use of surgical procedures, because of the very tough, interlocking annular folds

of the cervix (Almquist, 1959).

Dilution

Even mild dilution of ram semen usually can be demonstrated to have negative effects on the sperm cells. Percent motility is reduced regardless of the diluent, particularly as the dilution rate is increased. Greater dilution accounts in part for the lower resistance to cold shock of sperm collected by electro-ejaculation (Inskeep and Cooke, 1968).

A variety of diluents have been used by research workers, from cows' milk to mineral water. The egg yolk-citrate, egg yolk-phosphate and cows' milk diluents have been studied most extensively. The yolk-citrate diluents have contained many different concentrations of both yolk and sodium citrate along with several different sugars or alcohols as additives and various antibiotics in some cases (Inskeep and Cooke, 1968).

Roberts and Houlahan (1961) reported using, 100 mls. glass distilled water, 2.8 gms sodium citrate, 0.80 gms glucose and 20 gms of egg yolk. This, they say, is the diluent identical with the standard formula prescribed by the Soviet Ministry of Agriculture for use throughout the U.S.S.R. for artificial insemination of sheep.

Emmens and Robinson (1962) have pointed out that the egg yolk-citrate and phosphate diluents were developed initially for bull semen rather than for the ram. However, the Soviet Union, Rumania

and Poland appear to be using yolk-citrate-glucose diluents, usually with penicillin and stroptomycin added, with considerable success. Dilution rates are no higher than 1:6, and the reported conception rates in these countries to a single insemination range from 50 to 90% (Inskeep and Cooke, 1968). However, no report is given as to how these percentages are arrived at.

Salamon (1967) used a diluent containing 83 mM glucose, 100 mM sodium citrate, and 15% (v/v) fresh egg yolk. The semen was diluted with the first non-glycerolated portion at +30° C (1:1), cooled to +5° C in 1.5 hours, further diluted with the glycerol containing fraction and equilibrated for 4 hours. The final dilution rate and glycerol concentration was 1:2 and 6% (v/v).

Heated milk of both cows and ewes has been tested as a diluent for ram semen. Cows' milk was superior to ewes' milk in trials by Salamon and Robinson (1962). Dilution of semen 1:4 in either whole or skimmed, heated cows' milk followed by insemination at a dose of 0.30 ml did not depress fertility from that found with 0.1 ml undiluted semen. Comparisons of milk with yolk-citrate and yolk-phosphate diluents have shown the milk to be equal or slightly better (Inskeep and Cooke, 1968).

The inclusion of glycine seems to be beneficial especially when storage is anticipated. The diluent, yolk-citrate glycol-mucinase has had some usage in Russia, and is said to be improved by the addition

of penicillin plus streptomycin plus steptocid, and further by vitamin B, or B_{12}. With this diluent, the 21 day non-return rate for 2,757 ewes was 84% to a single insemination (Emmens and Robinson, 1962).

The dilution rate is largely dependent on the density of the semen and the number of ewes to be inseminated. The usual rate is up to 1:5. The average number of ewes per ram in the first field trials in 1928 was 212. In 1935, 355,400 ewes were inseminated to 489 rams (1070 ewes per ram) Rostov Province. One Russian worker in 1937 reported individual rates of 11,600, 12,500 and 15,016 ewes to one ram. Another Russian worker, in 1958, has reported the use of one ram on 17,683 ewes in less than four months (Emmens and Robinson, 1962). Obviously, with such widespread usage of one ram, the individual should be extremely sure no harmful genes are present. Extensive progeny testing must be completed before one ram is genetically spread so widely.

Storage of Semen

The probable cause of the slow development of artificial insemination of sheep is the fact that ram semen is very difficult to store successfully for long periods of time. Despite occasional satisfactory results, work with frozen ram semen is still in an experimental stage, and it is generally held that a serious loss in fertilizing capacity of ram spermatozoa occurs during freezing. The cause of this loss in fertilizing capacity is still unknown (Salamon, 1967). The

eventual goal in studies concerning the preservation of semen is long term storage in the frozen state. In this manner, a readily accessible supply of high quality semen from genetically superior rams would be available to the breeder, just as it is available to the cattleman today. When Emmens and Robinson (1962) prepared their review, the most successful report they could cite was by the Russian, Pokatilova in 1960. He reported 46% of the ewes lambing from insemination with semen which had been frozen to -21° C and stored at that temperature for 5 days. The semen was combined with an egg yolk-citrate-arabinose diluent, cooled from 20° to 0° C in 30 to 60 minutes, held at 0° C for 4 hours, rediluted 1:1 with the same diluent containing 15% glycerol (final concentration, 7.5%) and frozen slowly to -21° C over at least 60 minutes. Since then, a Russian worker reported that 91% of 126 ejaculates diluted in glucose-egg yolk-citrate-glycerol showed not less than 40% motility after storage for three to five months at -196° C. He then inseminated approximately 100 ewes in each of three field trials with this semen diluted 1:4. From 44 to 67% of the ewes lambed from two inseminations of 0.2 ml each of this semen in the cervix at a 24 hour interval, as compared to 40 to 78% of ewes bred with semen kept at 0° C. The diluent contained 100 ml distilled water, 1.5 gm glucose, 3.5 gm sodium citrate and 15 ml egg yolk. Fresh semen was diluted 1:1 with this mixture and cooled to 2° to 5° C in one hour. After cooling, the semen was rediluted 1:1 with medium containing

88 ml of the same diluent and 12 ml glycerol by layering in portions. The semen was frozen at rates of 0.5° C per minute from +4 to -15° C, 2.0° C per minute from -15 to -50° C, 5 to 10° C per minute from -50 to -75° C and 100° to 200° C per minute from -75° to -196° C (Inskeep and Cooke, 1968).

