STUDIES ON EMBRYO TRANSPLANTATION PROCEDURES

USING BOOROOLA-MERINO X PERENDALE EWES

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ABSTRACT

A study was carried out to develop an embryo transfer programme under farm conditions. For this embryos were collected from donor ewes 5-6 days after single-sire mating by rams of the same crossbreed as the ewes. Then the embryos were transplanted soon after collection or after they had been initially frozen and later on thawed before their transfer. To enable this study to be carried out, one hundred and sixty-one Booroola-Merino x Perendale ewes aged between 3 and 6 years and of 34-69 kg live weight were examined. These animals were classified as either carriers (F+) or non-carriers (++) of the fecundity gene on the basis of the number of ovulations recorded at laparoscopy carried out a few days after progestagen sponge treatment to induce oestrus synchronisation, and in a few cases on the basis of previous lambing data. The aims of the study therefore were also to identify ewe carriers or non-carriers of the fecundity gene, and to evaluate their ovulatory response to several doses of Pregnant Mare Serum Gonadotrophin (T1=0, T2=350, T3=700 and T4=1050 i.u. of PMSG) given during the embryo transfer programme.

On the basis of the criteria used to classify F+ and ++ ewes, 76 ewes were considered as carriers and 85 as non-carriers of the fecundity gene.

Immediately after progestagen synchronisation treatment the incidence of oestrus and the distribution of onset of oestrus was similar in both groups of ewes, and also following PMSG treatment given in the succeeding cycle.

Ovulation rate after the progestagen sponge treatment was significantly affected by genotype and age of the ewes, but it was not associated with the live weight of the ewes. The least-squares means for number of ovulations in F+ and ++ ewes and in 3 and 4-6 years old animals were 3.02, 1.73, 2.14 and 2.45 respectively. Higher sensitivity to PMSG treatment was observed from the right ovary than from the left ovary (3.40 v. 2.82 ovulations respectively).

No significant effect of dose of PMSG was found on the percentage of embryos recovered, the overall recovery rate being 66%. Of the ova or potential embryos recovered 78% had been fertilised and had developed to embryos. There were no significant differences between the 3 rams in the fertilisation rate in the ewes compared on a per ewe basis.

The reproductive performance of donor ewes which had been flushed, was considerably influenced by the efficiency of the embryo recovery procedures, since every embryo not recovered represents a potential pregnancy and such a situation sometimes is not desirable. In this study 30% of the ewes that were flushed, subsequently became pregnant and produce lambs as a result of one or more embryos not being recovered at surgery.

The number of ovulations after PMSG treatment was significantly affected by the dose-level of PMSG and the genotype, age and live weight of the ewes. F+ ewes recorded a significantly higher ovulation rate than ++ ewes. Their respective least-squares means were 3.61 and 2.31 corpora lutea. No significant difference was found between treatments 1 and 2 and between treatments 3 and 4. However, the response from the last two treatments was significantly higher than that from the first two treatments. Their respective least-squares means were 2.10, 2.01, 3.74 and 4.37 corpora lutea. Ewes 4-6 years old recorded a significantly higher number of ovulations than younger ewes (3 years old). Small significant effect of live weight was found on the ovulatory response of the ewes. Analysis within each genotype showed a similar trend in both genotypes, but small significant effect of weight was only detected in the ++ ewes.

The pregnancy rate that occurred after the transfer of two fresh embryos (86%) was significantly affected by the genotype of the recipient ewe (F+ ewes 75% v. ++ ewes 96%). The number of ovulations in the recipients and whether or not they had received PMSG before transfer had no effect on the incidence of pregnancy.

The pregnancy rate achieved after the transfer of frozen embryos (35%) was significantly influenced by the number of embryos transferred, but not by the ovulation rate in the recipient ewe or the time elapsing from flushing until freezing. Pregnancy rate was significantly higher after the transfer of 2 embryos compared to the transfer of single embryos (43% v. 0%), but only 8 transfers were made

in the later category.

On the basis of the number of ovulations, it can be concluded that ewe carriers of the fecundity gene recorded significantly higher ovulation rate after oestrus synchronisation and were more sensitive to PMSG stimulation than non-carrier ewes.

There was an encouraging pregnancy rate obtained with frozen embryos, even although the small number of transfers carried out limits conclusions that can be drawn. However, the eighty-six percent pregnancy rate achieved after the transfer of two fresh embryos per recipient, shows the feasibility of the embryo transfer programmme under conditions where suitable recipients are available. Where recipients are limited then additional embryos might be frozen and stored until ready for transplantation.

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INTRODUCTION

The constant rise of the world population, demands a constant increase of food. As a consequence, an increase in the efficiency of the actual agricultural systems is required to increase the actual levels of production. New technologies need to be developed by all the scientists working on animal production systems to contribute in the solution of the problem.

Scientists in Reproductive Physiology have been trying to develop techniques to be used under farm conditions, with the aim of increasing the efficiency of the actual animal production systems. One of the most recent advances has been the development of the embryo transfer technique.

Embryo transfer is a technique by which fertilised embryos are collected from a donor female and transferred to a recipient female that serves as a surrogate mother for the remainder of pregnancy (Mapletoft, 1986).

The use of this technique has been successful in domestic species, exotic animals (including humans and primates) and in several species of wildlife animals. In farm animals under New Zealand conditions, transfers have been usually carried out in Beef and Dairy Cattle, Sheep and Goats. However, recently the technique is trying to be applied on other species of interes to New Zealand Farming, such as Alpacas,

Llamas and Angora Rabbit.

Embryo transfer in sheep consists of the following steps: selection of the ewes, oestrus synchronisation in donor and recipient ewes, superovulation, service and embryo recovery from the donors and embryo transfer to the recipient ewes.

The development of techniques of embryo preservation (embryo freezing) have significantly spread the use of embryo transfer, mainly in farm animals. Nowadays, embryos can be frozen and in this way maintained for an indefinite period of time, transported at low cost anywhere that is desired and transferred at any time when required. As an example, successful transport of frozen embryos from New Zealand to Australia has been reported by Trevit (1983); and Mapletoft (1986) has pointed out that whereas the itercontinental transport of a live animal may cost $ 1000 or more, an entire herd can be transported, frozen in the form of embryos, for less than the price of a single plane fare.

Embryo transfer technique, up to date has been usually used to increase the number of offspring from animals with desirables charactristics. The possibility of freezing embryos and keep them in storage for indeterminate period of time; opens the possibility to evaluate the genetic gain from a particular flock, once frozen embryos can be thaw after being conserved for several years and be used as a control group animals.

In the study now to be reported, it was attempted to develop the methodology of an embryo transfer programme under farm conditions,

using a selected group of Booroola-Merino x Perendale ewes. Some of these animals were known to have inherited the Booroola gene for high fecundity (F+). Thus in the course of the work it was possible to evaluate the ovulatory response to exogenous gonadotrophin stimulation when these animals were utilized as "Donors" for embryo transfer. The particular objectives of the investigation were as follows.

a) Identification of ewes carrying the high fecundity gene.

b) Evaluation of the ovulatory response to Pregnant Mare's Serum Gonadotrophin (PMSG) treatment in ewes classified as carriers and non-carriers of the high fecundity gene.

c) Development of an embryo transfer programme under farm conditions.

d) Development of an embryo freezing technique.

CHAPTER 2

CHAPTER 2

REVIEW OF LITERATURE

2.1. Identification of Ewes Carring the High Fecundity Gene.

The high prolificacy of some Booroola Merino ewes has been related to the presence of a gene or a group of genes. This was first postulated by Turner (1980) and subsequently by Piper and Bindon (1980, 1982) and Davis et al. (1981, 1982).

Piper and Bindon (1979) indicated that the mode of action of the postulated gene may be additive for ovulation rate although probably is completely dominant for litter size (Piper and Bindon, unpub., cited by Piper and Bindon, 1982).

Results reported by Davis et al. (1982) are in agreement with Piper and Bindon (1979), however in their study the effect of the gene appears to be partially dominant for litter size when segregation criteria of at least one record of ovulation rate ≤ 3 and ≥ 5 are applied to identify heterozygous and homozygous carriers respectively, in 3/4 Booroola ewes.

The criteria used to identify ewe carriers of the high fecundity gene has been the occurrence of one or more sets of triplets, or higher litter sizes in a ewe's lifetime record (Piper and Bindon, 1980; Piper and Bindon, 1982; Davis et al., 1982, 1983).

In 1982, Piper and Bindon indicated that differences in mean litter size between animals postulated to be carrying one and no copies of the high fecundity gene, are around one lamb more per lambing or two to three times the standard deviation for litter size in the Merino.

Davis et al. (1982) have pointed out the importance of classifying the ewes carrying the high fecundity gene on the basis of ovulation rate ≥3 corpora lutea instead of using litter size. Their suggestion is illustrated in Table 2.1.

They also suggested the classification of Booroola Merino ewes into three categories, on the basis of ovulation rate.

Genetic Group	Ovulation Rate
FF	≥5
F+	3-4
++	1-2

The main problems when data on litter size are recorded are the high incidence of embryo mortality recorded for breeds of high ovulation rate (Hanrahan, 1974; Hanrahan and Quirke., 1985; Bindon et al., 1980) and problems on lamb identification because of mis-mothering (Welch and Kilgour, 1970).

Table 2.1. Number of F1 progeny of several Booroola-type sires, and the proportion with at least one record of ≥3 litter size/ovulation rate in 3-6 records (Davis et al., 1982).

Sire	Litter size		Ovulations	
	No. of progeny	Proportion ≥3	No. of progeny	Proportion ≥3
A	41	0.61	37	0.62
B	44	0.30	39	0.46
C	46	0.48	32	0.50
D	41	0.27	39	0.31
E	31	0.45	36	0.56
F	40	0.35	37	0.49
G	25	0.56	25	0.88
H	41	0.34	38	0.55
Mean		0.41		0.53

2.2. Oestrus Synchronisation.

Oestrus synchronisation is an important step in any embryo transfer programme. There are basically two ways to get an adequate oestrus synchronisation between donors and recipients. One is the selection of ewes coming in heat normally, usually requiring a large number of ewes. The other one is through oestrus synchronisation by artificial means. The topic of oestrus synchronisation in ewes has been extensively reviewed by several researchers (Phillips et al., 1945; Robinson, 1954, 1959; Lamond, 1964) for that reason only some aspects in relation to the present study are mentioned.

Oestrus synchronisation in sheep with progesterone and some of it's analogues during the oestrus and anoestrous season has been reviewed by Lamond (1964). Although several options or ways of treatment have been evaluated, up to date the most common approach is the use of intravaginal dispositives (pessaries or sponges) impregnated with one of the progesterone analogues, either MAP (6α-methyl-17α-Hydroxiprogesterone acetate) or CAP (6-chloro -Δ-Dehydro- 17 - Acetoxyprogesterone), being generally inserted for 14 days.

