TRANSCERVICAL ARTIFICIAL INSEMINATION OF ROMNEY EWES

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ABSTRACT

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Transcervical AI was attempted in 178 mixed age Romney ewes. AI was performed between 48 and 52 h after synchronisation of oestrus with progesterone impregnated CIDRs. Fresh or frozen-thawed semen was used and each insemination dose contained approximately 100 million spermatozoa. Half of these ewes were treated prior to AI with clenbuterol hydrochloride (Panipart$_{TM}$) in an attempt to cause cervical softening to allow insertion of an inseminating pipette through the cervix. Reproductive tracts were recovered from 32 ewes in which transcervical insemination was achieved. After flushing each tract the spermatozoa were counted from the cervix and from the left uterine segment.

94% of ewes showed oestrus within 48 h of CIDR withdrawal. Variation in time of onset of oestrus prior to AI did not affect conception rate ($P>0.05$). The conception rate based on non-return to oestrus was 34%. A greater percentage of ewes conceived to insemination with fresh semen (42%) than that for frozen-thawed (24%) ($P<0.05$). Clenbuterol did not affect depth of cervical penetration but it did reduce bleeding at the cervical os observed at AI ($P<0.01$). Parity/age of ewe significantly affected depth of cervical penetration ($P<0.001$) and the time taken to inseminate ($P<0.01$), with few two tooth ewes successfully transcervically inseminated (76% were vaginally inseminated). Sperm numbers were significantly higher in the cervices of ewes that were inseminated with fresh rather than frozen-thawed semen ($P<0.05$). Numbers of spermatozoa recovered were low in comparison to similar studies and were related to the extent of cervical damage (recorded at dissection of reproductive tracts). Uterine sperm counts were significantly lower ($P<0.05$) for those ewes where cervical damage was observed.

The depth of insemination was estimated at the time of AI, and full cervical penetration was recorded in 68% of the mature ewes. However examination of the ewe cervices at dissection suggested that this figure was misleading. Anatomical evidence suggested

that the inseminating needle had frequently lodged in the cervical wall and successful passage had not been achieved. Therefore it was likely that semen was deposited frequently in areas of damaged cervical tissue which would have been detrimental to survival of spermatozoa. This was probably the main reason for the low sperm numbers in the reproductive tracts and consequently the low conception rate. For those ewes where full cervical penetration was recorded at AI, only 28% conceived.

Dedication:

I dedicate this work to my two sons,

Jeremy and John Paul.

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LIST OF ABBREVIATIONS

The following abbreviations have been used in the text without prior definition:

Units:

°C	degree Celsius
h	hour(s)
iu	international units
USP	United States Pharmacopoeia
kg	kilogram(s)
mg	milligram(s)
µg	microgram(s)
ml	millilitre(s)
%	per cent

Others:

CIDR(S)	Controlled internal drug release device(s)
SE	Standard error

CHAPTER I

Introduction

Artificial insemination is an important reproductive technology that is used for genetic improvement in animal production systems. Semen from superior sires can be disseminated over a larger number of females than would otherwise be possible by natural mating. Large artificial insemination programmes operate in several overseas sheep industries including France, Australia, Russia and China. The New Zealand sheep industry for a variety of reasons, including a shortage of schemes that identify superior sires and low product prices, has not made such widespread use of artificial insemination in commercial flocks. However in this country's ram breeding flocks there is an opportunity to utilise artificial insemination in conjunction with sire reference schemes to evaluate the progeny of many sires.

These schemes are frequently large, co-operative ram breeding programmes. A sire reference scheme avoids the cost of operating a central stud flock as is necessary with group breeding schemes. Reference sires are used via artificial insemination to link and compare the component flocks across different environments and management systems. This allows larger numbers of sires, mainly naturally mated, to be evaluated.

A major difficulty with artificial insemination in sheep is that the cervix of the ewe provides a physical barrier to the insertion of an inseminating pipette into the uterus. Consequently the cervical artificial insemination technique, which involves the deposition of fresh semen via an inseminating pipette at the external uterine ostium (cervical os), is widely used in sheep artificial insemination. The main problem with cervical artificial insemination is the interference of spermatozoa transport by the cervix, especially when frozen-thawed ram semen is inseminated. This means that the cervical procedure is only suitable for fresh semen, and relatively high numbers of spermatozoa are required to achieve adequate conception rates.

The fertility of fresh semen declines rapidly within hours of collection. Cervical

artificial insemination therefore makes the transport of semen to distant flocks difficult and requires that semen be collected on the day of use. The development of a diluent which extends the fertilising ability of fresh ram semen for several days would largely resolve these problems. In the absence of such a breakthrough, other insemination techniques aimed at delivering spermatozoa directly into the uterus need to be investigated.

The laparoscopic technique was developed to circumvent the detrimental effect of the cervix on sperm transport. This procedure achieves commercially acceptable conception rates using frozen-thawed semen but involves the surgical deposition of spermatozoa through the abdominal wall into the uterus of the ewe. Expensive equipment, skilled technicians and anaesthetic agents are required.

Attempts to develop a transcervical artificial insemination (TAI) technique are driven by the knowledge that such a procedure, if successful, would make sheep artificial insemination of greater use because it would combine the advantages of the cervical and laparoscopic techniques. Compared with cervical artificial insemination programmes, lower doses of fresh semen would be required so semen of superior genetic quality could be disseminated over more females. The use of frozen-thawed semen would become more popular as it could be inseminated without the economic and welfare costs associated with laparoscopic artificial insemination. This would allow increased semen collection and storage from rams out of the breeding season. It would also facilitate the transport of semen. In New Zealand the development of transcervical artificial insemination would be suited for use in sire reference schemes. Consequently it would become more cost-effective for ram breeders to participate in these schemes. If the rate of genetic gain in the nucleus ram breeding flocks of this country could be accelerated there would be obvious 'spin-offs' to the industry. One has to only glance at the New Zealand dairy industry to see the potential for its use.

CHAPTER II

Review of Literature

1. The development of transcervical artificial insemination

1.1 Artificial Insemination technique

Vaginal and cervical AI are two simple, non-surgical AI methods described by Wallace (1992). The vaginal technique, which is relatively quicker and less stressful for the ewe, involves the 'blind' insemination of fresh semen, usually undiluted, into the vagina. Cervical AI involves the deposition of fresh semen, with the assistance of a speculum and associated light source, into the first cervical fold. Fairnie and Wales (1982) and Maxwell and Hewitt (1986) reported that conception rates following fresh semen inseminations for these two AI methods were similar. However Harvey *et al.* (1986) stated that conception rates were lower and higher semen dose rates were required with the vaginal method. There have been reports of acceptable conception rates after cervical AI with frozen-thawed ram semen (Colas, 1975) but often double inseminations were performed and/or relatively high insemination doses were used. Most studies concluded that both these techniques were unsuitable for the insemination of frozen-thawed ram semen (Clarke *et al.*, 1984; Maxwell *et al.*, 1984).

Killeen and Caffery (1982) have described an AI technique which involved the insertion of inseminating instruments through the abdominal wall and the injection of semen into the uterine horn(s) with the aid of a laparoscope. Commercially acceptable conception rates of 50 to 80% were recorded after laparoscopic insemination of frozen-thawed semen (Maxwell and Hewitt, 1986). However laparoscopic Al involves minor surgery and veterinary supervision which makes it a relatively expensive and invasive procedure. McKelvey (1994) referred to pressure from various animal welfare groups in the United Kingdom to develop non-surgical insemination techniques for use on animals for productive purposes.

The use of cervical traction to allow an insemination needle to be inserted deep into the cervix was reported by Salamon and Lightfoot (1967). Anderson *et al.* (1973) demonstrated that intrauterine deposition of frozen-thawed semen after cervical traction could achieve acceptable fertility. Fukui and Roberts (1976a,b) reported that fertility following a single transcervical insemination of frozen-thawed semen was comparable to that achieved after cervical AI with fresh semen. However in those latter experiments relatively high doses of semen were inseminated.

Halbert *et al.* (1990b) refined TAI by developing specialised AI equipment and methods of ewe restraint. The transcervical insemination of 80 ewes with 150 million frozen-thawed spermatozoa resulted in lambing rates of 65% and 55% for laparoscopic and TAI respectively (Halbert *et al.*, 1990c). Windsor *et al.* (1994) inseminated 433 Merino ewes with frozen-thawed semen using three different AI techniques; laparoscopic AI resulted in a significantly higher pregnancy rate than TAI in one of two experiments. Cervical AI with frozen-thawed semen resulted in a zero pregnancy rate in both of these experiments. Smith *et al.* (1995) reported an average pregnancy rate of about 65% after insemination of frozen-thawed semen regardless of whether laparoscopic or TAI was used. Table 1 shows a summary of results from TAI studies.

Table 1: Results of experiments on transcervical insemination of ewes

Reference	Experiment	N	Full cervical passage[1] (% of N)	Frozen-thawed semen (10^{6})	Lambing rate (% of [1])
Lawrenz (1985)	1	97	72	-	71
Halbert *et al.* (1990b)	1	89	82	-	-
Halbert *et al.* (1990c)	1	40	45	150	55
	2	38	76	150	40
Buckrell *et al.* (1994)	1	2060	88	150	28
Windsor *et al.* (1994)	1[a]	771	76	80	34
	2[b]	555	43	80	40

where [a] = ewes inseminated by experienced operators

[b] = ewes inseminated by inexperienced operators

1.2 Animal restraint and equipment

Fukui and Roberts (1976a,b) used a duckbilled speculum with a headlight and 300 mm forceps for traction on the cervix to aid insemination. The semen was deposited into the uterus via a 100 mm, ball-tipped, 17 gauge hypodermic needle. The ewe was inseminated in a standing position and a forefinger was placed in the animal's rectum to guide the needle through the cervical canal.

The inseminating instruments that Halbert *et al.* (1990b) found most effective were: the Grave's duckbilled speculum, Bozemann 267 mm tissue forceps, and a malleable probe

with a bent tip for inseminating semen. Use of the Grave's speculum allowed the cervical os to be readily located and the vagina sufficiently opened for the introduction of instruments. The Bozemann forceps enabled cervical traction with minimal trauma to the reproductive tract. The bent-tipped, malleable probe was used for inseminating as it was the only device tested that could be readily manipulated through the cervix. Halbert *et al.* (1990b) reported that the Commodore foot-trimming cradle and the modified Weribee laparotomy cradle were effective animal restraints for transcervical insemination. Both cradles were labour efficient, allowed the ewes to be restrained in dorsal recumbency and ensured that the vagina was positioned correctly for AI. The Commodore foot-trimming cradle was slightly better for TAI because the ewes were less stressed (Halbert *et al.*, 1990b). A subsequent study also recommended the foot-trimming cradle as the superior method of restraint due to the greater ease of loading/unloading (Buckrell *et al.*, 1994).

2. Factors affecting cervical penetration

2.1 Anatomy of the cervix

The anatomy of the cervix has a large influence on the success of transcervical penetration (Fukui and Roberts, 1977b; Halbert *et al.*, 1990a). Anatomical details were reported after slaughter and collection of material by Moré (1984), who studied 20 cervices from Lacuane breed ewes, and Halbert *et al.* (1990a), who observed 80 to 100 cervices from crossbred ewes. Within the cervices there were approximately five to six caudally orientated, funnel-shaped folds. The second or third cervical folds from the vaginal end had the narrowest openings and were often eccentrically aligned with the other circular folds. The interlocking nature of the cervical folds was demonstrated in both experiments by resin casts of cervices and Halbert *et al.* (1990a) also showed this feature using xeroradiography. Both studies reported a large variation in cervical structure between ewes. Moré (1984) stated that the eccentric fold(s) made it impossible to introduce an instrument into the cervix. However Halbert *et al.* (1990a) showed that when the recovered cervices were stretched similar to a retracted state in the live animal, the rings were separated and the cervical folds were straightened. This confirmed the

findings of Salamon and Lightfoot (1970) who observed that cervical traction allowed a significantly greater depth of insemination.

Fukui and Roberts (1978) and Halbert *et al.* (1990a) referred to the location of the cervical os as a major problem confronting TAI. Halbert *et al.* (1990a) noted that the average diameter of the openings in the eccentric cervical folds (2.7 $\pm$ 1.1 mm) limited passage of an AI instrument through to the uterus. The significant relationship between type of cervical os and transcervical penetration reported by Salamon and Lightfoot (1970) and Fukui and Roberts (1978) was not confirmed in a later study by Halbert *et al.* (1990a).

2.2 Cervical softening

Prior to parturition the process of cervical softening usually occurs (Meijer and Fentener van Vlissingen, 1993). Porter (1993) referred to cervical dystocia or lack of cervical softening as a common problem for the ewe at lambing. Meijer and Fentener van Vlissingen (1993) described the cervix as a thick-walled muscular organ which contained both smooth muscle and collagenous connective tissue in its walls. Parry and Ellwood (1981) stated that the process of cervical dilation involved rearrangement of the collagen fibres and activation of fibroblasts in the cervical wall.

Hormonal changes associated with cervical softening included a sudden decline in progesterone, and an increase in oestrogen and prostaglandins (Parry and Ellwood, 1981). Ledger *et al.* (1985) treated ewes in late pregnancy with epostane, which inhibits the enzyme responsible for the formation of progesterone from pregnenelone. The result was cervical dilation and advanced birth without the associated rise in oestrogen concentrations usually noted at parturition. In that same study the inhibition of prostaglandin synthesis by infusion of mefenamic acid reduced uterine activity and prevented the cervical softening caused by epostane. Rickords and White (1988) reported that the insertion of a lipid-based suppository containing 10 mg of prostaglandin E_2 at the cervical os for up to 13 h caused partial softening in 10/15 ewes but complete passage of the cervix with a 5 mm, ball-tipped probe was achieved in only one ewe.

Barry *et al.* (1990) achieved 100% cervical penetration in a group of 10 ewes following administration of prostaglandin E_2 and oestradiol to 'ripen' the cervix. Owiny and Fitzpatrick (1992) administered clenbuterol around day 130 of gestation. The subsequent reduction in myometrial contractility did not prevent cervical dilation at term. However when a prostaglandin synthetase inhibitor (sodium meclofenamate) was given orally in conjunction with the intravenous clenbuterol infusion, cervical dilation was prevented.

Intravenous administration of oestradiol-17β in ewes in late pregnancy has caused cervical softening 18 to 24 h after treatment (Fitzpatrick and Dobson, 1981). Adams (1986) reported that the daily subcutaneous administration of approximately 300 ug of oestradiol for a 200 day period caused major morphological changes to the cervix. These included a softer, broader and more bicornate cervix with the endocervix changing to resemble the endometrium.