The most interesting recent work done has been by Salamon and Lightfoot (1967_a) of Australia. In one study, the effects of cold shock, liquid storage and pellet-freezing on successive ram ejaculates was studied. In this study, they used semen which had been diluted in hypertonic yolk-citrate-glucose, frozen in pellets on dry ice, stored in liquid nitrogen for 48 hours and thawed in 2.6% sodium citrate at 37° C. Cold-shocking undiluted semen resulted in a serious reduction in both motility score and percentage of spermatozoa impermeable to congo red-nigrosin stain. Considerable reductions in motility score and percentage of unstained spermatozoa occurred as a result of cold-shocking the diluted semen. The effect, however, was much smaller than that seen in undiluted semen, apparently owing to the protective action of egg yolk. It was reported that the addition of increasing amounts of accessory secretions to successive ejaculates profoundly increases the susceptibility of the spermatozoa to cold shock. It was also found that the third and fifth diluted ejaculates gave better viability during the storage period of 16 days at +2° C than the first or subsequent ejaculates. There were, however,

no marked differences in the storage ability of successive ejaculates up to 6 days. As ram semen can be stored at +3° to +5° C for 1 to 2 days only without serious loss in fertilizing capacity (Salamon and Robinson, 1962). It seems that more frequent ejaculates than have been collected in the past may be stored over a short period for practical use. In this experiment, egg yolk-glucose-citrate diluent containing 6% (v/v) glycerol was used for pellet-freezing, and the mean recovery rate after thawing was 58.3%. Despite good initial recovery rates after pellet-freezing in this study, there was a substantial loss in viability during the subsequent post-thawing incubation period. Recovery rate is percent live motile sperm after thawing. During incubation periods of 2, 4, and 6 hours, 47, 60 and 70%, respectively, of the original post-thawing motile spermatozoa became immotile. Attempts to estabilish a relationship between susceptibility to cold shock and subsequent performances during storage at +2° C, or recovery after pellet-freezing were fruitless.

In a second study by Salamon and Lightfoot (1967_b) ram semen was diluted in hypertonic egg yolk-citrate-raffinose, frozen in pellets on dry ice, stored in liquid nitrogen for 4 to 12 weeks and thawed in 2.6% sodium citrate at 37° C and used for insemination within 10 minutes. Four methods of insemination were used. For cervical inseminations, 0.30 ml of thawed semen was deposited into the entrance of the cervix of ewes 10 to 25 hours after the onset of estrus.

"Deep" cervical insemination was carried out by traction on the cervical papilla with forceps permitting semen deposition 1-3 cm inside the cervical canal. Uterine and tubal inseminations, following laporotomy, were performed on most of the ewes within 6 hours (range 1-8) after ovulation. Rates of fertilization were determined by egg recovery and examination for cell cleavage 48 hours after either uterine or tubal inseminations and 60 hours after cervical insemination. In one phase of the experiment, the fertilization rates were 25, 88, and 54% for cervical, uterine, and tubal insemination, respectively. In another phase of the experiment, 93% of 29 eggs were fertilized after uterine insemination, but only 23 of 68 (34%) ewes did not return to estrus within 22 days. This would seem to indicate an abnormally high loss of embryos, which may be caused by genetic damage to the sperm even though the motility and fertilizing ability was retained. No information was given on embryo loss after uterine insemination with fresh semen, so infection and surgical trauma cannot be ruled out. Also, aged eggs at the time of fertilization may have had an effect, and even under apparently ideal conditions after natural service, early embryonic loss is usually within the range of 10 to 20% (Quinlivan et al., 1966).

After the raw semen has been diluted, usually with egg yolk-citrate, it has been frozen in several ways, all of which yield fairly similar results. Salamon (1967) froze semen in ampules,

in synthetic straws and in pelleted form. All three sample methods were stored at -196° C for two to four weeks, until insemination. Results obtained from this experiment were as folows: Ampule method of freezing - 5.6% of ewes bred lambed; straw method of freezing - 4.4% of ewes bred lambed; pellet form - 8.9% of ewes bred lambed. The number of inseminations within one estrus did not effect fertility.

One factor which has differed and which could be a key to success or failure is the rate at which the semen is cooled to the storage temperature. Often this rate has not been known precisely, but a slow rate of cooling has been shown to be important for deep-freezing in several studies. In 1960, a Russian worker reported a conception rate of 67% with semen cooled from 20° to 0° C over 4 hours as compared to 54% with semen cooled by plunging the container directly into ice. The storage temperature in this experiment was 0° C (Inskeep and Cooke, 1968).

Some other factors, which were shown to be of importance in the pellet freezing of bull semen, were equilibration time, diluent used, and glycerol levels. There was no difference in survival rate after freezing on pellet size when frozen in yolk-glucose, however, a significant difference was shown when yolk-citrate was used as the freezing medium. No difference in survival was shown between the extender containing a 7.5% glucose, 7.0% glycerol and different

concentrations of glucose containing 3.5% glycerol, except when the concentration of glucose was 10%. When results in the study of glycerol equilibration time were evaluated, it was found that a significant difference existed on survival of spermatozoa on equilibration time only when yolk-glucose-7% glycerol extender was used as the freezing medium. The difference was between 2 hours and 10 hours. There was no significant difference, due to equilibration time in the other extender. Optimal glycerol equilibration time seemed to favor 5 hours. Survival was measured by percent motility after thawing in a 3% sodium citrate solution, pH adjusted to 7.0 with citric acid (Nagase et al. 1964).

Number of Sperm and Assessment

In evaluating ram semen, there are no extensive or comparative reports for comparing the value of different test combinations of tests, as has been done in the evaluation of bull semen. It is obvious that there is a relationship between the physical activity and fertility of ram semen and that these depend on concentration and the motility of individual cells, which may be measured in a variety of ways. In addition, the resistance of the cells to certain types of shock, (heat, cold or light shock) and their morphological appearance, may contribute significant information about fertility. Normally, the examination of ram semen has involved visual or more refined estimates of volume, activity and density, with perhaps a count of abnormal

of spermatozoa. Thus, several workers have adopted the following scale, which is still in use, as a substitute for counting the number of spermatozoa with a haemocytometer:

Type of Sperm	Approximate Sperm Count in 10^8 per ml
Very thick creamy	30
Thick creamy	25
Creamy	20
Thin creamy	15
Thick milky	10
Milky	5
Cloudy	1
Less than cloudy	<1

Similar scales are employed elsewhere. For example, in the U.S.S.R. a microsopial evaluation is employed where the following gradings may be used for rapid microscopical examination:

Dense - space between spermatozoa less than the length of a single spermatozoa.

Medium - space between spermatozoa equal to or two times the length of a spermatozoa.

Thin - more widely scattered than above.