Details on sponge elaboration and its feasibility to control oestrus activity in ewes has been reported by Robinson (1965). Three points have been emphasised by Lamond (1964) when an oestrus synchronisation is carried out: suppression, synchronisation and fertility, considering a satisfactory system when 80 to 90 % of the treated ewes come in heat within a period of 36 to 48 hours.

In general, reduction in fertility has been observed after oestrus synchronisation using sponges (Clarke et al., 1966; Robinson et al., 1967). Such problem has been attributed to an impaired sperm transport and survival (Quinlivan and Robinson 1967, 1969; Allison and Robinson, 1970), and probably can be enhanced by an abnormal pattern of cervical mucus secretion during the oestrus (Smith and Allison, 1971). The reduction in fertility is minimal the following oestrus after sponge removal (Allison and Robinson, 1970), and Robinson (1967) has indicated quite normal fertility at the second oestrus following synchronisation.

More recently, normal fertility has been reported after oestrus synchronisation using CIDRs (control internal drug release) by Harvey et al. (1984), and MAP for 7 days plus an injection of prostaglandins on day 6 by Fitzgerald et al. (1985).

2.3. Superovulation.

The superovulation of ewes has been carried out using two types of gonadotrophins. One type is the gonadotrophin isolated from the pituitary gland and the other is isolated from the serum of pregnant mare between 60 and 80 days of pregnancy (Walker, 1977).

In ewes the use of pregnant mare's serum gonadotrophin (PMSG) has been reported widely, either as a raw material (Hunter et al., 1955; Rowson and Adams, 1957; Gherardi and Martin, 1978; Gherardi and Lindsay, 1980) or as freeze dried preparation which was reconstituted immediately before use (Moore et al., 1960; Holst, 1969; Clarke,

1973; Eastwood and McDonald, 1975; Tervit et al., 1976; Hanrahan and Quirke, 1982; and others).

Induction of superovulation in ewes with pituitary gonadotrophins has been reported by Moore and Shelton (1962, 1964a), Crosby et al. (1980), Torres and Cognie (1984), Chupin et al. (1986), Heyman et al. (1986) and Torres et al. (1986).

All the authors working with PMSG administered the hormone in only one dose injection, which is possible because of its long half-life (McIntosh et al., 1975). However, the researchers inducing superovulation with pituitary gonadotrophins treated the animals for 2 or 3 days on a single daily or twice daily injections, mainly because the rate of clearance of pituitary gonadotrophins from the blood stream is very fast (Akbar et al., 1974).

An analysis of the mentioned studies shows that to induce superovulation in ewes, a follicle stimulating gonadotrophin can be administered either near the end of the luteal phase of the cycle (days 11 to 13) or around the end of treatments with progestagens designated to control the time of ovulation.

2.4. Factors which Affect the Ovulatory Response to PMSG Treatment.

There are some factors which have been shown to influence the ovulation rate after gonadotrophin treatment.

2.4.1. Dose-level of gonadotrophin.

The effect of hormone treatment on ovulation rate was reported by all the researchers mentioned above. However, induction of superovulation has been shown on different breeds when the ewes were treated with at least 750 i.u. of PMSG.

A dose-response relationship to PMSG treatment was reported by Robinson (1951), Holst (1969), Bindon et al. (1971), Smith (1976) and Evans and Robinson (1980). Similar results were reported after the induction of superovulation with pituitary gonadotrophins by Moore and Shelton (1964a) and Boland (1973) (Cited by Gordon, 1975).

Increase in the ovulation rate when the dose of gonadotrophin was increased has been reported in cattle by Gordon et al. (1962), Mauleon et al. (1970), McGaugh et al. (1974) and Moore (1975).

The main problem found by all the authors when superovulation was attempted, was the large variation in ovulation rate to standard doses of gonadotrophins, being larger when PMSG was used instead of pituitary gonadotrophins. The variation in the response generally increased as the dose of gonadotrophin was increased.

The results from two studies are shown in Table 2.2 to illustrate the ovulatory response to gonadotrophins and its variation.

Table 2.2. Mean and range ovulation rates in ewes treated with PMSG on day 12 of the cycle (Cahill, 1982).

Dose of PMSG	Bindon et al. (1971) (Merino ewes)	Smith (1976) (Romney ewes)
0	1.5 (1-2)	1.5 (1-2)
Moderate (750 i.u.)	3.8 (2-17)	4.2 (2-8)
High (1500 i.u.)	7.1 (1-17)	7.2 (2-12)

On the other hand, Tervit (1967) and Clark (1973) reported a range in ovulation rate from 1-19 and 1-36 corpora lutea respectively, when Romney ewes were treated with 1500 i.u. of PMSG. In agreement with them, wide variation (1-39 corpora lutea) was found by Moore and Shelton (1962) when Merino ewes were treated with 75 to 100 mg of horse anterior pituitary hormone extract (HAP).

Hyperstimulation of the ovaries has been related to a high incidence of persistent large follicles (Moore and Shelton, 1962; Boland and Gordon, 1977; Mutiga and Baker, 1982; Ryan et al., 1982; Armstrong and Evans, 1983).

The high oestrogen output from the large follicles (Quirke and Hanrahan, 1975; DuMesnil DuBuisson et al., 1977) is thought to increase the rate of transport of embryos through the oviducts and decrease recovery rates (Quirke and Hanrahan, 1975; Ryan et al., 1982).

Other problems reported after hyperstimulation of the ovaries are decrease in ovulation rate (Shelton and Moore, 1967; Boland and Gordon, 1977; Bindon and Piper, 1982; Mutiga and Baker, 1982; Ryan et al., 1982; Gordon, 1983) and fertilisation rate (Averill, 1958, 1959; Moore and Shelton, 1962; Cumming, 1965; Tervit, 1967; Whyman and Moore, 1980; Bindon and Piper, 1982; Armstrong and Evans, 1983).

Faulty transport of spermatozoa through the cervix has been pointed out as the main reason for the decrease in the rate of fertilisation (Trounson and Moore, 1974; Betteridge and Moore, 1977; Evans and Armstrong, 1984).

2.4.2. Season.

The ovulation rate of naturally ovulating animals has been recorded to be highest during autumn and lowest in spring (Radford, 1959; Dun et al., 1960).

During the normal breeding season (autumn) an increase in the ovulation rate has been reported from the first to the third oestrus,

when ovulation rate was recorded either on different ewes after they were slaughtered (McDonald, 1958; Averill, 1959, 1964) or on the same ewes after laparoscopy (McDonald and Chang, 1966).

Attempts to increase ovulation rate in ewes have been carried out during the breeding season and the anoestrous season as well. Gherardi and Lindsay (1980) reported higher ovulatory response to PMSG during autumn than during spring. Results which have been supported by Torres et al. (1986) after the ewes were treated with pig pituitary extracts. Braden et al. (1960) working within the breeding season found greater response to superovulation in late February than in early January. This situation has been suggested by Lamond (1962) to be due to a change in sensitivity of the neural-endocrine systems controlling ovarian activity.

The effectiveness of inducing superovulation during the non-breeding season using progestagen/gonadotrophin regimens has been reported by Moore (1982); and Averill (1958) and Evans and Robinson (1980), reported no significant difference in the ovulatory response to PMSG between ewes treated during the breeding and anoestrous season.

In agreement with them, Moore (1982) indicated little effect of season on the ovulatory response of Merino ewes to progestagen/PMSG, or HAP treatments, although it was suggested that this may not be so in those breeds which experience a period of deep anoestrous.

Evans and Robinson (1980) indicated that the effect of progestagen treatment prior to PMSG and the type of breed, which showed little

activity during the anoestrous season, could affect their results.

Higher ovulatory response after PMSG stimulation during the anoestrous season in comparison with the breeding season is reported by Holst (1969). This may have resulted from the effect of the higher total gonadotrophin levels thought to be found in the anoestrous ewe (Kammlade et al., 1952).

On the other hand, Savery and Berry (1953) found that sheep eggs from ovulations induced in anoestrous had undergone normal maturation and were presumably fertilisable.

2.4.3. Day of injection.

No significant difference in the response to PMSG has been reported when the ewes were treated from day 12 to 15 of the oestrous cycle (Robinson, 1951; Wallace, 1954; Braden et al., 1960; Cumming, 1965; Bindon et al., 1971; Larsen, 1971).

The effect of gonadotrophin injection, given on the day of sponge removal or 12 to 15 days after the ewes come in heat because of the oestrus synchronisation, was reported by Gordon (1966), cited by Hulet and Foote (1967) and Hulet and Foote (1967, 1969). They found a higher ovulation rate when the ewes were treated 12 to 15 days after the oestrus synchronisation than at the time of sponge removal. The results have been attributed to the influence of exogenous v. endogenous progesterone immediately prior to ovulation. On the other

hand, significant interaction between progesterone treatment and PMSG injection was reported by Lamond (1964).

2.4.4. Age.

The effect of age on the response to gonadotrophins has been studied by several researchers. Robinson (1951), Averill (1958) and Gordon (1963) did not find any significant effect of age on the ovulatory response to PMSG treatment, although Averill (1958) reported a consistently steeper slope of the regression line of ovulation rate on age studing mature and two-tooth ewes. These results were supported more recently by Torres et al. (1986) when ewes were stimulated with pituitary follicle stimulating hormones (pFSH).

2.4.5. Breed.

The effect of breed on the ovulation response to PMSG treatment has been reported by Wallace (1954), Averill (1958), Bradford et al. (1971), Tervit et al. (1976) and Armstrong and Evans (1983). Within breeds, differences between strains were reported by Braden et al. (1960), Bindon et al. (1971) and Trounson and Moore (1972) concluding that strains of high natural ovulation rate gave higher ovulatory response to PMSG treatments than strains with low natural ovulation rate. A similar trend was reported by Eastwood and McDonald (1975) working with Romney and Border Leicester x Romney ewes.

However, Newton et al. (1970) reported that a higher dose of PMSG was required to produce the same ovulation rate for breeds with a high mean litter size, compared to less prolific breeds. Differences in ovulation rate were reported in ewes of the same breed with similar origin when they were run together after four months in a common environment (McDonald and Chang, 1966). In the same study, ewes born as twins shed more ova than did single born ewes. The last information has been confirmed by Packham and Triffitt (1966) and Larsen (1971) although in the last study the difference was not significant.

