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A STUDY OF THE TRANSPORT OF SPERMATOZOA IN THE  
REPRODUCTIVE TRACT OF THE EWE AFTER  
ARTIFICIAL INSEMINATION

A thesis presented in partial fulfilment of the requirements  
for the degree of Master of Agricultural Science  
in Animal Science  
at  
Massey University

KERIN HARLE GILES

1975

A million million spermatozoa,

All of them alive:

Out of their cataclysm but one poor Noah,

Dare hope to survive.

---- ALDCUS LEONARD HUXLEY

## PREFACE

This investigation was conducted at the Sheep Production Centre of the Sheep Husbandry Department, Massey University. The experimental work was conducted in July, 1974 and from February till June 1975, and represents original research by the author under the supervision of Dr. M.F. McDonald, Reader, Sheep Husbandry Department, Massey University.

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## ABSTRACT

Two preliminary experiments were conducted with artificially inseminated ewes to examine the considerable variation in counts of spermatozoa recovered in flushings from the reproductive tract. In the second trial, road transport stress immediately after insemination caused a reduction in the number of spermatozoa reaching the Fallopian tubes.

In the third trial, sperm transport after insemination with fresh, undiluted semen at two sites in the tract at predetermined times relative to synchronized ovulation (progestagen-HCG treatment) was investigated. Deposition of semen at the uterine end of the cervix or within the uterus using a specially adapted inseminating pipette, resulted in the recovery of higher numbers of spermatozoa from the uterus and Fallopian tubes 4 hours after insemination, than did insemination at the external cervical os. Twenty-four hours after insemination similar numbers of spermatozoa were found in all segments for both sites of insemination. Deposition of semen before or after the expected time of ovulation, or at both times, had little effect on the numbers of spermatozoa recovered. More ova with accessory spermatozoa were recovered from oviducts containing high numbers of sperm.

The transport of spermatozoa after deposition of fresh or frozen semen into the uterus by surgical and non-surgical methods, was studied in a fourth experiment. The number of spermatozoa recovered from the uterus and isthmus was similar for both types of semen, but only 'fresh' spermatozoa deposited at surgery, reached the ampulla in significant numbers. 'Frozen' sperm deposited by both methods, and fresh sperm deposited non-surgically,

did not reach the ampulla within 4 hours of insemination.

Impaired transport of 'frozen' spermatozoa to the site of fertilization appeared to be a result of reduced longevity of these cells within the female tract, and may involve factors such as breakage of spermatozoa in the uterus, and delayed transport in the oviducts.

The improvement of sperm transport in the ewe through the development of non-surgical, intrauterine inseminations and using either freshly extended or frozen semen, are discussed.

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INTRODUCTION

## INTRODUCTION

Among several advantages of artificial breeding of farm animals, is the rapid improvement that can be made following the increased selection pressure applied to individuals.

While it is recognised that under natural mating conditions, some rams are capable of outstanding reproductive performance, the dissemination of superior genetic material over a large number of ewes, is only possible with the use of artificial insemination.

Such practices have resulted in the successful grading-up of sheep in the Soviet Union and some South American countries. Australian sheep breeders have used artificial breeding as a means of improving the national wool clip, and a few New Zealand farmers have bred their sheep artificially.

The practical application of artificial insemination requires simple and effective methods that result in satisfactory conception rates, and depends on the development of a successful method of long-term preservation of ram semen, used in conjunction with synchronization of oestrus in ewes.

The reduction in fertility at induced oestrus after progestagen synchronization may well be associated with impaired sperm transport in the female tract, as could be the low fertility after deposition of frozen semen.

An understanding of the transport of spermatozoa in the female reproductive tract after artificial insemination may help in overcoming these physiological problems, and especially as in the future, timed inseminations without regard to detection of oestrus, are envisaged.

CHAPTER I

REVIEW OF LITERATURE

## CHAPTER ONE

### REVIEW OF LITERATURE

#### SYNCHRONIZATION OF OVULATION AND FERTILITY TO INSEMINATIONS AT PREDETERMINED TIMES.

Insemination without detection of oestrus has resulted in satisfactory levels of fertility being achieved in cattle (Wishart & Young, 1974) and to a lesser extent in swine (Dziuk & Polge, 1962) and sheep (Robinson, 1958; 1961).

Synchronization of oestrus after a series of progesterone injections was achieved by Dutt & Casida (1948) and O'Mary, Pope & Casida (1950), and Robinson (1965) introduced a convenient intravaginal method of progesterone administration that is more suitable for treatment of large numbers of sheep. An abundance of literature has appeared on dose responses and time relationships required to achieve optimum results (e.g. Robinson, 1967).

Exploitation of artificial breeding of sheep expected with the development of this technique, has not occurred, in part due to the poor fertility often associated with the induced oestrus (Robinson, 1969).

A wide range in the time of ovulation after progesterone injections resulted in low fertility after a single insemination at a predetermined time (Robinson, 1958). The administration of Pregnant Mare Serum Gonadotrophin (PMSG) after progesterone, failed to induce ovulations in the majority of ewes over a short period (Robinson, 1961).

The use of Human Chorionic Gonadotrophin (HCG), with and without PMSG, has given variable results. Killeen & Moore (1970c)

used both gonadotrophins, and though ovulation was not well synchronized, HCG appeared to exert its Luteinizing Hormone-like properties (see Gatchpole, 1964; Hutchison, 1964) 27 to 30 hours after its administration.

An extensive combination of treatments was investigated by Braden, Lamond & Radford (1960). The best response was achieved after daily injections of progesterone with PMSG given on the day of the last progesterone injection, followed 24 hours later by HCG.

Lamond (1962) observed no seasonal effects on the ability of PMSG-HCG treatment to synchronize ovulation.

Behavioural oestrus was almost completely inhibited when PMSG was given concurrently with the last progesterone injection (Braden et al, 1960; Robinson, 1962). The incidence of oestrus increased when PMSG followed progesterone by up to 24 hours (Southcott, Braden & Moule, 1962).

Dziuk, Hinds, Mansfield & Baker (1964) fed 6-methyl-17-acetoxy progesterone (MAP), followed by an injection of HCG without PMSG, and ovulation was synchronized between 24 and 28 hours after HCG. This was in agreement with Ortavant, Thibault & Wintenberger (1949). The method was repeatable (Hinds, Dziuk & Lewis, 1964; Dziuk, 1965; 1970).

Silicone-rubber, impregnated with progesterone and implanted subcutaneously for 14 days, followed 24 to 30 hours after removal by 250-500i.u. HCG, has proven an acceptable method for synchronizing ovulation in sheep (Dziuk, Cook, Miswender, Kaltenbach & Doane, 1968; Dziuk, Lewis & Graham, 1970; Dziuk, Ellicott, Weber & O'Reilly, 1970; Dziuk, Lewis, Graham & Moyer, 1972; Dziuk, O'Reilly, Ellicott, Weber, Garner, & Doane, 1972). Preliminary trials using the same techniques have been conducted

with swine (Dziuk & Polge, 1962; Dziuk, 1970) and cattle (Graves & Dziuk, 1968). Ovulation occurred in the majority of animals 40 hours after HCG, in both species.

Synchronization of ovulation with HCG given after intravaginal sponge administration of progestagen has not been investigated.

Fertility after PMSG-HCG-induced ovulations has been satisfactory during the breeding season (Cullen & Shearer, 1964) but not in anoestrous ewes (Braden & Moule, 1962).

After progestagen-synchronized oestrus, acceptable fertility followed a single insemination performed 48 hours after sponge removal (Robinson, Moore, Lindsay, Fletcher & Salamon, 1970). Equivalent conception resulted after inseminations with, and without, detection of oestrus (Robinson & Moore, 1967).

One or two inseminations at predetermined times, irrespective of oestrus, caused 48 and 62 percent of ewes to lamb (Robinson, 1970a, b) and even greater success has been achieved with small numbers of ewes (McClelland & Quirke, 1971).

Fertility to set-time insemination after implant-HCG treatments has been consistently high (60 to 85% Conception) to AI or natural mating.

#### METHODS USED FOR THE STUDY OF SPERM TRANSPORT

The principal method employed for investigating the transport of spermatozoa in the female reproductive tract has involved the recovery of sperm from the vagina, cervix, uterus and Fallopian tubes at progressive intervals after mating or AI.

The proposal of mechanisms of sperm transport has been based on information on distribution of spermatozoa in individual tract segments.

Artificial as opposed to natural breeding, conferred the advantage of depositing a known number of sperm cells, while techniques enabling control of the oestrous cycle aided the study of endocrinological factors affecting sperm transport.

Livability of spermatozoa within the tract (measured by duration of motility) has been most widely studied in vivo by deposition of sperm into ligated and non-ligated tract segments (Quinlan, Mare & Roux, 1933; Edgar & Adsell, 1960b) and in vitro by incubation in isolated tract sections (Mattner, 1963b).

Fallopian tube flushings, containing small numbers of spermatozoa have generally been counted by direct microscopic observation at high magnification. Haemocytometric determination of sperm cell numbers in other regions of the tract is possible because of the higher concentrations found.

Warbitton, McKenzie, Berliner & Andrews (1937) counted sperm only in 'representative areas' on evaporated slides; Braden (1953) centrifuged the flushings prior to resuspension and examination with haemocytometers. The accuracy of these methods is questionable as sampling error is likely to be high in the former, and haemocytometers exaggerate cell concentration when few sperm are present (Bane, 1952).

Flushings placed directly onto a series of slides and observation with phase-contrast after a settling period (Mattner, 1963a) proved less variable and achieved higher recovery of sperm than the method of Braden (1953).

The cervix of the ewe appears to be the region of the tract from which spermatozoa are most difficult to recover. Mattner (1966) calculated a 50% retention of sperm in the folds of the cervical

mucosa after a single flushing. Thus Lightfoot & Salamon (1970 a) used the method of dividing each section of the cervix into six pieces. These were stored in a known volume of saline and vigorously shaken before sampling, to facilitate sperm recovery.

Quinlivan & Robinson (1969) counted sperm on a slide following deep freeze storage of the flushings; this method might introduce extra losses of sperm on the additional glassware needed, and damage to sperm cells.

Variability is associated with what ever method is used, but the assumption that numbers of sperm recovered are representative of the actual numbers in the tract before flushing, must be accepted if conclusions are to be drawn and hypotheses constructed.

#### SPERMATOCOA IN THE REPRODUCTIVE TRACT

Spermatozoa in the female tract are considered as invasive organisms by Robinson (1973). The mechanisms by which they are protected and transported within this potentially hostile environment must therefore be effective for a sufficient length of time to enable spermatozoa to make contact with the ovum in the upper Fallopian tube.

In the ewe, at coitus, semen is deposited in the anterior vagina.

Diversity of opinion on the time taken for spermatozoa to reach the Fallopian tube is apparent (Table 1.1) and has lead to the hypothesis that two types of transport mechanisms exist: Rapid transport (sperm in oviducts within minutes of insemination); and a more gradual progression of sperm from the lower tract.

#### SPERMATOCOA IN THE VAGINA

In the ewe, spermatozoa survive for only a few hours after copulation, in the vagina. By this time, virtually all sperm remaining there are either dead or have very sluggish motility

TABLE 1.1

ESTIMATIONS OF THE TIME TAKEN FOR SPERMATOZOA  
TO TRAVEL FROM THE ANTERIOR VAGINA TO THE  
FALLOPIAN TUBES IN THE EWE

<u>Authors</u>	<u>Method of Mating</u>	<u>Time Taken</u>
Quinlan, Mare & Roux, 1932	NM	5 Hrs.
Green & Winters, 1935	NM	30 mins.
Kelley, 1937	NM	5 Hrs.
Phillips & Andrews, 1937*	NM	30 mins.
Lopyrin & Loginova, 1939	NM	8 Hrs. 40 mins.
Schott & Phillips, 1941*	NM	20 mins.
Starke, 1949	NM	6 mins.
Dauzier & Wintenberger, 1952	NM	8 Hrs.
Edgar & Asdell, 1960b	NM	2 Hrs. 30 mins.
Mattner, 1963a	NM	15 mins.
Mattner & Braden, 1963	NM	8 mins.
Mattner & Braden, 1963	AI	15 mins.
Oh & Lang, 1969	NM	4 Hrs.
Quinlivan & Robinson, 1969	AI	1 Hr.
Lightfoot & Salamon, 1970a	AI	30 mins.

NM = Natural Mating

AI = Artificial Insemination

\* Cited by Edgar & Adsell 1960b

(Quinlan et al, 1933; Starke, 1949).

Within a short time after insemination, spermatozoa move from the vagina to a more favourable environment in the cervix, in sufficient numbers to allow further progression to the upper tract.

The interval from insemination to the establishment of a cervical sperm population in the ewe, has been estimated to be as short as 15 minutes (Starke, 1949; Mattner, 1963a), though Ch & Lang (1969) measured maximal cervical numbers at 2 hours after coitus.

Mechanisms of movement of spermatozoa from vagina to cervix appear to be a function of sperm motility and contractility of the cervico-vaginal musculature.

Non-motile sperm deposited into the distal vagina have been reported as unable to progress to the cervix even after 8 hours (Dauzier, 1953) and the retention of abnormal spermatozoa in the vagina (Starke, 1949) indicates sperm motility as the sole motivator of passage into the cervix (Lightfoot & Restall, 1971).

However, other work (e.g. Mattner & Braden, 1963) suggests tract contractions as the predominant force.

In other species, a similar situation exists, (Walton, 1930; Bedford, 1971).

Radio-opaque material has been seen to move into the cervix of rabbits by virtue of vaginal contractions (Kriebiel & Carstens, 1939; Edgar & Adsell, 1960b; Akester & Inkster, 1961) but Moyes, Adams & Walton (1958) were unable to confirm this. In the cow Rowson (1955), noted no penetration of the cervical canal after depositing radio-opaque oil into the anterior vagina. Dead sperm have been found in the oviducts after  $2\frac{1}{2}$  minutes in the oxytocin-treated bovine tract (Van Demark & Noellier, 1951).

In women, vaginal contractions play the major role in movement of sperm into the cervix (Egli & Newton, 1961).

Some spermatozoa can survive for up to 18 to 24 hours in the vagina of the ewe, but the majority remain motile for only 12 hours (Quillan et al, 1953). However, Quinlivan & Robinson (1969) state that a vaginal sperm population remained viable for from 12 to 36 hours after artificial insemination, but they did not differentiate between live and dead cells.

Considerable loss of spermatozoa occurs from the vagina. Allison, (1972b) detected no difference in the number of sperm recovered from the vaginae of ewes inseminated naturally or artificially. This loss is due primarily to drainage of semen directly, and to leakage of cervical mucus containing numerous spermatozoa (Lightfoot & Salamon, 1970a).

Ovarian steroid influence on vaginal sperm populations has been studied by Allison & Robinson (1970, 1972) using progesterone-oestrogen injections in ovariectomised ewes. Oestrogenic effects were negligible, but progesterone decreased the number of sperm recovered.

#### SPERMATOZOA IN THE CERVIX

##### (a) Duration of Motility.

The cervix appears to offer the most favourable environment for sperm livability in the female tract. Quinlan et al (1953) found the maximum life span of spermatozoa in the ewe to be in the cervix, where they remained motile for up to 48 hours; in vitro life of sperm was maintained for longer in cervico-vaginal fluid than in fluids from other regions of the tract (Restall, 1969).

(b) The Cervix as a Spermatozoa Reservoir.

The work of Mattner (1963a,b) with the ewe, established that the cervix in this species has the ability to retain large numbers of spermatozoa which are gradually released to be transported to the Fallopian tubes. This reservoir ensures sperm in the oviducts over a period of time that would normally include ovulation. This function has been observed in other species where the anterior vagina is the site of semen placement: rabbit (Morton & Glover, 1974a); goat (Mattner, 1966;1968); cow (Mattner, 1966;1968); human (Nicholson 1965).

Following the rapid establishment of a cervical population, the number of spermatozoa remains high in the cervix and although it has begun to decline 15 minutes after mating, it is always higher than in the uterus. Positive correlations have been established between the number of sperm in the cervix and uterus, of the ewe (Quinlivan & Robinson, 1969); and of the rabbit (Morton & Glover, 1974a). This relationship exists at a significant level for up to 12 hours after insemination in the ewe and emphasises the importance of the cervix in providing the uterus with spermatozoa.

The cervical sperm pool is maintained at high levels for from 1 to 16 hours after coitus, and after this time, a linear decline occurs till 48 hours after mating, (Oh & Lang, 1969).

In acting as a reservoir, the cervix also constitutes a major barrier to the progression of large numbers of sperm to the upper tract, and only a few million ever reach the cervix from the vagina.

Within the cervix, a caudal to cranial gradient of sperm numbers exists (Allison, 1972b).

Movement of sperm into the uterus assists the decline of cervical sperm numbers. Replenishment from the vagina appears to occur for only a short time after insemination.

(c) Cervical Mucus and Sperm Penetration.

During the time that sperm are normally in the genital tract, the cervix of the ewe is full of mucus. Sperm entering the cervix and passing to the uterus, must traverse this fluid which undergoes changes in its physical and chemical properties during oestrus.

The physical appearance of cervical mucus during oestrus has been described by Dun & Restall (1961). As mucus viscosity increased, fertility to AI was reduced.

The chemical properties of cervical mucus are reviewed by Vickery & Bennett (1968).

Despite several reports, there is little evidence that any component of mucus is responsible for the prolongation of survival of sperm in the cervix (Gibbons & Mattner, 1966), and several reducing sugars measured in mucus (glucose, sorbital, fructose) do not seem to act as extra-cellular energy sources for spermatozoa in the mucus, (Gibbons, Collis & Sellwood, 1974).

Mechanisms of spermatozoa migration through cervical mucus are not completely resolved. Mattner & Braden (1969a) implicated active flagellation of spermatozoa by noting that live sperm, by virtue of their motility, were found mostly along the cervical mucosa; immotile sperm were found in highest concentration in the lumen only.

The physical properties of cervical mucus appear to aid both the movement of sperm through the cervix, and their alignment in the direction of the uterus. Tampion & Gibbons (1962) observed that spermatozoa are preferentially orientated in the direction along which

the threads of mucus are drawn out. They move along the lines of least strain imposed by the molecular anisotropy of the mucus.

Further, the work of Moghissi, Dabich, Levine & Newhaus (1964) has shown a mechanism which aids initial sperm penetration of mucus. Formation of phalanxes, a physical phenomenon that occurs when two fluids of differing viscosity come into contact, results in the accumulation of sperm at the tips of the formations, and facilitates penetration into the mucus. In addition, they implicated an enzymatic (protease) depletion of mucus viscosity which increased the degree of penetration by spermatozoa.

Cervical contractions are much less intense than those of the uterus, at least in the ewe (Lightfoot, 1970) and thus seem to play a less important role in sperm migration through the cervix. Other workers have implicated drainage of mucus into the uterus, digestion of the mucoid and consequent release of sperm into the uterine lumen (see Thibault, 1973).

Ciliated cells in the cervix (Restall & Lightfoot, 1970) may also play a part in orienting mucus threads, or in directing the movement of sperm through the cervical canal.

#### (d) Endocrine Control of Cervical Mucus Production.

Several attempts have been made to correlate mucus characteristics with the occurrence of oestrus and ovulation (Roland, 1952; Cohen, Stein & Koge, 1952; Scott-Blair & Glover, 1957; McDonald & Raeside, 1958; Lamond & Shanahan, 1969). The relative success of many of these studies has indicated direct control of production, secretion and composition of cervical mucus by ovarian hormones.

The infertility of sheep grazing oestrogenic pastures has been shown to be due in part, to a low cervical population of spermatozoa,

resulting in significantly fewer tubal sperm at the time of fertilization (Lightfoot, Croker & Neil, 1967). An Hyper-oestrogenic environment has been implicated (Lightfoot, Smith, Cumming, Marshal Wroth & Hearnshaw, 1974). The elevated oestrogen levels cause an excessive production of thin, watery mucus (Smith, 1971a) that impairs sperm transport in the cervix (Smith, 1971b).

Lindsay & Francis (1968) noted a linear response in the weight of cervical mucus produced, by spayed ewes, to injected stilboestrol. They concluded that progesterone sensitises the animal to respond to oestrogens, and also maintains a constant level of sensitivity. In agreement, Allison (1972a,b) and Allison & Robinson (1972) showed, using a series of progesterone and oestrogen combinations in ovariectomised ewes, that sperm transport was affected by the production of mucus in the cervix which was under control of a critical balance between the steroids. Croker, Robinson & Shelton (1974) noted a relationship between the number of sperm in the cervix of spayed ewes and the dose of oestrogen administered.

Similarly, Smith & Allison (1971) have pointed to an abnormal endocrine state of entire ewes after progestagen-induced oestrus, that causes a reduced passage of sperm in the cervix and therefore into the Fallopian tubes. Maximum production of mucus coincided with minimum viscosity and reduced the build up of a cervical sperm reservoir.

The administration of relaxin had no effect on the success of a cervical insemination (Salamon & Lightfoot, 1970).