These are combined with assessments of motility, grades 5 to 1 representing 100% falling progressively by 20% steps to 20% motile (total sperm motile). For example, grade 4 has a motility of 80% live sperm, grade 3 has 60% live sperm, and so on. In the ram, only dense semen of grades 4 or 5 is usually used for insemination (Emmens and Robinson, 1962).

The percentage of living spermatozoa may be estimated by 1) visual appraisal, 2) the so called vital stains, 3) or by the rarely employed and tedious method of direct counting.

It can be demonstrated in numerous ways that only sperm which are non-motile and cannot be reactivated are stained. If a mixture of stain and fresh semen is placed on a slide and observed under a microscope, it can be seen that only non-motile sperm take the stain. If one continues to observe the sperm until the mixture dries, one can also see that none of the non-stained sperm take the stain at the time of drying. Occasionally a non-active sperm is observed which does not stain. However, inactivity of sperm cells does not mean that they are dead. After staining, all cells in a field are counted. Cells which have stained are the dead cells, usually 200 total cells are counted and the live-dead percentages computed (Lasley, Easley and McKensie, 1942).

It is recommended that standardized conditions should be followed in applying the stain. This makes it possible to eliminate various variables in comparing semen samples. It is recommended that both the stain and the semen should be brought to a definite temperature between 20-30^{o} C and the smears made and dried at this temperature.

The stain used by these researchers was, 2% water soluble eosin in M/8 phosphate buffer (pH of 7.3) mixed in equal parts with a solution containing one part opal blue (undiluted) and 1 part M/8

phosphate buffer (pH 7.4) final pH of about 6.7, which is approximately isotonic with semen (Lasley, Easley and McKensie, 1942). Mixner and Sarof (1954) indicate that a low level of glycerol contained in frozen semen affects vital stains, however, Emmens and Robinson (1962) indicate that no stain was seriously affected by glycerol percentages up to 15%.

The number of sperm required for insemination will vary with the percentage of live, motile cells in the sample and the site and time of insemination. Some success has been reported with as few as 5 million sperm (Terrill, 1968_a). The usual practice in most studies where artificial insemination has been used as a tool, has been to use 0.1 ml of fresh semen, either undiluted or diluted 1:1 or 1:2 with such diluents as milk or egg yolk-citrate. Volumes greater than 0.4 ml are seldom used, but there are reports from Russia of lambing rates of 47 to 56% from vaginal insemination of 0.5 ml or 1.0 ml semen diluted 1:10 or 1:20 and 70 to 72% from 1.0 ml diluted 1:10, or 0.2 ml diluted 1:2 or 1:4. Similarly, French workers obtained lambing rates of 50 to 83% in limited numbers of ewes with 1.0 ml of semen diluted 1:30 or 1:40 (Inskeep and Cooke, 1968). Emmens and Robinson (1962) have concluded in their review of the earlier literature that a dose of 50 to 150 million sperm in 0.05 to 0.10 ml of sperm, undiluted or diulted, should yield a conception rate of 55 to 60%, if deposited into the cervix. In another study cited by Inskeep and Cooke (1968) insemination of 0.1 ml of

fresh semen diluted 1:2 with heated, whole cows' milk using alternate ejaculates from Merino rams, which were collected 11 times daily for 5 days, was tried. It was found that the decline in fertility with successive ejaculates could be accounted for entirely by the decline in sperm numbers. It was concluded that at least 120 to 125 million normal sperm were needed for maximum fertility from fresh semen and that each 25 million less, down to a dose of 25 million would reduce lambing by about 13%.

An ejaculate of 1 cc containing 3 billion spermatozoa diluted 1:1.5 and inseminated in 0.1 cc doses permits 25 ewes to be inseminated in the cervix with 120 million sperm each. Dilutions above 1:10 are not recommended. Motility of ram semen is greatly reduced or lost at relatively high dilutions of 20 million to 0.06 million per ml. Dilution of ram semen often results in reduced fertility (Terrill, 1968_a).

Insemination

Salamon (1967) observed that frozen ram spermatozoa survive only six or seven hr in the female tract. Mattner and Branden (1963) indicate that fresh semen will survive up to three days in the cervix, 12 hr in the vigina, and 30 hr in the uterus and oviducts. However, Salamon and Lighfoot (1967_b) conducted an experiment using frozen ram semen inseminated by four different methods. Cervical, "deep" cervical, uterine, and tubal inseminations were

used. They found that uterine inseminations gave the highest number of eggs fertilized per eggs recovered, and non-return per ewes inseminated. Their results indicated that an impaired pattern of sperm transport through the genital tract of the ewe was the principal cause of low fertilization rates after cervical insemination with frozen semen.

As stated before, the number of non-return ewes is not a good indication of the fertilizing capacity of semen. However, the fact remains that semen does not travel as well after it has been frozen. With this in mind, it is vital that a maximum number of sperm should be placed in the optimum site in the reproductive tract of the ewe. This site of fertilization has not been established precisely in the ewe, but it is presumed to be the upper half of the oviduct. To achieve optimum fertility the time of ovulation, the number of sperm deposited, the time required for sperm and egg transport, the life span of the sperm and eggs, and possible need for capacitation of sperm must all be taken into consideration. Furthermore, season of the year and age of the ewe were important time factors (Inskeep and Cooke, 1968). Single inseminations were less effective by 19 percentage points in the spring than in the fall. Conception rates to first service in virgin ewes were 8 to 15% lower than in older ewes. This latter effect may be due to age differences in the rate of egg transport. The lower incidence of multiple births in young ewes is

accounted for by the lower ovulation rate compared with mature sheep. Also, a greater proportion of dividing ova die in the younger ewes (Edgar, 1962).

Timing of insemination with relation to ovulation is a primary concern. Both the duration of overt behavioral estrus and the time of ovulation relative to the beginning or end of estrus have been shown to vary considerably both within and between breeds, leading Robinson (1959) to state that "the only reliable conclusion to be drawn..... is that ovulation occurs at about the end of estrus." Other researchers have indicated that ovulation generally occurs a minimum of 24 hr after the onset of heat (Inskeep and Cooke, 1968). McKenzie and Terrill (1937) laporotomized over 240 ewes and observed the follicular development up to and through ovulation. Ovaries were first observed from 15 to 35 hr after the onset of estrus, in most cases several hours elapsed before ovulation occurred. Conspicuous external changes in the development of the follicle prior to ovulation were confined to less than four hours. Ovulation occurred as early as 12 hr and later than 41 hr after the onset of estrus.