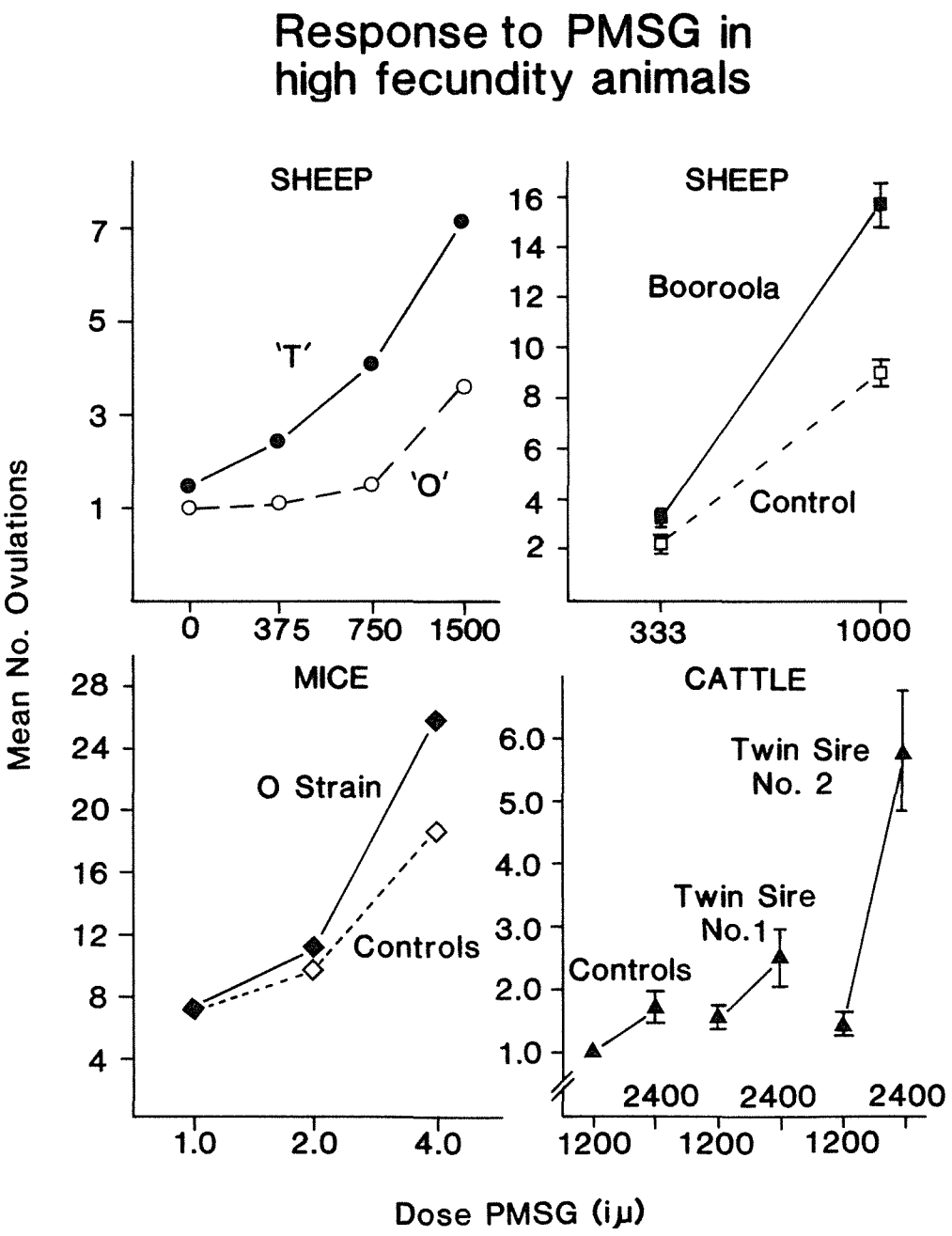
Bindon and Pennycuik (1974) working with hypophysectomised mice demonstrated that difference in ovarian sensitivity do represent part of the response to selection for ovulation rate.

In a review made by Bindon et al. (1986) it was concluded that Merino sheep, cattle and mice that are genetically high in fecundity are more sensitive to PMSG (Figure 2.1).

These results have been confirmed by Smith (1976) in Romney ewes selected for prolificacy.

In Merinos derived from Booroola crosses, the ewes carrying the high fecundity gene (F+) were more sensitive to PMSG than ewes without the high fecundity gene (Bindon and Piper, 1982; Piper et al., 1982; Kelly et al., 1983/84), although superovulation with FSH showed no difference in response from Booroola and control Merino ewes (Bindon and Piper unpub. results cited by Bindon et al., 1986).

Figure 2.1 Ovulatory response to PMSG by animals selected for fecundity (Bindon and Piper 1982)



2.4.6. Live weight.

Several studies have been carried out in an attempt to understand the influence of live weight or condition of the ewes on the response to PMSG stimulation.

Under normal conditions without PMSG stimulation higher ovulation rate has been reported in heavier ewes (Coop, 1962; Allison, 1968, 1975), but the ovulatory response of ewes with different weight to PMSG stimulation has been variable.

While most of the researchers have reported no significant effect of nutritional differences of live weight on the response to PMSG (Wallace, 1954; Allen and Lamming, 1961; Bellows et al., 1963; Bindon et al., 1971; Tait, 1971; Allison, 1973; Eastwood and McDonald, 1975), some others have indicated high ovulatory response to PMSG treatment in heavier ewes (Keane, 1973; Allison, 1975). However, the first author reported significant effect of weight on ovulatory response only in two out of three experiments carried out during the anoestrous season. Allison (1975) mentioned that the reasons to explain his results were obscure, but could be due to the ovaries of the lighter animals being less sensitive to a fixed amount of circulating gonadotrophin and/or due to a reduction of pituitary output in those animals.

2.5. Service of Donor Ewes.

Generally the service of the donor ewes has been carried out by natural mating. The use of rams kept with the ewes at the time of oestrus has been reported by most of the researchers (Hunter et al., 1955; Moore et al., 1960; Moore and Shelton, 1962, 1964b; Allison, 1975; Eastwood and McDonald, 1975).

However, in some studies the use of single sire by hand mating has been also reported (Keane, 1973; Tervit et al., 1976; Torres et al., 1986).

Even when all the researchers have emphasised the importance of using high proven fertility rams, failure of fertilisation occurs frequently in superovulated ewes and has been attributed to a decreased sperm transport through the cervix associated with the superovulatory treatments (Trounson and Moore, 1974; Evans and Armstrong, 1984).

On the other hand, Killeen and Moore (1971) and Trounson and Moore (1974) reported that if semen is introduced directly into the uterus (intrauterine insemination), high fertilisation rates (above 90%) result even in highly superovulated ewes. Reduction in embryo recovery rate was reported as a consequence of surgical insemination by laporotomy, in the study carried out by Trounson and Moore (1974). However, such effect was not found after intrauterine insemination with frozen semen through laparoscopy (Armstrong and Evans, 1984).

Failure of fertilisation after superovulation has been considered as the main factor limiting embryo transfer in sheep (Betterridge and Moore, 1977; Armstrong and Evans, 1983) and Evans and Armstrong (1984) suggested the use of artificial insemination by laparoscopy to overcome the problem.

2.6. Embryo Recovery from Donor Ewes.

The recovery of the embryos can be carried out from the oviduct or from the uterus and the time is usually decided on the basis of oestrus detection of the donor ewes.

Usually embryo recovery from the oviducts has been carried out from a few hours after ovulation until 3 to 4 days after oestrus detection (Robinson, 1951; Hunter et al., 1955; Averill, 1958; Cumming, 1965; Cumming and McDonald, 1967; Tervit, 1967; Larsen, 1971; Tervit and Havik, 1976). The flushings carried out 3 to 4 days after oestrus, normally include the last portion of the uterine horns. The technique used for embryo recovery at this time was developed by Hunter et al. (1955).

Although great variation (45% to 82%) in the percentage of embryos recovered by flushing the oviducts has been reported; Trounson and Moore (1974) have indicated that irrespective of the time after oestrus at which collections are attempted, flushing through oviducts results in high rates of recovery of embryos (around 80%).

Several researchers (Averill, 1958; Killeen and Moore, 1971; Tervit and Havik, 1976; Boland and Gordon, 1978) after flushing the oviducts have indicated a decrease in embryo recovery as the time after oestrus increases. Accelerated tubal transport following superovulation in sheep has been reported as the main reason for decreasing the embryo recovery (Robinson, 1951; Averill, 1958).

Recovery of embryos from the uterus can be carried out from day 5 until day 14 (Willadsen, 1979). However, under practical conditions, embryo recoveries are normally attempted between day 5 and 6 after the ewes come in heat. The efficiency of embryo recovery at this time has been reported to be from 69% to 86% (Tervit and Havik, 1976; Torres and Cognie, 1984; Torres et al., 1986). However, Bondurant (1986) has indicated that recovery rates tend to decrease as oestrus-to-collection interval increases, which may result from the early death of genetically non-viable embryos, the increased difficulty in removing a microscopic embryo from a relatively voluminous uterus or the premature luteal regression and expulsion of the embryo.

2.7. Embryo Searching and Evaluation.

The searching and evaluation of embryos is carried out with the help of a normal or stereoscopic microscope.

Studies of the cleavage stages in sheep embryos during the first 5 days have been reported by Hancock and Hovell (1961), Tervit (1967),

The evaluation of embryos has been done normally under practical conditions by embryo morphology. Linder and Wright (1983) proposed the following classification on that basis.

Good quality embryos : this is an ideal embryo, spherical, symmetrical with cells of uniform size, colour and texture. Embryos with trivial imperfections such as few extruded blastomeres, irregular shape and few vesicles are included in this category as well.

Fair quality embryos : embryos with definite shape and no severe imperfections; of extruded blastomeres, vesiculation and few degenerated cells.

Poor quality embryos : Embryos with severe problems, numerous extruded blastomeres, degenerated cells, cells of varying sizes, large numerous vesicles but a viable appearing embryo mass.

At the time of flushing, embryos at different stages of development, fertilised embryos of good, fair or poor quality; unfertilised ova and abnormal embryos can be found.

Several studies have been carried out to evaluate the incidence and type of abnormalities in sheep.

The incidence of morphological abnormalities was reported to be between 4 and 23% (Dutt, 1954; Hart, 1956; Averill, 1958; Braden, 1964; Allison, 1967; Tervit and McDonald, 1969) the most common abnormalities being fragmentation, degeneration, involution of vitellus and broken zona pellucida.

On the other hand, the presence of chromosomal abnormalities was indicated by Long and Williams (1980), although its incidence was low (6%). This type of abnormalities have been reported in cattle (8%) and pigs (11%) by McFeely (1967) and McFeely and Rajakoski (1968) respectively.

A common characteristic found after flushing superovulated animals has been the presence of embryos at different stages of development in the same flush.

Sheep embryos should be considered retarded when they are one (Moore, 1982; Bondurant, 1986) or more cleavage divisions behind their more advanced litter mates (Willadsen, 1979). Lack of development, indicating that ebmryos are not viable was reported by Moore (1982)

after in vitro embryo culture; confirming his earlier findings in 1976 when he indicated that relatively underdeveloped embryos are probably better left out of transfer attempts. On the other hand, Mapletoft (1986) mentioned that wide variation in embryo quality and stages of development are signals that the existing embryos are not entirely normal and pregnancy rates may be disappointing.