#### SPERMATOCYTES IN THE UTERUS

Sperm enter the uterus from the cervix in two possible ways. Firstly by means of some cervical mucus entering the uterus (Lineweaver, Hafez, Ehlers, Dickson & King, 1970), and secondly by gradual migration

out of the cervix along the mucosal lining (see Hartman, 1957).

Migration of spermatozoa through the uterus appears almost unequivocally a result of contractions of the myometrial tissue (Van Demark, 1958), the intensity and frequency of which, are highest during oestrus (Reynolds, 1965). The absence of uterine fluid in the ewe and rabbit (Reynolds, 1965), reduced the role played by flagellation by spermatozoa.

Sperm are rapidly distributed throughout the uterus, irrespective of their motility (Dauzier, 1955a; Mattner, 1963b; Ch, Lang & Dugenzich, 1969). Motile cells are able to move in both cranial and caudal directions out of the uterus of the ewe (Mattner, 1963b).

The rapid dispersal of radio-opaque material through the uterus has been noted in other species (Kriebiel & Carstens, 1939; Van Demark & Moellier, 1951; Rowson, 1955; Bickers, 1960).

Smooth muscles of the bovine uterus are responsive to oxytocin (Van Demark & Hays, 1952) and a similar response has been recorded in the ewe (Thibault & Wintenberger-Torres, 1967; Lightfoot, 1970) and sow (Stratman, Self & Smith, 1959).

Stimulation of the tract soon after mating has increased the number of sperm in the Fallopian tubes (Austin, 1964). This has been observed in the rabbit (Bedford, 1971), the ewe (Lightfoot, 1970) and the cow (Van Demark, 1958). The stimulus has taken the form of sterile matings and tactile stimulation of external genitalia, and involves the central nervous system (Cross, 1959). The subsequent release of endogenous oxytocin initiates the smooth muscle contractions (Van Demark & Hays, 1952; Hays & Van Demark, 1953).

Roberts & Share (1968) and Fox & Knaggs (1969) have recorded elevated levels of plasma oxytocin soon after coitus in the ewe and

woman, respectively.

However, Lightfoot (1970) considered that the contractions of the ovine tract induced by mounting and mating were entirely different in character from those induced by intra-venous injection of oxytocin.

Bedford (1971) offers two theories to account for the increase in contractility observed at, or soon after, mating. Either the coital stimulus increases the activity of the tract through neural pathways, or, a seminal plasma constituent may be responsible.

Ram and human semen is known to contain an 'oxytocic-like' substance (Hawker, Roberts & Walmsley, 1960). A prostaglandin known to stimulate contractions of the uterus, is implicated.

During a natural oestrus in the ewe, contractions of the uterus move in both cranial and caudal directions, originating from any point along the uterine horns. In the exteriorised uterus at laparotomy 58% moved toward the tubes and 16% toward the cervix (Brinsfield & Hawk, 1969).

Oestrogens have two effects on sperm transport in the uterus. Motility of the tract is increased (Noyes et al, 1959b; Bishop, 1961; Brinsfield, 1968) but the rate of sperm breakage and disappearance is greater after oestradiol injections (Hawk & Cooper, 1971).

Progesterone appears to have the opposite effects (Rowson, 1955; Hawk & Cooper, 1971).

Synergism between the two ovarian steroids is also probable with progesterone affording the necessary sensitivity to oestrogen that is seen in other reproductive functions. Thus the altered oestrogen patterns associated with synchronised oestrus (Henricks, Hill & Dickey, 1973), reduced fertility through decreasing sperm transport to the Fallopian tubes.

The uterus has been identified as the main site of leukocytic invasion of the reproductive tract in many species: rabbit (Mahajan & Menge, 1966; Morton & Glover, 1974a); goat (Mattner, 1968); ewe (Oh et al, 1969) and cow (Mattner, 1968; Howe & Black, 1963). Leukocytes are present in greatest number in the uterus during the first 2 to 4 hours after deposition of semen.

Significant relationships between the number of leukocytes in the uterus and the number of sperm inseminated, have been found in the rabbit (Mahajan & Menge, 1966). Mattner (1968) found no such correlation in goats or cows.

Leukocytes are present in greater numbers during oestrus than in the progestational phase of the cycle (Howe, 1967; Conley & Hawk, 1970). The influence of ovarian hormones on leukocyte populations in the tract are described by Brinsfield, Hawk & Richter, (1964).

Oh et al (1969) suggested a differential phagocytosis of dead sperm in the uterus of the ewe, but live sperm and separated heads and tails are engulfed by these cells.

Peak uterine populations of spermatozoa have generally been recorded about 12 to 24 hours after insemination in the ewe (Mattner, 1963a; Oh & Lang, 1969; Quinlivan & Robinson, 1969; Killeen & Moore, 1970a). Estimates of mean uterine sperm numbers at this time have ranged from tens to hundreds of thousands.

#### SPERMATOZOA IN THE FALLOPIAN TUBES.

##### (a) The Utero-tubal Junction as a Barrier to Sperm Movement.

The junction between the uterine horns and the Fallopian tubes has been considered as the second major barrier to sperm transport in the ewe (Dauzier & Wintenberger, 1952; Killeen & Moore, 1970a; Thibault,

1973) which may act as another reservoir of spermatozoa for the oviducts.

Motility of spermatozoa appears to be an advantage for passage across the junction in sheep (Mattner, 1963b; Ch et al, 1969) and rabbits (Inkster, 1964), though these same authors report that dead sperm do occasionally reach the ovarian side. Leonard & Perlman (1949) considered sperm motility essential for passage across the utero-tubal junction in the rat.

The closure of the utero-tubal junction (UTJ) appears to be under endocrine control. Edgar & Asdell (1960a) concluded that at, and for three days after oestrus, closure of the UTJ in the ewe, prevents tubo-uterine movement of fluid, due to an oestrogen-induced oedema of the tissue near the junction. However, Lopyrin & Loginova (1939) claim that the Fallopian tubes are impervious to sperm for only 15 to 17 hours before ovulation.

Oestrogens also promote closure of the UTJ in rabbits (Noyes et al, 1959a).

Reports on the progestational effects on the UTJ are few. Opening of the lumen in sheep on the 4th day of the cycle (Edgar & Asdell 1960a) occurs at a time when progesterone secretion from the corpus luteum is increasing. Hunter (1972) injected progesterone locally into the utero-tubal junction of sows and found an increase in the number of polyspermic eggs. He suggested that opening of the sphincter allowed greater numbers of spermatozoa into the Fallopian tubes.

(b) Spermatozoa Migration in the Fallopian Tubes.

Sperm survive for relatively short periods in the oviducts of ewes. Quinlan et al (1933) reported that motility was maintained for

no longer than 12 hours, while Mattner (1963b) observed degenerative morphological changes 5 hours after tubal insemination.

It is probable that spermatozoa are aided by forceful muscular contractions in their ascent of the isthmus (Hafez & Black, 1969), with sperm-tail flagellation being more important for movement in the ampulla (Dauzier, 1955b) and for fertilization (Austin, 1964).

Directional flow of Fallopian tube secretions have been studied by Bellve & McDonald (1968) and McDonald & Bellve (1969), who showed that peak isthmal flow occurred on days 3 to 5 of the cycle. Flow out of the ampulla end of the tubes reached a maximum on day 2 and accounted for the major part of total tubal secretions. Fluid flow may therefore offer resistance to sperm ascent in the isthmus, but aids it in the ampulla.

Cilia lining the oviducts of mammals are more important for directing the ovum down the ampulla (Hafez & Black, 1969) against the direction of fluid moving out of the ovarian end of the oviduct (Bellve & McDonald, 1968).

Spermatozoa are lost from the oviduct by passage into the peritoneal cavity (Rowlands, 1958; Edgar & Asdell, 1960b; Horne & Thibault, 1962; Mattner, 1963a; Mattner & Braden, 1963; Inkster, 1964), but are able to enter from the opposite direction (Adams, 1969).

The number of spermatozoa in the Fallopian tubes appears to be a function of the efficiency of transport through the lower reproductive tract. Mattner (1963a) and Quinlivan & Robinson (1969) found peak sperm numbers in the tubes 24 hours after insemination, while Ch & Lang (1969) showed a build up in the isthmus from 4 hours, rising to a peak at 16 hours after coitus. No data were presented for sperm in the ampulla in the latter report.

Estimates of maximum numbers of tubal sperm have varied considerably, but are in agreement in that only a very small percentage of the sperm deposited ever reach the site of fertilization.

#### SPERM NUMBERS AND FERTILIZATION

A relationship between the efficiency of sperm transport and fertilization rates, has been found in many species: rat and mouse (Braden & Austin, 1954); rabbit (Braden, 1953; Overstreet, 1970); ewe (Lightfoot & Salamon, 1970a).

Quinlivan & Robinson (1967) described the chances of fertilization occurring in the ewe as slight, moderate or high, when 200, 200 - 6000, or 6000 spermatozoa, respectively, are in the oviducts at about the time of ovulation. Kennedy & Kennedy (1972) also observed a relationship between the number of tubal sperm and the likelihood of fertilization in young Merino ewes.

Maximum tubal sperm populations appear to occur approximately 16 to 24 hours after their deposition in the anterior vagina. Ovulation occurs about 24 to 36 hours after the onset of oestrus. The time of arrival of the ovum in the ampulla and maximum sperm numbers in the tube is a critical period that governs the probability of fertilization. Peak numbers of sperm in the ampulla are not maintained for long (Quinlivan & Robinson, 1969) and are likely therefore, to change with the time of insemination relative to onset of oestrus.

Ageing of sperm and/or ova can lower fertilization rates (Roche, Dziuk & Lodge, 1968). Therefore, inseminations at times that result in the sperm 'awaiting' the ovum, or vice versa, are likely to contribute to reproductive wastage (Hafez, 1966).

## FACTORS CONTRIBUTING TO FAILURE OF SPERM TRANSPORT IN THE EWE.

PROGESTAGEN-INDUCED OESTRUS

Hancock (1962) postulated that an alteration in the pattern of distribution of spermatozoa in the female reproductive tract, and hence in the numbers available for fertilization, may cause the lowered fertility often observed following progestagen treatment for the synchronization of oestrus in sheep.

Quinlivan & Robinson (1967) found that sperm recovery from the Fallopian tubes of the ewe, was reduced at the first post-withdrawal oestrus after synchronization with intra-vaginal sponges. Further, Quinlivan & Robinson (1969) attributed the changed pattern to a failure to establish a spermatozoan population in the cervix.

Four mechanisms operating to reduce the efficiency of sperm transport at the induced oestrus have been identified as a result of the progestational effects of the hormone treatment:

(i) Failure to establish a cervical population sufficient to ensure prolonged migration of sperm to Fallopian tubes (Quinlivan & Robinson, 1969; Hawk & Conley, 1972).

(ii) Increased sperm cell disappearance from the vagina (Oh & Lang, 1969; Hawk, 1972a; 1973).

(iii) Increased sperm breakage and disappearance from the uterus (Hawk & Conley, 1971; 1973).

(iv) Reduced frequency, intensity and reversal of direction, of uterine contractions (Hawk & Ekternkamp, 1973).

Prostaglandin-induced oestrus maintains a greater efficiency of sperm transport than progestagen-induced oestrus, but is still less favourable than sperm passage in the naturally cycling ewe (Hawk, 1973).

However, fertility to induced oestrus is not always low

(McDonald, 1966; Jennings & Crowley, 1970) and this appears to be a function of achieving the correct steroid balance (Robinson, 1969).

#### AGE & BREED EFFECTS

Spermatozoa from the male of a closely related species can traverse the female reproductive tract (Mahajan & Menge, 1966; Hancock & McGovern, 1968), though fertilization is low.

Comparisons of sperm transport between breeds of sheep have not been undertaken, though they are likely to arise as a result of different endocrine environments.

Young Merino ewes displayed a reduced capacity to transport sperm to the Fallopian tubes as compared to mature ewes (Kennedy & Kennedy, 1972), but Ch & Lang (1969) found no age difference in Romney sheep.

#### PRESENCE OF FOREIGN MATERIALS

The insertion of an intra-uterine-device into one uterine horn of the ewe causes an ipsilateral impairment of sperm transport through the uterus. The foreign body enhances leukocytic emigration to the uterus and reduces the number of contractions moving toward the oviducts (Brinsfield & Hawk, 1969; Hawk, 1970; Conley & Hawk, 1970; Brinsfield, Clark & Hawk, 1971).

#### FACTORS AFFECTING SPERMATOCYTA TRANSPORT IN THE FEMALE AFTER ARTIFICIAL INSEMINATION

In view of the number of mechanisms operating to transport spermatozoa to the Fallopian tubes, it is apparent that many factors will affect the efficiency of this movement when relatively few sperm are placed in the genital tract of the ewe, as in artificial insemination.

#### NUMBERS OF SPERMATOCYTA INSEMINATED

The quantity of sperm cells required for insemination will

vary with the percentage of live, motile cells in the sample and with the site and time of insemination. Emmens & Robinson (1962) concluded that a dose of 50 to 150 million sperm in 0.05 to 0.1 ml of semen should yield an acceptable conception rate to a single insemination. Low dilution does not alter this appreciably (Mattner, Entwistle & Martin, 1969; Entwistle & Martin, 1972). Salamon (1962) observed that for every decrease of 25 million sperm below 120 million, 13% fewer ewes lambed.

The effect of numbers of sperm deposited on the number of sperm reaching the Fallopian tubes is emphasised at low sperm doses.

The difference in sperm transport between progestagen-treated and untreated ewes was eliminated when large numbers of spermatozoa were inseminated (Quinlivan, 1970).

The importance of the effect of sperm numbers inseminated is probably more important when frozen ram semen is used (Lightfoot & Salamon, 1970a,b; Salamon & Lightfoot, 1970).

Two inseminations within one oestrus (12 to 24 hour interval) have generally resulted in higher fertility than a single insemination (Osikowski, 1956; Colas, Dauzier, Courot, Crtavant & Signoret, 1968; Linge, 1970; Prahov, Doiceva, Doicev, Barkov & Hadziiski, 1970), though some workers have shown no such advantage (e.g. Zeltobrijuh, 1968).

It is probable that two inseminations are advantageous due to a greater number of sperm in the Fallopian tubes over a much longer period, increasing the chance of an egg-sperm contact. However, the magnitude of the difference between one and two inseminations will depend on the time and site of insemination, and on the number of sperm per dose (Salamon & Lightfoot, 1970).

### SITE OF INSEMINATION

The usual site of semen placement in the ewe, is just within the cervix, through the external cervical os. Insemination at this site allows a greater chance of establishing an adequate cervical sperm population. Deposition into the anterior vagina is considered unfavourable due to a less suitable environment for sperm livability. However, Koger (1951) and Tjupic (1959) have achieved satisfactory levels of fertility after vaginal inseminations.

Dun (1955) has recorded the variation found in the complexity of the cervical papillae of sheep of all ages, and has related this to the difficulty of locating the opening to the cervix. In aged ewes especially, the numerous folds obscured the opening and reduced the chances of a successful insemination. In most other cases, there was little difficulty in finding the orifice with the aid of a speculum and light source.

Depth of penetration of the cervix is normally limited to less than 2cm, though Salamon & Lightfoot (1967) achieved up to 3cm depth with 'cervical traction'.

Under surgery, intra-uterine inseminations have resulted in the fertilization of large numbers of ova (Lightfoot & Salamon, 1970a; Trounson & Moore, 1974). The by-passing of the cervix eliminates a large source of loss of sperm from the tract and yields a significantly higher rate of fertilization (Killeen & Moore, 1970a,b).

Little attention has been given to the possibility of an intra-uterine placement of semen in field trials.

### TIME OF INSEMINATION

Most workers have observed higher fertility to inseminations during early to mid oestrus (Warbitton et al, 1937; Carbonerro Bravo,

1955; Csikowski, 1956; Schindler & Amir, 1973) though others have not found any stage-of-oestrus effects (Sinclair, 1957; Jones, 1968; Lightfoot & Salamon, 1970a).

Changes in the properties of cervical mucus account for the differential sperm transport and fertility that has been observed (Dun & Restall, 1961).

Time of insemination has no effect on transport efficiency when sperm are placed into the uterus under surgery (Killeen & Moore 1970a,b).

#### INSEMINATION WITH FROZEN SEMEN

Fertility to inseminations with frozen semen is invariably low (Visser, 1974). Factors responsible for this have not been extensively studied.

It appears that longevity of 'frozen' spermatozoa is reduced in the female reproductive tract (Mattner et al, 1969) and because of this, the mechanisms of sperm transport are less effective (Lopyrin & Loginova, 1958; Lightfoot & Salamon, 1970a,b).

#### PURPOSE AND SCOPE OF STUDY

The present work was conducted as a series of trials, investigating sperm transport in the reproductive tract, after artificial insemination at predetermined times following hormone treatment designed to synchronize ovulation in ewes.

The use of an inseminating pipette enabling the deposition of semen into the uterus will be assessed in terms of the distribution of sperm throughout the tract, to determine the advantages of by-passing the cervix, which appears as the major site of sperm transport failure in the ewe.

A study of the transport of spermatozoa after insemination with frozen ram semen is included in an attempt to identify factors contributing to sub-fertility observed after inseminations with such material.

CHAPTER II

MATERIALS AND METHODS

## CHAPTER TWO

### MATERIALS AND METHODS

#### EXPERIMENTAL ANIMALS

A series of four trials was carried out on the No. 2 Sheep Farm, Massey University, in 1974 and 1975, involving 132 Romney and Border Leicester X Romney ewes.

Oestrus was synchronized using polyurethane intravaginal sponges impregnated with 40mg 6-methyl-17-acetoxy-progesterone (MAP).

Rotational grazing on predominantly perennial ryegrass-white clover pastures, was aimed at maintaining rather than increasing, liveweights.

Prior to the start of each experiment, ewes were ring-crutched to minimize accumulation of faeces around the tail. This facilitated the insertion of sponges and inseminations.

Seven mature Romney rams were used for semen collection.

#### SEMEN COLLECTION & EVALUATION

##### (a) Training of Rams.

Twelve ovariectomised ewes were available for use as teaser ewes for ram training in 1974. When required, ewes were subcutaneously injected with 10mg progesterone in 2ml peanut oil every second day for 4 days, and 60µg of oestradiol benzoate injected on the 5th day. The ewes were run with a harnessed, vasectomised ram.

Rams were accustomed to the collection site surroundings by allowing them access to this area. Following this familiarization process, a teaser ewe was placed in a head-crush crate from which, the rear of the ewe extended far enough to allow mounting by the ram without interference. Each ram in turn, was allowed access to the ewe

without using the artificial vagina (AV).

Within a week, all rams were well adapted to ejaculating into the AV.

In the breeding season of 1975, hormonal treatment of spayed ewes was not adopted as naturally cycling ewes were available.

Rams accustomed to serving into the artificial vagina, did so regardless of the stage of the cycle of the ewe, and the presence of a ewe was usually sufficient to elicit mounting and ejaculation.

It was found essential that individual ram behaviour patterns be known to the collector, and that a constant routine be adhered to. Any deviation from the procedure to which the rams had adapted, meant either a prolonged period till ejaculation, or failure to collect a sample.

(b) Preparation of the Artificial Vagina.

Plate 2.1 illustrates the components of the artificial vagina.

The AV was prepared for use in the manner described by Milovanov (1960). The temperature most suited to all rams was between 40 and 45°C. Water-soluble jelly was used for lubrication.

Collection of semen was performed by deflection of the protruding penis into the end of the AV. Knowledge of the mating characteristics of each ram was sufficient to determine the success of a collection. The ram was removed from the collecting area when service was completed.

(c) Assessment of Semen Quality.

The collecting tube containing the semen was placed in a water bath at 35°C and stoppered with cotton wool. As soon as possible, a small drop of the semen was placed on a warm slide between two coverslips. A third coverglass was placed over the drop of semen and motility of the

sample scored on a 0 to 5 scale. A sample of indistinct waves with no motion scored 0; while 5 represented a sample of highly distinct and rapidly swirling waves.

A live/dead stain (Eosin-nigrosin) was prepared (Plate 2.2) and an haemocytometric determination of sperm cell concentration was conducted. In conjunction with this, a spectrophotometric reading of the amount of light absorbed by a semen sample diluted 1:500 with 2% saline, was made. Appendix I contains the regression analysis of spermatozoa concentration (Haemocytometer), on absorption reading. This regression was employed as a means of rapid assessment of sperm density when speed of operations was necessary.

Only those samples reaching a motility score of 4 or better, and a reading of 0.25 on the spectrophotometer ( $3 \times 10^9$  sperm per ml), and with 90% live cells, were used for inseminations.

#### INSEMINATION OF EWES

All inseminations were completed within one hour of semen collection. A duck-billed speculum and the inseminating pipette shown in Plate 2.3 were used.

Pooled semen samples from all rams were used throughout.

Mini-straws (0.25ml) were filled with semen using a disposable syringe. The straw was placed in the pipette and the extending tip was cut off. A volume of approximately 0.2ml of semen remained in the straw.