Attempts to determine ovulation time in relation to the end of estrus was also unsuccessful.

The estrual cycle of the ewe is normally about 17 days in length, with from two-thirds to three-quarters of the cycles falling within the range of 16 to 18 days and only about 10% falling outside the

range of 14 to 19 days. From 5 to 10% of the ewes in a band may be expected to come in heat in each of the first 14 to 16 days, provided inseminations are not commenced before the breeding season is well under way. Ewes normally remain in heat about 30 hr, although the duration may vary from a few hours up to three days or longer (Terrill, 1968_a).

It has been generally observed that if more inseminations are made per heat period, a higher degree of fertility results. If ewes are to be inseminated only once during estrus, a safe plan is to inseminate about 12 to 24 hr after they are first observed in estrus. In practice, it is desirable to allow for considerable flexability above the minimum requirements, both in the number of inseminations per heat period and the number of sperm per insemination, depending on variations in the number of ewes in estrus, length of the estrus, and the number of spermatozoa collected (Terrill, 1968_a).

One interesting problem with respect to artificial insemination is the effect of association or lack of association with rams on the time of ovulation after the beginning of estrus. Some earlier work has indicated that coitus shortened estrus. Russian workers have reported that multiple matings at 2 hr intervals shortened both duration of estrus and the interval to ovulation as compared to ewes separated from rams for 24 hr after each mating. Continuous association with rams also shortened estrus as compared to teasing at 4 hr

intervals in Merino ewes in South Africa (Inskeep and Cooke, 1968). However, the interval from the onset of estrus to ovulation was lengthened by 5 hr to an average of 30 hr by the continuous presence of rams (Parsons, Hunter and Rayner, 1967). Parsons and Hunter (1967) found that synchronization of estrus with progesterone had no effect on the length of estrus or its reduction by the ram, but they did not determine ovulation time in the ewes treated with progesterone. The presence of rams may aid in the conception rate of ewes bred artificially. Further study should be undertaken to determine what, if any, effect this might have on ovulation time in the ewe. If ovulation time could be timed to synchronize with the ovulation and thus increase conception rate.

Jones (1968) attempted to increase the conception rate of ewes by injecting oxytocin into the ewes at an early stage after artificial insemination. The theory being that the oxytocin would stimulate uterine contractions to increase the transport of spermatozoa through the genital tract of the estrous ewe. His experiment did not uphold his theory. This does not mean that oxytocin may not be important, and by no means should this possibility be discarded, since other researchers, Hays, Van Denmark and Ormiston (1958) and Stratman, Self and Smith (1959), found that the injection of oxytocin into cows or pigs, respectively, just after mating or insemination increased the conception rate. Timing and the duration of stimulation by oxytocin

may be important factors in testing the effects of this hormone on sperm transport (Jones, 1968).

Conception Rate

To date no concrete evidence has been presented as to what causes the embryonic death loss and low conception rates or just what specific steps can be taken to alleviate the situation.

Evidence has been presented that the use of aged semen in artificial insemination increases the incidence of embryonic death. However, few investigations have been conducted to determine the changes in the sperm cell which might lead to this decrease in embryo survival (Anand, Hoekstra and First, 1967).

The non-return rate has been used by several researchers as an indication of the number of ewes settled. Non-return rate is simply the number of ewes which do not recycle and there are reports of a discrepancy between non-return and actual lambing of 9%. In 1962, a Russian workers reported up to 40% difference between non-return and lambing rates, and claimed that a high incidence of embryonic mortality occurs using frozen ram semen (Salamon, 1967). This seems to indicate that conception rate may not be accurately predicted by non-return rate.

In a study conducted by Salisbury et al. (1961) DNA content of bovine sperm cells was studied by means of microspectrophotometry after storage at 5^{o} C for 2, 3, 5, and 10 days in egg yolk-citrate

diluent permitting slow aerobic metabolism. A subsample of sperm cells from each of five bulls was subjected to the Feulgen technique on each of the storage days selected. The cells sampled on each of these days received a standard 12 minute, 60° C hydrolysis. Absorption measurements at 546 mμ of the individual cells indicated a marked progressive decrease in the Feulgen-DNA content of the stored spermatozoa. The loss of 30% of the initial DNA at the end of 5 days storage was highly significant statistically. This decrease approximately parallels the known decrease in fertility of stored sperm cells, as well as the increase in apparent embryonic mortality resulting from the use of similarly aged spermatozoa for artificial insemination (Salisbury et al. 1961).

In a similar study using boar semen, Anand, Hoekstra and First (1967) found that the DNA content of boar spermatozoa decreased significantly during 24 hours of storage. The average decrease was 15.4%. The decrease in DNA upon in vitro aging of the cells may be related to the known decrease in fertility of aged spermatozoa, as well as, the apparent increase in embryo mortality resulting from the use of aged spermatozoa in artificial insemination (Anand et al. 1967).

To date no data has been published with regard to the DNA loss of ram spermatozoa. This is one aspect of ram semen storage which must be investigated further.

MATERIALS AND METHODS

Rams

Four mature rams were trained to service an artificial vagina. The rams were a Dorset crossbred, a Hampshire, a Columbia and a Rambouillet, and they ranged from two to four years of age. The rams were confined by themselves and fed grass hay daily. No ewes were ever in close proximity, except when the rams were being ejaculated. A ewe, which was in estrus, was placed in a specially designed wooden crate, which held the ewe securely in place while collecting and breeding. The technician, working alone, would release one ram in the same pen as the confined ewe. The technician knelt on the right side of the ewe, holding the artificial vagina in his right hand. As the ram mounted, the artificial vagina was slipped in front of his penis. As soon as the ram made entry into the artificial vagina, he would thrust, ejaculate, and dismount. He was released from the pen at that time and another ram brought in. This procedure was used on all four rams. Within a training period of six to eight days, three of the rams would mount and ejaculate within 30-45 seconds. The fourth ram would never mount a ewe in the presence of a technician. The temperature of the water contained in the artificial vagina was initially maintained at 42^{o} - 44^{o} C. The artificial vagina was not cleaned following the service of any one ram and if all three rams serviced quickly, the water did not need to be re-warmed. Care was taken that the artificial vagina was not too warm. The individual

ram's particular preferences were quickly realized and the ram preferring the warmest artificial vagina was trained to go first and so on. The rams quickly became accustomed to their order and almost always came in that order.