Oxytocin may also be involved in cervical softening, as Porter (1993) stated that there is a relationship between oxytocin and prostaglandin production. Khalifa *et al.* (1992) dilated the cervix by the intravenous injection of oxytocin (200 to 600 USP units) 44 h and 52 h after progesterone pessary removal. This effect was repeated by a single oxytocin injection (400 USP units) nine days after pessary removal and 6 h and 12 h after oestradiol administration. McKelvey (1994) reported that the treatment of ewes with oxytocin (25 units) five to seven minutes prior to TAI did not facilitate complete cervical penetration or reduce the number of times the needle passed through the wall of the cervix.

It is generally accepted that relaxin is involved in the cervical relaxation that occurs at birth (Parry and Ellwood, 1981). Salamon and Lightfoot (1970) injected ewes with relaxin prior to attempted TAI and reported no improvement in fertility or ease of cervical penetration.

2.3 Other factors affecting cervical penetration

Halbert *et al.* (1990c) and Harvey (personal communication) suggested that the ease of transcervical AI was affected by breed. Halbert *et al.* (1990b) recorded cervical penetration in a group of animals from seven separate breeds but unfortunately the numbers of ewes in each category were too few to show any breed differences.

Buckrell *et al.* (1992) and Buckrell *et al.* (1994) reported that increased skill of AI technician significantly increased penetration rates through the cervix.

Smith *et al.* (1995) and Buckrell *et al.* (1994) reported that cervical penetration rates were higher when *postpartum* periods were shorter.

3. Factors affecting fertility to artificial insemination

3.1 Timing of artificial insemination

AI at a 'fixed time' following oestrous synchronisation produced no significant decline in conception rate compared with 'on oestrus' programmes (Harvey *et al.*, 1984; Harvey *et al.*, 1986). The optimum time for artificial insemination of ewes, which was estimated by double mating oestrus-induced ewes, was 15 to 11 h before ovulation (Dziuk, 1970). The time of ovulation will vary with method of synchronisation, and Shackell (1991) has shown this to be 59 h after CIDR withdrawal. Fixed time AI should therefore take place 44 to 48 h after CIDR removal (Shackell, 1991). However Paton *et al.* (1993) reported higher conception rates when the AI interval was extended to 48 to 54 h. Harvey (1989) recommended fixed time cervical AI at 48 to 52 h following CIDR removal.

Fixed time laparoscopic intrauterine AI of CIDR-synchronised ewes should take place 50 to 58 h post CIDR removal (Harvey, 1989), which is about 4 to 9 h prior to ovulation. Maxwell (1986a) reported that laparoscopic AI with frozen-thawed semen was more successful after ovulation. The lower percentage of ewes lambing when AI

took place prior to ovulation was attributed to higher embryonic mortality and greater tract disturbances near the time of ovulation (Maxwell *et al.*, 1984). Dziuk (1965) estimated that the fertilisable life of the sheep egg was about one to two days. It was suggested that provided spermatozoa reached the ampulla within this period fertilisation could occur. The lower conception rates reported by Maxwell *et al.* (1984) following AI 22 h after ovulation compared with 18 h supports this theory.

3.2 Number of spermatozoa inseminated and site of uterine deposition

Maxwell (1986a,b) recommended using a minimum dose rate of 40 million spermatozoa when inseminating frozen-thawed semen via laparoscopic intrauterine AI. In the large laparoscopic AI programmes operated by French Meat Breeding Organisations, 80 million frozen-thawed spermatozoa were inseminated per ewe (Cognie, 1990). It was suggested that a suitable range of fresh semen insemination doses for cervical AI was 100 to 400 million motile sperm (Clarke *et al.*, 1984). Other recommendations were that with synchronised AI approximately 200 million motile sperm should be used, while this could be reduced to about 100 million in natural oestrous programmes (Fairnie and Wales, 1982; Langford and Marcus, 1982; Harvey, 1989).

Following laparoscopic AI, McKelvey *et al.* (1985b) reported that 2/13 ewes only produced fertilised ova from that uterine horn that was inseminated. Consequently it was recommended that both uterine horns should be inseminated to prevent this unilateral fertilisation. Maxwell (1986b) compared fertility after the laparoscopic insemination of Merino ewes in either the uterine horn epsilateral to the corpus luteum or in both horns using the same total dose of semen. A higher percentage of ewes lambed when both uterine horns were inseminated. However these results were probably distorted by the additional tract interference in the single horn inseminations, owing to manipulation at laparotomy to find the uterine horn ipsilateral to the corpus luteum. Robinson *et al.* (1989) reported that deposition of spermatozoa in one uterine horn ensured fertilisation in the contralateral horn. It was concluded in that study that spermatozoa entering one uterine horn migrated readily to the other. Mattner (1963b) stated that regardless of their motility sperm that arrived at the uterus were rapidly

dispersed.

3.3 Depth of semen insemination

Salamon and Lightfoot (1967) reported that degree of cervical penetration influenced non-return rate. In that investigation the percentage of ewes not returning over those inseminated was cervical 0%, deep cervical 15%, and uterine 34%. Fukui and Roberts (1976a) also found a significant relationship between depth of frozen-thawed semen insemination in the ewe's reproductive tract and lambing rate. Fertility was 51% following intrauterine transcervical semen deposition and 20% for deep cervical inseminations. Halbert *et al.* (1990c) observed a trend for higher lambing rates as site of insemination progressed from mid cervical to deep cervical to uterine. Ritar *et al.* (1990) recorded a significant increase in pregnancy rate of cashmere goats with frozen-thawed semen as depth of AI increased. Windsor *et al.* (1994) reported that a greater depth of cervical insemination significantly improved conception rates in an experiment where inexperienced operators performed the insemination. In a concurrent trial there was a trend towards a higher pregnancy rate with increased depth of cervical penetration when AI was conducted by experienced operators. Smith *et al.* (1995) stated that conception rates following AI with fresh semen were significantly higher when full rather than partial cervical penetration was achieved (full 68%, partial 58%).

Conversely Salamon and Lightfoot (1970) reported lower fertility when depth of insemination increased. However in that study when depth of cervical penetration was less than 1 cm and mucus was scarce, lambing rate was significantly lower than for deeper inseminations.

3.4 Embryonic mortality

Buckrell *et al.* (1994) recorded a lambing rate of 26% for parous two tooth ewes compared with 52% for mature ewes following TAI. The difference in lambing rates could be due to the relatively high embryonic mortality rates for two tooth ewes reported by Blockey *et al.* (1975) and Edgar (1962).

Halbert *et al.* (1990c) suggested that embryonic mortality was higher following laparoscopic compared with transcervical AI.

3.5 Postpartum interval prior to Transcervical AI

Halbert *et al.* (1990c) suggested that an interval of only three weeks between weaning and transcervical AI could have lowered fertility. Buckrell *et al.* (1992) reported a markedly lower lambing rate in ewes successfully inseminated through the cervix when the *postpartum* period was less than four months.

4. Transport of spermatozoa in the reproductive tract

4.1 The dynamics of sperm transport

Sperm transport mechanisms were defined by Hawk (1983) as flagella activity of sperm, and muscular contractions, fluid movement and ciliary beats of the female reproductive tract. Allison and Robinson (1972) stated that failure of sperm transport was a major source of reproductive wastage in sheep. In sheep during natural mating semen is ejaculated into the cranial end of the vagina against the external uterine ostium (Evans and Maxwell, 1987). Sperm only survive for a few hours in the vagina so it is important that sufficient numbers of spermatozoa rapidly move through to the more favourable cervical environment (Hafez, 1974; Giles, 1975). Quinlivan and Robinson (1969) reported a positive correlation between the number of sperm in the cervix and the uterus for up to 12 h after insemination. Low sperm numbers in the anterior cervix two hours after AI reduced the number of sperm in the oviducts at ovulation (Croker *et al.*, 1975; Hawk and Conley, 1975). The consequence of this low number of healthy sperm in the uterine tubes around the time of ovulation due to interference with sperm transport mechanisms was reduced fertility.

Mattner (1963a) reported that a small number of spermatozoa were transported to the uterine tubes within minutes of semen deposition. Lightfoot and Restall (1971) and Hawk and Conley (1975) stated that this rapid phase of sperm transport did not always

occur and was not important for fertilisation. The slow phase of sperm transport resulted in the formation of selective sperm reservoirs at the uterotubal junction and in the cervical crypts (Hawk, 1983; Hafez, 1974). These reservoirs sequentially released spermatozoa that moved anteriorly to increase and maintain sperm populations in the uterus and oviducts (Hawk, 1983; Hafez, 1974). Hafez (1974) noted that these reservoirs also act as barriers to the cranial progress of large numbers of spermatozoa and consequently only several million of the billion spermatozoa deposited at the cervical os following natural mating managed to traverse the cervix. These numbers were still in excess of that required for fertilisation (Hafez, 1974).

Mattner and Braden (1963) suggested that sperm motility was an insignificant component of sperm passage into the cervix. However Lightfoot and Restall (1971) reported that sperm motility was important for the efficient movement of sperm into the cervix and for the migration of sperm through cervical mucus. When non-motile sperm were inseminated at the cervical os, no cells were recovered from the muscosal folds (Lightfoot and Restall, 1971). Sperm of low motility, although able to ascend the uterus via the cervical lumen, may be more susceptible to drainage to the exterior (Lightfoot and Restall, 1971). Hafez (1974) suggested that the functions of the cervix included the protection of spermatozoa from leucocytes and the filtration of defective and/or immobile sperm. The physical properties of cervical mucus appeared to assist the movement and orientation of sperm in the cervix (Gibbons and Mattner, 1966; Adams, 1981). Cervical mucus is also involved in the establishment and maintenance of a reservoir of sperm in the cervix (Gibbons and Mattner, 1966).

Muscular contractions of the ewe's reproductive tract probably moved spermatozoa through to the uterus and uterine tubes (Mattner and Braden, 1963; Mattner, 1963b; Lightfoot and Restall, 1971). Approximately 26 to 48 h after the onset of oestrus the direction and origin of these contractions changed, with the waves travelling caudally and originating from the uterotubal junction instead of the uterus (Croker and *et al.*, 1975; Hawk and Conley, 1975).

Hunter and Nichol (1983) reported that following mating sperm may reside in the lower

isthmus for 17 to 18 h until ovulation was near. Spermatozoa are restricted in the caudal area of the isthmus possibly to avoid uterine leucocytes and to avoid the egg being fertilised by more than one spermatozoa (Hunter and Nichol, 1983). The movement of sperm from the uterotubal junction to the ampulla was triggered at the time of ovulation (Parrish and First, 1993).

4.2 Measurement of sperm transport

It was suggested that the best method of assessing sperm transport was to slaughter the ewe, remove the entire reproductive tract, and count the number of sperm in different segments of the tract, ideally over a range of times (Hawk, 1983). Sperm numbers peaked in the uterus 12 to 24 h after insemination (Mattner, 1963a; Quinlivan and Robinson, 1969).

4.3 Sperm loss factors

Hawk (1983) stated that there was little information on the loss of sperm from the uterus and uterine tubes via drainage to the exterior, loss to the peritoneal cavity, and phagocytosis. Normally these sperm loss factors described above were not high enough to lower fertility (Hawk, 1983). Mattner (1963a) attributed drainage to the peritoneal cavity as a major source of sperm loss following mating. There was a large loss of sperm from the vagina following cervical AI due mainly to drainage of semen and leakage of cervical mucus to the exterior (Lightfoot and Salamon, 1970a). Quinlivan and Robertson (1969) reported that 24 h after cervical AI with 500 million spermatozoa, only 0.25% were recovered from the uterine tubes. Approximately 80% of the spermatozoa flushed from the reproductive tract after deposition at the uterocervical junction had passed caudally to the vagina (Lightfoot and Restall, 1971). Following AI, leucocyte activity was more pronounced in the uterus than in other areas of the tract and these white blood cells attacked live, healthy sperm cells (Hafez, 1974; Giles, 1975).

4.4 The effect of fresh or frozen-thawed semen on fertility

Salamon and Lightfoot (1967) recovered on average only 24 spermatozoa per uterine tube after cervical insemination of 12 ewes with frozen-thawed semen. Several thousand spermatozoa were recovered from the uterine tubes following natural service or cervical AI with fresh semen. Salamon and Lightfoot (1967) recorded an egg fertilisation rate of 25% following cervical insemination with frozen-thawed spermatozoa. In conjunction with that experiment a number of ewes were concurrently cervically inseminated with 100 million freshly diluted spermatozoa and 80% fertility resulted.

Mattner *et al.* (1969) recorded the distribution of spermatozoa in the genital tract of the ewe at 4 to 24 h following cervical AI with 100 million fresh or frozen-thawed spermatozoa. Four hours after AI, significantly more spermatozoa were found throughout the reproductive tract of ewes inseminated with fresh compared with frozen-thawed semen. Numbers of spermatozoa in the tracts examined 24 h after insemination (Table 2) increased in the uteri and uterine tubes of those ewes that were inseminated with fresh semen. No spermatozoa were found, 24 h after AI, in the uteri or uterine tubes of ewes that were inseminated with frozen-thawed semen.

Lightfoot and Salamon (1970b) used three AI techniques to inseminate ewes and recorded lower fertility for frozen-thawed rather than fresh semen.

Fukui and Roberts (1977a) reported that there were significantly less spermatozoa recovered from the uterine tubes following insemination (cervically or transcervically) of ewes with frozen-thawed compared with fresh semen (Table 2).

Table 2: Studies of spermatozoa transport in the ewe examined *postmortem*

Experimental study	Segment of tract[a]	Type of semen deposition		
		Cervical AI (fresh)	Cervical AI (frozen-thawed)	Transcer-vical AI (frozen-thawed)
Mattner *et al.* (1969): AI at 2nd oestrus after synchronisation, with 200 million spermatozoa.	cervix	233000	1700	-
	uterus	30500	-	-
	uterine tubes	12000	-	-
Fukui and Roberts (1977a): AI at natural oestrus, with 350 million undiluted spermatozoa.	cervix	859000	8800	14100
	uterus	445000	3800	11500
	uterine tubes	22500	1600	1700

where [a] = tracts recovered 24 h after AI

Lightfoot and Salamon (1970a) showed that poor fertility following AI with frozen-thawed ram semen was due to reduced viability and retention of spermatozoa in the cervix. Pursel *et al.* (1978) stated that the poor retention of frozen-thawed boar semen in sows was associated with freezing damage to sperm membranes. It was suggested in that trial that structural damage to the spermatozoa interfered with their ability to enter cervical mucus or adhere to the tract epithelium. Pursel *et al.* (1978) maintained that the rapid loss of frozen-thawed boar semen could not be attributed to phagocytosis.