Each ewe was inseminated by placing her hind quarters 'over the rail' (Miller, 1961). For a vaginal insemination, the tip of the pipette was placed just within the cervical canal and semen flooded over the external os. An intra-uterine insemination involved the manipulation of the pipette to penetrate the cervix as deeply as

possible (up to 5 or 6 cm depth). The plunger was forced forward to expel the semen from the straw. Plate 2.4 shows an insemination in progress.

A note was made of the apparent success of each insemination, together with a brief description of the appearance of the cervical mucus.

After insemination, ewes were left undisturbed in covered pens or were run on paddocks close to the laboratory till the time of slaughter.

#### SLAUGHTER OF EWES

The animals were slaughtered at the prescribed times following a period off pasture. The abdomen was opened by a ventral incision through the mammary gland and the reproductive tract removed with minimum disturbance.

Ligatures were tied between the oviducts and uterus; uterus and cervix; and cervix and vagina.

#### POST-SLAUGHTER TREATMENT OF REPRODUCTIVE TRACTS

##### (a) Ovarian Observations.

The number and size of follicles and corpora lutea were recorded for each ovary. Abnormalities such as cystic or luteinised follicles were also noted.

##### (b) Flushing the Fallopian Tubes.

In experiment 1, each Fallopian tube was flushed from the uterine end with two 1ml aliquots of 0.9% NaCl. Air was forced through the tubes between each volume of saline.

Equivalent volumes of flushing fluid were placed onto a series of 5 slides, directly from the oviducts. A cover slip (22 x 50 mm) supported by vaseline was placed over the fluid and sperm allowed to settle for 20 minutes. Counting was performed on a light microscope

at X259 magnification. Between 4 and 10 traverses across the entire width of the fluid were made and the number of sperm on each slide calculated, according to the area observed.

In experiments 2 and 3, the procedure was modified to facilitate ovum recovery. Instead of flushing directly onto slides, the entire volume of oviduct washings was collected in a small glass bowl which was examined for the presence of ova. Eggs were removed in as small a volume as possible and placed onto separate slides which were then examined at high magnification (X450) for the presence of spermatozoa on the zona pellucida. The remaining flushings were stained with a few drops of 1% aqueous eosin, and evenly distributed onto a series of slides and examined as before for the number of spermatozoa.

In experiment 4 the same procedure was used, except that each Fallopian tube was severed into isthmus and ampulla. Each of these segments were flushed with 1ml of saline into a glass bowl.

(c) Flushing the Uterus.

Each side of the uterus was flushed with 5ml of saline. Due to the large numbers of sperm found, the number of sperm present was calculated from the mean of 5 haemocytometric determinations.

(d) Flushing the Cervix.

Each cervix was flushed from the uterine end with 10ml of saline. The washings were collected in a vial, vigorously shaken, and sperm numbers estimated by haemocytometer.

(e) Flushing the Vagina.

The anterior half of the vagina was opened and placed in the top of a small funnel. The exposed lumen was washed with 5ml of saline which drained into a collecting vial. Haemocytometric determination of sperm numbers in the flushings was performed after the addition of

aqueous eosin to aid identification of spermatozoa, as cellular material was often present.

(f) Ovum Recovery.

When an ovary showed signs of an ovulation, the corresponding Fallopian tube was flushed and examined for ova. The eggs were removed and placed on slides under a coverglass supported by four drops of a vaseline-paraffin mixture. Pressure was applied to the coverslip till the egg was trapped between the slide and coverglass. The ova were examined under a light microscope and most were photographed. Presence and the number of accessory spermatozoa were recorded, as was the stage of cleavage.

All reproductive tracts were flushed for sperm within two hours of slaughter.

PLATE 2.1 Components of the Artificial Vagina

- A. Lined protection cover. B. Collection tube  
C. Rubber liner. D. PVC casing.

PLATE 2.2 Photomicrograph of Spermatozoa after  
Live-dead Staining (eosin/nigrosin)  
(x900).

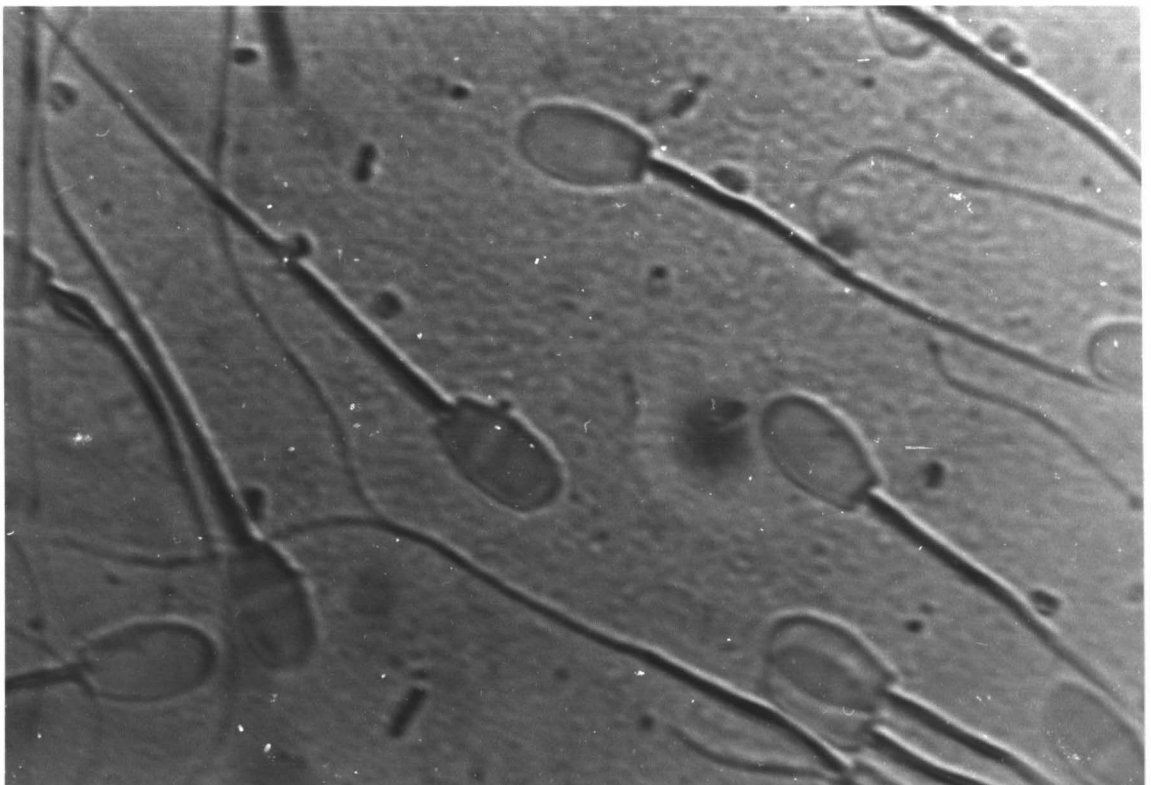
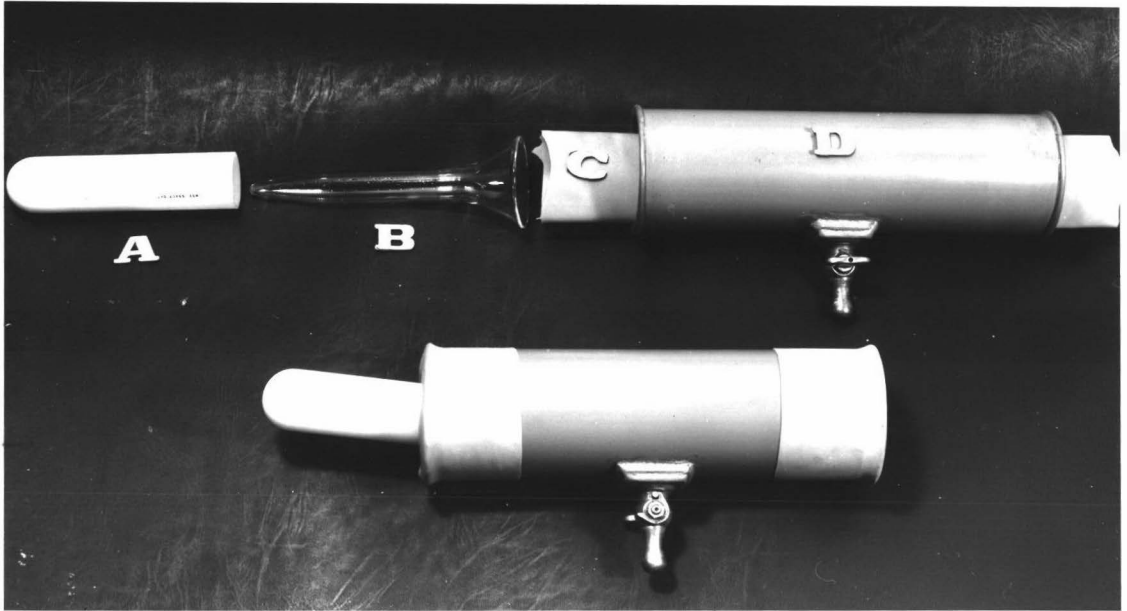


PLATE 2.3

Inseminating Instruments.

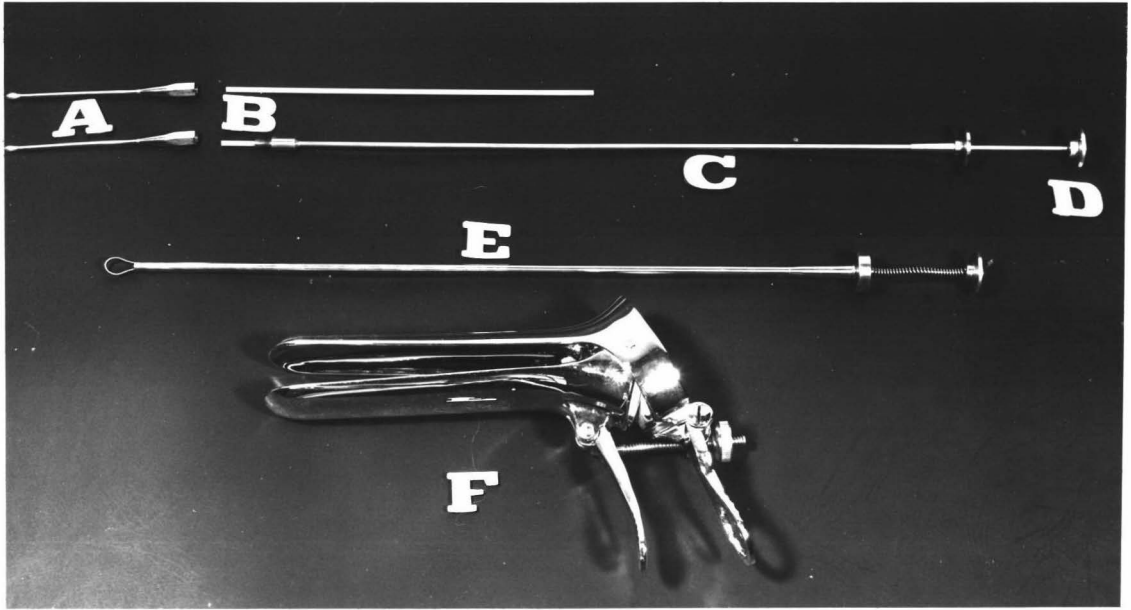
A. Detachable tip B. Extending straw.

C. Pippette barrel D. Plunger.

E. Cervical grips F. Vaginal Speculum

PLATE 2.4

Insemination in progress.



CHAPTER III

EVALUATION OF TECHNIQUES

## CHAPTER THREE

### EVALUATION OF TECHNIQUES

To gain experience in the use of techniques, two preliminary trials were conducted in July, 1974 (Experiment 1) and February-March 1975 (Experiment 2).

Among several aspects, the methods of recovery of spermatozoa from the reproductive tract and estimation of their numbers, received most attention.

#### EXPERIMENT 1

##### THE DISTRIBUTION OF SPERMATOZOA IN THE GENITAL TRACT AFTER ARTIFICIAL INSEMINATION EARLY OR LATE IN OESTRUS.

Recovery of spermatozoa from the reproductive tract by Mattner & Braden (1969b) has indicated that there is a difference in the distribution of numbers of sperm within the tracts of ewes inseminated early or late in oestrus. This may explain the several reports of the achievement of higher fertility to inseminations at various times during oestrus (e.g. Csikowski, 1956).

This trial was initiated to investigate sperm distribution in the cervix, uterus and Fallopian tubes of ewes after insemination early or late in oestrus.

#### Materials & Methods.

Thirteen Romney and Border Leicester X Romney ewes were treated with intravaginal sponges for 16 days. Harnessed, vasectomised rams were run with the ewes at the end of progestagen treatment. Observation for mating marks at 0800, 1200 and 1800 hours, allowed ewes to be allocated to groups described in Table 3.1.

Each ewe was inseminated at the external cervical os, with 0.2 ml of fresh, undiluted semen (approx.  $7 \times 10^8$  spermatozoa).

TABLE 3.1

TREATMENT GROUPS + EXPERIMENT 1

(n=3)

Group	Time of Insemination *	Interval Insemination to Slaughter (hrs)
1	Early	4
2	Early	24
3	Late	4
4	Late	24

\*Early = 0-12 hrs after onset of oestrus

Late = >18 hrs after onset of oestrus

Results.

Seven of the 13 ewes (56%) exhibited oestrus by the fifth day after sponge removal one on day 2; six on day 3.

One ewe was found to be in-lamb when slaughtered, and so all ewes not showing oestrus by day 7 were laparotomised.

Examination of the ovaries, showed that six ewes failed to exhibit oestrus for a variety of reasons, i.e. pregnancy (2 ewes), silent heat (1 ewe), lost sponge during treatment (1 ewe), absence of follicular development (2 ewes).

Spermatozoa were recovered from six ewes (Table 3.2) which exhibited oestrus. The animal that was inseminated and slaughtered

TABLE 3.2

NUMBERS OF SPERMATOZOA RECOVERED FROM  
THE GENITAL TRACTS OF EWES IN EXPERIMENT 1

Group	Tag Number of ewe	Number of spermatozoa recovered from:		
		Cervix	Uterus	Fallopian Tube
1	129	162,500	62,500	0
	257	3,500	0	0
2	318	160,000	40,000	30,000
3	297	1,087,500	0	110
4	134	225,000	50,000	0
	502	7,500	0	0

and found to be pregnant, did not yield spermatozoa from the cervix.

The flushings from the first animal (257), were collected in a vial and stored immediately under deep freeze. After thawing, counts were very low in the cervix and non-existent in the Fallopian tubes. Due to the difficulty of detecting spermatozoa, this method was subsequently abandoned in favour of the direct slide counting technique already described.

There were no significant effects of Time of insemination or Time of slaughter, on the number of sperm recovered from any segment of the tract.

#### Discussion.

In ewe 313, (inseminated in early oestrus and slaughtered 24 hours later), sperm were distributed throughout the tract. But in late-inseminated ewes, spermatozoa had not reached the Fallopian tubes in high numbers. At 4 hours after insemination, 'early' ewes had sperm in the uterus, but 'late' ewes did not.

These trends are in agreement with the data of Mattner & Braden (1969b). They suggested the difference in spermatozoa distribution to be a result of the increased viscosity of cervical mucus late in oestrus causing slower progression of sperm up the tract. No assessment of the amount or viscosity of mucus was made in the present study.

#### EXPERIMENT 2

THE EFFECT OF ROAD TRANSPORT IMMEDIATELY AFTER ARTIFICIAL INSEMINATION, ON THE DISTRIBUTION OF SPERMATOCOA IN THE REPRODUCTIVE TRACT OF THE EWE

Physiological effects of stress imposed on the ewe, may influence spermatozoa in the genital tract, through inhibition of contractions of the uterus.

The stress imposed by a 24 hour train journey, induced ovulations in the anoestrous Merino (Braden & Moule, 1964), and Mattner (1963a) has demonstrated following natural mating of 'conditioned' and 'unconditioned' ewes, significantly fewer sperm in the Fallopian tubes of the latter group, 15 minutes after coitus. By 4 and 48 hours after mating, the difference was undetectable.

On the basis of these results Mattner concluded that only the rapid transport of spermatozoa was affected by the stress, and overall there was no difference in the gradual progression of spermatozoa from the cervix to the site of fertilization. A cervical reservoir was established within a short time and only within 15 minutes of mating were tract contractions sufficiently reduced to cause an impairment of sperm transport mechanisms.

No studies appear to have been reported on the effect of stress on sperm transport, after artificial insemination in sheep. If the stress reduces sperm transport in the naturally mated ewe, then its presence during insemination, or soon after, may in part contribute to poor fertilization results.

The second preliminary trial was conducted to determine whether the conveyance of ewes by truck to an abattoir, after artificial insemination had any effect on sperm transport to the Fallopian tubes.

#### Materials & Methods.

Twelve Romney ewes were treated with intravaginal sponges for 7 days, and each received 500iu PMSG at sponge removal. All ewes were inseminated with 0.2 ml of fresh, undiluted semen, 48 hours after PMSG.

Oestrus detection by vasectomised rams was omitted to avoid the effect of mounting and mating on sperm transport that has been observed in sheep by Restall (1961).

Ewes were allocated at random, to two groups of six. The 'Trucked' group were inseminated at the field laboratory and then trucked by road (20 minutes) to an abattoir where they were slaughtered at a time corresponding to 4 hours after insemination. The second group (Non-trucked) were inseminated and slaughtered at the field laboratory.

### Results.

Follicular stimulation following FMSG was not great. The average number of large follicles per ewe in the two groups was 2.0 and 2.5 ( $P > 0.1$ ). No ovulations had occurred at slaughter.

Not all ewes yielded spermatozoa from every region of the tract. Failure to recover sperm from any segment of the tract of ewe 823 necessitated that analysis be confined to only 5 sheep in the non-trucked group.

More ewes yielded sperm from the cervix in trucked than non-trucked groups (6/6 vs 2/5 respectively). The difference approached a significant level, ( $\chi^2 = 4.72$ ;  $0.05 < P < 0.1$ ).

Sperm were recovered from the uteri of all ewes, and from the oviducts of 4 ewes in both groups (Table 3.3).

A comparison of the mean numbers of sperm in the oviducts of all ewes in each group, failed to show a significant difference, ( $P > 0.1$ ). However, the means based on only those ewes from which tubal sperm were recovered, showed that more sperm had reached the Fallopian tubes in the non-trucked sheep ( $P < 0.02$ , Table 3.4). No differences were detected between the mean numbers of sperm in the uterus or cervix of trucked and non-trucked ewes.

### Discussion

The stress imposed by transporting sheep by road immediately after artificial insemination, had reduced sperm numbers in the

TABLE 3.3

ESTIMATED NUMBERS OF SPERMATOZOA RECOVERED  
 FROM THE REPRODUCTIVE TRACTS OF EWES  
 SLAUGHTERED 4 HOURS AFTER INSEMINATION.  
 (EXPERIMENT 2)

TRUCKED EWES (n-6)				NON-TRUCKED EWES (n-6)			
Ewe Tag Number	Cervix	Uterus	Fallopian Tube	Ewe Tag Number	Cervix	Uterus	Fallopian Tube
472	3,722,500	9,260,000	5412	305	4,300,000	226,000	0
106	280,000	140,000	1390	535	25,000	960,000	21,205
530	5,120,000	180,000	0	192	0	185,000	7,093
766	135,000	110,000	0	107	0	136,540	8684
186	225,000	70,000	1320	793	0	50,000	12,724
755	112,500	111,500	3745	823	0	0	0

TABLE 3.4  
 NUMBERS OF SPERMATOZOA RECOVERED FROM  
 WITHIN THE REPRODUCTIVE TRACTS OF TRUCKED AND NON-TRUCKED EWES  
 (EXPERIMENT 2)

Reproductive Tract Segment	TRUCKED		NON-TRUCKED		Significance level
	Number Ewes with Sperm	Mean Number Sperm $\pm$ SE <sup>+</sup>	Number Ewes with Sperm	Mean Number Sperm $\pm$ SE <sup>+</sup>	
Cervix	6	512,800 $\pm$ 199,600	2	327,800 $\pm$ 131,200	N.S.
Uterus	6	241,800 $\pm$ 209,000	5	194,000 $\pm$ 161,000	N.S.
Fallopian tubes	4	24.69 $\pm$ 14.25	4	11,350 $\pm$ 1272	<0.02

<sup>+</sup> Adjusted means, reversion from log<sub>10</sub> data

N.S. = Not significant

Fallopian tubes.

Spermatozoa migrate through the uterus by means of uterine contractions (Dauzier & Winterberger, 1952; Mattner & Braden, 1963). The release of endogenous adrenaline caused by the imposed stress, may be sufficient to disrupt uterine migration by spermatozoa, and hence the numbers in the Fallopian tubes were reduced.

Mattner (1963a) considered this effect to be transitory, lasting for 15 minutes, but not 4 hours, after natural mating.