Ewes

Approximately forty non-pregnant ewes of mixed ages and breeds were used. A mature vasectomized ram with an ochered brisket was released with the ewes, which were checked twice daily for estrus. A ewe having a well ochered rump was considered in estrus. A ewe in estrus in the morning was bred that evening and a ewe in estrus that evening was bred that next morning. Terrill (1968) indicates that ewes should be inseminated 12 to 24 hr after they are first observed in estrus. Ewes were trucked approximately six miles to the breeding facilities, where they were bred artificially. If raw semen was needed for a new replication, the ewe which was to be bred was also used as a teaser. If no ewes were in estrus and raw semen was needed for a new replication, a ewe which was not in estrus was used as a teaser. The rams serviced the artificial vagina just as readily using either method. The ewe to be bred artificially was placed in the crate and her head secured. The semen to be used was drawn up into a standard artificial inseminating pipette (1/16" inside diam., 3/16" outside diam., by 18" long). A duck-billed specula was used to part the vagina and locate the cervix. On occasion a

flashlight was necessary to illuminate and find the cervix. The end of the pipette was inserted into the cervix as far as was gently possible, the semen was deposited and the ewe was released. Some of the ewes had their posteriors elevated after insemination for five minutes.

Storage and Freezing of Semen

Semen from the three trained rams was collected and pooled. Immediately after collection it was placed in a water bath, which was maintained at 30° C. It was equilibrated for seven to ten minutes. Egg yolk citrate maintained at 30° C was then added (1:4) by volume, and the dilution was placed in a beaker of water from the water bath and put in the refrigerator maintained at +3° C, allowed to equilibrate and cool there for 2 hr.

The egg yolk citrate used consisted of a solution prepared as follows: One liter of distilled water was placed in a volumetric flask and 23.53 g of sodium citrate and 7.54 g of glucose was added to it. This solution was refrigerated at +3° C. When a diluent was needed, 16 ml of this solution was added to 4 ml of pure egg yolk, to give a final volume of 20 ml egg yolk citrate at a concentration of 20% egg yolk (v/v). One part of raw semen to three parts egg yolk citrate yielded a 1:4 dilution. This dilution was refrigerated for 2 hr. Glycerol was added to the remaining unused egg yolk citrate. One and one-half ml of glycerol in 11 ml of 20% egg yolk citrate.

yields a 12% solution. This solution was used to redilute the 1:4 dilution to a final dilution of 1:8. One ml of 1:4 dilution + 4 ml egg yolk citrate (12%) glycerol yields a 1:8 dilution, with a final concentration of glycerol of 6% (v/v). This final dilution was equilibrated for 3 hours at +3° C, at which time it was ready for use.

The temperature of both the semen and the diluent were carefully controlled at all times, from collection to freezing to insemination. Care was taken to avoid exposure to the sunlight as well. The flask containing the semen was sealed, so the possibility of water contamination was reduced, and a small weight placed over the flask so that only the neck of the flask protruded from the water in the beaker. This beaker was then placed in the refrigerator and allowed to equilibrate there for 2 hr. After the semen was rediluted 1:8 and the final equilibration time was past, the semen was ready for placing in ampules and freezing. Ampules were kept in the refrigerator at all times at +3° C. These were removed from the refrigerator, as well as the beaker of water containing the flask of diluted semen. An empty canister was then removed from the nitrogen tank and placed on the table. The flask of semen was opened and a pipette was used to draw semen from the flask and deposit it in the ampules. As the ampules were filled, they were placed next to the canister. After the ampules were all filled, they were sealed with an oxygen-acetylene cutting

torch. After sealing, the ampule was again placed near the canister. This was done to reduce the temperature fluctuation. When all ampules had been sealed they were placed on canes, put in the canister and lowered part way into the nitrogen tank. The depth the canister was lowered depended on the amount of liquid air present in the tank, since the ampules must freeze in the vapors over the liquid air and not the liquid. No more than two ampules were placed on any one cane. In this way, all ampules could be in approximately the same amount of vapors, at the same temperature, at the same time.

Experiment I

In the first experiment, the 1:8 diluted semen was placed in ampules, sealed and frozen in the vapors of liquid air over a 30 minute period. This semen was then used to inseminate ewes. An ampule was removed from the nitrogen tank and placed immediately in a beaker of ice water. After it had thawed, it was drawn up into a pipette and used to inseminate as previously described. A motility score was assigned to the raw semen, the 1:4 dilution, the 1:8 dilution, and also the thawed semen.

Experiment II

In the second experiment, effects of aging of ram semen was considered. Two ewes were bred artificially each day. The semen was diluted 1:8 by methods previously described. The first day, two ewes were inseminated with fresh diluted (1:8) semen. The second day,

two ewes were inseminated with the same semen, now approximately 24 hr old. The third day, two ewes were inseminated with the same semen, now approximately 48 hr old. One ewe from each set was laporotomized 50-60 hr after the onset of estrus. Eggs were recovered by flushing the oviducts with serum from the ewe being flushed, if the ova were to be replaced in the uterus, and these ova were examined under a stereomicroscope to determine if fertilization had occurred. If fertilization had not occurred, the ova was discarded. Cleavage of the ova was the criteria determining fertilization. Saline solution (0.9%) was used to flush the uterine horns when the ova was not to be replaced in the uterus.

Ewes were also inseminated with frozen semen intrauterally. This was accomplished by first laporotomizing the ewe (Hulet and Foote, 1968). After the incision was made through the abdominal wall, the reproductive tract was exteriorized and the ovulation site was determined. The semen to be used was drawn up in a sterile disposable insulin syringe. The uterine horn on the side adjacent the corpus luteum was punctured and the semen was injected directly into the lumen of the uterine horn, thus by-passing the cervix. If ovulation had occurred on both ovaries one-half ml was placed in each uterine horn.