The presence of anucleated particules in sheep embryos has been reported by Killeen and Moore (1970, 1971) and (1979). However, because those embryos were able to develop normally it was suggested to consider them as atypical rather than abnormal.

2.8. Embryo Transfer.

The reproductive tract of the recipient is exposed by midventral laparotomy under general or local anaesthesia (Moore, 1982) restraining the ewes in a laparotomy cradle such as the one described by Lamond and Urquhart (1961).

The transfer of the embryos can be done either to the oviducts or to the uterine horns. For transfer to the oviduct, the embryos should be taken with a Pasteur pipette attached by flexible tubing to a 2 ml syringe in a very small volume of medium (less than 0.02 ml), inserted into the oviduct 3-5 cm and the fluid containing the embryo expelled gently while the pipette is slowly withdrawn. For transfers to the uterine horns, the embryos are taken with the Pasteur pipette in approximately 0.04 ml of medium. The deposition of the embryos is

carried out 3 to 4 cm from the utero-tubal junction, inserting the pipette 2 to 3 cm into the uterine lumen and the medium containing the embryo gently expelled (Hunter et al., 1955; Willadsen, 1979; Moore, 1982).

The transfer of sheep embryos through a laparoscope has been reported recently. Working with very small number of animals McKelvey and Robinson (1984) and Mutiga and Baker (1984) were able to get some ewes pregnant through laparoscopy.

The suggestion from Moore in 1982, that laparoscopic embryo transfer can result in pregnancy rates comparable to those achieved following the use of conventional surgical techniques has been confirmed by Schiewe et al. (1984), McKelvey et al. (1985) and Walker et al. (1985). They reported pregnancy rates from 33 to 75%.

2.9. Factors which Affect the Success of Embryo Transfer.

2.9.1. Number of embryos transferred.

The effect of number of embryos transferred on embryo survival or pregnancy rate has been widely reported. Most of the researchers have concluded that an increase in the number of embryos transferred per ewe do not markedly affect the proportion of ewes which became pregnant (Moore et al., 1960; Moore and Shelton, 1962; Cumming, 1965; Tervit, 1967).

Similar trends have been reported by Tervit et al. (1976) although the statistic comparison between the transfer of one and two embryos was not reported. However, Larsen (1971) reported a significant increase in pregnancy rate when the number of embryos transferred was increased from one to three (66% and 90% respectively).

The success or failure of any transfer depends more on the inherent ability of the recipient ewe to support a pregnancy than on the number of eggs transferred (Moore et al., 1960).

On the other hand, Larsen (1971) indicated that the chance of at least one embryo being in phase with the recipient tract, and hence a successful pregnancy occurring, might increase as the number of embryos transferred per ewe is raised; and Moore and Shelton (1964b) indicated that when the maternal environment was suitable, embryo losses were at random and not excessive.

Regarding embryo survival, there is a general agreement amongst most of the researchers (Moore et al., 1960; Cumming, 1965; Tervit, 1967; Moore, 1968; Cumming and McDonald, 1970; Larsen, 1971; Land and Wilmut, 1977) that increasing the number of embryos transferred causes a decrease in embryo survival.

However, Moore et al. (1960) reported that although embryo survival was depressed after the transfer of 5 embryos in comparison with 2, the litter size was still significantly greater after the transfer of 5 embryos.

Bindon et al. (1971) found that as ovulation rate increased, the proportion of normal embryos present on day 20 decreased.

Low concentration of progesterone on day 0, 1 as well as during the luteal phase in ewes has been related to significantly reduced embryo survival (Ashworth et al., 1984). Accordingly, in cattle, animals subsequently found to be pregnant had a higher concentration of progesterone on day 0 to 3 (Lee and Ax, 1984). However, no difference in the survival of embryos in ewes with high or low plasma progesterone was reported by Evans and Robinson (1980), concluding that over a wide range, progesterone has no quantitative effect on fertility.

2.9.2. Synchronisation.

There is no doubt that adequate oestrus synchronisation between donors and recipients is a very important step in the success of any embryo transfer programme.

The highest pregnancy rate has been reported when oestrus synchronisation between donors and recipients is exact (Hancock and Hovell, 1961; Moore and Shelton, 1964b; Cumming, 1965) on the basis of oestrus detection.

However, an acceptable pregnancy rate can be obtained when there is a synchronisation of ± 1 day (Moore and Shelton, 1964b; Rowson and Moore, 1966; Hunter et al., 1955) and even ± 2 days as it has been reported by Averill (1956), Moore and Shelton (1964b) and Rowson and

Moore (1966).

A difference of 3 days between the onset of oestrus in the donor and recipient appears to be incompatible with pregnancy (Moor, 1965, cited by Newcomb, 1977) or to result in extremely low conception rates (8%) (Rowson and Moore, 1966).

The importance of the high degree of synchronisation between donors and recipients has been shown when transfers have been carried out from day 2 to day 12 of the cycle (Moore and Shelton, 1964b; Moor, 1965, cited by Newcomb, 1977).

The establishment of pregnancy depends upon a precise sequence of hormonal changes, which induce changes in uterine secretions and endometrial structure that are critical for embryo development and implantation (Wilmut et al., 1985). Rowson and Moore (1966) have indicated that the most likely reasons of why an "out-of-phase" embryo dies is because the uterine environment may not be suitable for it, or because the "out-of-phase" embryo is incapable of secreting a sufficient luteotropic action on the recipient's corpus luteum, with the result that the corpus luteum is not maintained. Accordingly, Wilmut et al. (1985) mentioned that embryos, which are too asynchronous to compensate for an inappropriate uterine environment, become abnormal, fail to inhibit luteolysis and are lost from the uterus.

The effect of uterine environment on the embryos depends upon the stage of development at the time of transfer (Wilmut and Sales, 1981).

In sheep, embryos transferred, so that they were retarded relative to the uterine environment, developed more quickly than they would have done in a synchronous environment. On the other hand, the development of embryos that were relatively advanced was slowed down (Wilmut and Sales, 1981; Lawson et al., 1983). The same effect has been observed in pigs (Pope et al., 1982, cited by Pope et al., 1986, and Pope et al., 1986). Regardless of the breed, better embryo development and survival was found when older embryos were transferred to younger uterus. Similar results had been reported by Hunter et al. (1955) and Tervit (1967) although the last author carried out only 7 transfers.

Hancock (1962) indicated that marked variation between ewes can occur in the time of ovulation and fertilisation in relation to the onset of oestrus. Because of that, it is unlikely that the transferred embryo and the recipient genital tract stages of development are closely synchronised even if the onset of oestrus in the donor and recipient ewes are exactly synchronised (Tervit, 1967). The above probably could be the reason why some ewes do not become pregnant to transfer even though the conditions are considered to be optimum (Larsen, 1971). Furthermore, Cumming (1965) suggested that there is a specific lower and upper limit of embryonic and endometrial development that will allow the development of transferred embryos. Wilmut et al. (1985) suggested that in a situation of perfect synchronisation, the proportion of pregnancies should be greater than that achieved at the present.

2.9.3. Site of transfer.

There are basically two places in which the sheep embryos can be transferred and this should be done according to the embryo's age or more adequately according to its physiological development. Embryos collected on day 3 (Moore, 1982) or embryos with less than 8 cells (Moore and Shelton, 1962) should be transferred into the oviduct and embryos collected after day 3 or embryos with more than 8 cells should be transferred into the uterus.

Several studies (Moore et al., 1960; Moore and Shelton, 1962; Shelton and Moore, 1966; Tervit, 1967) reported high embryo survival after uterine transfers. However, in these studies. age of the embryo and place of transfer were confounded, once only older embryos were transferred into the uterus. So the results should be taken with caution. One study in which age of the embryo and place of transfer were not confounded was carried out by Moore and Shelton (1964b). They found that tubal transfers were more successful than uterine transfers.

No significant effect of place of embryo transfer on pregnancy rate was reported by Moore and Rowson (1960); and Killeen (1976) and Averill and Rowson (1958) indicated that no 2-cell embryos and only 16% of the 4-cell sheep embryos developed into lambs when transferred to the uterine horns. On the other hand, several researchers have reported an increase in embryo survival as the age of the embryo increases (Averill and Rowson, 1958; Moore and Shelton, 1964b; Shelton and Moore, 1966).

2.10. Preservation of Embryos.

2.10.1. Medium for freezing.

The medium most commonly used for embryo freezing has been Dulbecco's Phosphate Buffered Saline (PBS), to which a cryoprotectant is added. The most widely used have been dimethyl sulphoxide (DMSO) and glycerol; DMSO has been generally used at 1.5 M concentration (Willadsen, 1977; Lehn-Jensen and Greve, 1978; Trounson et al., 1978; Willadsen et al., 1978a; Massip et al., 1979; Bilton, 1980; Tervit and Goold, 1984) and glycerol at concentrations between 1.4 to 1.5 M (Lehn-Jensen and Greve, 1981; Bouyssou and Chupin, 1982; Kennedy et al., 1983; Bui-Xuan-Nguyen et al., 1984; Pettit, Jr., 1985). Attempts have been done to improve pregnancy rate through the inclusion of 10-20% of fetal calf serum (Bouyssou and Chupin, 1982; Bui-Xuan-Nguyen et al., 1984), sheep serum (Tervit and Goold, 1978; Chupin et al., 1984), new born calf serum (Leibo, 1984) and steer serum (Kennedy et al., 1983).

2.10.2. Cryoprotectants.

Several compounds have been included in the freezing medium, with the aim to increase survival rate of embryos during deep freezing. These compounds are commonly known as cryoprotectants, usually the most used being DMSO and glycerol (Lehn-Jensen, 1981).

Ethylene glycol and propanediol have been used for the same purpose (Maurer, 1978; Tervit, 1983 ; Tervit and Goold, 1984) as well as polyvinylpyrrolidone (PVP) (Maurer, 1978). All these compounds have been used in a concentration between 1M and 2M.

A common characteristic from all these compounds is their ability to reduce the amount of ice which may be formed in an aqueous solution at any subzero temperature and maintain solutes in the liquid phase of the medium (Vos and Kaalen, 1965).

Two factors have been identified as causing freezing injury: solution effects and intracellular ice formation (Mazur, 1977). Cellular damage because of solution effects, occurs when the cooling rate is lower than the optimal, whereas intra cellular ice formation injures the cells when the cooling rate is faster than the optimal. The optimal cooling rate will be one in which the rate of cooling is slow enough to prevent ice formation within the cells and fast enough to avoid the exposure of the cells to a high concentration of solutes. Although cryoprotectants help to reduce ice formation in the freezing medium, they can themselves cause cellular damage by osmotic trauma (Mazur, 1977). For that reason, the cryoprotectant is added and removed from the freezing medium in small amounts, which allows the embryos to equilibrate to the change in intra and extra-cellular osmotic pressure (Maurer, 1978).