The present study indicates that the effect of stress lasts for at least 4 hours in the artificially bred ewe.

On the basis of this preliminary experiment, it was decided that insemination and slaughter of ewes would take place at the same location.

#### CONCLUSIONS

The great variability in recovery and estimation of numbers of sperm in the reproductive tract was a notable feature of both experiments.

The experience gained, suggested several areas where careful attention in technique is necessary:

- (a) The success of an insemination into the cervix might influence the estimated sperm numbers recovered soon after AI. The flooding or penetration of the cervical os would result in greater cervical sperm than an insemination into the anterior vagina.
- (b) A very low percentage of cells deposited, are recovered.
- (c) The method of direct counting immediately after slaughter, limited the number of tracts that could be analysed effectively on one day. (Between 6 and 8 tracts by one investigator).
- (d) Stress imposed on the ewe can reduce the transport of sperm to the upper reproductive tract.

CHAPTER IV

EXPERIMENT 3

SPERMATOCYTES IN THE REPRODUCTIVE TRACT AFTER ARTIFICIAL  
INSEMINATION WITH FRESH, UNDILUTED SEMEN

## CHAPTER FOUR

### SPERMATOCYTES IN THE REPRODUCTIVE TRACT

#### AFTER ARTIFICIAL INSEMINATION WITH

#### FRESH, UNDILUTED SEMEN

This trial investigated the distribution of spermatozoa in the tract following artificial insemination at predetermined times after hormone treatment and without the detection of oestrus.

#### MATERIALS & METHODS

##### (a) General

Seventy-two 6- and 7-year-old Romney ewes were treated with intravaginal sponges for 14 days.

Each ewe was intramuscularly injected with 500i.u. HCG 24 hours after the removal of sponges. On the basis of previous work (Dziuk & Coworkers) the expected time of ovulation was predicted to occur 30 hours after HCG.

##### (b) Experimental Design

A completely randomized blocks design (2 Blocks) incorporating a 2 X 3 X 2 factorial, was used. The factors were:

- (1) Site of Insemination.....Vaginal (just within the external cervical os) vs Uterine (Deep as possible into cervical canal).
- (2) Time of Insemination.....Single insemination 12 hours before expected time of ovulation (-12), vs single insemination 12 hours after ovulation (+12), vs two inseminations (one before and one after ovulation ( $\pm$  12)).
- (3) Time of Slaughter after insemination..... 4 hrs vs 24 hrs.

Figure 4.1 shows the daily experimental procedures for block 1, and Figure 4.2 indicates the sequence of inseminations and slaughter,

FIGURE 4.1

PLAN OF EXPERIMENT 3 (BLOCK 1)

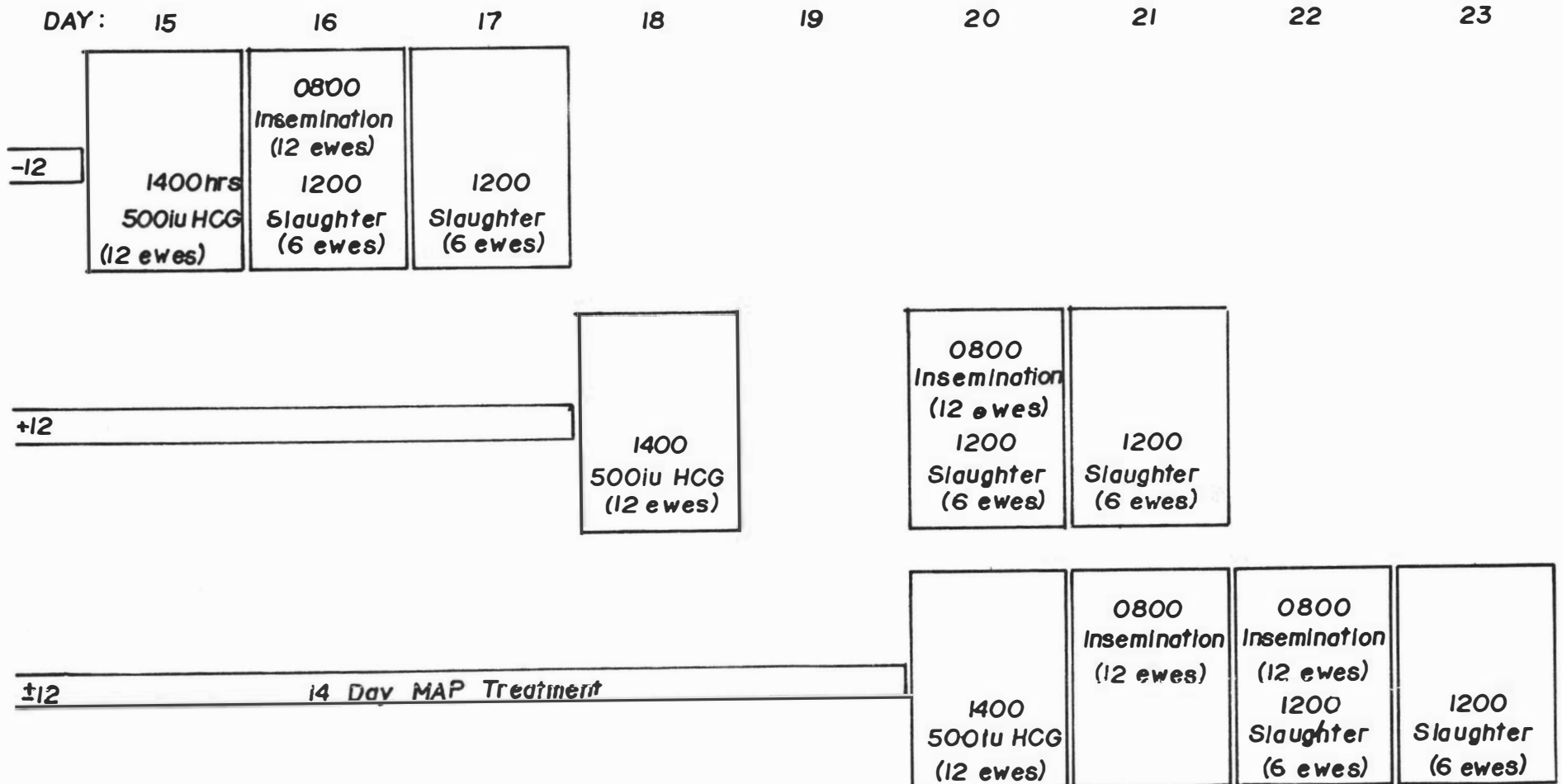
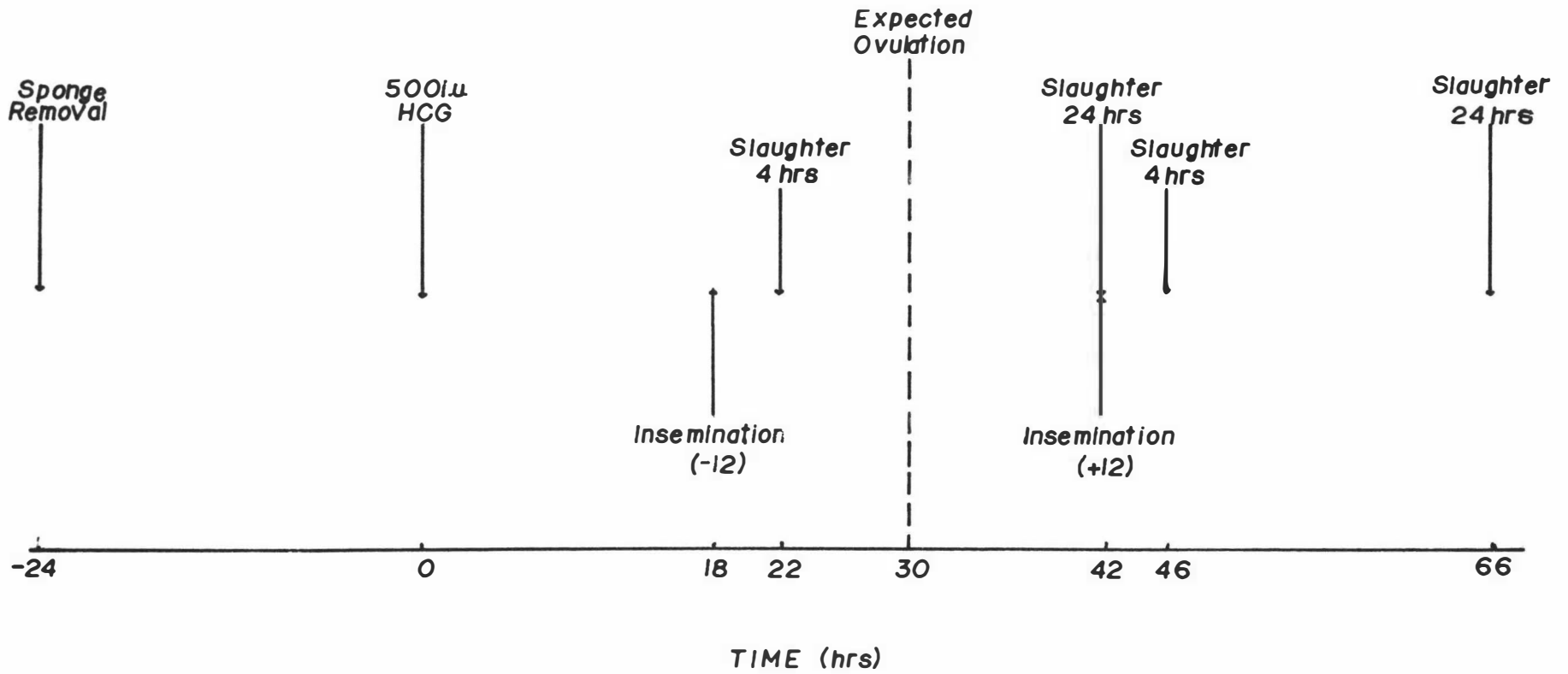


FIGURE 4.2

SEQUENCE OF PROCEDURES RELATIVE TO  
ADMINISTRATION OF HCG:- EXPERIMENT 3



relative to the administration of HCG.

At each single insemination, between  $7.4$  and  $8.0 \times 10^9$  spermatozoa were deposited; at each of the two inseminations, half this number were inseminated ( $3.3$  to  $3.8 \times 10^9$ ).

(c) Statistical analysis.

Data were examined after logarithmic transformation (sperm count + 1) to overcome zero values. Unless otherwise stated, analyses of variance were performed on the transformed data using the following linear model:

$$Y_{jklmn} = \mu + R_j + S_k + T_l + K_m + (ST)_{kl} + (SK)_{km} + (TK)_{lm} + (STK)_{klm} + e_{jklmn}$$

where,  $Y$  is the transformed number of sperm recovered from the segment of the tract of  $n$ th ewe in the  $j$ th block receiving the  $klm$ th treatment combination.

$n = 1, 2, \text{ or } 3.$

$\mu$  is a common mean

$R_j$  is the  $j$ th effect of Block:  $j = 1, 2$

$S_k$  is the  $k$ th effect of Site of insemination;  $k = 1, 2$

$T_l$  is the  $l$ th effect of Time of insemination;  $l = 1, 2, 3$

$K_m$  is the  $m$ th effect of Time of Slaughter;  $m = 1, 2$

Factors in parentheses are interactions

$e_{jklmn}$  is the random error associated with each observation.

The following component analysis (Fixed factors, Model 1--Snedecor, 1956), was used for testing variance ratios.

<u>Source of Variation</u>	<u>Expectation of Mean Squares</u>
Blocks	$\sigma^2 + klmn\sigma^2$
Site of Insemination (Site)	$\sigma^2 + jlmn\sigma^2$
Time of Insemination (Time)	$\sigma^2 + jkmn\sigma^2$
Time of Slaughter (Slaughter)	$\sigma^2 + jlmn\sigma^2$
Site X Time	$\sigma^2 + jmn\sigma^2$
Site X Slaughter	$\sigma^2 + jln\sigma^2$
Time X Slaughter	$\sigma^2 + jkn\sigma^2$
Site X Time X Slaughter	$\sigma^2 + jn\sigma^2$
Error	$\sigma^2$

$$F = \frac{\text{Mean Square}}{\sigma^2}$$

## RESULTS

### I. TIME OF OVULATION

The 12 ewes slaughtered 22 hours after HCG were not expected to have ovulated, but one had done so (Table 4.1). The ovum recovered from this ewe still retained its cumulus cells, indicating ovulation very close to the time of slaughter.

Synchronized ovulations appeared to have occurred. Firstly, the incidence of ewes ovulating did not differ significantly between slaughter at 42 and >46 hours after HCG (Table 4.2). Secondly, the appearance of corpora lutea on ovaries of ewes slaughtered 66 hours after HCG, suggested that ovulation had occurred substantially before the time of slaughter.

TABLE 4.1  
 INCIDENCE OF OVULATION AT INTERVALS  
 AFTER HCG - EXPERIMENT 3

Interval from HCG to slaughter (hrs)	Number of ewes	Number of ewes ovulating	Percentage of ewes ovulating
22	12	1	8.3
42	12	11	91.7
46	24	19	79.2
66	24	17	71.0
Σ 42-66	60	47	78.3

TABLE 4.2  
 EFFECT OF INTERVAL FROM HCG TO SLAUGHTER  
 ON THE NUMBER OF EWES OVULATING

Interval from HCG to slaughter (hrs)	Number of Ewes: With      Without C.L.      C.L.		Total Number of Ewes	% Ovulations
42	11	1	12	91.7
>46	36	12	48	75.0

C.L. = Corpora Lutea

Chi<sup>2</sup> = 0.743 - Not Significant

Each of the 11 ewes which had not ovulated 22 hours after HCG contained at least one large follicle, the majority of which were pre-ovulatory in appearance.

Twelve ewes had not ovulated as expected at slaughter; 5 had two quiescent ovaries; 4 had been slaughtered just prior to ovulation (each of these ewes had preovulatory follicles); and 3 showed little ovarian stimulation (only one small or medium follicle).

Of the 4 ewes which had not ovulated but contained preovulatory follicles, 3 were from treatments slaughtered at 46 hours after HCG.

Ewes inseminated 12 hours before ovulation, generally showed thin, clear mucus in the vagina, similar to that found in early oestrus during a normal cycle. Observation of cervical mucus 12 hours after ovulation, tended to show a thicker, less abundant mucus.

## II. RECOVERY OF SPERMATOCYTES

### (a) From the Vagina.

Spermatozoa were not recovered from the vagina of any ewe inseminated into the uterus.

Table 4.3 shows the mean numbers of spermatozoa recovered from vaginally inseminated ewes. More sperm were found in the vagina at 4 hours ( $5.908 \pm 0.084$ ) than at 24 hours ( $4.922 \pm 0.310$ ) ( $P < 0.025$ ).

### (b) From the Cervix.

Table 4.4 shows the effect of Site of insemination on the number of ewes from which sperm were recovered from the cervix. The 5 ewes that did yield cervical sperm after insemination into the uterus, all had cervixes in excess of 7.0cm length. The 31 ewes that did not have sperm in the cervix all had cervixes of 6.0cm or less.

TABLE 4.3

NUMBERS (TRANSFORMED) OF SPERMATOCYTES RECOVERED FROM  
THE ANTERIOR VAGINA OF VAGINALLY INSEMINATED EWES (n=6)

Time of Insemination	Time of Slaughter (hrs after Insemination)	Numbers of Sperm Recovered (Mean $\pm$ S.E.)
-12	4	6.111 $\pm$ 0.154
	24	5.276 $\pm$ 0.095
+12	4	5.797 $\pm$ 0.084
	24	5.492 $\pm$ 0.123
+12	4	5.815 $\pm$ 0.170
	24	4.209 $\pm$ 0.875

ANALYSIS OF VARIANCE

Source of Variation	d.f.	Mean Squares
Blocks	1	1.775 N.S.
Time	2	1.755 N.S.
Slaughter	1	7.548 *
Time X Slaughter	2	1.261 N.S.
Error	29	0.818

N.S. Not Significant

\* P < 0.05

TABLE 4.4  
EFFECT OF SITE OF INSEMINATION ON THE NUMBER  
OF EWES YIELDING SPERM FROM THE CERVIX

Site of Insemination	Number Yielding sperm From Cervix	of Ewes: Not yielding sperm From Cervix	Total Number of ewes	Ewes Yielding Sperm
Uterus	5	31	36	13.9
Vagina	35	1	36	99.2

$\chi^2 = 50.625, P < 0.005$

The transformed mean numbers of sperm recovered from the cervixes of vaginally inseminated ewes, are given in Table 4.5. The analysis of variance revealed no sources of significant variation, but 50% of total variance was due to the Time of Slaughter.

(c) From the Uterus.

Preliminary analysis of the numbers of sperm recovered from the uterus showed that significant heterogeneity of variance existed (See Appendix IIa). The most appropriate way of examining the data was therefore to "pool", in each block, the three observations per cell and analyse these means. Table 4.6 records these data and the analysis of variance.

Flushing tracts 4 hours after a vaginal insemination resulted in fewer sperm in the uterus, than were present after 24 hours, or at either time after intra-uterine deposition (Table 4.7).

Table 4.8 shows that it was the Site of Insemination X Time of Slaughter that varied with the Time of insemination.

Fewest sperm were in the uterus 4 hours after a single

TABLE 4.5

NUMBERS (TRANSFORMED) OF SPERMATOCYTES RECOVERED FROM  
THE CERVIX OF VAGINALLY INSEMINATED EWES (n =6)

Time of Insemination	Time of Slaughter (hrs after Insemination)	Number of sperm Recovered (Mean $\pm$ SE)
-12	4	5.289 $\pm$ 0.185
	24	5.130 $\pm$ 0.119
+12	4	5.238 $\pm$ 0.129
	24	4.919 $\pm$ 0.071
$\pm$ 12	4	5.367 $\pm$ 0.107
	24	4.541 $\pm$ 0.917

ANALYSIS OF VARIANCE

Source of Variation	d.f.	Mean Squares	Partition of Variation (%)
Blocks	1	0.1577 N.S.	4.7
Time	2	0.1984 N.S.	5.9
Slaughter	1	1.6977 N.S.	50.4
Time X Slaughter	2	0.3645 N.S.	10.8
Error	29	0.9496	28.2
			<u>100.0</u>

N.S. Not Significant

TABLE 4.6

NUMBER (TRANSFORMED) OF SPERMATOZOA RECOVERED FROM THE UTERUS  
(EXPERIMENT 3)

Site of Insemination	Time of Insemination	Interval to Slaughter (hrs)	Block 1	Block 2	
Vagina	-12	4	1.593	1.687	
		24	5.379	5.330	
	+12	4	1.684	1.629	
		24	5.095	5.259	
	±12	4	3.061	5.218	
		24	5.279	3.147	
	Uterus	-12	4	5.643	5.616
			24	5.224	5.430
+12		4	5.344	5.259	
		24	5.238	4.926	
±12		4	5.304	5.537	
		24	4.834	4.783	

ANALYSIS OF VARIANCE

Source of Variation	d.f.	Mean Squares	
Blocks	1	0.001	N.S.
Site	1	14.681	xxxx
Time	2	0.229	N.S.
Slaughter	1	6.347	xxx
Site X Time	2	0.614	N.S.
Site X Slaughter	1	11.886	xxxx
Time X Slaughter	2	2.533	x
Site X Time X Slaughter	2	1.707	0.05 < P < 0.1
Error	11	0.430	

N.S. Not Significant  
 x P < 0.05  
 xxx P < 0.005  
 xxxx P < 0.001

TABLE 4.7

THE INTERACTION OF SITE OF INSEMINATION AND TIME OF  
SLAUGHTER ON THE NUMBERS OF SPERM RECOVERED FROM THE UTERUS  
(EXPERIMENT 3)

Site of Insemination	Time of Slaughter (hrs after insemination)	
	4	24
Vagina	2.470 $\pm$ 0.595 $\neq$	4.915 $\pm$ 0.356
Uterus	5.451 $\pm$ 0.069	5.073 $\pm$ 0.107

$\neq$  Mean  $\pm$  S.E.

ANALYSIS OF VARIANCE

Source of Variation	d.f.	Mean Squares
Between Sites Within 4hrs	1	26.490 <b>xxxx</b>
Between Sites Within 24hrs	1	0.080 N.S.
Error	11	0.430

N.S. Not Significant

**xxxx** P < 0.001

vaginal insemination. Two inseminations into the vagina resulted in fewer sperm at 4 and 24 hours, than were found 24 hours after a single insemination at this site (Table 4.8).

Following uterine deposition of semen, spermatozoa were recovered in similar numbers from the uterus at both times of recovery, regardless of the placement of one, or two lots of semen.

The superiority of uterine inseminations occurred within 4 hours of deposition of semen, but by 24 hours, sperm had migrated to the uterus in similar numbers, irrespective of the site at which they were deposited.

(d) From the Fallopian Tubes.

Equivalent numbers of sperm were recovered from both oviducts of each tract, (see Appendix IIb). Results are therefore presented as the number of sperm recovered from both Fallopian tubes.