Experiment III

Semen was collected and frozen using a 2^3 factorial design. The factors which varied were equilibration time in glycerol (2 hr and 6 hr), glycerol concentration (2% and 6%) and method of freezing (ampule and pellet). When using the pelleted method, semen which had gone through a final equilibration of 2 hr or 6 hr was dropped in small droplets directly on a cake of dry ice as described by Salamon and Lightfoot (1967_a). After the pellets were frozen solid, they were transferred to the liquid nitrogen tank where they were stored in direct contact with the liquid air. A small pocket was formed in the end of a strip of aluminum foil. The pellets were placed in this pocket and the foil was hung inside the canister and the canister lowered into the liquid nitrogen tank.

After storage in liquid nitrogen for 24 hr the semen was evaluated for the following: Motility (score), progressive motility (score), live (count), dead (count), detached heads (count), coiled tails (count) other abnormalities (count) and normal sperm (count).

Motility of the ejaculate was assigned an arbitrary number by assessing under the microscope the amount of motion of the live semen sample. A score of one was very low, indicating the lowest possible percentage of sperm moving. A score of ten indicated the highest percent of motile sperm, as was indicative of a thick creamy sample having very thick, fast, swirls and eddies. Progressive motility was also an

arbitrary number assigned by assessing the individual sperm cell rather than the whole sample. A score of five indicated extreme motility while a score of one indicated no movement at all. After these two scores were assigned, a slide was made of the sample using live-dead stain. Stains, slides and staining techniques were as described by Lasley, Easley and McKenzie (1942). This stain only enters dead cells, therefore when viewed under a microscope, the live cells and the dead cells could be counted. Also, detached heads and coiled tails were counted. Two hundred total cells were counted to determine the live-dead count, and 200 were counted to determine abnormal sperm numbers.

The technique for thawing ampules was the same as previously described, but a different method was used to thaw the pellets. When the egg yolk citrate used for diluent was made, an excessive amount was prepared. Usually twice as much as was normally needed (about 40 ml). The final glycerol portion, which was not used in diluting the semen 1:8, was saved. Two ml of this egg yolk citrate glycerol was placed in a watch glass and lowered to approximately $+3^{o}$ C. The pellets to be thawed were then placed directly into this solution and placed in the refrigerator at $+3^{o}$ C. After the pellets had thawed (usually about 15 minutes) the watch glass was warmed to body temperature by holding it in the hand. After reaching body temperature, motility and progressive motility were recorded along

with the other observations. Usually all pelleted semen was analyzed before analyzing the ampuled semen. In that way, one pelleted sample could be thawing as another was being assessed. All eight samples from one replication were analyzed at one time, thus minimizing any effects which may have been caused from longer storage of any one sample.

RESULTS AND DISCUSSION

Experiment I

Six collections were made from the three rams and 100 vials of semen (diluted 1:8) were frozen. After freezing and storage for 24 hr., approximately one-half of the vials were thawed and scored for motility. Average motility of the six collections was less than one (Table I). With no frozen semen with which to inseminate ewes, experiment I was abandoned. Two days after the sixth collection, a motility score of five was observed in a thawed sample of semen. Placing the semen in the upper levels of the tank allows it to freeze slowly over a 30 to 40 minute period in the vapors above the liquid air, rather than freezing it rapidly in the liquid air. This proved to be the critical factor in freezing ram semen. Salamon (1967) and Salamon and Lightfoot (1967_a) indicated that slow freezing in the vapors of liquid nitrogen was important in survival of ram semen. The techniques required for sealing the ampules are relatively simple, but until they are learned, the ampules can be heated too extensively and cause death of the semen from overheating. Speed and dexterity in handling and sealing the semen ampules seem to play an important role in the survival of the semen. Light, heat shock, and cold shock are all detrimental to the semen, and all are preventable once the techniques and skills required for ampule freezing of semen are acquired.

TABLE I. MOTILITY SCORE OF POOLED EJACULATES BEFORE AND AFTER FREEZING.

	Before	After
November 21, 1969	6	0
November 24, 1969	6	0
December 3, 1969	7	0
December 3, 1969	9	0
December 4, 1969	9	1
December 8, 1969	8	1
December 10, 1969	10	5
January 14, 1970	8	4

Experiment II

The effects of aging ram semen on fertility and embryonic mortality were explored by observing twenty-two ewes bred in three different ways, (naturally, cervically, and intrauterally) and using three different semen dilutions and ages of semen (Table II).

Five ewes were bred intrauterally with fresh semen diluted 1:4 (v/v) and with a motility score of nine or more. Three of these ewes were pregnant and one was open when laporotomized approximately two months later. One ewe (No. 43) had a corpus luteum (CL) at breeding, but 60 hr later the ova was not recovered. For reasons unknown, she was not pregnancy checked nor was a special effort made to observe

her in estrus.

Four ewes were inseminated with fresh 1:8 diluted semen in or near the cervix. Two of these ewes were laporotomized at 60 hr after breeding, with a total of three unfertilized ova recovered. The remaining two ewes were laporotomized and found to be open approximately two months after insemination (Table II). These four ewes were bred the same as the five ewes discussed previously with respect to the timing of insemination. The timing and method of insemination may be one explanation for these results. The four ewes inseminated with the 1:8 semen were inseminated in or near the cervix, and thus the semen had to pass through the cervix to reach the ova, however, the cervix was bypassed on the ewes inseminated intrauterally with the fresh 1:4 semen. Also, since all five ewes bred with 1:4 semen intrauterally had ovulated at the time of insemination (Table II) it may be assumed that since the other ewes were handled the same, they too had ovulated. In that case, it was entirely possible that the semen did not reach the ova in time to fertilize it in the case of the 1:8 bred ewes. This leads one to the conclusion that ewes bred in or near the cervix should be bred earlier with respect to their estrous cycle, than should ewes bred intrauterally. Inskeep and Cooke (1968) indicate that transport of spermatozoa from the vagina to the upper half of the oviduct varies from a few minutes to several hours, the normal being several hours. Although Terrill

(1968_b) indicates semen may reach the ampullae of the oviducts in less than 15 minutes. Another possible explanation is the dilution rates of the two semen samples used (1:4 and 1:8). Terrill (1968_b) indicates that motility of ram sperm is greatly reduced or lost at relatively high dilutions of 20 million to 0.06 million per ml. Dilutions above 1:10 are not recommended. The 1:8 dilution rate placed in the vagina may not have had enough motility, after navigating the cervix, to fertilize the decending ova. Inskeep and Cooke (1968) also indicate that a capacitation in the oviduct of 1.5 hr may be necessary for the sperm to be able to fertilize the egg. Further research in this area is necessary before any concrete recommendations may be made.