Maxwell (1984) suggested that the problems of sperm transport after cervical AI with frozen-thawed semen can be largely overcome by using laparoscopic AI.

4.5 The effect of oestrogen and progestagins on sperm transport

Allison and Robinson (1972) stated that two critical areas of sperm transport, the physical properties of cervical mucus and uterine motility, were largely regulated by ovarian hormones. The main hormones involved were progesterone and oestrogen which operated in tandem to allow successful gamete transport and fertilisation.

Allison and Robinson (1972) reported that efficient sperm transport required the ewe either to be in oestrus or receiving an oestrogen injection if ovariectomised. Hawk (1983) reported that in some experiments the administration of oestrogen at mating increased spermatozoa numbers in the uterine tubes and anterior cervix, while in another experiment an injection of oestrogen at AI had no effect on fertilisation rate. Progestagens (potent synthetic agonists of progesterone) or oestrogen administered to the ewe around the time of mating may change the quality or the physical nature of cervical mucus and consequently interfere with sperm retention, survival and transport (Hafez, 1974). The contractility of the cervix and uterus is higher during the oestrogen dominated follicular phase and lower during the progesterone regulated luteal phase (Allison and Robinson, 1972). The movement of spermatozoa into the cervix and the formation of a stable cervical spermatozoa population depended on a period of progesterone priming followed by high levels of oestrogen (Allison and Robinson, 1972).

This delicate endocrine balance is upset by artificial synchronisation of oestrus with progestagins. Hawk *et al.* (1981) reported that the use of progestagen to synchronise oestrus inhibited the movement of sperm through the reproductive tract and caused death and immobilisation of sperm in the cervix and uterus. Reductions in sperm numbers were most severe in the uterus and anterior cervix. Consequently the formation of sperm reservoirs in the cervix two hours after insemination and in the uterine tubes near the time of ovulation were restricted. Quinlivan and Robinson (1969), Boland *et al.* (1978a), and Hawk *et al.* (1987) also reported a dramatic reduction in sperm survival and transport following progestagen synchronisation of oestrus in ewes. Fertilisation failure observed by Hawk *et al.* (1987) was probably due to the low numbers of sperm,

especially healthy sperm capable of fertilising the egg, in the uterine tubes at ovulation.

4.6 The effect of sympathomimetic compounds on uterine motility

The smooth muscle of the female reproductive tract is innervated by the sympathetic branch of the autonomic nervous system and there are α and β receptors (adrenergic receptors) in these muscle cells which react with the catecholamines (De Nooij, 1984). Stimulation of α receptors causes muscular contraction of uterine smooth muscle while activation of β_2 receptors causes relaxation (Zerobin and Kündig, 1980).

Clenbuterol is a specific β_2 sympathomimetic compound (De Nooij, 1984). It is used to delay parturition in sheep, cattle and pigs by relaxing the uterus (Zerobin and Kündig, 1980). Plant and Bowler (1988) observed that the use of clenbuterol delayed parturition by 10 h in most ewes. Zerobin and Kündig (1980) stated that three separate trials had shown that a single intravenous injection of 100 and 300 μg of clenbuterol completely suppressed contractions of the myometrium of the ewe. Hassett and Sloss (1984) noted that one of clenbuterol's applications in bovine obstetrics was treatment of incomplete dilation of the cervix. This therapeutic action was presumably due to myometrial inhibition delaying parturition and consequently allowing more time for the relaxation of cervical muscle.

Clenbuterol is a rapid-acting tocolytic compound that has a large and prolonged effect (Zerobin and Kündig, 1980). Hassett and Sloss (1984) reported that it was difficult to predict when maximum uterine muscle relaxation occurred following intramuscular administration of tocolytic compounds in conjunction with caesarean operation in cattle. An intravenous injection of 10 ml clenbuterol produced optimal myometrial relaxation less than three minutes after administration (Hassett and Sloss, 1984). De Nooij (1994) stated that relaxation of the bovine uterus occurred 10 to 20 minutes after intramuscular injections of clenbuterol. De Nooij (1984) suggested that the response of uterine contractility to clenbuterol could be influenced by an animal's state of excitement which could affect the circulation of the compound.

Zerobin and Kündig (1980) reported that oxytocin bound to receptors in uterine smooth muscle and that its action was antagonistic to clenbuterol. Both exogenous and endogenous oxytocin modified or prevented the action of clenbuterol (Zerobin and Kündig, 1980). Lightfoot and Salamon (1970a) and Salamon and Lightfoot (1970) reported that an oxytocin injection prior to AI did not affect sperm transport. Those experiments showed that an injection of relatively high levels of oxytocin (5.0 iu) at mating depressed fertilisation rates. However Stratman *et al.* (1959) reported that the injection of oxytocin into gilts at the time of AI improved fertilisation rates.

Hawk *et al.* (1982) administered two α adrenoceptor agonists, methoxamine and phenylephrine, to rabbit does at mating. Both substances significantly increased uterine contractions. Phenylephrine consistently increased numbers of spermatozoa throughout the female reproductive tract while methoxamine had no effect. Phenylephrine also increased fertilisation rate, presumably due to the higher numbers of spermatozoa in the uterine tubes at ovulation.

5. The purpose and scope of the investigation

A recently modified procedure for transcervical AI in sheep was evaluated. Fertility was measured and observations were made to identify factors influencing the success of TAI. The effects of a possible cervical softening agent on various parameters that could influence the reproductive success of TAI were recorded. Fresh or frozen-thawed semen was inseminated and subsequent effects on fertility were measured. Reproductive tracts from a proportion of those ewes successfully transcervically inseminated were recovered, near the time of ovulation, for anatomical observation and for estimation of numbers of spermatozoa.

The specific objectives of the experiments involved in this programme were:

1. To evaluate and develop the transcervical AI technique in conjunction with attempted cervical relaxation.

2. To record fertility after 'fixed time' transcervical AI (48 to 52 h after progesterone treatment) of mixed age Romney ewes within the normal breeding season.

3. To compare the effect of the transcervical insemination of 100 million fresh or frozen-thawed spermatozoa on fertility.

4. To record the effect of clenbuterol administration and transcervical AI of fresh or frozen-thawed spermatozoa on sperm transport and the macro-anatomy of the reproductive tract.

CHAPTER III

Materials and Method

1. Experimental design and animal management

The experimental animals consisted of 199 Romney ewes (29 maiden, two tooth ewes and 170 parous, mature ewes) from the Massey University progeny test flock. The animals were grazed on the 'Terrace Block' with herbage mass on offer maintained between 800 to 1400 kg DM/ha. Facial eczema spore counts conducted on adjacent paddocks gave very low to zero readings. Ewe live weights averaged 52 kg at the start of the trial and 50 kg at scanning.

The trial had two experimental components. An AI experiment evaluated the transcervical procedure and then a sperm transport study, which involved the slaughter of 32 of the inseminated ewes, focused on the subsequent numbers of spermatozoa in the ewe reproductive tracts. Both experiments were 2 x 2 factorial designs. Due to the constraints of labour and facilities, animals in each experiment were randomly allocated to four replicates which corresponded to the four consecutive days of AI or slaughter (referred to as day 1,2,3,4).

The two experimental factors were type of semen (freshly diluted or frozen-thawed) and the administration of the tocolytic compound clenbuterol hydrochloride[a] (Planipart$_{TM}$ Boehringer Ingelheim Ltd). Either 0.25 ml of frozen-thawed semen or 0.125 ml of fresh semen was inseminated with the dose constant at 100 million spermatozoa per insemination. The clenbuterol treatments involved either a 7 ml intramuscular injection five minutes prior to AI, or no injection.

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[a]= each ml contains 0.03 mg of 4 amino-α- [(tert.-butyl amino) methyl] -3,5, dichlorobenzyl - alcohol hydrochloride.

The AI experiment involved the staggered synchronisation of ewes to allow 'fixed time' AI over a four-day period. Animals allocated to each day of AI were balanced for age and live weight and randomly allocated to each treatment. At the end of each of the four AI days, eight ewes which had been successfully transcervically inseminated were selected for slaughter and collection of reproductive tracts the following morning. For each slaughter day two mature, parous ewes that were balanced for age were randomly selected from each treatment.

2. Synchronisation and oestrous detection

Two vasectomized rams wearing mating harnesses fitted with marking crayons were put with the ewes to stimulate cycling activity prior to synchronisation of oestrus. Synchronisation involved the intravaginal insertion of a type-G CIDR device (EAZI-breed, Carter Holt Harvey Ltd, Hamilton, New Zealand) into each ewe. The CIDRs, impregnated with 9% progesterone, were inserted for 12 days. All rams were kept away from the ewes during this period to improve synchronisation via the 'ram effect'.

Six vasectomized rams fitted with mating harnesses and marking crayons were joined with the ewes 18 h after CIDR removal. Tupping marks were recorded every six hours up to the start of AI from 30 h to 48 h after CIDR withdrawal. Marked ewes were removed at each recording. All vasectomised rams used were greater than three years of age and were of proven infertility.

3. Semen

3.1 Collection and examination of fresh semen

Two rams (Finnish Landrace x Romney) were trained to serve into an artificial vagina as mounting was attempted, with the aid of an ovariectomised ewe restrained in a headbail. The method is described in detail by Evans and Maxwell (1987). Fresh semen was collected each day from both rams in the hour preceeding the start of AI. The ejaculates were placed in a waterbath at 32°C following collection.

A 0.1 ml sample of semen was removed from the ejaculate immediately following collection and used to determine semen quality. Motility was assessed by viewing movement of spermatozoa under a microscope (x40 magnification). A score of 1 to 5 was given (1 = poor, 5 = excellent) and any samples scoring less than three were rejected. A visual assessment of concentration was also made by scoring for colour. A score of 0 to 5 was given (0 = insignificant number of spermatozoa/ml and 5 = 4.5 to 6.0 thousand million spermatozoa per ml). Ejaculates from both rams were pooled if assessment of quality was satisfactory. The concentration of spermatozoa was determined in a 0.01 ml sample of semen using a haemocytometer as outlined by Evans and Maxwell (1987). The pooled ejaculate volume was then measured and an equal volume of diluent was added. The pooled semen was divided into two test tubes for safety and hygiene reasons.

3.2 Fresh semen dilution

The final dilution to 800 million spermatozoa per ml for insemination took place within half an hour of collection. An error on the first day of AI resulted in a final dilution to 861 million spermatozoa per ml. Test tubes containing the freshly diluted semen were then placed in a beaker of water from the waterbath and the temperature was allowed to fall gradually to approximately 15°C.

The diluent (Evans and Maxwell, 1987) was prepared by mixing 10 g skim milk powder with 100 ml distilled water and heating at 90°C for ten minutes. After cooling to 15°C, Streptopen liquid penicillin was added. Two batches of diluent were made over the four-day AI period but used within 48 h.

3.3 Frozen semen

The frozen ram semen obtained had been collected from two progeny-tested Coopworth rams, diluted to 400 million spermatozoa per ml, packaged in 0.25 ml Cassou straws, and frozen by a one-step method in liquid nitrogen. Any ejaculates at collection containing less than 80% live sperm or with a motility score of less than four were

rejected prior to dilution and freezing. A tris-glucose-egg yolk diluent with glycerol and 1000 units/ml of antibiotic added (Evans and Maxwell, 1987) was used for the one-step dilution of the rams' semen. Periodically during the AI period the frozen-thawed semen was checked for motility by examining a drop under low microscopic power. There was a non-random allocation of frozen semen from sires between replicates. Semen from one sire was used exclusively on day 1 and 3 of AI and it was also used for some inseminations performed on day 4. Semen from the other sire was used exclusively on day 2 and also for some day 4 inseminations.

4. Artificial Insemination.

4.1 Technique

Ewes were inseminated between 48 and 52 h following CIDR withdrawal regardless of the timing of oestrus. An attempt was made to inseminate all ewes by the TAI method described by Halbert *et al.* (1990b,c).

A Cassou inseminating gun [Figure 1b(a)] was used with a specially designed needle [Figure 1a(b)] . The tip of the 19 gauge (89 mm) epidural spinal needle was bent to an angle of 45°, 10 mm from the end. The original opening was soldered closed and a new hole was constructed for semen release immediately anterior to the bend. There was a special connection necessary to fix the needle to the pipette. The Luer-lok hub (Becton, Dickinson and Co., Rutherford, N.J.) of the needle was fixed to the distal segment of a 2 ml syringe which was packed with a piece of rubber tube and glued to the barrel of the inseminating gun.

The ewe was restrained horizontally in a dorsally recumbent position on a modified laparoscopy cradle. The hind legs were tied cranially and a cushion was placed under the ewe's lower back to elevate the hindquarters. A pair of (267 mm) Bozemann uterine

Figure 1a Equipment for transcervical artificial insemination.
Unmodified insemination needle (a), modified needle (b), modified needle - not used in this experiment (c)

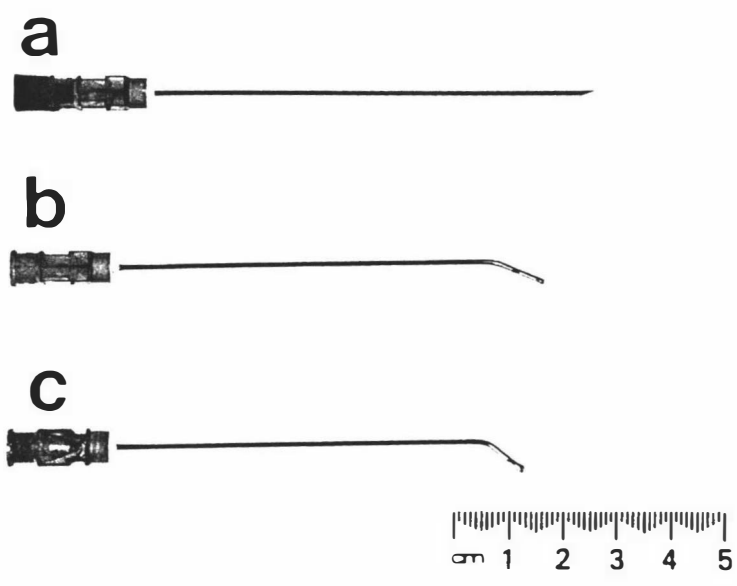


Figure 1b Equipment for transcervical artificial insemination.
Cassou inseminating gun (a) and Bozeman forceps (b)