The variability of the data was highly heterogenous (Appendix IIc). 'Pooling' observation within each cell of each block resulted in the data given in Table 4.9. The analysis of variance disclosed a significant Site of insemination X Time of slaughter interaction.

A short interval from vaginal insemination to slaughter, resulted in few sperm in the Fallopian tubes. Only one of 12 ewes inseminated once at the cervical os, yielded sperm from the tubes 4 hours after insemination. All ewes receiving two lots of semen in the vagina had sperm recovered from the oviducts.

Compared to vaginal deposition of semen, intrauterine insemination resulted in a 4-fold increase in sperm in the Fallopian tubes 4 hours after a single insemination. However, by 24 hours, the numbers of sperm transported to the tubes were equivalent, for

TABLE 4.8

THE INTERACTION OF SITE OF INSEMINATION AND  
TIME OF SLAUGHTER WITH TIME OF INSEMINATION

Time of Insemination	Time of Slaughter after Insemination (hrs)	Site of Insemination	
		Vagina	Uterus
-12	4	1.640 ± 0.047	5.629 ± 0.014
	24	5.355 ± 0.025	5.327 ± 0.103
+12	4	1.657 ± 0.028	5.302 ± 0.043
	24	5.177 ± 0.082	5.082 ± 0.156
±12	4	4.139 ± 1.079	5.421 ± 0.117
	24	4.213 ± 1.066	4.809 ± 0.026

± Mean ± S.E.

ANALYSIS OF VARIANCE

Source of Variation	d.f.	Mean	Squares
Between Sites Within 4hrs for Time -12	1	15.916	****
Between Sites Within 24hrs for Time -12	1	0.001	N.S.
Between Sites Within 4hrs for Time +12	1	13.286	****
Between Sites Within 24hrs for Time +12	1	0.009	N.S.
Between Sites Within 4hrs for Time ±12	1	1.640	0.05 < P < 0.1
Between Sites Within 24hrs for Time ±12	1	0.355	N.S.
Error	11	0.430	

N.S. Not Significant

\*\*\*\* P < 0.001

TABLE 4.9

NUMBERS (TRANSFORMED) OF SPERMATOZOA RECOVERED FROM THE OVIDUCTS  
(EXPERIMENT 3)

Site of Insemination	Time of Insemination	Interval to Slaughter (hrs)	Block 1	Block 2
Vagina	-12	4	0.000	0.000
		24	3.599	3.282
	+12	4	1.053	0.000
		24	2.986	1.025
	±12	4	1.099	2.021
		24	3.643	2.652
Uterus	-12	4	3.785	2.309
		24	2.010	3.089
	+12	4	3.300	3.279
		24	2.399	2.035
	±12	4	2.262	3.449
		24	4.029	3.809

ANALYSIS OF VARIANCE

Source of Variation	d.f.	Mean Squares
Blocks	1	0.4305 N.S.
Site	1	8.6338 ***
Time	2	1.5693 N.S.
Slaughter	1	6.0010 **
Site X Time	2	0.1252 N.S.
Site X Slaughter	1	8.1737 ***
Time X Slaughter	2	0.9631 N.S.
Site X Time X Slaughter	2	1.2712 N.S.
Error	11	0.5373

N.S. Not Significant  
 \*\* P < 0.01  
 \*\*\* P < 0.005

both sites of insemination (Table 4.10).

Classification of ewes into four groups according to the number of spermatozoa recovered from the oviducts is shown in Table 4.11.

Four hours after insemination into the vagina, all ewes had either none, or low numbers of spermatozoa in the tubes. At the same time after uterine deposition of semen, ewes were distributed in all categories, with the majority falling in the moderate and high classes (Table 4.11). Twenty-four hours after insemination, the distribution were similar for both sites of insemination.

(e) Total Sperm Recovered.

An average of 0.09% (range 0.005 to 0.84%) of sperm inseminated, were recovered from the entire reproductive tract. Table 4.12 shows the mean percentage sperm recovered for each treatment group.

A greater recovery occurred in vaginal (0.11%) than in uterine (0.04%) inseminated ewes ( $P < 0.005$ ) and at 4 than at 24 hours after insemination (0.1% vs 0.05%,  $P < 0.005$ ). The interaction approached statistical significance ( $0.05 < P < 0.1$ ). Recovery of sperm after a single insemination was higher than after two inseminations (0.08 vs 0.06%;  $P < 0.05$ ).

### III. THE DISTRIBUTION OF SPERM IN THE WHOLE REPRODUCTIVE TRACT

Figure 4.3 shows the mean numbers of sperm recovered from each segment of the tract after single or double inseminations.

(a) Vaginal Insemination.

Four hours after a single insemination, a marked caudal to cranial decline occurred in the reproductive tract (Figure 4.3a). At the same time after two inseminations, this decline was less noticeable due to the higher uterine sperm population (Figure 4.3b).

Twenty-four hours after a single insemination, the decline

TABLE 4.10

THE INTERACTION OF SITE OF INSEMINATION AND TIME  
OF SLAUGHTER ON NUMBERS OF SPERM IN THE OVIDUCTS  
(EXPERIMENT 3)

Site of Insemination	<u>Time of Slaughter after Insemination</u>	
	4 hrs	24 hrs
Vagina	0.696 $\pm$ 0.342 $\neq$	2.865 $\pm$ 0.398
Uterus	3.064 $\pm$ 0.257	2.895 $\pm$ 0.362

$\neq$  Mean  $\pm$  S.E.

ANALYSIS OF VARIANCE

Source of Variation	d.f.	Mean Squares
Between Sites within 4hrs	1	16.829 <b>xxxx</b>
Between Sites within 24hrs	1	0.003 N.S.
Error	11	0.537

N.S. Not Significant

**xxxx** P < 0.001

TABLE 4.11

THE DISTRIBUTION OF EWES CLASSIFIED ACCORDING TO THE  
NUMBER OF SPERMATOCYTES RECOVERED FROM THE FALLOPIAN TUBES

Time of Slaughter (hrs after Insemination)	Site of Insemination		<u>Numbers of Spermatozoa in Tubes</u>			
			High ( $>5000$ )	Moderate ( $2000-4999$ )	Low ( $\leq 2000$ )	None
4	Vagina	No. of Ewes:	0	0	4	14
		Percent:	0	0	22.2	77.8
	Uterus	No. of Ewes:	3	8	5	2
		Percent:	16.7	44.4	27.8	11.1
24	Vagina	No. of Ewes:	3	5	8	2
		Percent:	16.7	27.8	44.4	11.1
	Uterus	No. of Ewes:	6	1	8	3
		Percent:	33.3	5.6	44.4	16.7

TABLE 4.12

TREATMENT EFFECTS ON THE PERCENT RECOVERY OF SPERMATOCYTES FROM THE GENITAL TRACT

Site of Insemination	Time of Insemination	Time of Slaughter (hrs after Insemination)	% Sperm Recovered (Mean $\pm$ S.E.) (Angular Transformation)	
Vagina	-12	4	2.868	$\pm$ 0.547
		24	1.815	$\pm$ 0.173
	+12	4	1.925	$\pm$ 0.160
		24	1.392	$\pm$ 0.223
	$\pm$ 12	4	2.183	$\pm$ 0.458
		24	1.330	$\pm$ 0.326
Uterus	-12	4	1.613	$\pm$ 0.221
		24	0.973	$\pm$ 0.087
	+12	4	1.018	$\pm$ 0.107
		24	0.805	$\pm$ 0.170
	$\pm$ 12	4	1.130	$\pm$ 0.085
		24	1.177	$\pm$ 0.143

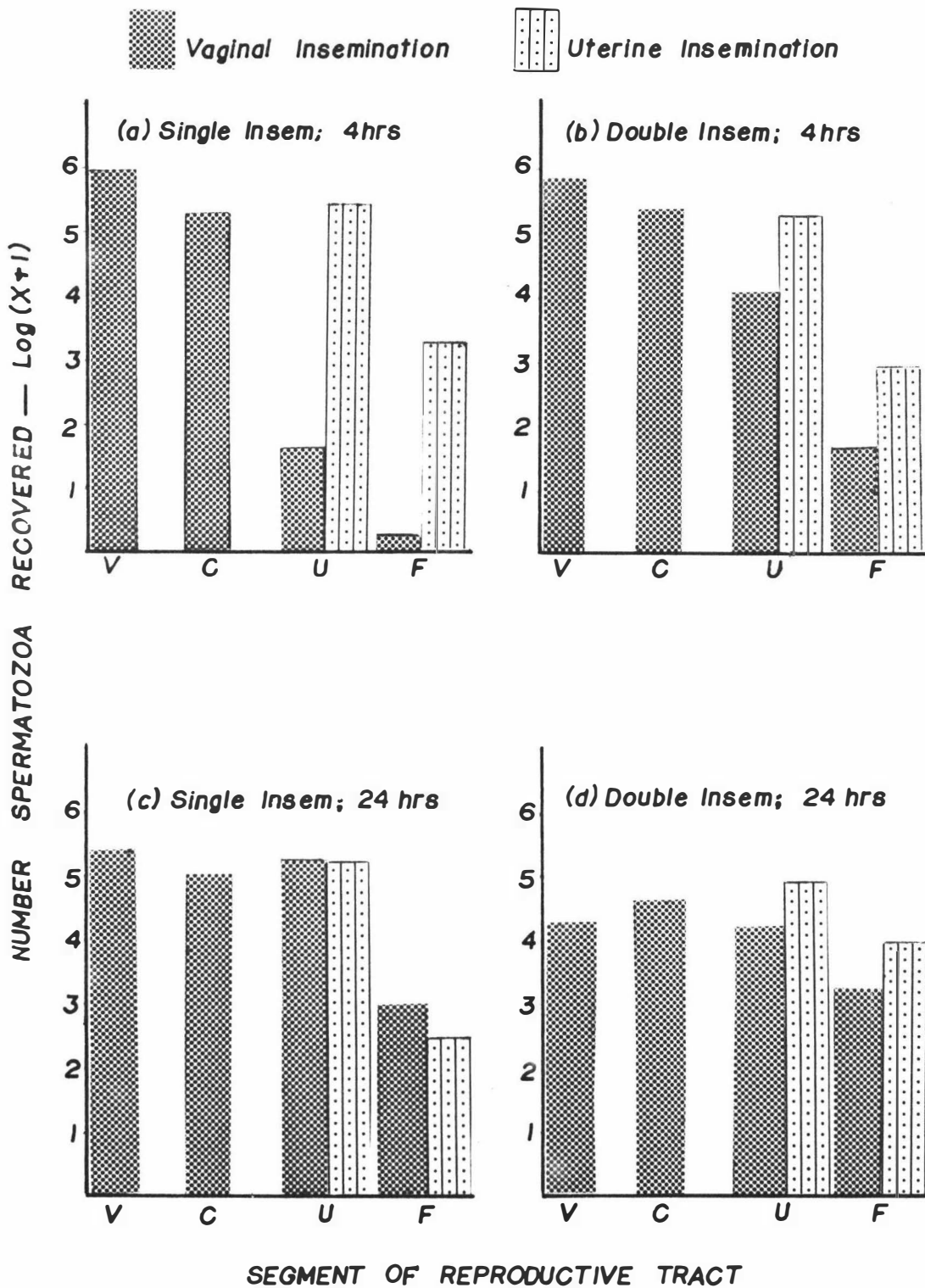
ANALYSIS OF VARIANCE

Source of Variation	d.f.	Mean Squares	
Site	1	11.504	xxx
Time	2	1.776	x
Slaughter	1	5.270	xxx
Site X Time	2	0.310	N.S.
Site X Slaughter	1	1.330	0.05 < P < 0.1
Time X Slaughter	2	0.384	N.S.
Site X Time X Slaughter	2	0.125	N.S.
Error	59	0.130	

N.S. Not Significant  
 x P < 0.05  
 xxx P < 0.005

FIGURE 4.3

DISTRIBUTION OF SPERM IN THE REPRODUCTIVE TRACT  
(Experiment 3)



V = Vagina C = Cervix U = Uterus F = Fallopian Tubes

existed only between the uterus and the oviducts (Figure 4.3c). Following a double insemination, there was little difference between regions (Figure 4.3d).

Tubal spermatozoa numbers were high 24 hours after insemination (single or double), due to the high uterine sperm numbers at this time (Figure 4.3c,d).

(b) Uterine Insemination.

Spermatozoa were in the uterus in similar numbers irrespective of number of inseminations or time of slaughter. Only 24 hours after the second of two inseminations, was the difference between uterine and tubal sperm number reduced.

Overall, uterine inseminations resulted in higher numbers of sperm in the uterus and Fallopian tubes 4 hours after insemination, but not at 24 hours.

#### IV. OVUM RECOVERY AND FERTILIZATION

Table 4.13 shows that from the 47 ewes which ovulated, 56 ovulations were recorded. Thirty (59%) ova were recovered, of which, 9 (27.3%) had sperm attached to, or in, in the zona pellucida. Only one ovum was cleaved.

There tended to be a greater recovery of ova with sperm on the zona pellucida at 24 hours than at 4 hours after insemination ( $0.1 < P < 0.25$ ).

The relationship between the proportion of ova recovered with sperm on or in the zone pellucida and the number of spermatozoa per tube is given in Figure 4.4.

TABLE 4.13

INCIDENCE OF THE RECOVERY OF OVA WITH AND WITHOUT SPERM ON OR IN THE ZONA PELLUCIDA

Site of Insemination	Time of Insemination	Time of Slaughter (hrs. after Insemination)	Number of ova Recovered	Number of Ova with Sperm on Zona
Vagina	-12	4	1	0
		24	3	2
	+12	4	3	0
		24	4	1
	±12	4	2	0
		24	5	2*
Uterus	-12	4	0	0
		24	4	1
	+12	4	2	0
		24	3	1
	±12	4	4	1
		24	2	1

\* 1 ovum cleaved (2 cells)

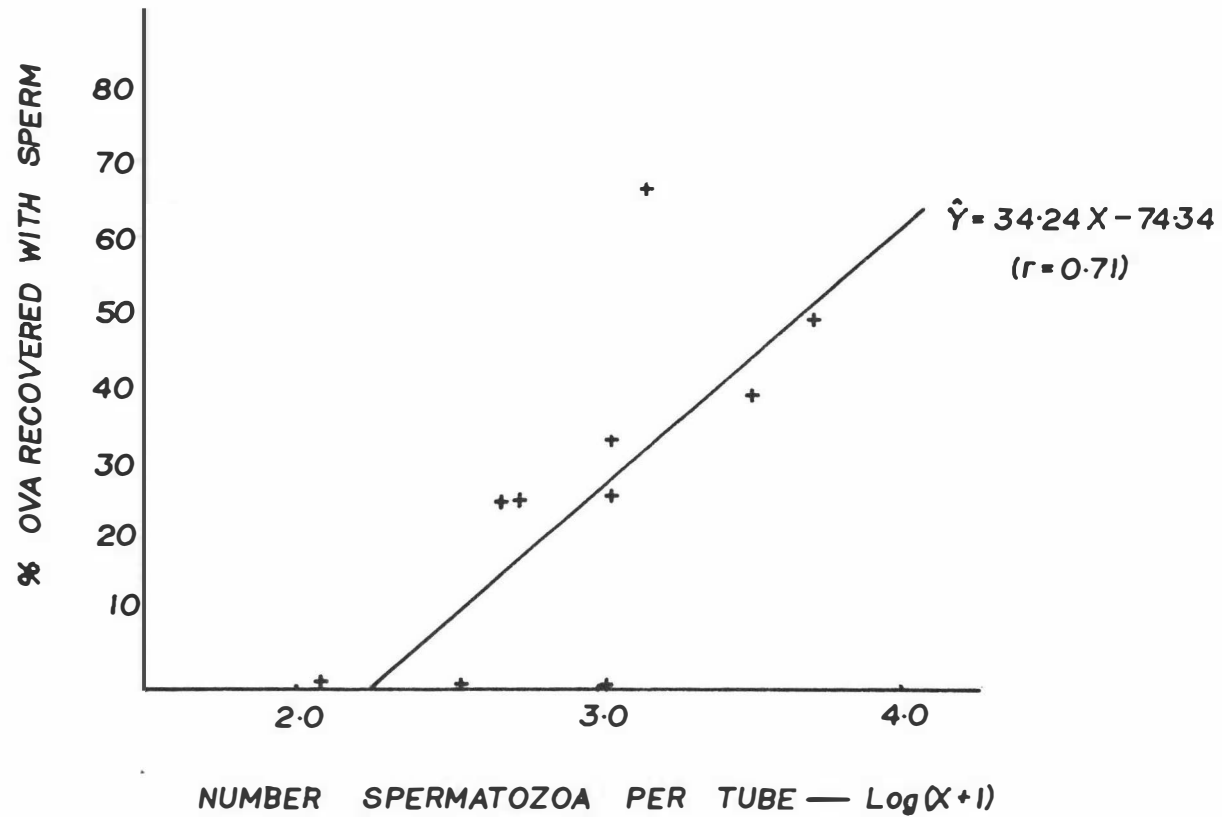
ANALYSIS OF CHI<sup>2</sup>

Main Effects	Value of chi <sup>2</sup>	of	chi <sup>2</sup>	of	chi <sup>2</sup>
	Cva Recovered		Cva With Sperm		
Site	0.016	N.S.	0.103	N.S.	
Time	0.360	N.S.	1.210	N.S.	
Slaughter	0.813	N.S.	2.080	0.1 < P < 0.25	

N.S. Not Significant

FIGURE 4.4

RELATIONSHIP BETWEEN NUMBER OF TUBAL SPERMATOZOA AND  
PROPORTION OF OVA WITH SPERM ON OR IN ZONA PELLUCIDA



DISCUSSION

## (a) Synchronization of Ovulation.

The combination of progestagen and HCG appeared to successfully synchronize ovulation in the majority of ewes in this trial. Because no animals were slaughtered close to the expected time of ovulation, the degree of synchronization could only be estimated to occur between 22 and 42 hours after HCG.

Facial eczema was prevalent on the property during the investigation and of the 5 ewes that had inactive ovaries, one had liver damage, and 4 displayed the external lesions characteristic of this disease. In other experimental ewes a similar retardation of ovarian activity by facial eczema, has been observed (M.F. McDonald & K.C. Eastwood, pers. comm.).

## (b) Spermatozoa Transport.

The percentage sperm recovered from the reproductive tract of the progestagen-treated ewe, in the present work, is in agreement with Quinlivan & Robinson (1969) and Hawk & Conley (1972). Vaginally inseminated ewes yielded a higher recovery rate, presumably due to the retention of sperm in the cervical mucus, or because of disappearance of cells from this region (Conley & Hawk, 1969). Significantly fewer sperm were recovered from the vagina 24 hours after insemination than at 4 hours, the difference was not accounted for by sperm movement into the cervix.

The recovery rate after single inseminations was higher than after two inseminations. This was probably due to greater disappearance of spermatozoa from the first volume of semen, which by the time of recovery had been in the tract for between 28 and 52 hours.

Direct uterine deposition of semen might be inferred from the

number of ewes yielding cervical sperm after 'intra-uterine' insemination (Table 4.4). But the method of flushing may also have contributed to reduce the number of ewes from which sperm were recovered from the cervix. A cranial to caudal direction was necessary due to the mucosal folds. If semen was deposited at the junction of the cervix and uterus, then the insertion of the flushing needle from the uterine end may have missed most of the sperm in this region.

As in the naturally mated ewe (Mattner, 1963a,b; Mattner & Braden, 1963), the cervix in the artificially bred ewe appeared to act as a reservoir of spermatozoa for the upper regions of the tract. No differences were detected in cervical sperm populations of vaginally inseminated ewes, but numbers were generally higher 4 hours after insemination, than at 24 hours. Two inseminations within 24 hours, did not enhance the numbers of sperm in the cervix, indicating that a maximum sperm population was established, irrespective of the numbers of sperm deposited. A high correlation between cervical and uterine sperm numbers at 4 hours ( $r = 0.8$ ) but not at 24 hours ( $r = 0.4$ ) indicated a dissociation between the two sperm populations by the later time.

The provision of oviduct sperm from the uterus was shown by a significant correlation between sperm numbers in these tract segments at 24 hours ( $r = 0.87$ ;  $P < 0.01$ ), but not at 4 hours, and by greater tubal sperm 24 hours after a single vaginal insemination that had occurred with a concomitant increase in uterine sperm numbers (see Figure 4.3).

There appeared to be little resistance to sperm transport through the utero-tubal junction, as sperm were distributed in the uterus and oviducts at 4 and 24 hours after uterine insemination,

in a similar pattern.

The recovery of ova suggested that spermatozoa were not well distributed throughout the Fallopian tube, as only 1 of 30 ova was cleaved, and only 27% had sperm in the outer egg layers. This points to a delayed sperm movement into the ampulla after artificial insemination as compared to natural mating (Ch & Lang, 1969).