Four ewes were bred in or near the cervix with semen diluted 1:8, and aged for 24 hr, and two ewes were bred similarly with 1:8 semen aged for 48 hr (Table II). At laporotomy, two ova were recovered from the ewe inseminated with the semen aged for 24 hr and neither were fertilized, nor were either of the other two ewes pregnant at 60 days post insemination. The same was true of the two ewes inseminated with 48 hr old semen. Two corpora lutea were present on one ewe, and although neither egg was recovered this ewe returned to estrus, thus indicating non-pregnancy. The other ewe bred with 48 hr old semen was aslo non-pregnant at the pregnancy check two months later. A similar explanation may be presented for

these results as was presented for the fresh 1:8 diluted semen, with the additional handicap of this semen being aged from 24 to 48 hr. Terrill (1968_a) indicates that ram semen when extended and stored for a day tends to lose its fertilizing capactity. Inskeep and Cooke (1968) and Terrill (1968^b) reported similar findings.

Six ewes were inseminated with 1:8 diluted semen, which had been frozen in ampules and stored in liquid air from 3 days to 3 weeks (Table II). All six ewes were bred intrauterally with semen which ranged in motility score from 2 to 5. Four of the ewes had CL when bred. Two of the ewes had no structures on the ovaries at breeding. Two ewes were laporotomized at 60 hr past insemination and one unfertilized egg was recovered. When the remaining four ewes were checked for pregnancy, approximately two months later, none were pregnant. Salamon (1967) cites several reports of successful results from insemination with frozen ram semen. In one, lambing rates by Russian workers were reported as high as 55-67%, however, in the same report Salamon stated that he got only 5.6% lambing rates with a single insemination of frozen semen. He concluded that spermatozoa of rams did not maintain their fertilizing capacity when frozen. In a comparison of cervical insemination with uterine and tubal using frozen semen Salamon and Lightfoot (1967_b) indicated a higher percent of fertile eggs with uterine insemination. However, only 34% of the ewes having fertile eggs did not return to estrus. This high rate of

embryos suggests that the frozen sperm may have suffered genetic damage despite the fact that motility and fertilizing ability were retained. Furthermore, Salamon (1967) reports that frozen spermatozoa survive only six or seven hours in the female tract. All of these factors apparently are responsible for the results reported in this paper using frozen semen.

One ewe was allowed to breed naturally. Timing of natural service was the same in relation to her estrous cycle as was used for all ewes in the study. That is, she was bred approximately 12 hr after the detection of estrus. When a fertility check was made, one ova was recovered and it was fertile. This would indicate that the methods used to determine cleavage of the ova were accurate and that the problem was either the timing, diluting or insemination procedure.

Estrus check procedures may have also accounted for some of the discrepancies seen in this report. Ewes were checked for estrus twice daily, at 8:00 A.M. and 5:00 P.M., or every nine and fifteen hours. Thus, a ewe coming into estrus at 6:00 P.M. would be considered in estrus at 8:00 A.M., and not bred until that evening. Earlier and later estrus checks would be an advantage, as well as, three or more checks per day, rather than two.

Another problem that may have been of consequence was that it was at the latter part of the normal breeding season, February for sheep, and it may be assumed that some ewes did not recycle for this

reason. It is also possible that ewes may have come in estrus without ovulating. Terrill (1968_a) indicated that Columbia type ewes have a breeding season that ranges from 85 to 153 days, with a mean of 119 days. Seasonal fluctation in day length is an important factor affecting the length of the breeding season. There is a negative correlation between the length of day and the number of ewes in estrus. Only five ewes were bred with 1:4 fresh semen intrauterally and only one of those was fertility checked at 60 hr after breeding. More ewes should be used and other dilutions and inseminating procedures used. No ewes were bred intrauterally with 1:8 semen for example. More research along these lines needs to be conducted.

Experiment III

Ejaculates of three trained rams were pooled and diluted 1:8 (v/v) final concentration, frozen in liquid air, thawed after 24 hr storage and evaluated for survival. The criteria used to evaluate a sample of semen for survival were: Percent motility, progressive motility, live count, detached head count, and coiled tail count. Semen was collected, frozen and evaluated over a seventy-four day period. Eight complete replications were collected and a total of seventy two samples of semen evaluated, thirty-six of these samples being in the pelleted form and thirty-six in the ampuled form.

TABLE II. EFFECT OF INSEMINATION METHODS, DILUTION RATES AND TYPE OF SEMEN ON FERTILIZATION RATE AND PREGNANCY IN EWES.

Ewe No.	Type Semen	Type Insemination	Motility Score	Treatment	Insemination	Fertilization	Pregnancy Check
26		Natural		F.C.		1-8 cell	
35	Fresh 1:8	I.C. (a)	8+	F.C.		2-1 cell	
9	Fresh 1:8	I.C.	8	F.C.		1-1 cell	
11	Fresh 1:8	I.C.	8+	S.C.			Open
18	Fresh 1:8	I.C.	8	S.C.			Open
43	Fresh 1:4	I.U. (b)	9	F.C.	1 C.L.	no recovery	
1	Fresh 1:4	I.U.	9	S.C.	1 C.L.		Pregnant
35	Fresh 1:4	I.U.	9+	S.C.	1 C.L.		Open
33	Fresh 1:4	I.U.	9	S.C.	1 C.L.		Pregnant
28	Fresh 1:4	I.U.	9+	S.C.	1 C.L.		Pregnant
1	24 hr 1:8	I.C.	5	F.C.		1-1 cell	
7	24 hr 1:8	I.C.	4+	F.C.	1 C.L.	no recovery	
39	24 hr 1:8	I.C.	5	S.C.			Open
15	24 hr 1:8	I.C.	4+	S.C.			Open
38	48 hr 1:8	I.C.	4	F.C.	2 C.L.	no recovery	
36	48 hr 1:8	I.C.	4	S.C.	N.S.S.		Open
37	Frozen 1:8	I.U.	5	F.C.	1 C.L.	1-1 cell	
14	Frozen 1:8	I.U.	5	F.C.	N.S.S.	no ova	
24	Frozen 1:8	I.U.	4+	S.C.	N.S.S.		Open
19	Frozen 1:8	I.U.	3-	S.C.	1 C.L.		Open
34	Frozen 1:8	I.U.	3-	S.C.	2 C.L.		Open
8	Frozen 1:8	I.U.	2	S.C.	1 C.L.		Open