2.10.3. Addition of cryoprotectant.

Slow addition of DMSO and glycerol has been used in most studies. It is, however, established that the bovine blastocysts stages will survive direct transfer into PBS containing 1.5 M DMSO or 1.4 M glycerol (Willadsen, 1979; Renard et al., 1981).

In most instances the concentration of the cryoprotectant in the medium has been added at room temperature in 0.5 M steps every 10 minutes until the final concentration is attained (Lehn-Jensen, 1980, 1981; Chupin et al., 1984; Tervit and Goold, 1984; Pettit, Jr., 1985; Wright, 1985). After that, an additional period between 10 and 30 minutes is allowed for the complete permeation of the embryonic cells to occur before freezing.

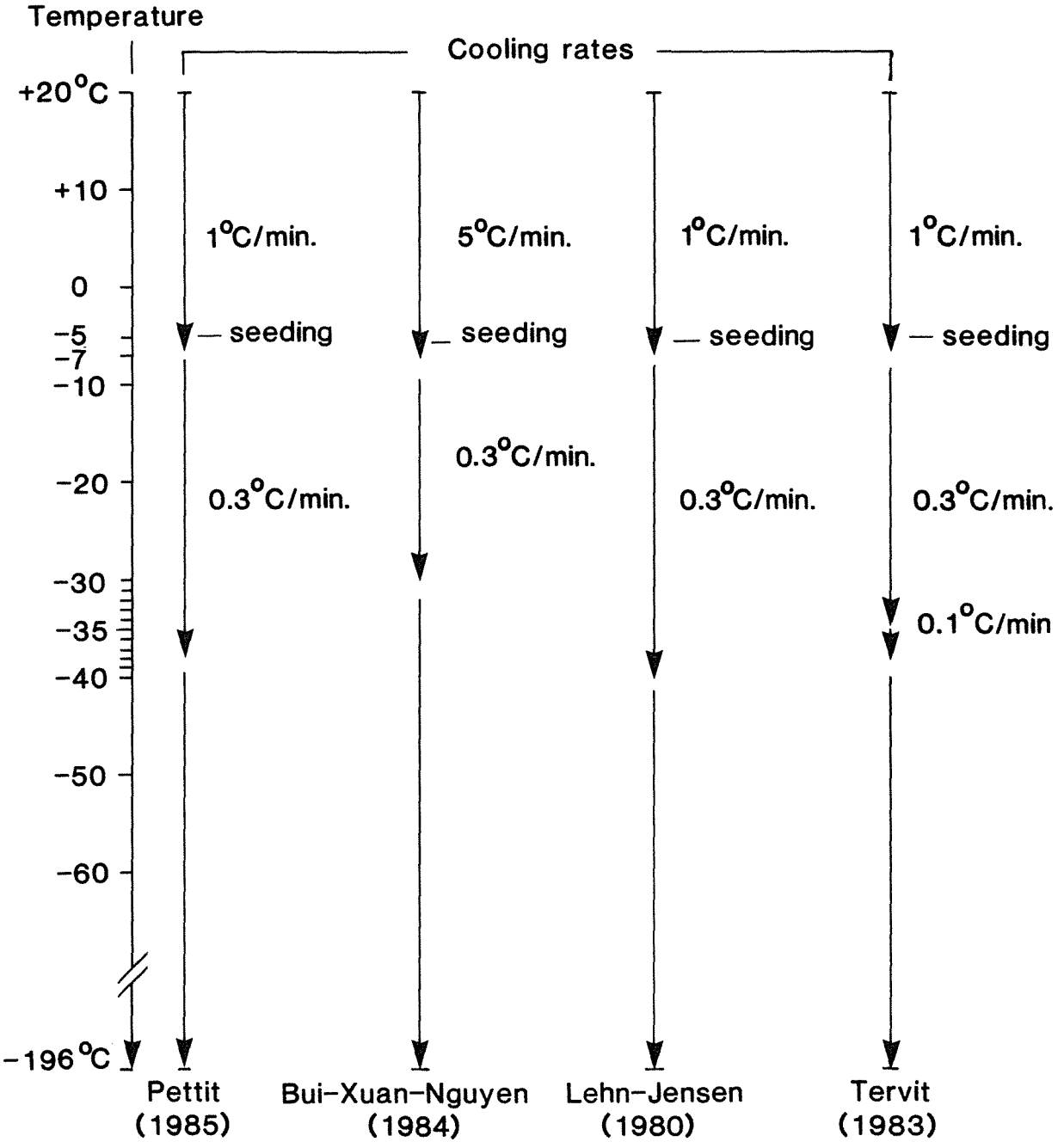
2.10.4. Containers for freezing embryos.

Successful results have been obtained freezing embryos in both straws and ampoules (Massip et al., 1979; Lehn-Jensen, 1980; Pettit, Jr., 1985; Wright, 1985). The straws can be sealed with vinyl plastic putty (Wright, 1985) or polivinilic alcohol powder (Massip et al., 1979). On the other hand, ampoules are usually sealed over a gas flame (Massip et al., 1979; Pettit, Jr., 1985).

2.10.5. Process of freezing.

Two freezing procedures have been basically developed to preserve embryos from laboratory and domestic animals. The first technique which relies on slow cooling (0.2 - 2 °C/min), to a temperature of -60 °C or below, before plunging the embryo directly in liquid nitrogen at -196 °C and slow thawing (4-25 °C/min) was originally developed with mouse embryos (Whittingham et al., 1972; Wilmut, 1972). The same procedure has been successfully used to freeze sheep (Willadsen et al., 1976a; Willadsen, 1977), cattle (Bilton and Moore, 1977; Willadsen et al., 1978b; Lehn-Jensen and Greve, 1978; Massip et al., 1979), goat (Bilton and Moore, 1976) and rabbit embryos (Maurer and Haseman, 1976; Tsunada and Sugie, 1977). However, nowadays a second technique is most widely used; this technique relies on a relatively short interval of slow cooling and a plunge temperature of between -30 and -40 °C combined with rapid thawing (Willadsen, 1977; Willadsen et al., 1978b). The second technique apart from being much less time-consuming, is less demanding in special apparatus than the former one (Lehn-Jensen, 1981). A significant improvement in embryo quality which was reflected in better pregnancy rate was reported by Elsden et al. (1982) when the cooling rate between -35 °C and -38 °C was decreased from 0.3 to 0.1 °C/min. Some of the freezing procedures used for several researchers are shown in Figure 2.2.

Figure 2.2 Embryo freezing procedures used successfully



2.10.6. Seeding.

The induction of seeding or ice crystallization has been carried out at -5 to -7 °C through one of the following approaches: by means of a supercooled pair of forceps (Bilton and Moore, 1976; Willadsen et al., 1978b) supercooled fine metallic bar, 2mm long (Bui-Xuan-Nguyen et al., 1984), supercooled piece of copper (Chupin et al., 1984) and supercooled metal spatula (Leibo, 1984). Seeding has been induced also with a crystal of sterile ice in the tip of a Pasteur pipette (Willadsen, 1977) and with a hemostat (Wright, 1985). Supercooling is obtained by placing the metallic dispositives in liquid nitrogen and seeding by touching the freezing medium with the supercooled dispositives.

The samples are kept at seeding temperature for 5 to 10 minutes to allow for the crystallization of the medium to progress to equilibrium (Schneider and Mazur, 1986).

2.10.7. Thawing process.

There are two possibilities to thaw the embryos; slow thawing and fast thawing, but the method used is determined by the cooling rate during freezing. Embryos frozen slowly should be thawed slowly and embryos frozen fast should be thawed quickly.

Nowadays fast freezing procedures are the most commonly used, which have originated as a consequence the development of fast thawing

procedures. The most common method of thawing fast frozen embryos is to place the embryos directly in a 37 °C water bath until all the ice melts (Tervit, 1983; Bui-Xuan-Nguyen et al., 1984; Chupin et al., 1984), although, embryos have been successfully thawed at 25 °C and 35 °C for 20 seconds (Pettit, Jr., 1985).

2.10.8. Removal of cryoprotectant.

Cyroprotectant exerts a large osmotic pressure on the blastomeres and if the extra- and intra-cellular gradient is too large, cellular damage can occur. Thus, after thawing the cryprotectant is diluted in several steps, thereby reducing the danger of osmotic damage to embryos. There are three possibilities to do that; adding small volumes of an isotonic diluent (Wittingham et al., 1972), transferring the embryos to solutions with decreasing concentrations of the cryoprotectant (Willadsen et al., 1978b) and dilution with solutions of differing osmolarities (hyper-to hypo-tonic) which quickly decreases the concentration of cryoprotectant while changing the osmolarity of the solution in small increments (Bank and Maurer, 1974).

The most common approach to remove the cryoprotectant from the freezing solution after thawing has been through 6 steps in decreasing concentrations of cryoprotectant (Lehn-Jensen, 1980; Pettit, Jr., 1985; Wright, 1985).

The removal of cryoprotectant with solutions of differing osmolarities was reported by (Merry et al., 1983; Renard et al., 1983;

Chupin et al., 1984; Tervit and Goold, 1984).

2.10.9. Post-thaw embryo assessment.

Embryos need to be evaluated after freezing and thawing, before the decision to transfer them or not is taken. Maurer (1978) mentioned three different approaches to evaluate embryo viability after freezing: embryo morphology, in vitro embryo culture and transfer the embryos directly to a foster mother. The use of dye tests have been reported by Whittingham (1978) and Schilling et al. (1979), measurement of embryonic metabolic activity by Renard et al. (1978) and examination of embryo morphology after culture in vitro by Trounson et al. (1976) and Renard et al. (1978).

Maurer (1978) suggested the use of in vitro embryo culture on the basis that most mammalian embryos can develop under such conditions for at least 24 hours. However, Willadsen et al. (1976b) cited by Maurer (1978) indicated that post-thaw assessment may not be necessary if reliable freezing techniques are used. More recently Greve et al. (1979) and Lehn-Jensen et al. (1981) indicated that none of the above approaches appear to have any practical advantage over the assessment of survival by a purely morphological examination, whether this takes place before direct transfer without freezing or immediately after removal of the cyroprotectant.

2.11. Factors Affecting Efficiency of Freezing Technique.

2.11.1. Embryo quality.

The effect of the quality of the embryos on their ability to support freezing procedures and develop pregnancies has been reported in several studies.