It is concluded that deposition of semen closer to the uterus than is normally possible is advantageous for up to 4 hours after insemination, as greater numbers of sperm reach the Fallopian tubes. As a result, such inseminations close to the time of ovulation, are likely to result in superior levels of fertilization. Two inseminations within 24 hours appear to be useful only when deposition is at the external os of the cervix uteri and when sperm distribution in the tract is examined 4 hours after insemination.

CHAPTER V

EXPERIMENT 4

THE TRANSPORT OF SPERMATOZOA AFTER SURGICAL AND  
NON-SURGICAL DEPOSITION OF FRESH OR FROZEN SEMEN

## CHAPTER FIVE

### THE TRANSPORT OF SPERMATOZOA AFTER SURGICAL AND NON-SURGICAL DEPOSITION OF FRESH OR FROZEN SEMEN

This trial was designed to compare the transport of spermatozoa to the ampulla of the oviduct after deposition of fresh or frozen semen into the uterus, in an attempt to characterize some factors that may be contributing to cause a reduction in fertility to inseminations with frozen ram semen.

#### MATERIALS AND METHODS

Thirty-six 6-and 7-year-old Romney ewes were subjected to progestagen-HCG treatment to synchronize ovulation.

A 2 X 2 X 3 factorial design (n = 3) was used; the factors were:

- (1) Type of Semen .....Fresh vs Frozen
- (2) Method of Insemination .....Surgical vs Non-surgical
- (3) Time of Slaughter after insemination ..4 vs 24 vs 48 hrs.

Figure 5.1 shows the plan of the experiment.

Frozen semen<sup>\*</sup> was processed in mini-straws (0.25ml).

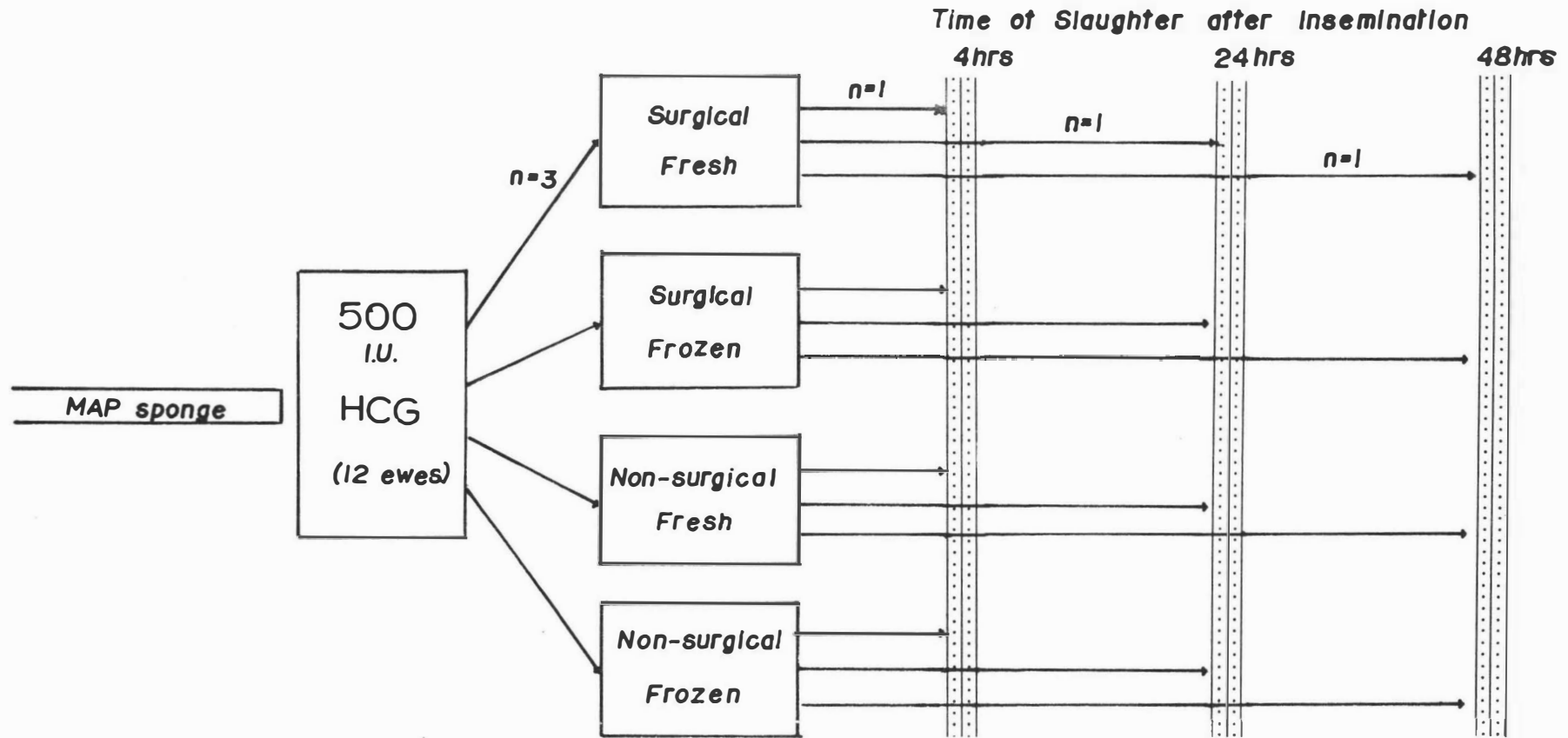
Immediately before insemination, the straws were thawed in a water bath at 36°C for 12 seconds. The percent motile cells was determined by staining a thawed sample on each day of insemination.

Approximately equivalent numbers of live spermatozoa (100 to 120 X 10<sup>6</sup>) were deposited for both types of semen, by varying the

<sup>\*</sup>Courtesy of Dr. J.F. Smith, Ruakura Agricultural Research Centre.

FIGURE 5.1

PLAN OF EXPERIMENT 4



Repeated 3 times giving 3 ewes per treatment

volume inseminated.

All inseminations took place 12 hours before the predicted time of ovulation.

Non-surgical inseminations were performed as in Experiment 3. For surgical inseminations, mid-ventral laparotomy was performed under light anaesthesia (Nembutal), and the uterus was exteriorised. A 16-gauge hypodermic needle was used to pierce the wall of the uterine cornua. Semen was expelled into the corpus uteri by carefully forcing the inseminating pipette through the puncture and inside the lumen. Plate 5.1 shows the exteriorised uterus and inserted pipette. The peritoneum and abdominal tissue were sutured and ewes placed in recovery pens. Slaughter took place 4, 24 or 48 hours after insemination.

Statistical treatment of data was as in experiment 3.

## RESULTS

### I. TIME OF OVULATION

Of the 24 ewes expected to have ovulated, only 62% had done so (Table 5.1). Nine ewes failed to show an ovulation point on either ovary for various reasons, i.e. quiescent ovaries (2 ewes), cystic follicles (1 ewe), slaughtered before ovulation as judged by unruptured, preovulatory follicles (6 ewes).

Four, and 2 ewes, slaughtered at 42 and 66 hours after HCG, respectively, had preovulatory follicles, and there appeared to be little difference in the appearance of corpora lutea on the ovaries of ewes slaughtered 42 and 66 hours after HCG injection.

TABLE 5.1

## INCIDENCE OF OVULATIONS AT INTERVALS AFTER HCG

(EXPERIMENT 4)

Interval from HCG to Slaughter (hrs)	Number of ewes	Number of ewes ovulating	% ewes ovulating
22	12	0	0
42	12	7	58.3
66	12	8	66.7
Σ 42-66	24	15	62.5

These observations indicate a spread of ovulations over the time-range considered rather than a narrow interval during which the majority of ovulations occurred.

Table 5.2 shows that surgery had no effect on the incidence of ovulations or the degree of follicular development, as the number of corpora lutea and large follicles were similar for all ewes.

## II. RECOVERY OF SPERMATOZOA

## (a) From the Cervix.

Sperm were recovered from the cervixes of more ewes after surgical than non-surgical inseminations (13/18 vs 8/18;  $\chi^2 = 2.86$ ,  $0.05 < P < 0.1$ ).

The mean numbers of spermatozoa recovered from the cervix of ewes in each treatment, are recorded in Table 5.3. Surgical deposition of semen into the uterus, resulted in higher numbers of sperm in this region of the tract, than did non-surgical insemination

TABLE 5.2

TREATMENT EFFECTS ON FOLLICULAR DEVELOPMENT AND THE  
NUMBER OF CORPORA LUTEA ON OVARIES AT SLAUGHTER

Type of Semen	Method of Insemination	Time of Slaughter (hrs after Insemination)	Number of:		
			CL	LF	MF
Fresh	Surgical	4	0	7	0
		24	2	4	0
		48	1	5	0
	Non-surgical	4	0	2	1
		24	2	5	0
		48	3	5	0
Frozen	Surgical	4	0	2	3
		24	2	2	0
		48	2	2	1
	Non-surgical	4	0	2	2
		24	1	2	1
		48	2	7	2
Total			15	45	8

CL Corpora Lutea  
LF Large Follicles  
MF Medium Follicles

TABLE 5.3

NUMBERS (TRANSFORMED) OF SPERMATOCYTES RECOVERED FROM  
THE CERVIX OF EWES AFTER INTRA-UTERINE INSEMINATIONS

Type of Semen	Method of Insemination	Time of Slaughter (hrs after Insemination)	Numbers of Sperm Recovered (Mean $\pm$ SE)	
Fresh	Surgical	4	4.456	$\pm$ 2.242
		24	3.345	$\pm$ 1.678
		48	3.085	$\pm$ 1.545
	Non-surgical	4	3.129	$\pm$ 1.569
		24	1.434	$\pm$ 1.434
		48	1.593	$\pm$ 1.593
Frozen	Surgical	4	3.993	$\pm$ 1.999
		24	2.951	$\pm$ 1.483
		48	4.377	$\pm$ 0.100
	Non-surgical	4	2.852	$\pm$ 1.438
		24	1.492	$\pm$ 1.492
		48	1.392	$\pm$ 1.392

ANALYSIS OF VARIANCE

Source of Variation	d.f.	Mean Squares	
Type of Semen (Semen)	1	0.0049	N.S.
Method of Insemination (Method)	1	26.5954	0.05 < P < 0.1
Time of Slaughter (Slaughter)	2	5.5640	N.S.
Semen X Method	1	0.1776	N.S.
Semen X Slaughter	2	0.6915	N.S.
Method X Slaughter	2	0.7580	N.S.
Semen X Method X Slaughter	2	0.9492	N.S.
Error	24	7.4350	

N.S. Not Significant

(3.700  $\pm$  0.586 vs 1.982  $\pm$  0.528). The difference approached statistical significance ( $0.05 < P < 0.01$ ).

(b) From the Uterus.

Table 5.4 shows the treatment mean numbers of sperm recovered from the uterus. There were no significant effects. However, 30.5% of total variation was due to the Time of slaughter, and 22% due to the interaction of Method and Slaughter. Examination of treatment means suggested that after surgical insemination, higher mean numbers of sperm were found in the uterus at 4 hours.

(c) From the Isthmus.

Data are presented as the sum of the number of sperm in the isthmus of the left and right oviducts. The variance of the data was homogeneous ( $\chi^2 = 12.54$ ; 11 d.f. — NS).

Table 5.5 presents the treatment mean numbers of spermatozoa recovered from the isthmus. There was a significant interaction of Method of insemination and Time of slaughter.

Regression analysis of this interaction revealed distinct changes in isthmal sperm populations after surgical and non-surgical insemination (Table 5.6). Figure 5.2 shows that following surgical placement of semen into the uterus, there was a significant linear decline in the number of spermatozoa recovered from the isthmus with time ( $P < 0.005$ ). Opposed to this, an increase in isthmal sperm from 4 to 24 hours, followed by a decline to 48 hours, occurred after non-surgical deposition of semen; the quadratic component of which was highly significant ( $P < 0.005$ ).

(d) From the Ampulla.

Table 5.7 shows that significant heterogeneity of variance existed among the data for numbers of sperm recovered from the ampulla.

TABLE 5.4

NUMBERS (TRANSFORMED) OF SPERMATOZOA RECOVERED FROM  
THE UTERUS AFTER INTRA-UTERINE DEPOSITION OF SEMEN

Type of Semen	Method of Insemination	Time of Slaughter (hrs after Insemination)	Number of Sperm Recovered (Mean $\pm$ SE)
Fresh	Surgical	4	6.299 $\pm$ 0.593
		24	3.557 $\pm$ 1.794
		48	1.684 $\pm$ 1.684
	Non-surgical	4	3.356 $\pm$ 1.679
		24	4.799 $\pm$ 0.052
		48	3.483 $\pm$ 1.743
Frozen	Surgical	4	5.602 $\pm$ 0.301
		24	3.366 $\pm$ 1.680
		48	3.233 $\pm$ 1.619
	Non-surgical	4	3.074 $\pm$ 1.550
		24	3.333 $\pm$ 1.676
		48	1.566 $\pm$ 1.566

ANALYSIS OF VARIANCE

Source of Variation	d.f.	Mean Squares	Partition of Variation (%)
Semen	1	2.2554 N.S.	5.2
Method	1	4.2616 N.S.	9.8
Slaughter	2	13.3261 N.S.	30.5
Semen X Method	1	4.6773 N.S.	10.7
Semen X Slaughter	2	0.3121 N.S.	0.7
Method X Slaughter	2	9.6537 N.S.	22.0
Semen X Method X Slaughter	2	2.8409 N.S.	6.5
Error	24	6.3709	14.6

N.S. Not Significant

TABLE 5.5

NUMBERS (TRANSFORMED) OF SPERMATOZOA RECOVERED FROM THE ISTHMUS

Type of Semen	Method of Insemination	Time of Slaughter (hrs after Insemination)	Number of Spermatozoa Recovered (Mean $\pm$ S.E.)
Fresh	Surgical	4	4.345 $\pm$ 0.152
		24	3.358 $\pm$ 0.485
		48	0.563 $\pm$ 0.563
	Non-surgical	4	1.936 $\pm$ 0.969
		24	3.125 $\pm$ 0.223
		48	0.734 $\pm$ 0.734
Frozen	Surgical	4	4.152 $\pm$ 0.096
		24	3.043 $\pm$ 0.348
		48	0.733 $\pm$ 0.733
	Non-surgical	4	0.771 $\pm$ 0.771
		24	2.615 $\pm$ 0.369
		48	1.249 $\pm$ 0.631

ANALYSIS OF VARIANCE

Source of Variation	d.f.	Mean Squares	
Semen	1	0.5659	N.S.
Method	1	8.2842	x
Slaughter	2	17.7904	xxx
Semen X Method	1	0.1720	N.S.
Semen X Slaughter	2	0.8445	N.S.
Method X Slaughter	2	8.7676	xxx
Semen X Method X Slaughter	2	0.3296	N.S.
Error	24	0.9924	

N.S. Not Significant      x P < 0.05      xxx P < 0.005

TABLE 5.6

INTERACTION OF METHOD OF INSEMINATION AND TIME  
OF SLAUGHTER ON NUMBERS OF SPERM IN THE ISTHMUS

Time of Slaughter (hrs after Insemination)	Method of Insemination $\neq$	
	Surgical	Non-surgical
4	4.248 $\pm$ 0.127 a	1.353 $\pm$ 0.612 c
24	3.201 $\pm$ 0.285 ab	2.874 $\pm$ 0.226 b
48	0.648 $\pm$ 0.415 c	0.992 $\pm$ 0.448 c

$\neq$  Mean  $\pm$  S.E. Sub scripts in common, not significantly different.  
(Duncan's Multiple Range Test ---- Little & Mills,  
1972).

REGRESSION ANALYSIS

Source of Variation	d.f.	Mean Squares
Linear:		
Slaughter: Surgical	1	38.8854 <b>xxx</b>
Slaughter: Non-surgical	1	0.3920 N.S.
Quadratic:		
Slaughter: Surgical	1	2.2634 N.S.
Slaughter: Non-surgical	1	11.5751 <b>xxx</b>

N.S. Not Significant

**xxx** P < 0.005

FIGURE 5.2

SPERMATOZOA POPULATIONS IN THE ISTHMUS

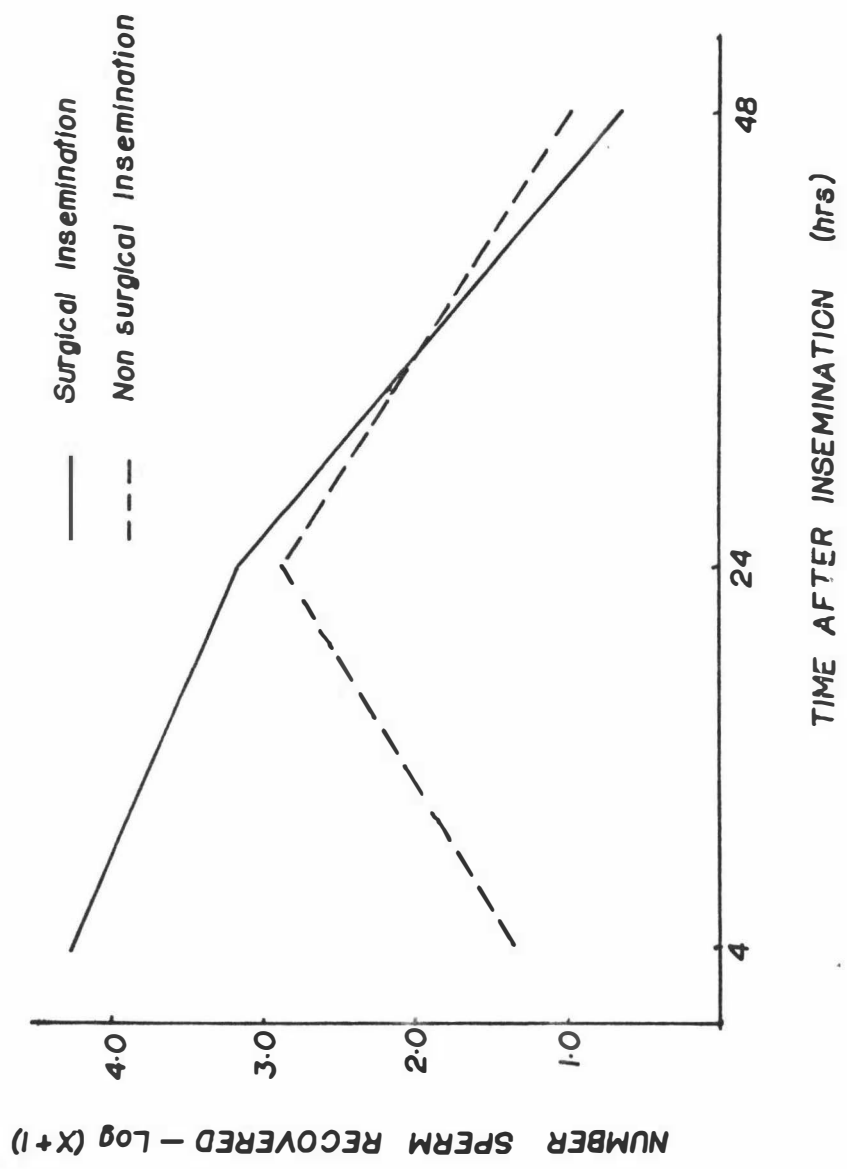


TABLE 5.7

NUMBERS (TRANSFORMED) OF SPERMATOCYTES RECOVERED  
FROM THE AMPULLA

Type of Semen	Method of Insemination	Time of Slaughter (hrs after Insemination)	Number of Sperm (Mean $\pm$ S.E.)
Fresh	Surgical	4	3.549 $\pm$ 0.109
		24	3.186 $\pm$ 0.406
		48	1.468 $\pm$ 0.754
	Non-surgical	4	0.000
		24	0.836 $\pm$ 0.836
		48	1.553 $\pm$ 0.780
Frozen	Surgical	4	0.000
		24	1.997 $\pm$ 1.002
		48	1.300 $\pm$ 0.663
	Non-surgical	4	0.000
		24	1.586 $\pm$ 0.836
		48	1.204 $\pm$ 0.604

Heterogeneity  $\chi^2 = 28.5248$ ,  $P < 0.005$

This was caused by the inconsistent data from animals slaughtered 4 hours after insemination.

Consideration of the data collected at 24 and 48 hours after insemination, showed the variation to be homogeneous (Bartlett's Test). Analysis of variance of these data showed no significant main effects or interactions (Table 5.8).

Of the treatment groups in which the ewes were slaughtered 4 hours after insemination, 3 had zero variance and therefore, no analysis was attempted.

Figure 5.3 presents the trends observed in sperm populations in the ampulla. Only after surgical insemination of fresh semen were sperm present in high numbers. They remained high till 24 hours after insemination, and although analysis revealed no statistical significance, a substantial decline occurred from 24 to 48 hours.

None of the other treatments resulted in sperm in the ampulla 4 hours after insemination, and by 24 hours, they were still lower than at the corresponding time after surgical deposition of fresh semen.

#### (e) Total Sperm Recovered

Table 5.9 contains the mean percent recovery of sperm from the tracts of ewes in each treatment. The analysis of variance of these data after angular transformation, shows several significant effects.