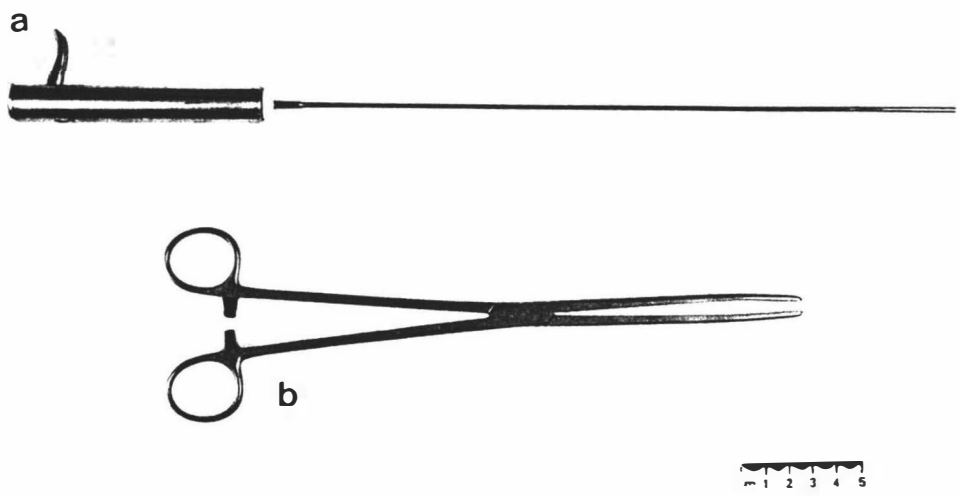


Figure 2a Transcervical artificial insemination - the handles of the forceps displaced from the speculum to allow visualisation of the cervical os and insertion of the inseminating needle

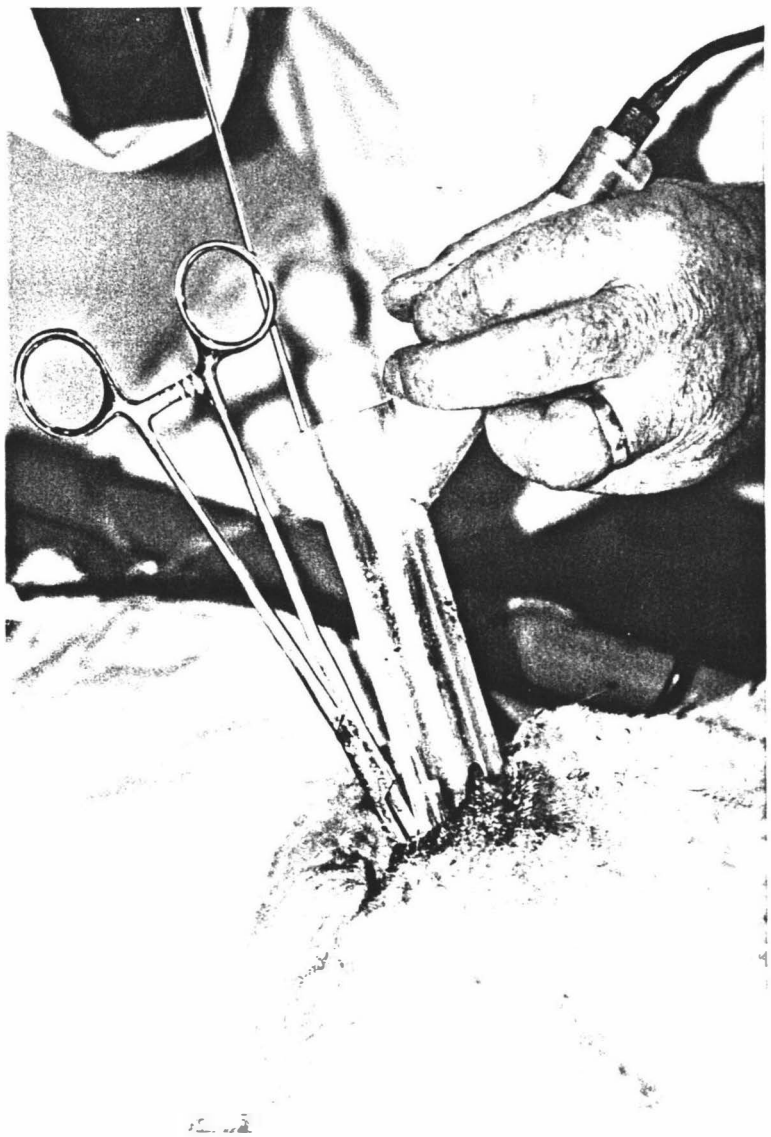
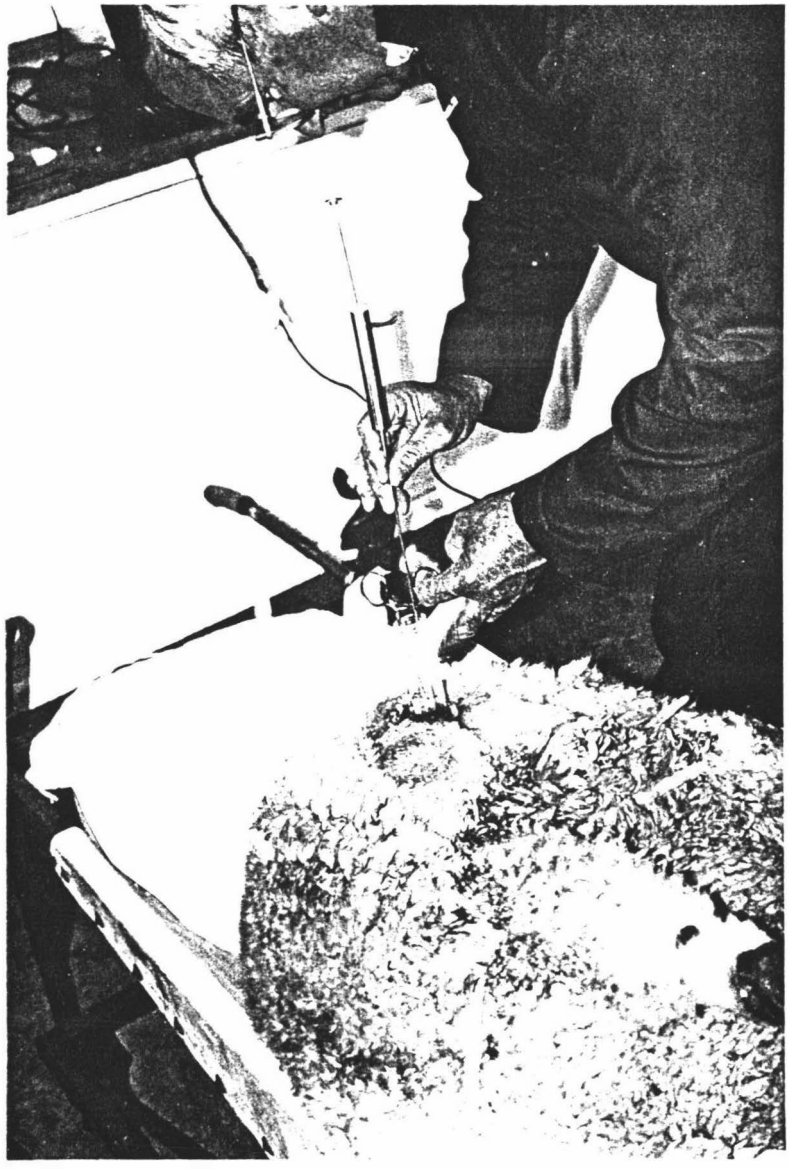


Figure 2b Transcervical artificial insemination - cervical traction applied via forceps to assist penetraton of the cervix with the inseminating needle



dressing forceps [Figure 1b(b)] were inserted into the ewe's vagina via a speculum and were fastened to tissue surrounding the cervical os. The perspex speculum had a narrow slit along one side and a Welsh Allyn light in the handle. A plastic rod was inserted into the speculum and smeared with lubricant prior to insertion to assist vaginal entry. The speculum was wiped clean of mucus between ewes with sterile gauze swabs.

Once attached to the cervical os the handles of the forceps were displaced sideways through the open slit on the side of the speculum (Figure 2a). This allowed visualisation of the cervical os for the introduction of the needle of the inseminating pipette. The handles of the forceps were then moved back inside the speculum so that the angles of cervical traction and needle insertion were aligned (Figure 2b). Slight tension on the forceps straightened the cervix to assist manipulation of the needle of the inseminating pipette through the cervix to the uterus (Figure 2b). The pipette was then rotated slowly in both directions while simultaneously applying slight downward pressure. Occasionally the pipette was withdrawn slightly as the needle became lodged in one of the several blind pockets lining the cervical canal. It was easier to perform the insemination with assistance as there were three instruments to hold simultaneously (speculum, forceps and pipette). The forceps could be let go to allow operation of the pipette plunger.

Most inseminations were performed by one experienced technician while a second operator also with previous AI experience inseminated ten ewes. Three inexperienced operators who were being trained in the TAI technique inseminated a further 13 ewes.

The observations made at artificial insemination were:

Name of technician - five technicians;

Sire (frozen-thawed inseminations only) - two sires;

Appearance of cervical os at time of AI (subsequently known as condition of cervix) - dry, wet, clear fluid, cloudy fluid;

Depth of penetration of pipette - vaginal (blind), cervical (at the cervical os), one quarter cervical penetration, one half cervical penetration, three quarters cervical penetration, full cervical penetration;

Time taken to inseminate - less than 0.45, between 0.45 and 1.5, between 1.5 and 2.25, between 2.25 and 5.0 (minutes);

The amount of bleeding at the cervical os following AI - no bleeding, slight bleeding, bleeding, heavy bleeding;

Time of day when insemination was completed - range 1.00 to 5.00 pm.

4.2 Semen handling

For the fresh semen inseminations a 0.25 ml Cassou straw was attached to a 1 ml tuberculin syringe and 0.25 ml of freshly diluted semen was drawn into the straw. Half a straw (0.125 ml), which contained 100 million spermatozoa, was inseminated into each ewe. This was achieved by marking a midway point on the plunger handle of the inseminating gun. The gun was then attached and the pipette was primed for use.

Prior to frozen-thawed inseminations 0.25 ml straws of semen were removed from the liquid nitrogen canister with dental forceps and thawed rapidly in a waterbath at 37°C for 15 seconds. Excess water was wiped from the straw with a sterile swab before loading it into the inseminating pipette. The distal crimped tip of the straw was cut with a special strawcutter 5 mm from the end and the pipette was then primed. Each ewe was inseminated with 0.25 ml of semen containing 100 million spermatozoa.

5. Pregnancy data

Four entire rams wearing mating harnesses and crayons were joined with the ewes on the 15th March 1993, which was one week after artificial insemination commenced. The rams remained with the ewes for 38 days and 'tup marks' were recorded either once or twice weekly.

Experienced commercial operators determined pregnancy details 77 to 81 days after insemination by real-time ultrasonography. Ewes pregnant to artificial insemination had larger foetuses and greater cotyledon development compared with those ewes mated naturally.

6. *Postmortem* examinations and counts of spermatozoa

6.1 Ewe reproduction tract: removal, dissection and flushing

The ewes were slaughtered by captive bolt and exsanguination approximately 18 h after AI. The genital tract of the ewe was removed, placed in a tray, and ligatures applied at the uterotubal junctions and at both ends of the cervix within five minutes (Figure 3a). The tracts were then put into individual plastic bags for transport to the laboratory for further processing. The broad ligament and other connective tissues were removed from the reproductive organs with scissors. Each genital tract was divided into five segments by cutting at the ligatures and bisecting the uterus (Figure 3b). The cervices were visually assessed for evidence of damage caused by the insemination needle. This was recorded as either present (Figure 4a) or absent (Figure 4b). Techniques for flushing segments of the reproductive tracts were based on methods outlined by Mattner and Braden (1963), Allison and Robinson (1972), and Hawk *et al.* (1978).

The uteri and uterine tubes were immediately flushed with physiological saline while the cervices were initially frozen. Each uterine tube was flushed twice from the uterotubal end by injecting 1.5 ml of saline followed by 1 ml of air. Each uterine segment was flushed twice by injecting 5 ml of saline followed by 'hand-stripping' to expel the fluid. Uterine and tubal flushings were frozen in 40 ml plastic vials until the time of sperm counting.

Prior to flushing the cervices were thawed and quartered longitudinally through the lumen followed by 15 to 20 cross-cuts. The pieces of cervices were then placed in a beaker with 50 ml of physiological saline, agitated and left to soak for 24 h. After thorough mixing the fluid was subsampled for sperm.

Figure 3a Ewe reproductive tract with ligatures.
Uterotubal junctions (a), cranial cervix (b), caudal cervix (c)

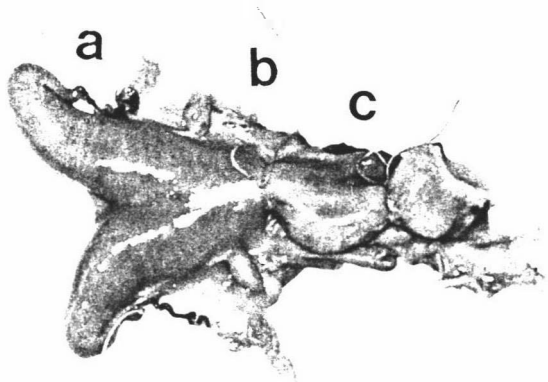


Figure 3b Ewe reproductive tract dissected.
Uterine tubes (a), uterus (b), cervix (c)

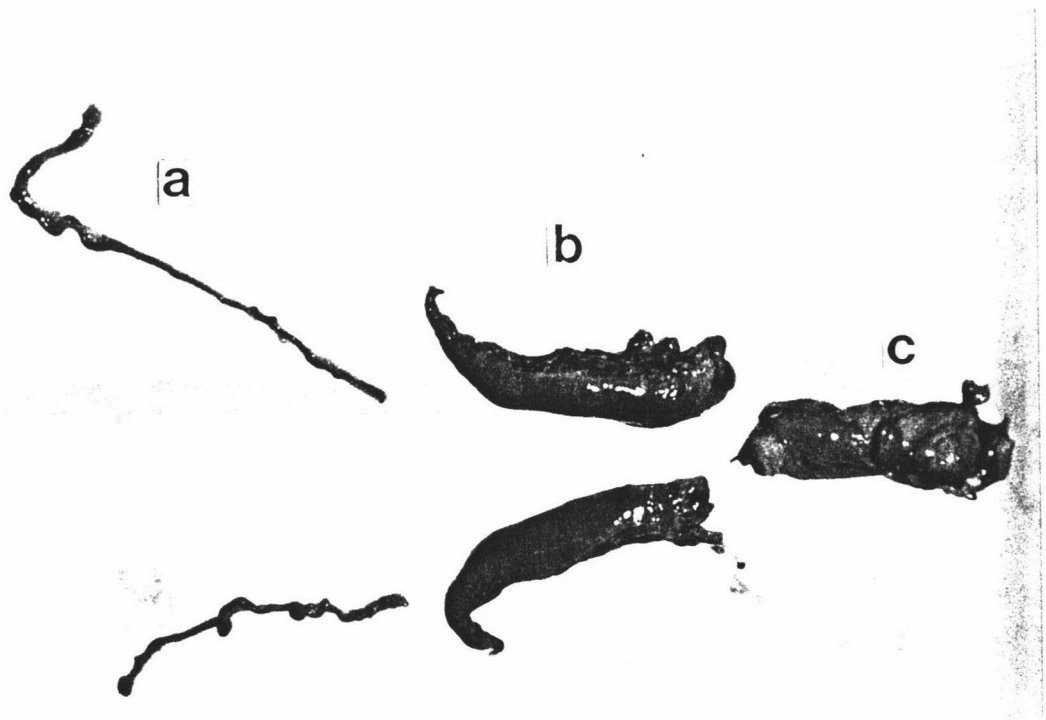


Figure 4a Dissected cervix of ewe 410/89 with a prominent area of haemorrhage in the cranial part