Wright (1985) indicated that good quality embryos experience higher pregnancy rate than fair or poor quality embryos (43, 26 and 23% respectively). Freezing procedure decreased the quality of the embryos, the poor quality embryos being more affected. Similar findings were reported by Pettit. Jr. (1985). In agreement with the above results, Elsden et al. (1982) reported effect of embryo quality before freezing on pregnancy rate. All the researchers were unable to find a significant effect of embryo development (early morula, morula, early blastocyst or blastocyst) on pregnancy rate after thawing.

On the other hand, Bui-Xuan-Nguyen et al. (1984) did not find any significant differences between morula and blastocysts after their culture in vitro for 24 to 48 hours after freezing.

2.11.2. Time from collection to freezing.

Studies in which the time from flushing the embryos until the freezing procedure was started, has been evaluated recently by Pettit. Jr. (1985) and Wright (1985). Results from the first study showed a

steady decrease in pregnancy rate as the period from flushing to freezing was increased by more than 3 hours. In the second study, the same trend was observed after a 4-hour period from flushing to freezing.

CHAPTER 3

CHAPTER 3

MATERIALS AND METHODS

3.1. Experimental Animals and their Management.

The experimental animals came from two flocks maintained at Massey University.

Booroola-Merino x Perendale ewes (BMxP).

A group of 161 BMxP ewes aged between 3 and 6 years and of 34 - 69 kg live weight were used as donors and/or recipients in embryo transfer studies. This group of animals had been under selection for the presence of the Booroola gene since the origin of the flock in 1980.

Drysdale ewes.

Forty-three Drysdale ewes, between 3 and 7 years of age were used as recipients for embryo transfer.

Rams and teasers.

Three BMxP rams were used as single sires to mate the donor ewes of the BMxP group. Two vasectomised Suffolk rams (teasers) were used to detect oestrus in the recipient ewes.

Management of animals.

The animals were grazed on ryegrass-white clover pastures and it

was intended to maintain their body weight constant. Drinking water was supplied ad libitum. All the ewes were identified individually with plastic ear tags and serial numbers sprayed on the flank. The entire and vasectomised rams were fitted with Sire Sine harnesses using crayons of different colors once the ewes came on heat. Detection of oestrus in the ewes was carried out at 08.00 h and 17.00 h and often at midday at periods when ewes were expected to be in oestrus. A ewe was considered as showing heat when she was well marked with crayon on her rump.

3.2. Experimental Plan.

The BMxP ewes were allocated into 2 groups of carriers (F+) or non-carriers (++) of the high fecundity gene, on the basis of the previous lambing history and after laparoscopy to determine ovulation rate following oestrus synchronisation. These ewes were stimulated with several treatments of PMSG (T1, T2, T3 and T4) to record the ovulatory response from the Booroola genotype. The allocation and number of ewes in each of the treatments are indicated in Figure 3.1. Particular details are outlined in the text.

3.2.1. Oestrus synchronisation.

To enable embryo transfer to be carried out the oestrous cycles of all the ewes were synchronised using intravaginal sponges impregnated with 40 mg of M.A.P. (17α- acetoxy -6α- methylpregn - 4 ene - 3, 20

dione) inserted for 14 days. Details of the times when the oestrus synchronisation tretmant was applied to the BMxP groups of ewes are indicated in Table 3.1.

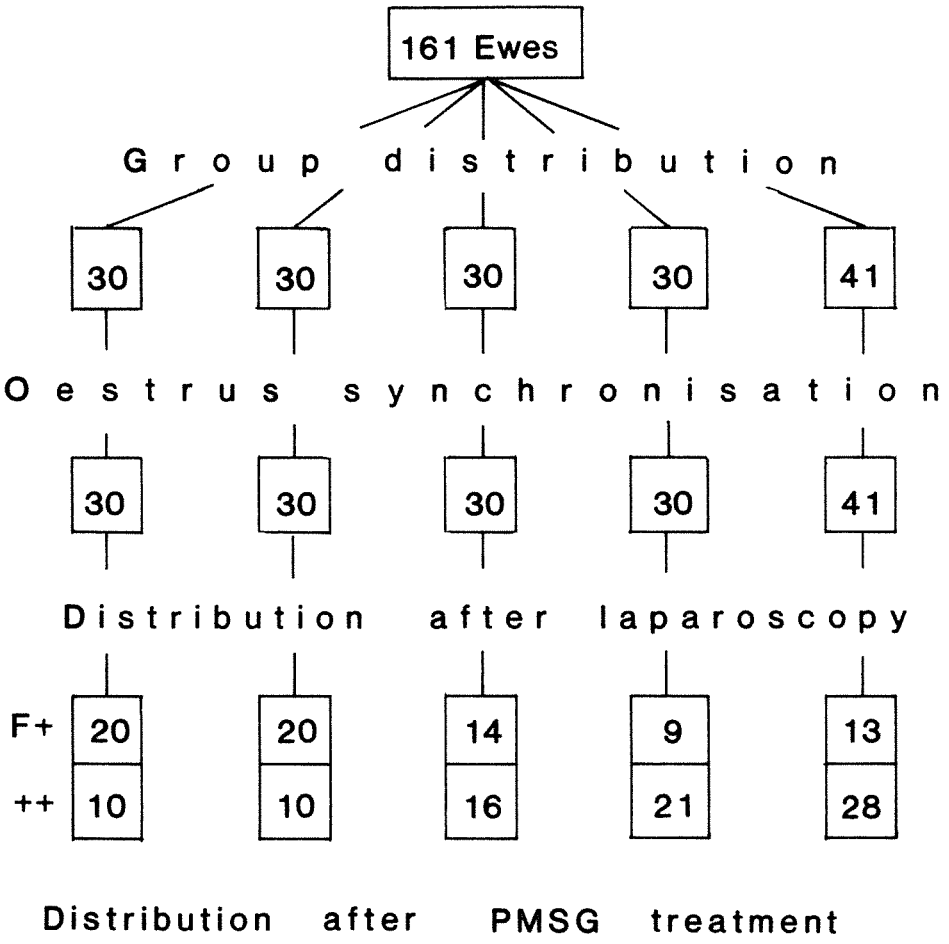
Table 3.1. Distribution of ewes among groups and oestrus synchronisation timetable.

Group	No. of ewes	Sponge insertion	Sponge removal
1	30	28-02-86	14-03-86
2	30	03-03-86	17-03-86
3	30	05-03-86	19-03-86
4	30	07-03-86	21-03-86
5	41	10-03-86	24-03-86

The difference in timing of the synchronisation procedure between groups was intended to allow recipients from one group which came into oestrus several days later than expected to be used as recipients in the succeeding treatments.

Oestrus synchronisation also was carried out in two other groups of Drysdale ewes. These ewes were used as recipients for the transfer of frozen embryos. The first group of Drysdale ewes was synchronised using the procedure described above. The second group was synchronised following the same procedure but with an injection of 500 i.u. of P.M.S.G., given at sponge removal.

Figure 3.1 Experimental plan of an embryo transfer programme using B μ x P ewes. Number of rectangles = No. of ewes allocated.



	T_1	T_2	T_3	T_4	T_1	T_2	T_3	T_4	T_1	T_2	T_3	T_4	T_1	T_2	T_3	T_4	T_1	T_2	T_3	T_4
F+	3	4	6	6	5	5	5	6	4	4	3	3	2	2	2	3	4	3	3	3
++	4	2	2	2	2	2	3	3	3	4	4	5	6	5	5	5	23	3	3	3

Note: Ewes allocated in treatments T_3 and T_4 were distributed at random to 3 entire Booroola rams for mating.

3.2.2. Laparoscopy.

All the BMxP ewes were laparoscoped between days 5 and 6 of the oestrous cycle (day of oestrus=day 0) to record the number of recently formed corpora lutea. Their number of corpora lutea Laparoscopy was also carried out on ewes not showing oestrus at the expected time after synchronisation and allocated to their respective groups. Ewes with an ovulation rate of 3 or more corpora lutea were considered to be carriers of the high fecundity gene (F+) and ewes with an ovulation rate less than 3 corpora lutea were considered as non-carriers (++) of the gene.

3.3. Embryo Transfer Procedure.

3.3.1. PMSG treatment.

The ewes were distributed at random to one of the following four treatments: Treatment 1 was the control and no PMSG was injected. Animals given treatments 2, 3 and 4 were injected with 350, 700 or 1050 i.u. of PMSG respectively, given on days 12 to 13 of the oestrous cycle after the ewes had been synchronised. The different doses of PMSG were diluted in a constant volume of fluid (6 ml) and injected subcutaneously. An additional group of ewes was injected with 1500 i.u. of PMSG (T 5) once the first five groups of flushings and transfers were finished. At this time the selection of the ewes and the time when they were treated was on the basis of normal oestrous activity. A few of these ewes had been used before as recipients.

Although only one batch of PMSG was used during the experiment, some ewes were injected with PMSG diluted and frozen for 2 to 4 days, but most of them were injected with fresh PMSG immediately after dilution.

3.3.2. Service of donor ewes.

The ewes located within the treatments 3, 4 and 5 were used as donors and mated by a known ram. The distribution of the ewes to the rams after PMSG injection was at random. However, care was taken to avoid high degree of inbreeding which had been already detected between some of them. Because the donor ewes were always kept with the rams when they came on heat after being treated with PMSG, they were mated several times. The ewes from treatments 1 and 2, used as recipients were always maintained with the teasers and used either with its own group or with one of the following groups according to the time when they came on heat.

3.3.3. Embryo recovery from donor ewes.

Surgical recovery of the embryos using the technique described by Tervit and Havik (1976) was carried out between days 5 and 6 after the ewes came on heat (day of heat = day 0). This was attempted by flushing the uterine horn ipsilateral to the ovary in which the ovulation was recorded, with 20 ml of modified Phosphate Buffer Saline (PBS) plus 5% sheep serum and warmed to 37 °C. Most of the donor ewes were flushed even when only 1 or 2 corpora lutea were recorded.

The sheep serum used was obtained from entire Suffolk rams and processed using the procedure described by Tervit (1967), but in this case the sterilization was carried out by passing the serum twice throughout cellulose filters of first 0.45 µm and 0.20 µm pore size. Prior to the recovery of the embryos the serum was heated to 56 °C for 30 minutes.

After embryo recovery was attempted, the donor ewes were returned to individual entire rams and kept in small paddocks. Observations to record "returns" to oestrus were made for at least a further cycle.

3.3.4. Embryo searching and evaluation.

The identification of the embryos was carried out with the help of a stereoscopic microscope, using low magnification (20X) for searching and embryo manipulation and a higher magnification (40X) for embryo evaluation. As an extra help a plastic cover petri dish with small squares was used under the glass petri dish containing the flushing to facilitate the finding of embryos. Once an embryo was found, it was immediately transferred into a small petri dish containing fresh PBS plus 10% sheep serum. After all the embryos from a particular ewe were found they were carefully evaluated, on the basis of normal embryo development and embryo morphology. The embryos were kept in the small petri dish at 37 °C in an incubator until they were transferred or processed for embryo freezing.