Insemination with fresh semen resulted in more than a 10-fold increase in the recovery rate than occurred after deposition of frozen semen (1.82% vs 0.13%;  $P < 0.05$ ). Recovery of sperm was higher after surgical (1.88%) than non-surgical insemination (0.07%).

A linear decline in percent recovery occurred with time after insemination ( $P < 0.01$ ). The high recovery rate of sperm after surgical

TABLE 5.8

ANALYSIS OF VARIANCE OF NUMBERS OF SPERM RECOVERED  
FROM THE AMPULLA 24 AND 48 HOURS AFTER INSEMINATION

Source of Variation	d.f.	Mean	Squares
Semen	1	0.3373	N.S.
Method	1	2.8968	N.S.
Slaughter	1	1.6102	N.S.
Semen X Method	1	1.1489	N.S.
Semen X Slaughter	1	0.0019	N.S.
Method X Slaughter	1	2.8209	N.S.
Semen X Method X Slaughter	1	1.7016	N.S.
Error	16	1.7092	

N.S. Not Significant

FIGURE 5.3

SPERM POPULATIONS IN THE AMPULLA AFTER DEPOSITION OF FRESH OR FROZEN SEMEN

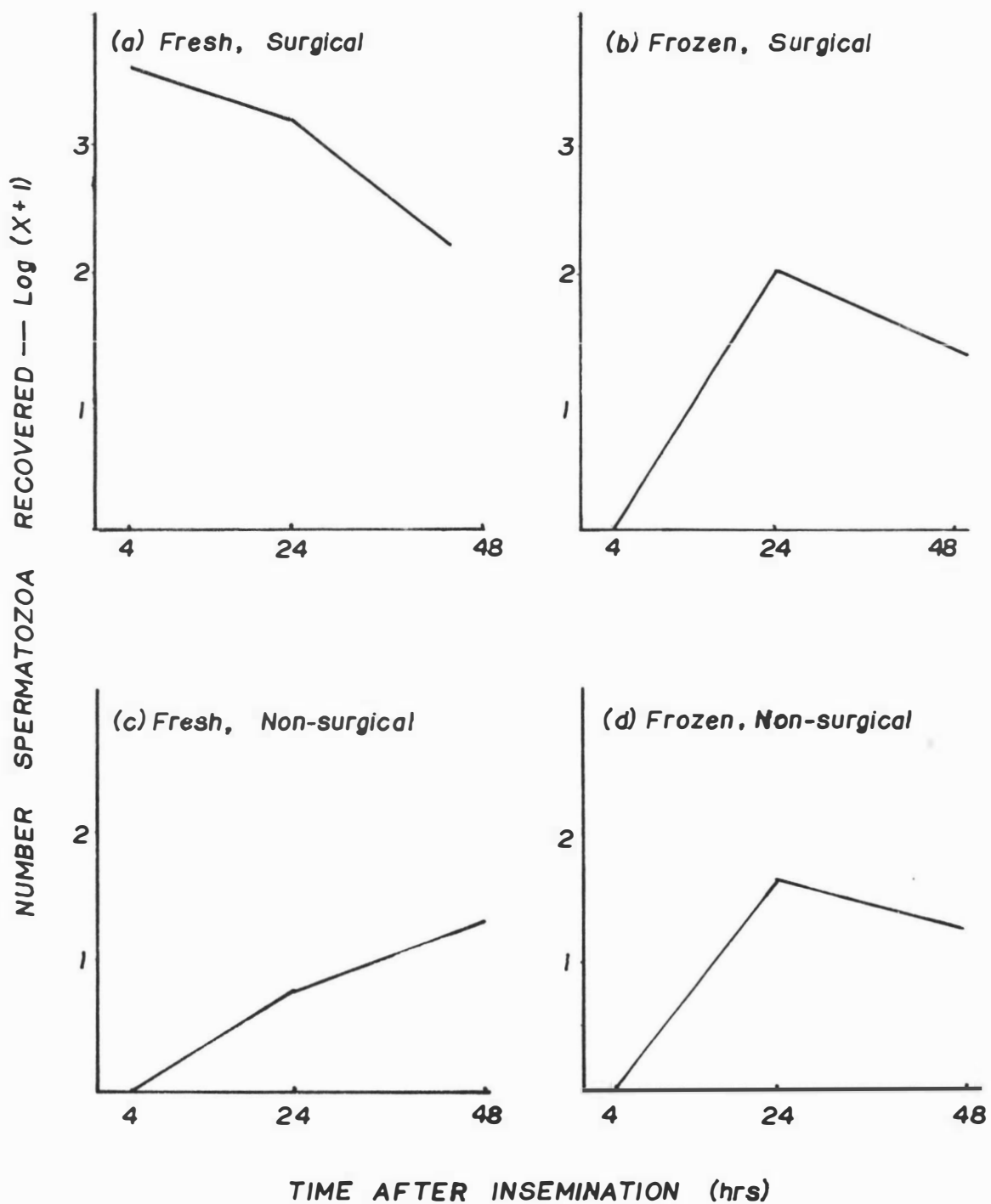


TABLE 5.9

MEAN PERCENT RECOVERY OF SPERMATOZOA FROM THE  
TRACT AFTER INSEMINATION WITH FRESH OR FROZEN SEMEN

Type of Semen	Method of Insemination	Time of Slaughter (hrs)	Percent Recovery of Spermatozoa
Fresh	Surgical	4	10.220
		24	0.310
		48	0.102
	Non-surgical	4	0.090
		24	0.072
		48	0.138
Frozen	Surgical	4	0.570
		24	0.050
		48	0.036
	Non-surgical	4	0.030
		24	0.050
		48	0.020

ANALYSIS OF VARIANCE

Source of variation	d.f.	Mean Squares	
Semen	1	66.124	x
Method	1	87.703	x
Slaughter	2	68.640	x
Semen X Method	1	30.952	N.S.
Semen X Slaughter	2	27.168	N.S.
Method X Slaughter	2	78.204	xx
Semen X Method X Slaughter	2	43.607	N.S.
Error	24	13.219	

N.S. Not Significant  
 x P < 0.05  
 xx P < 0.01

insemination with fresh semen, at 4 and 24 hours, caused a significant Method X Slaughter interaction.

### III. BREAKAGE OF SPERMATOZOA

During the counting of sperm in the tract flushings, it was apparent that in some ewes, a high degree of breakage of sperm cells, at the sperm head-midpiece junction had occurred.

Broken cells were counted as 'tails', but single sperm heads were often seen. Most broken cells appeared in uterine flushings, and to a lesser extent in the isthmus. Table 5.10 presents the treatment mean percent broken cells in the uterus. No separated heads or tails were found in the ampulla of any ewe.

TABLE 5.10

MEAN PERCENT BROKEN SPERMATOZOA IN FLUSHINGS OF THE UTERUS

Type of Semen	Method of Insemination	Time of slaughter after Insemination (hrs)		
		4	24	48
Fresh	Surgical	3.3	2.7	0.0
	Non-surgical	0.0	0.0	0.0
Frozen	Surgical	49.7	5.8	0.0
	Non-surgical	0.0	4.2	0.0

### IV. LEUKOCYTES IN THE REPRODUCTIVE TRACT

Table 5.11 gives the results of a subjective assessment of the presence of polymorphonuclear phagocytes in uterine flushings. Generally, fresh semen caused a greater leukocyte population in the uterus. Surgical insemination per se, appeared not to influence this

response as frozen semen deposited in this way did not induce high populations of phagocytic cells.

Plate 5.2 shows the phagocytosis of a spermatozoon.

#### V. OVUM RECOVERY AND FERTILIZATION

From the 15 ewes which ovulated, 14 ova (91%) were recovered.

Ova were judged to be fertilized by the presence of normal cleavage (7 ova) or by the presence of spermatozoa in the outer egg layers (3 ova) (Table 5.12).

Four single cell ova had no sperm on or in, the zona pellucida, and were considered unfertilized.

Plates 5.3 to 5.6, show some of the ova recovered.

Significantly more ova were cleaved among those recovered at 48 hours after insemination, than at 24 hours, ( $\chi^2 = 10.0$ ,  $P < 0.01$ ).

#### DISCUSSION

##### (a) Time of Ovulation.

Ovulation data for ewes in this trial suggested that the hormone treatment failed to synchronize ovulation. Not only did few ewes ovulate, but the distribution of ovulations, suggested that HCG had failed to induce rupture of follicles.

##### (b) Spermatozoa Transport.

Surgical insemination of semen, showed that spermatozoa are capable of moving into the cervix from the uterus, often in large numbers.

Within the uterus, spermatozoa were maintained in relatively high numbers for longer than 24 hours after insemination. Although no attempt was made to distinguish between motile and immotile sperm in flushings, the movement of sperm from the uterus into the cervix, and back into the uterus, may aid in maintaining uterine sperm numbers.

TABLE 5.11

PRESENCE OF LEUKOCYTES IN THE UTERUS

Type of Semen	Method of Insemination	Time of Slaughter (hrs after Insemination)	Leukocyte Count
Fresh	Surgical	4	+++
		24	++
		48	—
	Non-surgical	4	++
		24	±
		48	—
Frozen	Surgical	4	+
		24	—
		48	0
	Non-surgical	4	+
		24	±
		48	—

0 No score made      + Some but few  
 — None seen          ++ Many  
 ± Very Few            +++ Very Numerous

TABLE 5.12

INCIDENCE OF THE RECOVERY OF FERTILIZED OVA

Type of Semen	Method of Insemination	Time of Slaughter (hrs after Insemination)	Number of ova:		Criterion of Fertilization
			Recovered	Fertilized	
Fresh	Surgical	4	0	0	
		24	2	2	Single cell, many sperm; 2 cell
		48	1	1	2 cell
	Non-surgical	4	0	0	
		24	2	1	Single cell, sperm in vitellus
		48	3	2	6 cell, 2 cell
Frozen	Surgical	4	0	0	
		24	1	1	Single cell, many sperm;
		48	2	2	6 cell, 8 cell
	Non-surgical	4	0	0	
		24	1	0	
		48	2	1	4 cell
TOTAL			14	10	



Surgical insemination usually resulted in higher uterine sperm numbers than non-surgical insemination, and indicated that direct intra-uterine deposition of semen did not occur under non-surgical conditions.

The data recorded for sperm in the isthmus reflects little or no resistance to sperm movement imposed at the utero-tubal junction, as surgical insemination of fresh semen resulted in high numbers of sperm distributed throughout the upper reproductive tract within 4 hours of insemination. The quadratic component of non-surgical insemination (Figure 5.2) shows that the distribution of sperm within the isthmus was dissimilar to that which occurred after surgical deposition. This was a reflection of the site at which semen was expelled from the inseminating pipette. If non-surgical inseminations deposited semen at the junction of the cervix and uterus, then the distribution of sperm cells within the uterus was most probably less thorough than when semen was placed directly within the uterus at surgery. Consequently, a period of time (24 hours) was necessary for a build up of a maximum isthmal population.

If a barrier to sperm passage exists within the oviduct, it probably occurs either within the isthmus or at the isthmus-ampulla junction. The numbers of sperm in the isthmus declined from 24 to 48 hours following non-surgical insemination, and those in the ampulla rose or were unchanged, between these times. This suggests that peak populations in the top of the Fallopian tube did not occur till some time later than 24 hours after placement of semen. The distribution of fresh sperm throughout the oviduct 4 hours after surgery may indicate that the isthmus-ampulla barrier operates for only a certain time in response to changing ovarian steroid output, or that the

presence of large numbers of presumably viable spermatozoa in the isthmus, renders the barrier less effective.

Motility of sperm may therefore play the predominant role of sperm transport to the ampulla from the isthmus. No separated tails or heads of spermatozoa, seen in large numbers in the isthmus, were found in the ampulla of any ewe. Following placement of frozen semen into the tract, or fresh semen by non-surgical methods, sperm transport was much less effective in the initial period (4 hrs) after insemination. Even by 24 hours, few sperm were in the ampulla after these treatments.

This study has pointed to two factors that appear to be responsible for the reduction in numbers of frozen sperm in the top of the oviducts: the high degree of damage to cells occurring in the uterus, and, less effective sperm transport.

Breakage of sperm cells appeared to be a physical phenomenon. It was probably associated with semen preservation, as the degree of breakage was not reflected by the presence of leukocytes in the uteri of ewes receiving frozen semen.

Spermatozoa disappear within the tract, due in part, to phagocytosis by leukocytes. Most commonly, complete sperm were undergoing engulfment at the sperm head, but some separated tails were seen within leukocytes.

The proportion of cells deposited which were recovered was higher after surgical deposition of fresh semen and the percent recovery indicated that disappearance of sperm was considerably higher after insemination with frozen semen. The reduced transport of frozen sperm was reflected by the low recovery rate at all times except 4 hours after surgical insemination (Table 5.9).

A summary of the sperm transport mechanisms that appeared to have operated in the movement of sperm to the ampulla is given in Table 5.13.

TABLE 5.13

SUMMARY OF SOME MECHANISMS OF SPERM TRANSPORT  
IN THE REPRODUCTIVE TRACT

Type of Semen and Method of Insemination	Sperm in the Uterus & Isthmus:			Relative Numbers Sperm in Ampulla
	Apparent Distribution of sperm	Assessed proportion motile cells	Degree of damage	
Fresh; Surgical	Good	High	Nil	High (up to 24 h)
Fresh; Non-surgical	Poor	High	Nil	Low (initially)
Frozen; Surgical	Good	Low	High	Low (all times)
Frozen; Non-surgical	Poor	Low	High	Low (all times)

PLATE 5.3      Single-cell, unfertilized ovum  
recovered 24 hours after Non-  
surgical insemination with  
frozen semen.

PLATE 5.4      2-cell ovum recovered 24 hours after  
surgical insemination with fresh semen.

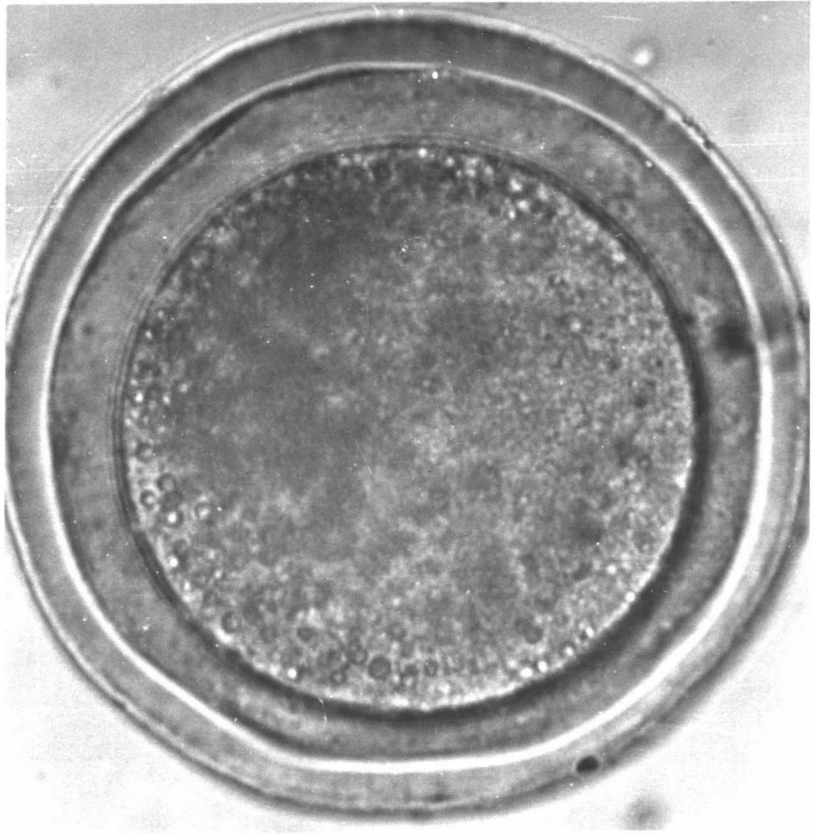
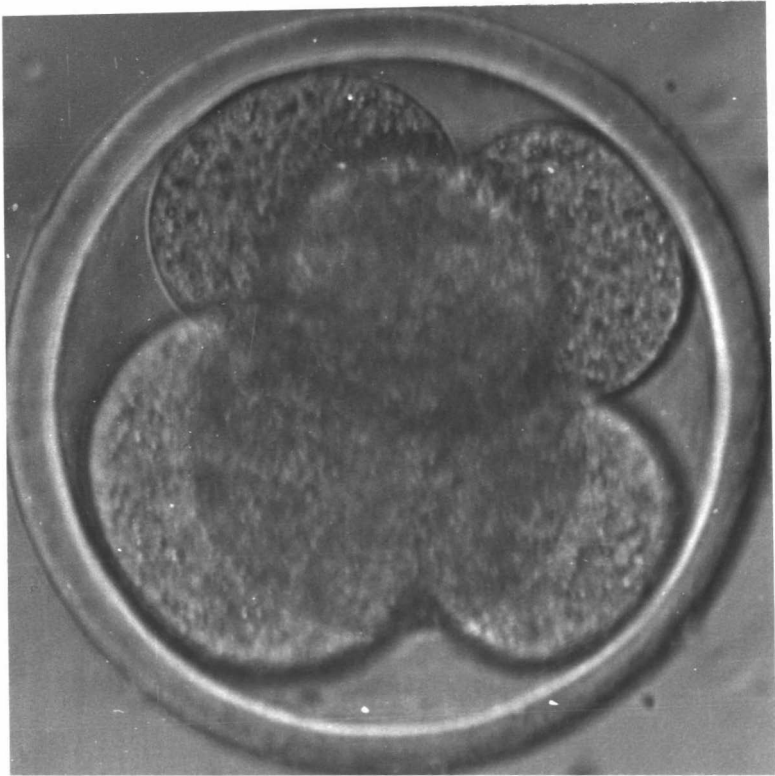


PLATE 5.5      6-cell ovum recovered 48 hours after  
non-surgical insemination with fresh  
semen.

PLATE 5.6      8-cell ovum recovered 48 hours after  
surgical insemination with frozen semen.



CHAPTER VI

GENERAL DISCUSSION

## CHAPTER SIX

### GENERAL DISCUSSION

#### THE VARIABILITY OF SPERM COUNTS

In spite of experience in the use of techniques of sperm recovery gained in the preliminary trials, the degree of variation in counts was not substantially reduced in Experiments 3 and 4. Seemingly uncontrollable variation in sperm counts between animals exists, and unless large numbers of experimental ewes are available, this problem is likely to hinder the identification of the physiological mechanisms involved in the transport of sperm in the female genital tract.

Transformation of the data did not always eliminate heterogeneity of variance which remained in the uterine and oviduct counts in Experiment 3, and in ampulla counts in Experiment 4. This indicates a source of variation other than that associated with enumeration data.

Careful attention to experimental techniques was adopted as far as practicable, but the present work indicated several factors that probably contributed to the variation in sperm counts between the same region of the tracts of different ewes:

- (a) Misidentification of sperm cells.

In flushings mainly from the oviducts and vagina, epithelial cells are often present and these can be mistaken for sperm. The addition of aqueous eosin to the washings in Experiment 3 and 4 facilitated the accurate identification of sperm. Mattner & Braden (1963) have pointed out the increased accuracy of detection of sperm

after allowing a settling period before examination and this procedure was followed.

(b) Differences in volume of fluid collected after flushing.

Considerable variation in the size of reproductive tracts occurred between animals. This led to the collection of differing volumes of fluid from similar segments of reproductive tract. For example, from the 2ml of saline used to flush each Fallopian tube in Experiment 3, the volume recovered after flushing varied from less than half, to almost the total quantity. Haemocytometer determinations of cell numbers were calculated on the basis of volume collected after flushing the uterus and cervix.

(c) Transference of sperm cells owing to contamination by equipment.

Clean equipment was used for each flushing. However, as considerable handling of the tracts was necessary using the described method, it is possible that sperm may have been transferred between segments. This appeared to be the case in Experiment 1 (see Table 3.2, ewe 297). Dauzier (1958) has suggested that reports of spermatozoa reaching the ovarian end of the oviducts within a few minutes of insemination (rapid transport) are attributable to contamination by sperm from elsewhere.

Careful experimental techniques aimed at reducing the error variance thus appear less effective than an increase in the number of experimental animals as an appropriate way of overcoming much of the variation observed with such work. It should not be forgotten however, that on any one day, only a limited number of reproductive tracts can be processed ~~if~~ prolonged microscopic observation is to be avoided.

INTRA-UTERINE INSEMINATION OF EWES.

Other than with the aid of surgical procedure, the uterus

of the ewe is almost impossible to reach due to the physical difficulty of penetrating the cervical canal. Very little work has been carried out on the development of equipment that would allow non-surgical intra-uterine inseminations. Inskip (1974) has used a 45cm tapered inseminating tube to deposit semen in the uterus of "some" ewes.