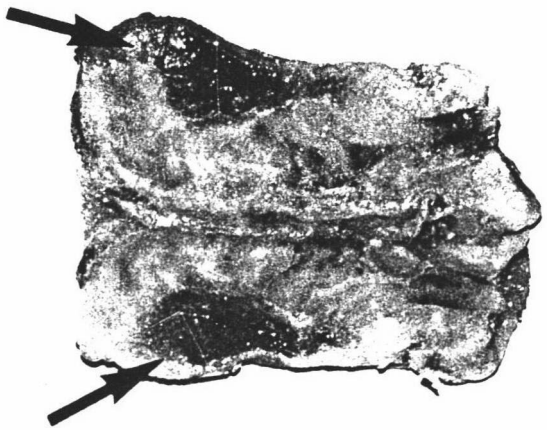
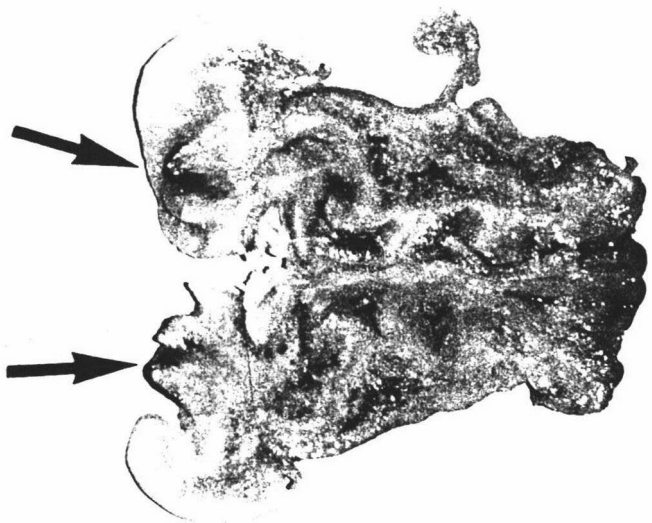


Figure 4b Dissected cervix of ewe 500/89 with evidence of haemorrhage in tissue surrounding the cervical os



6.2 Counting of spermatozoa

Owing to the expected low numbers of sperm in the uterine flushings, sperm were concentrated using a sedimentation technique developed by Anwar (1994). Following vigorous shaking of the original flushings, measured uterine samples of approximately 5 ml were removed for sedimentation. After a seven-day settling period the supernatant was discarded and the residual fluid was retained for subsampling. Spermatozoa in the cervical flushings were not concentrated prior to counting.

Spermatozoa were counted in flushings from only the left uterine segment and these results were used to predict total uterine sperm count. The extremely low sperm numbers recorded in the uterus indicated that tubal sperm numbers would be insignificant and these samples were not counted.

To assist with identification of sperm on the haemocytometer, one drop of eosin stain was added to the flushing vial prior to subsampling as described by Hackett and Macpherson (1965). Prior to the removal of each of six subsamples, the original flushings were shaken vigorously. A drop of fluid from each subsample was placed in the counting chamber of a haemocytometer beneath a coverslip. The slide was placed under a microscope at x400 magnification and sperm were counted in five of the nine large squares.

High levels of cleanliness were adopted to prevent cross contamination with sperm between different segments of the same tract and between different animals. Haemocytometer slides were washed twice in 70% alcohol followed by a rinse with distilled water between each count.

The formulae for calculating sperm numbers:

$$\text{no. of sperm/sample} = \frac{\text{average no. sperm/slide x residue volume}}{\text{volume of haemocytometer x sample volume}}$$

and

no. of sperm/tract = no. of sperm/sample x flushing volume.

The order of counting samples was organised to minimise sampling effects. On each counting day for a particular segment of the tract, all ewes of one age group from the same slaughter day were analysed.

7. Analyses of data

The data were analysed using the Statistical Analysis System (SAS Institute Inc., 1988).

The Catmod and Frequency Procedures were used for the analysis of fertility data.

Sperm count results were transformed using log(base 10) to reduce data variability. Any zero counts were replaced by half the lowest number of spermatozoa recorded in any sample from that segment of tract prior to log transformation (Hawk and Conley, 1975). An analysis of variance was conducted on the transformed sperm counts using the General Linear Model (GLM) procedure. The data are presented as both transformed means $\pm$ SE and retransformed means with 67% confidence interval.

The level of statistical significance was set at the 5% level ($P<0.05$) but actual probabilities were stated where appropriate.

The nomenclature used to denote levels of statistical significance were as follows:

 # P<0.10

*** P<0.05**

**** P<0.01**

***** P<0.001**

NS not significant.

The Results section lists the main effects and first order interactions tested for each

variable analysed. It was not possible to test the significance of some interactions due to low numbers of observations in a proportion of the cells. Models that described statistically significant relationships are shown in Table 3.

Table 3: Models that described statistically significant relationships.

Model	SAS. Proc.	Data set	N	Variable(s)
$Y_{ij} = \mu + Day_i + e_{ij}$	Catmod	A-NC	191	Time to onset of oestrus
$Y_{ij} = \mu + TS_i + e_{ij}$	Catmod	A-NC-IO-S	148	Non-return to oestrus
$Y_{ij} = \mu + TC_i + e_{ij}$	Catmod	A-NC-IO	178	Amount of bleeding
$Y_{ij} = \mu + Age_i + e_{ij}$	Catmod	A-NC-IO	178	Depth of AI, Time to AI
$Y_{ij} = \mu + Dam_i + e_{ij}$	GLM	S	32	Sperm count (uterus)
$Y_{ij} = \mu + TS_i + e_{ij}$	GLM	S	32	Sperm count (total), sperm count (cervix)

where Y = dependent variable,

μ = overall mean,

TS_i = fixed effect of insemination treatment i (i = fresh or frozen-thawed semen),

TC_i = fixed effect of clenbuterol treatment i (i = 0 ml or 7 ml),

Age_i = fixed effect of ewe age i (i = two tooth, four tooth, or six tooth),

Day_i = fixed effect of day of AI i (i = day 1-4),

Dam_i = fixed effect of damage to cervix i (i = yes or no),

e = random error,

and A = all ewes (N=199),

NC = ewes not synchronised by CIDR treatment (N=8),

IO = ewes inseminated by inexperienced operators (N=13),

S = ewes slaughtered for tract recovery (N=32).

CHAPTER IV

Results

Eight ewes were removed from the trial for the following reasons: lost CIDR (six ewes), CIDR not removed (one ewe), mated by rams (one ewe).

AI data from 13 ewes inseminated by three inexperienced technicians were excluded from the statistical analyses as it was considered that the results would not be comparable to those following AI conducted by technicians with practical experience.

The animals which were slaughtered did not provide data for analysis of conception rate as these ewes were euthanased before fertility could be determined. Information from these animals was included in the other statistical analyses conducted.

The pooling of categories with too few data was necessary prior to analyses of certain variables. These variables included condition of cervix, depth of AI, and cervical bleeding (Appendix 1, Tables 19-21).

1. The onset of oestrus

Ninety-four per cent of the ewes (179/191) came into oestrus within 48 h of CIDR withdrawal. Figure 5 shows the data for the onset of oestrus relative to the day of AI. The effect was highly significant, with a shorter interval occurring after CIDR

Figure 5: The effect of day of AI on interval from CIDR withdrawal to detection of oestrus.

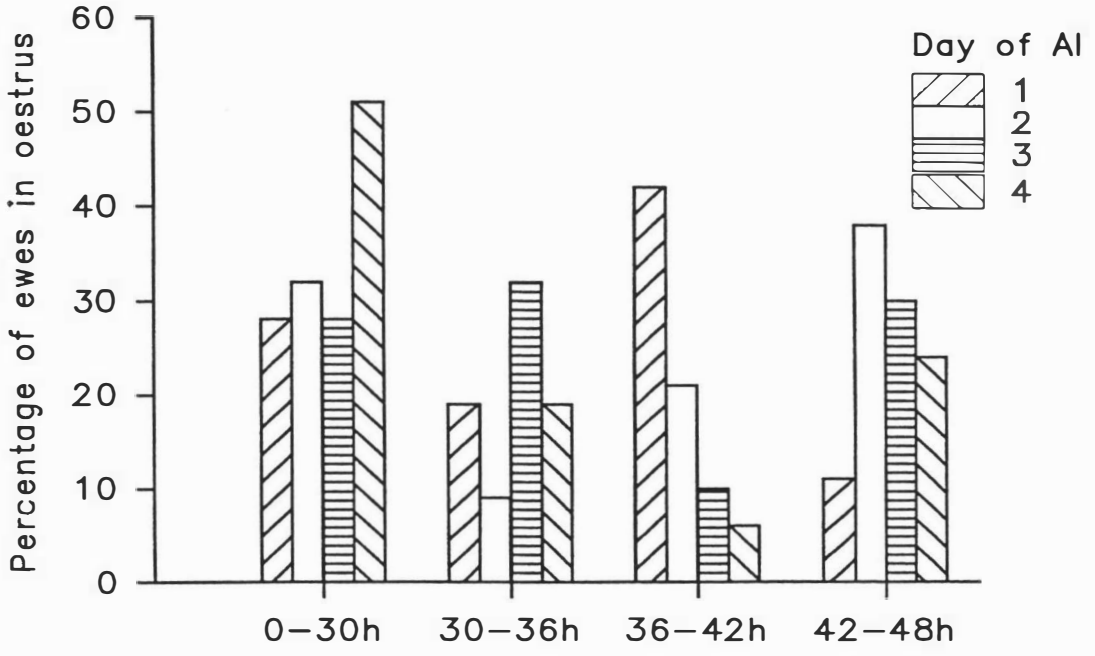
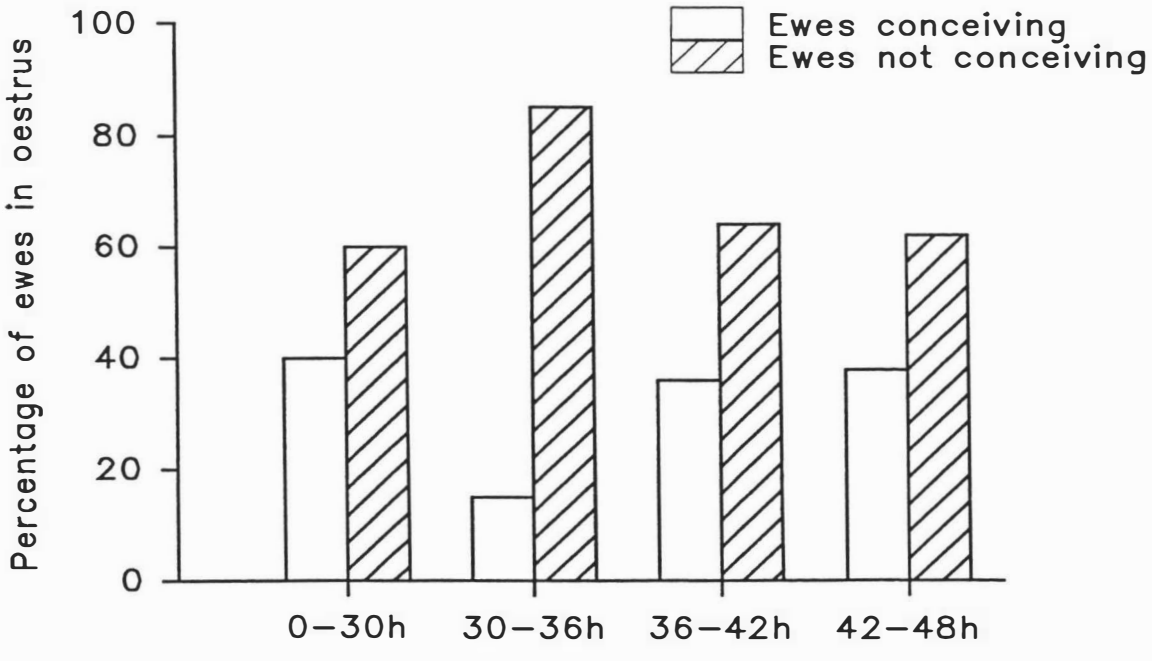


Figure 6: The effect of interval from CIDR withdrawal to detection of oestrus on conception rate.



withdrawal as the day of AI progressed. There was no significant interaction between age of ewe and day of AI.

Figure 6 shows that when oestrus was recorded between 30 and 36 h after CIDR withdrawal, conception rate was lower than for the other intervals. This result was not significant.

2. Condition of the cervical os

Day of AI, onset of oestrus, and level of clenbuterol had no significant effect on condition of the cervical os recorded at insemination. The effect of age of ewe approached statistical significance, with relatively few two tooth ewes showing cloudy cervical fluid (Table 4). There was no significant interaction between age of ewe and day of AI.

Table 4: The effect of age of ewe on condition of cervix.

Condition of cervix	Number of animals (%) per ewe age group			Signif.
	Two tooth	Four tooth	Six tooth	
Dry/clear fluid	20 (77)	41 (59)	49 (52)	
Cloudy fluid	6 (23)	29 (41)	46 (48)	#
Total ewes per age group	26	70	95	

3. Factors affecting conception rate to artificial insemination

Table 5 shows the distribution of ewes after pregnancy scanning and classified relative to data for non-return to oestrus after AI. The data indicated that three ewes recorded as 'marked' by the 'follow-up' rams were already pregnant to AI. Seven ewes in the non-return category were reclassified at scanning as not pregnant to AI. A further three ewes that did not return to service after AI were diagnosed as dry. The conception rate was estimated at 30% (43/145 ewes pregnant) by ultrasound scanning and 34% (51/148) by recording non-returns to natural service. Three ewes that were artificially inseminated were missing at time of scanning.

Table 5: A comparison of non-return and scan data.