3.3.5. Transfer of fresh embryos.

All the transfers with fresh embryos were made into BMxP ewes. The embryos were transferred into the recipient ewes which came on heat as close as possible with the donor ewes, regardless of the day of flushing. It was planned to transfer two embryos per ewe and place one embryo into each horn regardless of the number of ovulations and their distribution between ovaries. However, in a few recipients only one or three embryos were transferred mainly when only one embryo was left or sometimes two, but from different donors, or on a very few occasions when the number of embryos available was three but of poor quality. The medium used for embryo transfer was modified PBS plus 10% sheep serum. The numbers of corpora lutea in the recipient ewes were recorded at the time of transfer.

After the embryo transfer and completion of surgery, the recipient ewes were grazed with teaser rams and the possible occurrence of oestrus observed for at least the duration of a further cycle. Some of the ewes which 'returned' at the normal time after being used as recipients were used later, either as recipients or as donors.

3.4. Preservation of Embryos.

Embryos selected for preservation were frozen following a standard procedure for embryo freezing. The steps of the procedure to freeze the embryos and subsequently their transfer into adequate recipients are outlined in the following section.

3.4.1. Solutions for embryo preservation.

Two types of solution were prepared for embryo preservation. These were for (a) Short term embryo preservation, in which initially the embryos could be identified and manipulated prior to immediate transfer or later storage. The solution comprises modified PBS + 10% sheep serum at 37 °C; and (b) Long term embryo preservation, i.e. the freezing solution made with modified PBS, sheep serum and glycerol in the proportions indicated below, which is an example of how to prepare 20 ml of freezing solution.

Freezing solution (glycerol 10%)

PBS	15 ml
Sheep serum	3 ml
Glycerol	2 ml
Total	20 ml

3.4.2. Addition of the cryoprotectant.

The addition of the cryoprotectant was carried out in three steps, keeping the embryos always at 37 °C inside an incubator. Dishes containing the freezing solution were prepared and the embryos placed

following the next schedule.

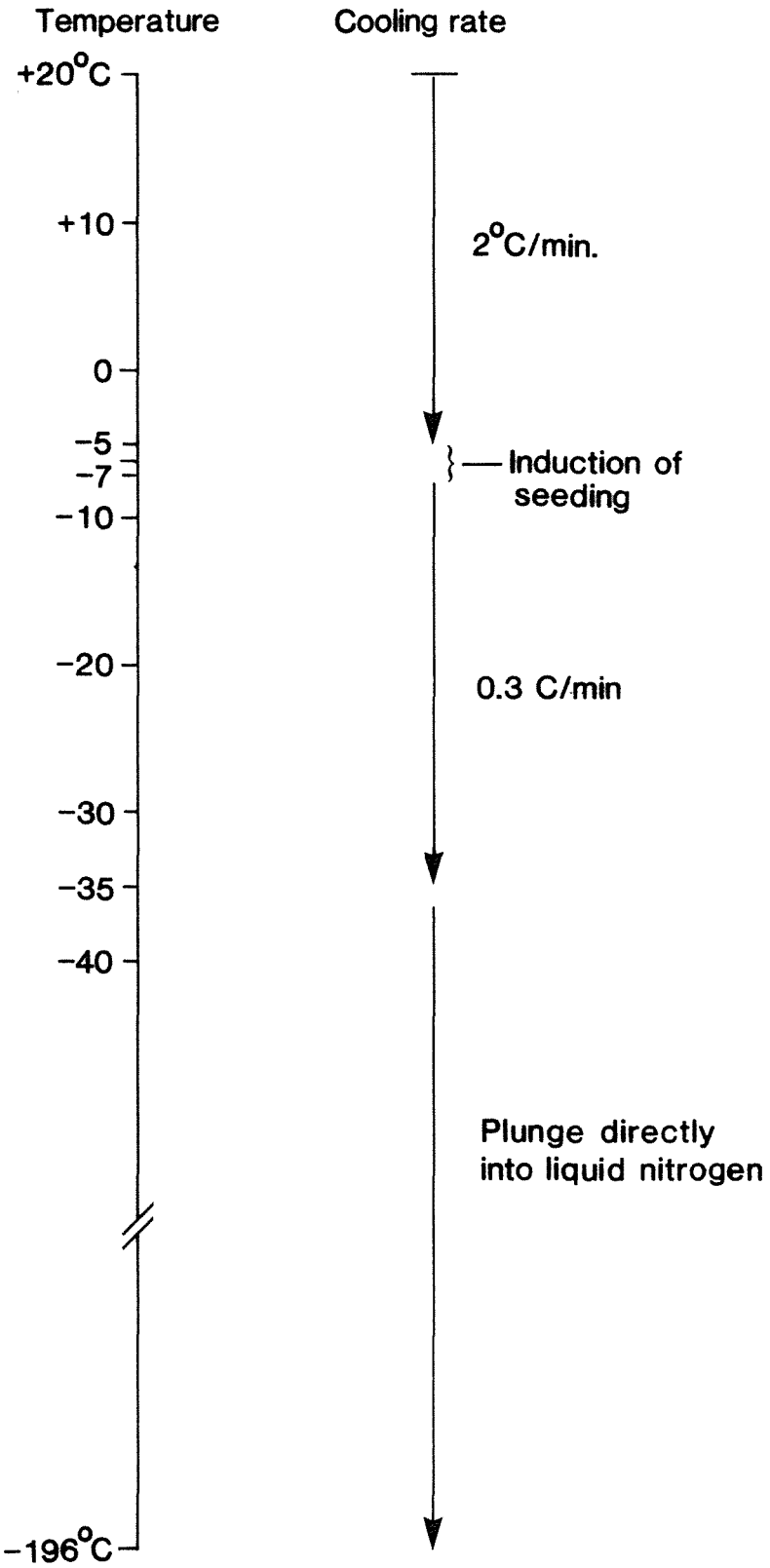
1. 3.3% glycerol in PBS: 8 - 10 min.
2. 6.6% glycerol in PBS: 8 - 10 min.
3. 10.0% glycerol in PBS: 10 - 15 min.

Following the last equilibration period the embryos were placed in straws (0.50 ml) between two drops of air using a small syringe (1 ml) attached to the straw by a small plastic rubber tube. The straws were sealed using small hot scissors and finally each straw identified with the number of the ewe and the number of embryos. The embryos were then ready to be frozen.

3.4.3. Freezing procedure.

The freezing of the embryos was carried out in a programmable freezing machine and the procedure is described in Figure 3.2.

Figure 3.2 Freezing procedure used in the present experiment.



Details of the freezing procedure.

The freezing procedure was started with a cooling rate of 2 °C/ min from ambient temperature until -5 to -7 °C was reached. At the beginning of the procedure each straw was placed with the hot seal down inside the freezing chamber and 5 min before induction of seeding the straw was inverted. Seeding was induced with a supercooled metal wire touching on both extremes of the straw, without touching the portion in which the embryos were placed. The embryos were kept at -5 to -7 °C for 5 min and the rate of cooling changed to 0.3 °C/ min until -35 °C. Then the embryos were plunged directly into liquid nitrogen at -196 °C. Once the embryos were in liquid nitrogen they were stored for variable times and transferd to the recipient ewes.

3.4.4. Thawing.

The embryos were thawed by removing the straws from the liquid nitrogen and plunging into a water bath at 37 °C until all the ice had melted.

3.4.5. Removal of the cryoprotectant.

Immediately after thawing, the embryos were placed in freshly prepared freezing solution containing 10% glycerol and the glycerol was removed in six stages, keeping the embryos always at 37 °C inside an incubator. Dishes containing the solution were prepared and the embryos placed in each for the following times.

1. 10.00% glycerol in PBS: 8 - 10 min.
2. 8.30% glycerol in PBS: 8 - 10 min.
3. 6.60% glycerol in PBS: 8 - 10 min.
4. 4.95% glycerol in PBS: 8 - 10 min.
5. 3.30% glycerol in PBS: 8 - 10 min.
6. 1.65% glycerol in PBS: 8 - 10 min.

After the last step in the removal of glycerol, the embryos were maintained for 8-10 minutes in a fresh solution of PBS + 10% sheep serum, before their transfer to recipient ewes.

3.4.6. Transfer of frozen embryos.

All the frozen embryos were transferred into Drysdale ewes. The technique used for transfer of frozen embryos was the same as described for the transfer of fresh embryos. Before the transfer was made all the embryos were evaluated on the basis of normal morphology and only embryos of good or fair quality were used.

3.5. Preparation of Material and Equipment.

All glassware was cleaned and sterilised according to standard tissue culture procedures. Most of the equipment and materials were sterilized by autoclaving. The serum was sterilised through Diapore filters and the straws for embryo storage were sterilised with gas (Ethylene oxide).

3.6. Analysis of Data.

The general linear model used for analysing ovulation rate was:

$$Yijk = M + Gi + Aj + Wk + Eijk.$$

where

M = general mean.

Gi = effect of high fecundity gene (i = 1 or 2).

Aj = effect of age (j= 3 or 4-6 years).

Wk = effect of weight (covariable).

Eijk = error peculiar to each Yijk.

The linear model for analysing ovulation rate after PMSG treatment also included the effect of PMSG treatment and the interaction between genetic group by treatment as shown below:

$Yijkl = M + Gi + Tj + (GT)ij + Ak + Wl + Eijkl.$

where

M = general mean.

Gi = effect of high fecundity gene (i = 1 or 2)

Tj = effect of PMSG treatment (j = 1, 2, 3 or 4).

(GT)ij = interaction effect.

Ak = effect of age (k = 3 or 4-6 years).

Wl = effect of weight (covariable).

Eijkl = error peculiar to each Yijkl.

The analysis of the factors affecting ovulation rate following PMSG treatment, but on a within genotype basis, was carried out using the following model.

$Yijk = M + Ti + Aj + (TA)ij + Wk + Eijk.$

where

M = general mean.

Ti = effect of PMSG treatment (i = 1, 2, 3 or 4).

Aj = effect of age (j = 3 or 4-6 years).

(TA)ij = interaction effect.

Wk = effect of weight (covariable).

Eijk = error peculiar to each Yijk.

The ovulation rate data were transformed using the Log10 (y) transformation and the significance of the variables affecting ovulation rate was tested using the F-test on the transformed data. Multiple comparisons of means was carried out using Duncan's multiple range test. Bartlett's test for homogeneity of variances was undertaken in each case.

The significance of the factors affecting pregnancy rate after the transfer of fresh or frozen embryos was tested using chi-square test.