High rates of fertilization have been achieved after surgical inseminations into the uterus and this is a reflection of the higher numbers of sperm transported to the tubes (Killeen & Moore, 1970a).

The present work has confirmed this by showing significantly high tubal sperm numbers 4 hours after insemination, but because relatively few fertilized eggs were recovered, the relationship between sperm transport and fertilization rate was not clearly shown.

Deep penetration of the cervix of ewes was not always successful but it was estimated that in at least 50% of ewes, a depth of 5cm was possible using this equipment. A pair of cervical grips were used occasionally to provide traction on the cervical papillae, but usually, this resulted in damage to the tissue by tearing.

Modification of the pipette to include a more flexible tip, and manipulation, via the rectum, of the pipette through the cervix, (as used by Andersen, Aamdal & Fougner, 1973) may assist in reaching the uterus in the majority of sheep. The results of Experiment 3 indicated that successful penetration of the cervix depended on the length of the cervical canal, which varied from 3 to 10cm in length. No reliable method for selecting ewes with a short-length cervix can be advanced. Approximately 2% of the ewes inseminated 'intra-uterine' suffered extreme haemorrhaging and oedema of the cervical tissue. The time taken for each insemination ( $2\frac{1}{2}$  minutes per ewe),

might reduce the effectiveness of this instrument if large numbers of sheep are to be inseminated.

SPERMATOCYTA TRANSPORT AFTER ARTIFICIAL INSEMINATION.

The numbers of sperm recovered from the tracts of ewes in the present experiments, are in general agreement with other reports (Mattner & Braden, 1963; Mattner, 1963a,b), but those recovered from the uterus were higher than those found after artificial insemination by Quinlivan and Robinson, (1969).

A mean recovery rate of less than 0.1% of sperm deposited after intravaginal progestagen sponge treatment, agrees with Hawk & Conley (1972), and it appeared that in vaginally inseminated ewes, the greatest loss of sperm occurred from the vagina by drainage of semen to the exterior. A ten-fold reduction in vaginal numbers occurred from 4 to 24 hours after insemination. Hawk & Conley (1971) estimated that more than 62% of cells deposited are lost by this means. The examination of thick, cheese-like mucus in the vagina of some ewes slaughtered 24 hours after insemination (Experiment 3), indicated that phagocytic destruction of spermatozoa in the vagina had also occurred. This source of loss was reduced by intra-uterine deposition of semen.

The cervix of the ewe acts as a reservoir of spermatozoa, and as a barrier to sperm migration. Experiment 3 showed that the advantage of by-passing the cervix, by placing the semen near to the uterus, was revealed in terms of the effectiveness of sperm transport to the oviducts within 4 hours of insemination. Twenty-four hours after vaginal insemination sperm had moved from this reservoir to the upper tract in numbers equivalent to those found after 'uterine' deposition of semen.

Similar numbers of sperm were present in the uterus at both 4 and 24 hours after intrauterine inseminations (Experiment 3). No statistical difference in cervical sperm numbers was evident after vaginal inseminations. This suggests that disappearance of sperm in the uterus between 4 and 24 hours, was low. Leukocytic engulfment (Experiment 4) suggested one source of loss, but Conley & Hawk (1971) estimated that not more than 5% of sperm loss in the tract is due to phagocytosis.

Results of Experiment 4 showed that sperm can move from the uterus into the cervix. A mechanism of sperm movement between these two sections of the tract may maintain high numbers of sperm in both segments for up to 24 hours. Thus Killeen & Moore (1970a) suggest that regions of the tract other than the cervix, may act as sperm reservoirs for the Fallopian tubes.

The degree of resistance imposed by the utero-tubal junction to sperm transport in the ewe, is not clear. Edgar & Asdell (1960a) suggest it has a valve-like action. Other workers have suggested that motility of sperm is an advantage for passage from the uterine cornua to the isthmus (e.g. Ch et al, 1969). The implication from the present work, of lack of restraint to sperm movement at this site, agrees with Quinlivan & Robinson (1969) who suggested that the isthmus posed a greater barrier to sperm progression than the utero-tubal junction. Bishop (1961) and Hafez & Black (1969) report that few mucosal folds are present at the utero-tubal junction of the ewe, and Anderson (1928) has shown that the structure requires very little pressure to allow fluid flow into the oviducts. Analogy from other species (Braden, 1953; Greenwald, 1963) also suggests the isthmus as posing greater resistance to transport of sperm due to its smaller

diameter. Direction of flow of oviduct secretions through the isthmus is against the forward movement of sperm in this region (Bellve & McDonald, 1968).

The existence of a barrier to ova transport at the isthmus-ampulla junction, has been shown by Holst & Braden (1972), the opening of which appears to be under steroid hormone control. The present work indicated that sperm motility is an advantage for passage from isthmus to ampulla. The fact that sperm from fresh semen deposited under surgery were found in high numbers in the ampulla at all times of recovery, suggests that the barrier may not be effective when large numbers of viable sperm are in the isthmus.

Either there is a barrier to sperm movement in the tubes that has accounted for the observed sperm distribution patterns, or some other factor is contributing to cause a delayed sperm transport to the ampulla in the artificially inseminated ewe.

The distribution of spermatozoa in the isthmus and ampulla of the oviduct (Experiment 4) indicated a peak ampulla sperm population later than 24 hours after insemination. At this time, considerably less than half of the total spermatozoa in the oviduct were in the upper half of the tubes. Similarly, when the whole Fallopian tube was flushed (Experiment 3) 4 hours after insemination, sperm were recovered in high numbers. But results from Experiment 4 suggest that at this time, nearly all of these sperm were probably in the isthmus and not the ampulla. This means that total tubal spermatozoa (reported by many workers) may not be a true indication of sperm transport to the site of fertilization and its relationship to fertility.

In Experiment 3, the absence of cleaved eggs suggested that

poor sperm transport to the site of fertilization had occurred, or insufficient time had elapsed to allow normal cleavage to take place. From the data of Dziuk (1965), when insemination took place 15 hours before ovulation, the first cleavage of blastomeres occurred about 20 hours after ovulation. Thus in the present study, those ova recovered from ewes inseminated before ovulation, probably had insufficient time for cleavage. Ova from ewes inseminated after ovulation and recovered 24 hours later, ought to have cleaved if fertilization was successful. However, only one was cleaved (recovered from a ewe slaughtered 24 hours after the second of two inseminations). This suggests a slow transport of sperm to the ampulla, where fertilization takes place (Holst & Braden, 1972).

The percent recovery of sperm from the tract was low, and drainage of semen probably accounted for much of this loss from vaginally inseminated ewes. However, the recovery of sperm from within the tract of uterine-inseminated ewes was also low and loss to the exterior through the vagina was considered unlikely as no ewes had sperm recovered from this section of the tract.

Mechanisms of sperm disappearance in the uterus are not clear. Phagocytosis is clearly one source and although some workers suggest that this accounts for very little of the loss (Conley & Hawk, 1971) others have noted that phagocytosis is difficult to observe using light and phase contrast microscopy, but that under the electron microscope, many of the sperm cells not seen with the less sophisticated equipment can be observed (Brinsfield, Clark, Gerich & Hawk, 1970). Incorporation of sperm into epithelial glands (Austin, 1975) is also another possible source of loss of sperm cells within the reproductive tract. Voidance to the peritoneal cavity is known to occur (Edgar & Asdell, 1960b) and in the present investigation

this may account for some disappearance of sperm, particularly from those ewes inseminated intra-uterine, as higher tubal sperm numbers existed soon after insemination. Retention of sperm after flushing is probably one of the largest sources of 'unaccounted-for' spermatozoa in the reproductive tract, especially in the cervix, as in Experiment 4, those ewes inseminated under surgery into the uterus, yielded a much higher percent recovery of sperm than occurred in Experiment 3.

Most workers comparing sperm transport after deposition of fresh or frozen semen at the external cervical os, have found a marked superiority of the former material (e.g. Mattner et al, 1969). The present investigation has revealed no difference in sperm transport in the reproductive tract when fresh, undiluted, and frozen semen were deposited through the cervix. Only when fresh semen was placed in the uterus under surgery, did more sperm reach the ampulla than after placement of frozen semen. This effect was observed at 4 hours after insemination. This suggests that the cervix is a site of sperm transport failure after insemination with frozen semen. This result is in agreement with Lopyrin & Loginova (1958) and Salamon & Lightfoot (1967).

Nearly half the sperm recovered from the uterus of ewes inseminated with frozen semen were broken. This appeared to be owing to a physical rather than physiological mechanism and may therefore be a result of the preservation process. Watson & Martin (1974) have identified other structural changes to ram spermatozoa occurring after deepfreeze storage. White, Blackshaw & Emmens (1954) have shown that ram sperm after freezing have a reduced longevity on incubation in vitro.

Although similar numbers of motile spermatozoa were deposited

under surgery, the difference in numbers of sperm in the oviducts after insemination with frozen as compared to fresh semen, suggest an impairment of transport of the former material. The implication that motility plays an important role for sperm passage in the ampulla (Experiment 4; Dauzier, 1955b), suggests that lower numbers of viable frozen spermatozoa were present in the isthmus, and therefore, eventually in the ampulla.

It is unlikely that the mechanisms of sperm transport external to the spermatozoon would differ for those that had been subject to deep-freeze storage. Thus it seems probable that reduced longevity of frozen sperm within the reproductive tract, renders these mechanisms less effective.

While the fertilizing ability of frozen sperm has been questioned (Smith, 1975), the reduced fertility of ewes inseminated with frozen semen, appears to be the result of reduced transport of sperm to the ampulla (Experiment 4). The time taken for the transport of frozen sperm was clearly longer than for fresh sperm. Both the percent of tubal sperm in the ampulla and the recovery rate of frozen sperm cells, reflects this slow passage in the tract.

Earlier inseminations with frozen semen relative to the time of ovulation, may therefore be of benefit when considering the time of maximum sperm populations at the site of fertilization.

#### THE EFFECTS OF STRESS ON SPERM TRANSPORT IN THE ARTIFICIALLY INSEMINATED EWE

The observation in Experiment 2 illustrated the effects that stress can have on one reproductive function in the ewe. The existence of stress on animals during the insemination procedure cannot be discounted. The following observations from the present study indicated retardation of sperm transport after artificial insemination.

(a) There was a slower progression of sperm to the site of fertilization than is apparent after natural mating (Experiment 3; Mattner, 1963a,b; Mattner & Braden, 1963; Ch & Lang, 1969).

(b) The distribution of sperm in the isthmus after non-surgical insemination (Experiment 4) showed that a build up occurred from 4 to 24 hours after insemination. If the semen was being expelled at the junction of the cervix and uterus, then the observed pattern in the isthmus could be due to a less thorough distribution of sperm in the uterus. Uterine contractions are considered to be segmental rather than peristaltic (Bishop, 1961), encouraging dispersal rather than mass movement. Stress during insemination may have caused a reduction in uterine contractions resulting in a slower dispersal of sperm through the uterine lumen. Laparotomy does not appear to reduce the contractility of myometrial tissue (Brinsfield & Hawk, 1969), hence insemination under surgery probably resulted in a thorough distribution of sperm through the uterus and the isthmus (Figure 5.2).

(c) No evidence of rapid transport after artificial insemination was observed. From a total of 42 ewes inseminated at the external cervical os, in only 2 ewes were sperm recovered from the Fallopian tubes within 4 hours of insemination.

(d) Two inseminations at the cervical opening (Experiment 3) did not enhance the number of spermatozoa recovered from the tubes 4 hours after the second insemination. This was unexpected as these ewes were slaughtered at a time equivalent to 28 hours after the first volume of semen, a result that was not reflected by those ewes receiving one insemination and slaughtered 24 hours later. It is unlikely that the number of sperm deposited was limiting sperm

transport to the tubes (Emmens & Robinson, 1962), as each of the two inseminations contained approximately  $3 \times 10^9$  spermatozoa. The effect of stress during the first insemination, and the added handling of these sheep, implicates a neural effect operating in some ewes to retard the transport of sperm in the tract.

Boland & Gordon (1972) have suggested that stress during insemination may cause the decrease in fertility of ewes receiving two doses of semen at appointed times after hormone treatment. The effects of stress on reproductive functions in the ewe are not well documented as suitable control conditions are difficult to establish. Thibault (1973) maintains that only under completely free mating conditions is there a large sperm ascent which occurs at the same speed in all ewes. Sheep are more apt to suffer some degree of trauma during insemination and are therefore not likely to exhibit the stimulatory response to artificial insemination that has been observed in cattle (Van Demark & Hays, 1952). In the present work, ewes were placed over a rail for insemination as other equipment such as an inseminating cradle (see Maule, 1962) was not available. Improved conditions for inseminations might help in reducing stress imposed on the ewe.

Any stress imposed on the ewe probably exerts its effect on sperm transport by reducing contractility of the tract, particularly that of the uterus. The stimulatory effect of oxytocin on uterine tonus (discussed in Chapter 1) is inhibited by the administration of exogenous epinephrine (Van Demark & Hays, 1952; Hays & Van Demark, 1953). The physiological reaction to stress is the endogenous release of epinephrine. Rough handling or fright can result in the release of sufficient adrenaline to inhibit contractions of the female tract.

That this effect can last for a long period of time was indicated in Experiment 2 where sperm transport was reduced even after 4 hours; and is supported by the finding of Warren & Hawk (1971) that the effect of an intra-uterine device on smooth muscle contractions in the ewe, can be observed for as long as 2 years after its removal. Stress-induced reduction of uterine contractility may therefore be the cause of the apparently poorer distribution of sperm in the uterus and isthmus after non-surgical inseminations (Experiment 4), and may explain the several reports that have failed to show evidence of rapid sperm transport or increased sperm movement to the tubes after injection of oxytocin (Jones, 1968; Jones, Martin & Lapwood, 1969; Salamon & Lightfoot, 1970) or following the sterile mating of artificially inseminated ewes (Hawk, 1972b).

The insertion of the inseminating pipette through the cervix of ewes in the present work may have been more traumatic than vaginal inseminations, and hence by 24 hours after inseminations, there was little difference between sites of insemination in the numbers of sperm recovered from the top of the reproductive tracts.

Future studies must therefore be aware, of the effects of stress on sperm transport and of the conditions under which animals may be subject to stresses. Past investigations that have involved the movement of experimental animals after treatment ( e.g. Quinlivan & Robinson, 1969) should be evaluated with this qualification.

#### CONCLUSIONS

Recovery of spermatozoa from all segments of the reproductive tract of the ewe, necessitates the slaughter of animals at progressive time-intervals after the deposition of semen. Assumptions are then made on the physiological mechanisms of sperm transport operating

between each time of recovery.

In other words, a dynamic system is investigated and evaluated on the basis of 'static' data. Recovery of sperm from each segment must therefore take place as often as possible in order to hypothesise on the movement of sperm through the tract. Thus, considerable expense of experimental resources is incurred, which may not always be justified due to the variability of the data collected.

A method that allows repeated recovery of sperm from the tract of the same animal is not available. As the transport of spermatozoa to the Fallopian tubes appears to be a function of the efficiency of movement through the lower tract segments, recovery of sperm from the oviduct might mean a more efficient method for investigation of factors affecting transport of spermatozoa in the female. Flushing the tubes at laparotomy (such as is done for egg transfer work) might be employed, or a modification of the method of collecting Fallopian tube secretions (Belly & McDonald, 1963) could be used as a means of repeated sampling from the tract of the same ewe, at various times after insemination.

The potential of an inseminating instrument allowing the deep penetration of the cervix, and possibly deposition of semen into the uterus, has been partially shown. The number of spermatozoa transported to the Fallopian tubes 4 hours after insemination into the uterus was significantly higher than after insemination at the external cervical os. The likelihood of fertilization therefore appeared to be higher.

This equipment may have greatest benefit for increasing the fertility of ewes inseminated with frozen semen. Field trials assessing the fertility to inseminations with this equipment are

needed to corroborate the evidence that sperm transport is enhanced by removing the retardation imposed by the cervix. In these experiments there was no difference between one insemination before or after ovulation, or between a single or double dose of spermatozoa. This could have been a result of the large numbers of sperm deposited or that deposition at the uterus removed the effect of the cervix where endocrinological effects have their major influence. Further work using variable numbers of sperm per insemination might indicate the limits to which freshly extended ram semen could be used.

It is desirable that future studies of sperm transport be supplemented with lambing results as the in utero viability of ova fertilized by frozen spermatozoa has not been investigated.

The development of a successful method of synchronizing ovulation and inseminating without observation for oestrus, might widen the scope of artificial breeding of sheep in New Zealand, even without the use of frozen semen. Diluted semen used on the day of, or day after collection has been very successfully achieved in the dairy cattle industry and such a technological development with ram semen, may have application in New Zealand.

Although the injection of HCG may not be entirely satisfactory for the induction of ovulations, the use of synthetic gonadotrophin-releasing factors might be more promising. However, as the cervix of the ewe appears to be the site of sperm transport failure after oestrus synchronization, the inseminating instrument used in this study might allow successful set-time inseminations after progestagen treatment, without the need for expensive ovulating hormones.

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APPENDICES

## APPENDIX I

### THE ASSESSMENT OF SPERMATOZOA CONCENTRATION

#### IN SEMEN SAMPLES

From 36 semen collections performed during the training of rams a significant linear relationship was established between the sperm density of the sample (measured by haemocytometer) and the turbidometric reading of the sample diluted 1:500.

Regression Equation:  $\hat{Y} = 0.22 + 11.35X$

where  $\hat{Y}$  is the concentration of sperm in the sample.

$X$  is the turbidometric reading.

Correlation Coefficient:  $r_{xy} = 0.955, P < 0.001.$

The following table was used for rapid evaluation of sperm density. The concentration is read as  $X \times 10^9$  per ml. of semen. (e.g. the concentration of a semen sample that registered a turbidity reading of 0.26, is  $3.171 \times 10^9$  per ml of semen).

	0	2	4	6	8
0.1	1.355	1.582	1.809	2.036	2.263
0.2	2.490	2.717	2.944	3.171	3.398
0.3	3.625	3.852	4.079	4.306	4.533
0.4	4.760	4.987	5.214	5.441	5.668

APPENDIX II (a)

SPERMATOCYTES IN THE UTERUS

(EXPERIMENT 3)

BARTLETT'S TEST OF HOMOGENEITY OF VARIANCE

Site of Insemination	Time of Insemination	Time of Slaughter (hrs)	Variance $s^2$	Log $s^2$
Vagina	-12	4	6.460	0.810
		24	0.042	-1.377
	+12	4	6.589	0.819
		24	0.010	-2.000
	±12	4	4.240	0.627
		24	4.524	0.655
Uterus	-12	4	0.080	-1.969
		24	0.057	-1.244
	+12	4	0.020	-1.699
		24	0.158	-0.801
	±12	4	0.029	-1.538
		24	0.480	-0.319

$\chi^2 = 112.544$ , 11 d.f.  $P < 0.001$

Variance is therefore significantly Heterogeneous.

APPENDIX II (b)

NUMBERS OF SPERMATOZOA RECOVERED FROM THE  
LEFT AND RIGHT FALLOPIAN TUBES  
TEST OF COMMON CORRELATION COEFFICIENT  
(Snedecor, 1956: p 178)

Treatment Group	n	n-3	r	z	Weighted z (n-3)z	Weighted Square (n-3)z <sup>2</sup>
Vagina, -12, 4						
	24	6	0.842	1.228	3.684	4.524
+12, 4						
	24	6	0.964	1.999	5.999	11.995
±12, 4						
	24	6	0.997	3.250	9.751	31.695
	24	6	0.989	2.599	7.796	20.259
Uterus, -12, 4						
	24	6	0.980	2.298	4.595	10.558
	24	6	0.967	2.044	6.132	12.533
+12, 4						
	24	6	0.843	1.232	3.695	4.556
	24	6	0.997	3.250	9.752	31.695
±12, 4						
	24	6	0.989	2.599	7.796	20.260
	24	6	0.996	3.106	9.319	28.947
	59	29			68.519	177.017

$\text{Chi}^2 = 14.558, 9 \text{ d.f. } P < 0.05.$

Therefore conclude that all correlations between left and right oviduct sperm are from a common population. Thus, combination of data for the Fallopian tubes is plausible.

APPENDIX II (c)

SPERMATOCOA IN THE FALLOPIAN TUBES

(EXPERIMENT 3)

BARTLETT'S TEST OF HOMOGENEITY OF VARIANCE

Site of Insemination	Time of Insemination	Time of Slaughter	Variance $s^2$	Log $s^2$	
Vagina	-12	4	0.010	-2.000	
		24	0.034	-1.469	
	+12	4	1.660	0.220	
		24	1.548	0.189	
	±12	4	2.881	0.459	
		24	2.295	0.361	
	Uterus	-12	4	2.274	0.357
			24	1.713	0.234
+12		4	0.019	-1.721	
		24	3.076	0.488	
±12		4	1.954	0.292	
		24	0.101	-0.996	

$\text{Chi}^2 = 59.83, 11 \text{ d.f. } P < 0.005$

Therefore Variance is significantly Heterogeneous