	Scanned pregnant to AI n = 43	Scanned not pregnant to AI n = 102
Ewes not returning n = 50	40	10
Ewes returning n = 95	3	92

Time of day when the ewe was inseminated, day of AI, age of ewe and condition of cervix did not significantly effect conception rate. First order interactions were tested for their effect on conception rate between: age of ewe, day of AI, semen type, level of clenbuterol, depth of AI, bleeding at cervical os, condition of cervix, and onset of oestrus. None of these interactions was significant.

3.1 Operator effect

Statistical comparison of operator success was inappropriate due to insufficient data from the second operator. The proportions of ewes conceiving for the two technicians were 3/10 (30%) and 47/148 (32%) and on the basis of these results operator data were pooled.

Although the data from those ewes inseminated by the inexperienced technicians were excluded from the analysis of conception rate, it was observed that none of these animals was subsequently pregnant to AI.

3.2 Sire effect

There was no significant effect on conception rate for the two sires that provided the frozen-thawed spermatozoa. The proportions of ewes conceiving were 6/29 (21%) and 12/45 (27%) for the two sires.

3.3 Type of semen

Conception rates after inseminations with fresh or frozen-thawed semen are shown in Table 6. The percentage of ewes that conceived to AI with fresh spermatozoa (42%) was higher than that recorded for inseminations with frozen-thawed spermatozoa (24%). When two tooth ewe data were removed from the analysis, the type of semen inseminated no longer had a significant effect on conception rate.

Table 6: The effect of AI with fresh or frozen-thawed semen on conception rate.

Animals	Type of semen	Number of ewes per treatment	Number of ewes (%)		Signif.
			Conceiving	Not conceiving	
All ewes	Fresh	73	31 (42)	42 (58)	
	Frozen-thawed	74	18 (24)	56 (76)	*
Mature ewes	Fresh	60	23 (38)	37 (62)	
	Frozen-thawed	63	15 (24)	48 (76)	#

3.4 The use of clenbuterol

Table 7: The effect of clenbuterol on conception rate following AI.

Animals	Clenbuterol treatment	Number of ewes per treatment	Number of ewes (%)		Signif.
			Conceiving	Not conceiving	
All ewes	0 ml	76	28 (37)	48 (63)	NS
	7 ml	71	22 (31)	50 (69)	
Mature ewes	0 ml	66	23 (35)	43 (65)	NS
	7 ml	57	15 (26)	42 (74)	

Table 7 shows the effect of clenbuterol administration prior to AI on conception rate. The trend in fertility, whether or not two tooth ewes were included, was for a higher percentage of control ewes conceiving to AI.

3.5 The effect of depth of insemination

The effect of depth of insemination on conception rate is shown in Table 8. There was a decline in conception rates as depth of insemination increased but this was not significant.

Table 8: The effect of depth of insemination on conception rate.

Insemination site	Number of ewes per category	Number of ewes (%)		Significance
		Conceiving	Not conceiving	
Vaginal/shallow cervical	38	16 (42)	22 (58)	
Deep cervical	32	12 (38)	20 (62)	
Full cervical penetration	78	22 (28)	56 (72)	NS

3.6 The effect of bleeding on conception rate

The detection of blood at the cervical os following the AI did not significantly influence conception rate (Table 9).

Table 9: The effect of bleeding at the cervical os after AI on conception rate.

Bleeding category	Number of ewes per category	Number of ewes(%)		Significance
		Conceiving	Not conceiving	
No bleeding	99	37 (37)	63 (63)	
Bleeding	48	13 (27)	35 (73)	NS

3.7 Time taken to perform artificial insemination

Table 10 shows the conception rates of four groups of experimental animals which were inseminated over different time intervals. There was no significant effect on conception rate of the time taken to inseminate. The trends revealed that conception rate was higher if inseminations were performed either quickly or slowly rather than at an intermediate speed.

Table 10: The effect of time taken to inseminate on conception rate.

Time category (minutes)	Number of ewes per category	Number of ewes(%)		Significance
		Conceiving	Not conceiving	
< 0.45	36	13 (36)	23 (64)	
> 0.45 and < 1.30	38	12 (31)	27 (69)	
> 1.30 and < 2.15	29	7 (24)	22 (76)	
> 2.15 and < 5.00	44	18 (41)	26 (59)	NS

4. Factors affecting the presence of bleeding after artificial insemination

There was no significant effect of age of ewe, day of AI, time of onset of oestrus, condition of cervix, type of semen, or depth of AI, on the presence of blood recorded at AI. First order interactions were tested for their effect on bleeding after AI between: level of clenbuterol, day of AI, age of ewe, depth of AI, condition of the cervix, and onset of oestrus. None of these interactions was significant.

A summary of the effect of clenbuterol on the presence or absence of bleeding following AI is given in Table 11. The percentage of ewes with no bleeding at the cervical os after AI increased from 60% to 78% when clenbuterol was used.

Table 11: The effect of clenbuterol on the number (%) of ewes with bleeding at the cervical os after AI.

Bleeding observation	Clenbuterol administration		Significance
	0 ml	7 ml	
	n = 92	n = 86	
No bleeding	55 (60)	67 (78)	
Bleeding	37 (40)	19 (22)	*

5. Factors affecting the depth of insemination

There was no significant effect on depth of AI of time to onset of oestrus, day of AI, type of semen, or level of clenbuterol. First order interactions were tested for their effect on depth of insemination between: level of clenbuterol, day of AI, age of ewe, condition of the cervix, and onset of oestrus. None of these interactions was significant. However the effect of the interaction between age of ewe and condition of cervix on depth of insemination approached significance.

5.1 Age of the ewe

Table 12 presents the effects of age of ewe on depth of insemination. There was a highly significant effect of ewe age on depth of insemination. The majority of two tooth

inseminations (76%) were performed at the vaginal or shallow cervical depth. This contrasted markedly to the results for four tooth and six tooth ewes where full cervical penetration was achieved in 70% and 67% of inseminations respectively.

Table 12: The effect of age of ewe on depth of insemination.

Depth category	Number of ewes (%)			Signif.
	Two tooth ewes n = 25	Four tooth ewes n = 66	Six tooth ewes n = 87	
Vaginal/ shallow cervical	19 (76)	10 (15)	9 (10)	
Deep cervical	2 (8)	10 (15)	20 (23)	
Full cervical penetration	4 (16)	46 (70)	58 (67)	***

5.2 Condition of the cervix

Table 13 shows how depth of insemination was influenced by different cervical conditions. Initially all age groups were analysed. There was almost a significant effect

(P=0.06) of condition of cervix on depth of AI. Full cervical penetration was achieved in 54% of those ewes inseminated when the cervix was dry/clear and in 70% of ewes when a cloudy fluid was observed. The effect of the condition of the cervix on depth of penetration was less when two tooth ewes were removed from the analysis (P=0.10).

Table 13: The effect of condition of cervix on depth of insemination.

Animals	Depth of insemination	Number of ewes (%)		Signif.
		Dry/clear fluid	Cloudy cervical fluid	
All ewes	Vaginal/shallow cervical	24 (23)	14 (19)	
	Deep cervical	24 (23)	8 (11)	
	Full cervical penetration	56 (54)	52 (70)	
	All depths	104	74	#
Mature ewes	Vaginal/shallow cervical	10 (12)	9 (13)	
	Deep cervical	22 (26)	8 (12)	
	Full cervical penetration	53 (62)	51 (75)	
	All depths	85	68	NS

6. Factors affecting time taken to inseminate

Day of AI, level of clenbuterol and type of semen did not have a significant effect on time taken to inseminate. First order interactions were tested for their effect on time taken to inseminate between: semen type, level of clenbuterol, age of ewe and day of AI. None of these interactions was significant. The effect of ewe age on insemination time is shown in Table 14. There was a highly significant effect of ewe age on 'time taken to inseminate'. The dominating result was that 60% of two tooth ewes were inseminated in less than 45 seconds. Only 32% of four tooth ewes and 27% of six tooth ewes took longer than two and one quarter minutes to inseminate.

Table 14: Time taken for insemination relative to age of ewe.

Time category (minutes)	Number of ewes (%)			Signif.
	Two tooth ewes	Four tooth ewes	Six tooth ewes	
<0.45	15 (60)	17 (26)	13 (15)	
>0.45 & <1.30	6 (24)	15 (23)	31 (36)	
>1.30 & <2.15	1 (4)	13 (19)	19 (22)	
>2.15 & <5.00	3 (12)	21 (32)	24 (27)	
All times	25	66	87	**

7. Spermatozoa transport following transcervical artificial insemination

Analyses were conducted on the influence of type of semen, clenbuterol administration, day of AI and degree of cervical tissue damage on the transport of spermatozoa in the tract. First order interactions were tested for their effect on spermatozoa counts between: day of AI, semen type, clenbuterol administration, and cervical damage. None of these interactions was significant.

7.1 The effect of type of semen

Table 15 shows how semen type influenced the number of spermatozoa recovered from the reproductive tracts. The sperm numbers in the cervix and in the cervix and uterus combined were significantly lower when frozen-thawed semen was used rather than fresh semen. The uterine counts were low and not significantly related to whether frozen-thawed or fresh material was used.

Table 15: The effect of insemination with fresh or frozen-thawed semen on numbers of spermatozoa recovered from the ewe reproductive tract.

Segment(s) of reproductive tract	Number of spermatozoa recovered		Signif.
	Fresh semen n = 16	Frozen-thawed semen n = 16	
	Mean logarithm ± SE[a]		
Cervix	3.77 ± 0.15 (1)	3.34 ± 0.17 (2)	*
Uterus	2.47 ± 0.19 (3)	2.55 ± 0.13 (2)	NS
Total[b]	3.84 ± 0.14	3.46 ± 0.15	*
	Geometric means[c]		
Cervix	5888 (4169, 8318)	2188 (1479, 3236)	*
Uterus	295 (191, 457)	355 (263, 479)	NS
Total[b]	6918 (5012, 9550)	2884 (2042, 4074)	*

[a] = Log transformed values ± SE. Figures in parentheses indicate number of ewes in the group from which no sperm were recovered.

[b] = Cervical and uterine sperm counts combined.

[c] = Geometric means are the anti-logs of the mean log numbers. The 67% confidence interval is displayed in parentheses.

Table 16: The effect of clenbuterol administration prior to AI on numbers of spermatozoa recovered from the reproductive tract of the ewe.

Segment of reproductive tract	Number of spermatozoa recovered		Signif.
	0 ml clenbuterol n = 16	7 ml clenbuterol n = 16	
	Mean logarithm ± SE[a]		
Cervix	3.57 ± 0.17 (2)	3.55 ± 0.17 (1)	NS
Uterus	2.53 ± 0.17 (3)	2.49 ± 0.16 (2)	NS
Total[b]	3.66 ± 0.15 (4)	3.64 ± 0.15 (3)	NS
	Geometric means[c]		
Cervix	3715 (2512, 5495)	3548 (2399, 5248)	NS
Uterus	339 (229, 501)	309 (214, 447)	NS
Total[b]	4570 (3236, 6457)	4365 (3090, 6166)	NS

[a] = Log transformed values ± SE. Figures in parentheses indicate number of ewes in the group from which no sperm were recovered.

[b] = Cervical and uterine sperm counts combined.

[c] = Geometric means are the anti-logs of the mean log numbers. The 67% confidence interval is displayed in parentheses.

7.2 The effect of clenbuterol

Table 16 shows the effect of clenbuterol administration on numbers of spermatozoa recovered from the ewe reproductive tracts. The numbers of spermatozoa counted in different segments of the tract were not significantly affected by clenbuterol treatment. The uterine counts were very low, with the geometric means (of spermatozoa numbers) for the uterus being less than 10% of those for the cervix.

7.3 The effect of cervical damage

Table 17 shows how damage inflicted on the cervix during transcervical AI affected spermatozoa numbers in the cervix and uterus of the reproductive tracts. Damage had a significant effect on spermatozoa counts for the uterus. The effects of cervical damage on spermatozoa counts for the cervix and for both segments combined approached significance (P=0.09 and P=0.07 respectively). Table 17 also shows that only 14% of ewes with no cervical damage, compared with 36% of ewes with damage, had segments of tract from which no sperm were recovered.

Table 17: The effect of cervical damage on numbers of spermatozoa recovered from ewe reproductive tracts.

Segment(s) of reproductive tract	Number of spermatozoa recovered		Signif.
	No cervical damage	Cervical damage	
	n = 21	n = 11	
	Mean logarithm ± SE[a]		
Cervix	3.67 ± 0.14 (1)	3.35 ± 0.21 (2)	#
Uterus	2.69 ± 0.15 (2)	2.17 ± 0.12 (3)	*
Total[b]	3.75 ± 0.13 (3)	3.45 ± 0.18 (4)	#
	Geometric means[c]		
Cervix	4677 (3388, 6457)	2239 (1380, 3630)	#
Uterus	490 (347, 692)	148 (112, 195)	*
Total[b]	5623 (4169, 7586)	2818 (1862, 4266)	#

[a] = Log transformed values ± SE. Figures in parentheses indicate number of ewes in the group from which no sperm were recovered.

[b] = Cervical and uterine sperm counts combined.

[c] = Geometric means are the anti-logs of the mean log numbers. The 67% confidence interval is displayed in parentheses.

7.4 The effect of day of artificial insemination

Table 18: The effect of day of artificial insemination on numbers of spermatozoa recovered from the ewe reproductive tract.

Part of ewe genital tract	Number of spermatozoa recovered				Signif.
	Day 1 n = 8	Day 2 n = 8	Day 3 n = 8	Day 4 n = 8	
	Mean logarithm ± SE[a]				
Cervix	3.36 ± 0.33 (2)	3.32 ± 0.22 (1)	3.65 ± 0.17	3.90 ± 0.17	NS
Uterus	2.70 ± 0.20	2.02 ± 0.20[b] (4)	2.79 ± 0.10	2.54 ± 0.30 (1)	#
Total[c]	3.53 ± 0.28 (2)	3.38 ± 0.21 (4)	3.76 ± 0.12	3.93 ± 0.18 (1)	NS
	Geometric means[d]				
Cervix	2291 (1072, 4898)	2089 (1259, 3467)	4467 (3020, 6607)	7943 (5370, 11749)	NS
Uterus	501 (316, 794)	105 (66, 166)[b]	617 (490, 776)	347 (174, 692)	#
Total[c]	3388 (1778, 6457)	2399 (1479, 3890)	5754 (4365, 7586)	8511 (5623, 12882)	NS

[a] = Log transformed values ± SE. Figures in parentheses indicate number of ewes in the group from which no sperm was recovered.

[b] = Significantly different from other treatments in the same row.