(a) Bred in or near the cervix
(b) Bred intrauterally
F.C. Fertility Check
S.C. Survival Check
N.S.S. No significant structures observed on ovary

The two levels of glycerol used in this study were, 3% and 12%. Results of this experiment (Table IV) tended to show that glycerol at the 3% level yielded the highest percentage of motile sperm, as well as the highest percentage of live sperm ($P < .01$) (Table III). Although, detached heads, and coiled tails were not significant, they tended to be more prevalent in the 3% glycerol samples than in the 12% glycerol samples. Progressive motility was virtually the same at both levels. It may be conjectured that the 3% glycerol level lended itself well to motility, but did not offer enough protection from cold shock (indicated by the coiled tails). Most researchers tend to agree that final diluent level of glycerol should be about 6% for optimum results. First, Henneman and Williams (1957), used a glycerol level of 7% and obtained a 17% conception rate. Inskeep et al. (1968) cites a study by Russian workers who reported an average conception rate of 55% using semen diluted at a 6.5% glycerol level. Terrill (1968_a) recommends an optimum glycerol concentration of 7 or 8% final diluent, and Salamon (1967) used a final glycerol level of 6%. However, in another study conducted by Salamon and Lightfoot (1967_b) a 93% fertilization rate was reported with a 3% glycerol level. It should be mentioned here that this fertilization rate was due primarily to method of insemination (intrauteral in this case) rather than glycerol level. Nagese et al. (1964) found no difference in glycerol level from 3.5% glycerol to 7.5%,

however, concentrations above 10% were shown to be detrimental.

TABLE III. MEAN SQUARES OF THE CRITERIA USED TO DETERMINE SPERM SURVIVAL AFTER FREEZING.

Source of Variation	d.f.	Motility	Progressive Motility	Live Count	Coiled Tails	Detached Heads
Total (corrected)	71					
Replication	9	4.07**	1.48**	1343.04**	384.29**	1227.43*
Percent Glycerol (G)	1	3.13*	0.13	2048.00**	72.00	72
Equilibration time (E)	1	17.02**	5.02**	747.55	43.55	401.39*
Freezing method (F)	1	25.68**	5.02**	4544.22**	107.55	0
G X E	1	0.01	0.67	180.50	1042.72**	264.50
G x F	1	1.12	0.01	2964.50**	364.50*	121.60
E x F	1	0.12	0.01	68.06	53.40	1404.50**
G x E x F	1	0.02	0.02	3813.55**	288.00**	1150.90**
Error	55	0.738	0.299	275.72	128.88	166.47

* ($P < .05$)
** ($P < .01$)

Results of this study would indicate that a 3% level was more condusive to survival of semen than was a 12% level. This is by no means a recommendation for the use of a 3% glycerol level over a 6% level. More research should be conducted along these lines before a concrete recommendation can be made as to exact glycerol level. Findings from this study would indicate that a 12% glycerol level was desirable as measured by motility.

Semen was equilibrated for either two hours or six hours in the glycerol. Results of this experiment indicate that a six hour final

equilibration time yielded a significantly higher percentage ($P < .01$) motile and progressive motile sperm (Table III). Live count, detached heads, and coiled tails were not significant in this study. Live count tended to be higher at the 6 hr equilibration and the percentage of detached heads and coiled tails were lower at the 6 hr equilibration. All criteria used to measure survival of the sperm after freezing agreed that the 6 hr equilibration period was the optimum final equilibration time (Table IV). Nagase et al. (1964) indicates in his study that the optimum final equilibration time was 5 hours. It may, however, vary from 5 to 10 hr and still yield very similar results. Other researchers, Salamon (1967) and Salamon and Lightfoot (1967_a), used equilibration times of 4 hr and 3 hr, respectively, with relatively good results.

Semen was frozen in pelleted or ampuled form. Results of this experiment (Table IV) tended to show that the ampuled form of freezing semen was superior to the pelleted form. There was a highly significant ($P < .01$) difference in percent motile sperm, progressive motility and live count due to the method of freezing and no significant difference between the two methods for coiled tails and detached heads (Table III). Ampuled method of freezing gave consistantly better results. These findings are not in close agreement with the literature. Salamon (1967) reported a lambing rate of 8.9% using pelleted semen as compared to 5.6% using ampuled semen. The same diluents were used for

TABLE IV. THE EFFECTS OF GLYCEROL LEVEL, EQUILIBRATION TIME, AND METHOD OF FREEZING ON SURVIVAL AFTER 24 HOUR STORAGE IN LIQUID AIR.

	Number of Observations	% Motility	Progressive Motility	Live Count	Detached Heads Count	Coiled Tails Count
Overall Mean	72	3.2	2.9	52.	47.	33.
Glycerol		*	N.S.	**	N.S.	N.S.
3%	36	3.4a	2.8a	57.a	48.a	34.a
12%	36	3.0b	2.9a	47.b	46.a	32.a
Equilibration Time		**	**	N.S.	N.S.	N.S.
2 hr	36	2.7a	2.7a	49.a	49.a	34.a
4 hr	36	3.7b	3.2b	55.a	45.a	32.a
Method of Freezing		**	**	**	N.S.	N.S.
Pellet	36	2.6a	2.7a	44.a	47.a	34.a
Ampule	36	3.8b	3.2b	60.b	47.a	32.a
Glycerol x Equilb.		N.S.	N.S.	N.S.	*	**
3% 2 hr	18	2.9a	2.6a	56.b	52.a	38.b
12% 2 hr	18	2.5a	2.7a	42.a	47.a	14.a
3% 6 hr	18	3.9b	3.3b	59.b	44.a	29.a
12% 6 hr	18	3.4b	3.1b	52.ab	45.a	35.b
Glycerol x Freezing		N.S.	N.S.	**	N.S.	N.S.
3% Pellet	18	2.7a	2.7a	43.a	49.a	37.a
12% Pellet	18	2.5a	2.6a	45.a	45.a	31.a
3% Ampule	18	4.1b	3.2b	72.b	47.a	30.a
12% Ampule	18	3.4c	3.2b	48.a	47.a	33.a