[c] = Cervical and uterine sperm counts combined.

[d] = Geometric mean is the antilog of the mean log number. The 67% confidence interval is displayed in parentheses.

Table 18 shows the variation in the number of spermatozoa recovered relative to the day of AI. There was no significant effect of day of AI on sperm counts for the cervix or the cervix and uterus combined. The difference in uterine sperm counts for animals inseminated on day 2 of AI approached statistical significance (P=0.06).

CHAPTER V

Discussion

1. Measurement of conception rate

Low fertility in the ewe can be due to a combination of factors: failure of oestrus, low numbers of fertile ovulations, poor sperm transport, or embryonic mortality (Edey, 1976). In the current study ewe ovarian function was conducive to reasonable fertilisation rates (Appendix 3) and a high level of oestrous activity was recorded (Figure 5). Detrimental effects on sperm transport were observed but embryonic mortality was not determined. The low fertility measured could not therefore be attributed solely to interference with sperm transport mechanisms.

A proportion of the decline in ewes conceiving to AI as diagnosed by scanning rather than non-return data may be attributed to embryonic mortality. Edey (1976) referred to a base level of embryonic mortality of approximately 20 to 30% with the majority of deaths occurring before day 18 of pregnancy. That loss rate included partial failure of multiple ovulations, so the actual effect on conception rate would be less. The slight discrepancy between scanning and return data could be associated with errors either in determining foetal age by ultrasound scanning or with recording 'tup marks'. Johns (1993) reported that in a pregnancy diagnosis trial the week of conception was correctly estimated for 71% of the ewes scanned. However in the present study the animals were synchronised and there was a non-mating interval to aid diagnosis.

2. Sire

Two rams provided semen for the fresh inseminations and another two for the frozen-thawed inseminations. Fresh semen was pooled prior to use so any effect on conception rate due to variation between sires used for the fresh inseminations was nullified. The fertilising ability of semen from different rams can have a significant effect on conception rate, especially following AI with frozen-thawed semen (Maxwell, 1986;

Epplestein and Maxwell, 1991), although not in all cases (Windsor *et al.*, 1994). The two sires that provided the frozen-thawed semen had been used successfully in an AI progeny test scheme in the previous breeding season and their semen had been checked pre-freezing for motility. However Epplestein and Maxwell (1991) reported that the motility of ram spermatozoa was unrelated to its fertilising ability.

In the present study there was no apparent effect on conception rate between the two sires used for the frozen-thawed inseminations but the small numbers of ewes inseminated by each sire limited the possibility of establishing a difference. Further, the sires used for frozen-thawed semen inseminations were not randomly allocated to AI days. Therefore sire effect on conception rate within the frozen-thawewd semen inseminations was confounded by day of AI. Nevertheless the conception rate results suggested that the effect of sire could be removed from the models tested.

3. Sperm transport and survival

The overall low fertility recorded was probably related to the low sperm numbers in the female reproductive tract near the time of ovulation. This relationship has a large bearing on fertilisation (Hawk *et al.*, 1987). The uterine placement of 500,000 sperm has resulted in a 30% conception rate (Maxwell, 1986b). In the ewes at *postmortem,* less than 2000 spermatozoa were recovered from the uteri of 26/32 ewes (Appendix 3). This was very low in relation to the average uterine counts of 11,500 frozen-thawed spermatozoa recovered by Fukui and Roberts (1977a) or 30,500 fresh spermatozoa recovered by Mattner *et al.* (1969). Results from these sperm transport studies (Table 2) can be compared with those of the current experiment (Appendix 3). Lower insemination doses and insemination at a synchronised oestrus may not entirely explain the overall lower sperm counts obtained in the current study. However these factors were almost certainly involved in the difference in fertility recorded following AI with the different semen types.

In the present study the numbers of spermatozoa inseminated (100 million) should not have limited fertility where fresh semen was used. According to recommendations for

laparoscopic intrauterine AI, these numbers of frozen-thawed spermatozoa are in excess of that required for acceptable fertility. Therefore it was assumed that this insemination dose was sufficient for TAI. However if transcervical passage was unsuccessful then this number of frozen-thawed spermatozoa deposited vaginally or cervically would have limited fertility due to the high rates of mortality and loss described by Lightfoot and Salamon (1970a). This effect would have been reinforced by the detrimental action of progesterone on sperm transport and survival (Quinlivan and Robinson, 1969; Hawk and Conley, 1975; Boland *et al.*, 1978; Hawk *et al.*, 1987).

Low sperm numbers and subsequent fertility may be related to the timing of AI. Laparoscopic intrauterine AI is recommended 50 to 58 h after CIDR removal (Harvey, 1989). In the present study attempted deposition of sperm in the uterus occurred much earlier and possibly resulted in high sperm mortality prior to ovulation due to an increased exposure to uterine leucocyte activity. Aged spermatozoa at fertilisation may have also reduced fertility. Conception rates were higher when fresh rather than frozen-thawed semen was inseminated. Other studies have recorded lower fertility after AI with frozen-thawed semen rather than fresh (Salamon and Lightfoot, 1967; Lightfoot and Salamon, 1970b). The numbers of spermatozoa were significantly higher in the cervix when fresh rather than frozen-thawed semen was inseminated. The difference in conception rates following insemination with the two different types of semen could be attributed to the corresponding differences in numbers of spermatozoa counted in segments of the reproductive tract 18.5 h after AI. Other sperm transport studies (Table 2) have highlighted similar differences.

For all analyses of uterine sperm numbers it should be considered that the haemocytometer method was not sufficiently sensitive to differentiate confidently between these low counts. For example one sperm counted on six slides corresponded to 90 sperm in the uterus after dilution rate and concentration calculations. There are several possible reasons for the lower uterine sperm numbers on the second slaughter day. The non-random use of sires which provided the frozen-thawed spermatozoa between AI days could have caused the difference, as one sire only was used on day 2 of AI. The decision to exclude animals inseminated by the inexperienced technicians

was made after the 32 ewes were selected for slaughter. Two of those ewes selected for the study of sperm transport were subsequently found to have been inseminated by an inexperienced technician and were slaughtered on day 2 after AI. The numbers of spermatozoa found in both of these ewes were low. The relatively low motility score (3) of one ejaculate used on day 2 may also have been a contributing factor.

The effect of semen type on conception rate cannot be separated from the effect of the sires providing the fresh semen and those supplying the frozen-thawed semen. Therefore the effect of semen type was confounded by sire.

4. Transcervical AI of maiden two tooth ewes

Transcervical AI of the two tooth ewes was often thwarted by the tightness of the vagina, which prevented correct insertion of the speculum and location of the cervical os. Consequently 76% of the two tooth ewes were inseminated at a shallow depth (one ewe cervically and the remaining 18 vaginally) and only 16% were successfully transcervically inseminated. Appendix 1, Table 20 shows the initial depth of AI results for two tooth ewes prior to the pooling of depth categories.

Observations made in the present study supported the conclusions of Fukui and Roberts (1978) and Buckrell *et al.* (1994) that maiden two tooth ewes were not suitable candidates for TAI. Salamon and Lightfoot (1970) reported that a high degree of vaginal constriction in barren ewes permitted only shallow insemination when using the cervical traction technique. The relatively low number of uterine inseminations achieved by Halbert *et al.* (1990c) in one experiment was no doubt due to the inclusion of a high proportion of maiden ewes. Buckrell *et al.* (1994) achieved a successful cervical penetration rate of 85% when attempting TAI on a group of parous two tooth ewes. However only those ewes where a speculum could be comfortably inserted into the vagina were included in the trial.

Despite the shallow penetration of the inseminating needle, the conception rate of two tooth ewes was not significantly different to that of mature ewes. This was due to the

relatively high conception rate (56%) for those two tooth ewes inseminated by vaginal AI. The current experiment was not designed to compare differences in AI techniques but the data from depth of AI can be related to the results of studies comparing AI method. Some studies have reported that vaginal AI was as effective as cervical AI (Fairnie and Wales, 1982; Maxwell *et al.*, 1986). Most experiments have reported lower conception rates when the vaginal method was used (Rival *et al.*, 1984; Harvey *et al.*, 1986).

The results of vaginal AI in the current trial were even more surprising when it was considered that AI took place at a progesterone synchronised oestrus and about half of the inseminations were with frozen-thawed semen. This success of vaginal AI suggested that there was a detrimental effect on fertility associated with the transcervical procedure that cancelled out any advantage of improved sperm transport with greater cervical penetration.

The quicker insemination of two tooth ewes ($P<0.001$) in the current experiment was related to the high percentage of vaginal inseminations. Vaginal AI is a very quick procedure (Wallace, 1992).

5. Depth of AI

The effect of depth of AI on conception rate was not significant and agreed with the findings of Halbert *et al.* (1990c). However the trend in the present trial was for a lower conception rate as depth of AI increased, while the opposite trend was observed by Halbert *et al.* (1990c). Salamon and Lightfoot (1970) found that inseminations of frozen-thawed ram spermatozoa assisted by cervical traction compared with normal cervical AI resulted in significantly lower fertility (24% versus 37% of ewes lambing respectively). Other studies reported improved conception rates as depth of AI increased in sheep (Salamon and Lightfoot, 1967; Fukui and Roberts, 1976a; Windsor *et al.*, 1994; Smith *et al.*, 1995) and goats (Ritar *et al.*, 1990).

The depth of cervical penetration at AI is probably closely related to the site where

sperm are temporarily stored in the selective reservoirs referred to by Hawk (1983) and Hafez (1974). Halbert *et al.* (1990a) showed that it is difficult to force fluid through the cervix of a ewe in oestrus. In that study fluoroscopy failed to detect contrast material in the uterus after it was injected into the caudal cervix. The reservoirs of spermatozoa in the cervical crypts form selective barriers to the cranial progress of large numbers of sperm (Hafez, 1974). Therefore the further into the reproductive tract the sperm is deposited, the less the restrictive effect of these reservoirs on spermatozoa transport and the higher the chance of fertilisation. Significantly more spermatozoa were recovered from the uteri of sheep inseminated at the site of the internal rather than external cervical os (Lightfoot and Restall, 1971).

Hawk and Conley (1975) and Croker *et al.* (1975) referred specifically to the anterior cervix as the initial region of failure of sperm transport. The ewes in the present study were synchronised with progesterone, which causes serious interference with sperm viability and transport especially in the anterior cervix (Hawk *et al.*, 1981). Therefore insertion of semen past this point where disruption of sperm transport was greatest should have been resulted in higher fertility.

There was no increase in conception rate as depth of insemination of frozen-thawed spermatozoa increased. This absence of a significant interaction between type of semen and depth of AI on conception rates was surprising, as Lightfoot and Salamon (1970a) reported that poor fertility after AI with frozen-thawed spermatozoa was due to low retention and high mortality of spermatozoa in the cervix. However Smith *et al.* (1995) reported that depth of AI significantly affected conception rates for fresh but not for frozen-thawed inseminations.

The absence of a convincing relationship between depth of insemination and conception rate in the present and other TAI trials could be due to the increased trauma inflicted on the cervix as the insemination needle is manipulated towards the uterus. The detrimental effects of cervical damage on spermatozoa transport may override a positive relationship between increased depth of AI and higher conception rates.

Buckrell *et al.* (1994) reported full penetration in 88% of ewes transcervically inseminated but the subsequent lambing rate was only 33%. Buckrell *et al.* (1992) recorded similar results with a full cervical penetration rate of 87% but a subsequent lambing rate of 41%. The ratio of successful pregnancies to full cervical penetration was 0.42 and 0.47 respectively in those trials. In the current study the ratio of pregnancies to full cervical penetrations was 0.28. The reason for the relatively low ratios above may be linked to the false recording of full cervical penetrations. This occurs when the insemination needle pierces the wall of the cervix rather than entering the uterine lumen. Some of the TAI trials which discuss cervical penetration rates may be misleading due to this feature.

There could be a tendency for the recording of more false penetrations with experienced AI technicians. Windsor *et al.* (1994) reported that although full penetration rates were lower when inexperienced technicians performed TAI, subsequent pregnancy rates were the same as for laparoscopic inseminations. In contrast, experienced AI technicians in that study achieved higher transcervical penetration rates but significantly less ewes became pregnant to TAI compared with animals laparoscopically inseminated. Until better instruments and techniques can be developed, it appears that a tentative insemination style with less emphasis on achieving full transcervical penetration may be beneficial to pregnancy rates in TAI programmes. The decline in insemination time and the increase in success of full cervical penetrations reported by Buckrell *et al.* (1994) as a TAI programme progressed may have been detrimental to pregnancy rates.

6. Cervical damage

Transcervical dye insemination followed by *postmortem* examination has shown deposition of dye in the tunica submucosa of the cervical wall in 1/10 (Halbert *et al.*, 1990b), 6/19 (Campbell *et al.*, 1995), and 41/73 (McKelvey, 1994) ewes inseminated. In those experiments AI was performed with a modified spinal needle identical to that used in the current study. Modification of the AI needle [Figure 1a(c)] so that it was ball-tipped like the needle used by Fukui and Roberts (1976a,b) reduced major damage to the cervical wall in 1/10 ewes inseminated (Campbell *et al.*, 1995).

Figure 4a illustrates the degree of cervical damage that was observed and Table 17 shows the detrimental effect of this type of injury to sperm transport. Campbell *et al.* (1995) reported that even when the AI needle remained within the cervix there was significantly more trauma caused as depth of penetration increased. The bleeding and inflammation following this degree of tissue insult would produce undesirable conditions for sperm survival. The indiscriminate killing of degenerating and healthy sperm cells by leucocytes which occurs mainly in the uterus does not normally have a major effect on sperm numbers in the female tract (Hafez, 1974). However the rapid increase in number of polymorphic neutrophils at a wound site such as shown in Figure 4a would result in a higher sperm mortality rate. Due to the timespan between uterine deposition and fertilisation, spermatozoa would be susceptible to leucocyte attack over a relatively long period. Changes in chemical composition of the tissue surrounding the damage site, including a decline in pH and an increase in spermicidal factors, would also be detrimental to sperm survival. There may also be loss of sperm following damage due to: (1) increased drainage to the exterior as the cervix becomes less able to maintain a sperm reservoir as the tonicity of the cervical mucus is altered, (2) passage right through the cervical wall to the peritoneal cavity, or (3) abnormally fast passage through the reproductive tract to the peritoneal cavity. Whatever the mechanism involved it appears likely that in the current experiment the trauma caused to the cervices during AI considerably reduced numbers of spermatozoa in the female tracts and this probably explained the low fertility recorded to TAI.

7. Clenbuterol administration

Clenbuterol administration had no significant effect on sperm numbers. Clenbuterol is a specific β_2 adrenoceptor that, like progesterone reduces activity of the myometrium. A decline in uterine motility around the time of mating would presumably have been detrimental to sperm transport and consequently fertility, as suggested by Lightfoot and Restall (1971). Hafez (1974) referred to the inherent motility of the female genital tract as the primary method of gamete transport. Conversely oxytocin, which is antagonistic to clenbuterol, has increased fertilisation rates in gilts (Stratman *et al.* 1959).

The possible negative effects of clenbuterol on sperm transport may have been compensated for by its beneficial effect of reducing bleeding. The administration of clenbuterol significantly reduced bleeding at the cervical os following AI. This was possibly due to a local vasoconstriction caused by this β_2 adrenergic compound. The reduction in bleeding observed at the cervical os probably also occurred at deeper sites where the insemination needle inflicted damage during the AI process.

Figure 4b suggested that bleeding at the cervical os may not be related to haemorrhage located deeper in the cervix. Other cervices observed at dissection showed a similar apparently unconnected bleeding pattern between the two sites. Bleeding at the cervical os may not therefore be of much value in trying to correlate damage to conception rate. This theory was supported by other experimental results. For example there was no significant effect of bleeding at the cervical os on conception rate despite the significant effect of cervical damage on sperm counts. Also depth of AI had no significant effect on the amount of bleeding observed at the cervical os. The results presented here would suggest that this damage caused by manipulation of the anterior cervix was not a major factor limiting the success of TAI.

The use of clenbuterol to cause cervical relaxation was unsuccessful. Depth of AI was not improved by clenbuterol treatment. The endocrine state of an animal close to parturition is very different to one at mating (Gilman *et al.* 1990). Therefore the action of clenbuterol may vary with stage of pregnancy (or oestrous cycle) due to its interaction with other hormones or to changes in the numbers and/or responsiveness of β receptors in cervical tissue. Dilation of the cervix that occurs at parturition is a complex process that is not fully understood and may involve more than the relaxation of the large amount of smooth muscle in this caudal extension of the uterus. Clenbuterol has been used to delay parturition by eliminating uterine contractions to allow more time for cervical dilation to proceed. Its use to 'soften' the cervix directly is not reported elsewhere in the literature. Barry *et al.* (1990) induced cervical relaxation using prostaglandin E_2 and oestradiol while Khalifa *et al.* (1992) dilated the cervix using oxytocin (and oestradiol), which suggested that the answer to cervical softening may lie more with these compounds. Clenbuterol administration would need to be tested over

a range of dose rates and injection times in relation to AI to evaluate fully its use to induce cervical relaxation.

8. Onset of oestrus

The decline in interval from CIDR withdrawal to the onset of oestrus as AI day advanced can be explained mainly by the 'ram effect' and 'social facilitation'. There could also be environmental factors which affected the timing of oestrus or the ability of the vasectomised rams to stimulate oestrus.

The introduction of rams early in the breeding season to ewes that were previously isolated from male sheep caused a more synchronous and earlier onset of oestrus - known as the 'ram effect' (Edgar and Bilkey, 1963). In the present experiment ewes assigned to each day of AI were placed in adjacent paddocks following CIDR removal. The teaser rams were introduced to the first group of ewes 18 h after CIDR removal. This meant that the second and consecutive groups of ewes were exposed to a possible 'ram effect' at the time of CIDR removal.

The 'ram effect' can be markedly augmented by the exposure of rams to oestrous ewes prior to joining with the main ewe flock (Pearce and Oldham, 1984). This process is known as 'social facilitation'. Therefore the rams' ability to stimulate oestrus would have increased as they were shifted into the consecutive groups of ewes assigned to each AI day.

There is a trend for lower fertility in those ewes that come into oestrus between 30 and 36 h after CIDR removal. Maxwell *et al.* (1984) reported higher embryonic mortality when ewes were inseminated 24 and 36 h after sponge removal due possibly to aged sperm at fertilisation. Several studies have recommended that AI takes place just prior to ovulation (Dziuk, 1970; Evans and Maxwell, 1987).

However the onset of oestrus did not have a significant effect on conception rate, which agreed with the findings of Amir and Schindler (1972). Harvey *et al.* (1984), Harvey

et al. (1986), and Maxwell (1986a) stated that cervical AI at a fixed time after synchronisation produced similar conception rates to 'on-oestrus' AI. These results therefore support the conclusions of Maxwell (1986a) and Shackell (1991) that despite a wide variation in onset of oestrus after CIDR removal, time of ovulation is highly synchronised.

9. Condition of cervix and depth of insemination

Cloudy rather than clear or no fluid observed at the cervical os was associated with deeper cervical penetration. This effect approached statistical significance when the two tooth ewes were included in the analysis. Further investigation showed that the effect of the interaction between age of ewe and condition of cervix on depth of insemination also approached statistical significance. These findings suggest that the physiological state of the cervix affects depth of insemination more in the young or non-parous ewe.

10. Operator

The skill of the AI technician can have an effect on conception rate (Windsor *et al.*, 1994). In the current trial there was a similar proportion of ewes conceiving following inseminations by either operator.

11. Time taken to inseminate

Buckrell *et al.* 1994 recorded an average AI time of just under six minutes including animal handling, but efficiency improved as AI technicians gained experience. Less than one third of the mature ewes in the current study took longer than two and one quarter minutes to inseminate. It would appear that a commercially acceptable insemination time of less than two minutes would be achievable with small refinements in technique and equipment. An output of 30 ewes per hour is comparable to that obtained in laparoscopic AI programmes.

Conclusion

In comparison with mature ewes the maiden two tooth ewes proved to be unsuitable candidates for transcervical AI. This was mainly because the insertion of the speculum into these young ewes was difficult. Therefore the majority of the two tooth ewes were inseminated vaginally, which explained the relatively quick insemination time for this group of animals.

A higher non-return rate in the two tooth compared with the mature ewes was surprising, especially when the greater number of vaginal inseminations in the younger ewes was considered. Most studies show that the deeper the deposition of semen into the reproductive tract, the higher the subsequent non-return rate. In this experiment increased cervical penetration with the inseminating needle was associated with greater tissue damage. This trauma inflicted on the tracts would have been detrimental to sperm transport. However the design of the trial precluded detailed assessment of the main factors responsible for the higher conception rate of the two tooth ewes.

Fresh semen rather than frozen-thawed semen gave the highest conception rate. This was most likely due to the higher number of healthy sperm passing into the uterine tubes with the fresh inseminations. The lower numbers of sperm recovered from ewes inseminated with frozen-thawed semen in the current study supports this conclusion. In the trial the total number of sperm in the fresh and frozen-thawed inseminating doses was approximately equivalent. However it should be noted different sires produced the two types of semen and the proportion of motile sperm was larger with the fresh semen inseminations.

Overall conception rate was unacceptably low from a commercial perspective. Examination of the reproductive tracts indicated that ovarian function at the time of AI was conducive to normal fertility. There was no measure of early embryonic mortality so the loss of embryos prior to assessment of non-return rate may partly explain the low fertility. However the low numbers of sperm flushed from recovered tracts implied that the low fertility was mainly owing to very low numbers of sperm reaching the site for

fertilisation, and this would be because of poor sperm transport. When ewes are mated at a synchronised oestrus such as in the current experiment, the potential fertility is reduced owing to the detrimental effect of the synthetic progesterone on sperm transport. The shallow depth of many inseminations, especially for the frozen-thawed semen, and the trauma sustained by the ewe cervices from the insemination needle, would be the other major factors disrupting normal sperm transport mechanisms.

The severity of cervical damage seen in the animals' *postmortem* was greater than expected. While in some of the previous trials occasional piercing of the tract wall had occurred, generally few complications were reported. Hence assessment and examination of the affected organs was not part of the original experimental design and limited observations were made. The presence of blood at the cervical os due to the attachment of forceps to cervical tissue was unrelated to bleeding at sites of deeper injury associated with the passage of the insemination needle. The observation of bleeding at the cervical os was of limited value in attempting to assess the more serious damage located further into the cervix.

High transcervical penetration rates in this and other experiments may be inversely related to high conception rates due to injury inflicted in the cervix by the inseminating needle. It is clear that the transcervical procedure using the current instruments must be performed with caution. Frequently a compromise must be reached between a prolonged attempt at passing the inseminating needle through the cervix and the need to avoid damage to reproductive tissue.

The development of an effective cervical softening agent for use with transcervical AI would be advantageous from both a technical and animal welfare perspective in relation to commercial development of the technique. The attempted cervical softening using clenbuterol in this experiment was unsuccessful. Other pharmacological studies of the cervix have indicated that there are a number of agents, including the prostaglandins, oxytocin and oestradiol, that may have more potential for causing relaxation of the genital tract and thus facilitate passage of the inseminating needle.

There was no negative effect of clenbuterol on sperm transport even though the compound is known to reduce the uterine contractions important for the movement of spermatozoa through the reproductive tract. There was a positive correlation between clenbuterol administration and reduced cervical bleeding following AI. This relationship would be beneficial to sperm survival and may have compensated for a clenbuterol-induced reduction in uterine contractions.

It was possible that physiological changes caused by clenbuterol were masked by the effects of cervical damage sustained at AI. Only one dose rate of clenbuterol and time of administration with respect to synchronisation and AI were tested in this experiment. Other injection regimes and dose rates may affect cervical softening and sperm transport differently.

The transcervical AI procedure does show promise as an alternative to laparoscopic AI, but further research will be necessary to develop this potential. Refinements in instrumentation and operator technique, and the development of an effective cervical softening agent, could lead to better results with this technique. Some breeds of sheep and individuals may be more suitable for transcervical AI than others. In addition other areas of research might include animal restraint for AI and timing of transcervical AI following synchronisation.

Appendix 1

The number of ewes conceiving (%) that were recorded in initial categories for condition of cervix, depth of AI, and bleeding at the cervical os.

Table 19: The effect of condition of cervix on conception rate.

Condition of cervix	Number of ewes	Number of ewes (%)		Pooled categories
		Conceiving	Not conceiving	
Dry	2	1 (50)	1 (50)	Dry/ clear fluid
Wet	6	2 (33)	4 (67)	
Clear fluid	82	29 (35)	53 (65)	
Cloudy fluid	58	18 (31)	40 (69)	Cloudy fluid
Totals	148	50	98	

Table 20: The effect of depth of AI on conception rate.

Depth of insemination	Number of ewes	Number of ewes (%)		Pooled categories
		Conceiving	Not conceiving	
No speculum penetration	20	10 (50)	10 (50)	Vaginal/ shallow cervical
No cervical penetration	5	1 (20)	4 (80)	
1/4 cervical penetration	13	5 (38)	8 (62)	
1/2 cervical penetration	16	4 (25)	12 (75)	Deep cervical
3/4 cervical penetration	16	8 (50)	8 (50)	
Full cervical penetration	78	22 (28)	56 (72)	Full cervical penetration
Totals	148	50	98	

Table 21: Bleeding at the cervical os recorded at insemination.

Bleeding category	Number of ewes	Number of ewes (%)		Pooled categories
		Conceiving	Not conceiving	
No bleeding	100	37 (37)	63 (63)	No bleeding
Slight bleeding	40	11 (27)	29 (73)	Bleeding
Bleeding	6	2 (33)	4 (67)	
Heavy bleeding	2	0 (0)	2 (100)	
Totals	148	50	98	

Appendix 2

Table 22: Assessment and dilution of fresh semen for insemination.

Date of semen collection	Ram no.	Vol.[a] (ml)	Col.[b] (0-5)	Mot.[c] (1-5)	Conc. sperm/ml (pooled)	Diluent added (ml)	Final conc. sperm/ml
8/3/93	2356	0.90	5	5	} $232x10^7$	5.61	$861x10^6$
8/3/93	1139	1.00	4	4	}		
9/3/93	2356	1.00	5	5	} $362x10^7$	8.10	$800x10^6$
9/3/93	1139	1.30	4	3	}		
10/3/93	2356	0.60	5	5	}		
10/3/93	1139	0.85	5	4	} $332x10^7$	6.60	$800x10^6$
10/3/93	2356	0.65	5	5	}		
11/3/93	2356	0.65	5	5	}		
11/3/93	2356	1.00	5	5	} $316x10^7$	7.90	$800x10^6$
11/3/93	1139	0.70	4	4	}		

where Vol.[a] = volume of ejaculate

Col.[b] = colour of ejaculate

Mot.[c] = motility of ejaculate

Appendix 3

Table 23: Numbers of spermatozoa recovered, cervical damage and ovarian structures.

Ewe number	Treat.[a] + day[b]	Number of sperm		Cervical damage	CL[d]	Follicles >4 mm	Follicles <5 mm
		Uter.[c]	Cervix				
341/89	FC1	279	833	no	-	1	1
120/90	FC1	282	37708	no	1	1	-
33/89	FN1	291	2083	no	1	-	-
71/90	FN1	277	0	yes	1	-	2
381/89	DC1	10867	46875	no	-	1	-
128/90	DC1	330	0	no	1	1	-
412/89	DN1	663	1458	no	-	2	4
25/90	DN1	269	6250	yes	-	1	2
408/89	FC2	93	500	no	1	-	-
48/90	FC2	207	2166	no	1	1	1
391/89	FN2	1918	3333	no	-	2	4
5/90	FN2	0	0	yes	1	-	1
9/89	DC2	90	1250	yes	-	2	1
130/90	DC2	0	6000	yes	-	1	4

Table 3 continued next page....

30/89	DN2	0	8833	no	1	1	4
146/90	DN2	0	9000	no	-	-	3
462/89	FC3	575	333	yes	2	-	4
239/90	FC3	1104	10166	no	1	1	1
500/89	FN3	1668	3000	no	1	6	-
262/90	FN3	576	10167	no	1	-	-
410/89	DC3	243	6333	yes	2	1	1
199/90	DC3	514	6167	no	1	1	1
594/89	DN3	799	6000	no	1	2	1
167/90	DN3	317	7000	no	1	1	1
36/89	FC4	3434	8667	no	-	2	-
21/90	FC4	255	7333	yes	2	-	1
16/89	FN4	107	1833	no	2	1	4
223/90	FN4	107	4000	no	-	1	3
321/89	DC4	95	4500	yes	1	1	1
198/90	DC4	0	7333	yes	-	2	-
40/89	DN4	13602	86833	no	1	-	2
123/90	DN4	345	11667	yes	1	-	-

Table 3 continued next page...

where:

Treat.[a] = D = freshly diluted semen,

F = frozen-thawed semen,

N = no clenbuterol administration,

C = clenbuterol administration,

and

Day[b] = 1-4 corresponds to day of AI,

Uter.[c] = number of spermatozoa estimated present in the left uterine segment,

CL[d] = total number of corpora lutea present on both ovaries.

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