CHAPTER 4

CHAPTER 4

RESULTS

4.1. Ewes Carring the High Fecundity Gene.

4.1.1. Distribution of ewe carriers (F+) and non-carriers (++) of the high fecundity gene.

Table 4.1 shows the distribution of ewes classified as carriers or non-carriers of the high fecundity gene. In total, 76 ewes (47%) in the flock were classified as carriers (F+) of the fecundity gene and 85 ewes (53%) were considered to be non-carriers (++) of the high fecundity gene. Only ewes in which both ovaries were observed at laparoscopy were included to generate these data.

Table 4.2 indicates the total and average number of ovulations among the ewe carriers (F+) and non-carriers (++) of the fecundity gene by groups. The mean ovulation rate of the F+ ewes (3.1) was considerably higher than the average ovulation rate in non-carrier ewes (1.8).

Table 4.1. Distribution of ewe carriers (F+) and non-carriers (++) of the high fecundity gene by group.

	G R O U P					Total number of ewes
	1	2	3	4	5	(%)
F+	20	20	14	9	13	76(47)
++	10	10	16	21	28	85(53)
	30	30	30	30	41	161(100)

Table 4.2. Ovulation rate in ewe carriers (F+) and non-carriers (++) of the high fecundity gene by group.

Group	Ovulation rate in ewes: Genotype of the ewe					
	F+			++		
	Number of ewes	Number of Corpora Lutea		Number of ewes	Number of Corpora Lutea	
		Total	Mean		Total	Mean
1	16	52	3.25	8	17	2.12
2	17	58	3.41	10	19	1.90
3	9	30	3.33	16	30	1.87
4	7	24	3.42	17	26	1.52
5	13	41	3.15	26	47	1.80
Total	62	205	3.10	77	139	1.80

4.1.2. Distribution of ovulation rate.

Data from 139 BMxP ewes of which both ovaries were observed at laparoscopy were included to calculate the distribution of ovulation rate. No significant difference in the rate of ovulation was found between left and right ovaries (2.12 v. 1.99 respectively).

4.1.3. Factors affecting ovulation rate.

Data from 141 BMxP ewes were used to evaluate the effect of genotype, age and weight of the ewes on the normal ovulation rate after oestrus synchronisation. Results are presented as raw and transformed data in Appendix 1. Their respective analysis of variance is shown in Table 4.3.

4.1.3.1. Genotype.

The analysis of variance (Table 4.3) showed highly significant effect ($P < 0.01$) of genotype on the natural ovulation rate. The least-squares means for ewe carriers (F+) and non-carriers (++) of the fecundity gene were 3.02 and 1.73 respectively.

4.1.3.2. Age.

The analysis of the results showed a highly significant effect

(P<0.01) of age of the ewes on their ovulation rate (Table 4.3). The ovulation rate was significantly higher in 4-6 year old ewes than in 3 year old ewes. The least-squares means for 3 and 4-6 year old ewes were 2.14 and 2.45 respectively.

4.1.3.3. Live weight.

The analysis of the results did not show significant effect of weight of the ewes on their ovulation rate (Table 4.3).

Table 4.3. Factors affecting ovulation rate after oestrus synchronisation: Analysis of variance.

Source of variation	D.F.	S.S.(A)	
Genotype	1	2.3928	*
Age	1	0.0905	*
Weight	1	0.0041	N.S.
Error	137	1.7517	
Total	140	4.2391	

(A) = Transformed data.

* P 0.01

N.S. = Not significant.

4.2. Incidence and Distribution of Oestrus After Hormonal Treatment.

4.2.1. Efficiency of the intravaginal dispositive.

From the 161 sponges inserted only 4 were not recovered at the time of sponge removal, which represent a 97.5% of sponge retention. It was not possible to establish at what time the sponges had been lost.

4.2.2. Incidence and time of onset of oestrus after progestagen sponge treatment.

4.2.2.1. Effect of genotype on the incidence of oestrus.

The incidence of oestrus after sponge removal from 161 BMxP ewes is shown in Table 4.4. The statistical analysis of these results did not show any significant difference between ewes classified as carriers (F+) or non-carriers (++) of the high fecundity gene, in the incidence of heat after sponge removal.

Table 4.4. Effect of genotype of the ewe on the incidence of oestrus after sponge removal.

Genetic group	Total number of ewes	Number of ewes detected on heat	% of ewes detected on heat
F+	76	70	92
++	85	80	94
	161	150	93

$chi^2 = 0.255$ N.S.

The time when oestrus occurred in the ewes after sponge removal is shown in Table 4.5. Sixty-four percent of the ewes came into heat between 70-80 hours, 19% up to 56 hours and 17% between 94-104 hours relative to the end of treatment.

Table 4.5. Distribution of onset of oestrus after sponge removal.

	Hours after sponge removal					
	56	70	80	94	104	Total
Number of ewes	29	49	47	21	4	150
%	19	33	31	43	3	100

The number and percentage of F+ and ++ ewes showing heat after sponge removal are shown in Table 4.6 and Figure 4.1. No significant difference was found between F+ and ++ ewes in the pattern of onset of oestrus.

Figure 4.1. Distribution of oestrus after sponge removal from ewes classified as carriers (F+) or non-carriers (++) of the fecundity gene.

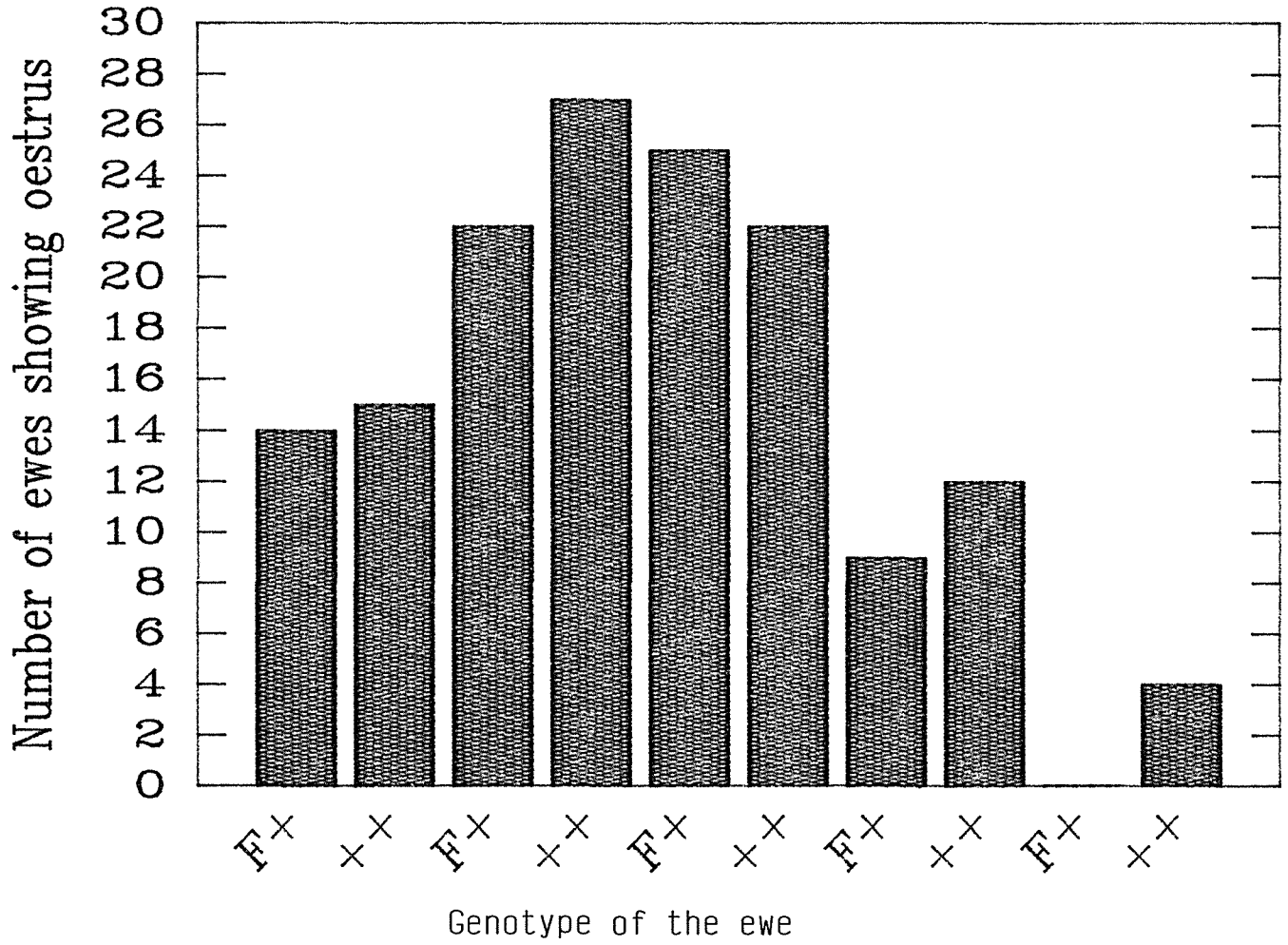


Table 4.6. Effect of genotype of the ewe on the incidence of oestrus after sponge treatment.

Genetic group	Hours after sponge removal					Total number of ewes
	56	70	80	94	104	
	No (%) of ewes in oestrus					
F+	14(20)	22(31)	25(36)	9(13)	0	70
++	15(19)	27(34)	22(31)	12(14)	4(2)	80
	29	49	47	21	4	150

chi^2 = 4.518 N.S.

4.2.3. Incidence and distribution of oestrus after PMSG treatment.

4.2.3.1. Effect of genotype on the incidence of oestrus after PMSG treatment.

The incidence of oestrus after PMSG treatment from 109 BMxP ewes is shown in Table 4.7. No significant difference was found between ewes classified as carriers (F+) or non-carriers (++) of the high fecundity gene, in the incidence of oestrus after PMSG treatment.

Table 4.7. Effect of genotype of the ewe on the incidence of oestrus after PMSG treatment.

Genetic group	Number of ewes		% of ewes detected in oestrus
	Total	detected in oestrus	
F+	58	53	91
++	51	49	96
	109	102	93.5

chi ^{2} = 0.997 N.S.

The occurrence of oestrus after PMSG treatment is shown in Table 4.8. Eighty three percent of the ewes were detected in heat on day 3 and 4 after PMSG treatment, 8% on day 2 and 9% on day 4 and 5.

Table 4.8. Distribution of onset of oestrus after PMSG treatment.

	Hours from PMSG treatment									
Ewes	48	60	72	84	96	108	120	132	166	Total
Number	4	4	13	36	23	13	7	1	1	102
%	4	4	13	35	22	13	7	1	1	100

The number and percentage of F+ and ++ ewes showing heat after PMSG treatment are shown in Table 4.9 and Figure 4.2. The analysis of these data did not show any significant difference between F+ and ++ ewes, in the number of ewes detected in heat after PMSG treatment.

Figure 4.2. Distribution of oestrus after PMSG treatment in ewes classified as carriers (F+) or non-carriers (++) of the fecundity gene.

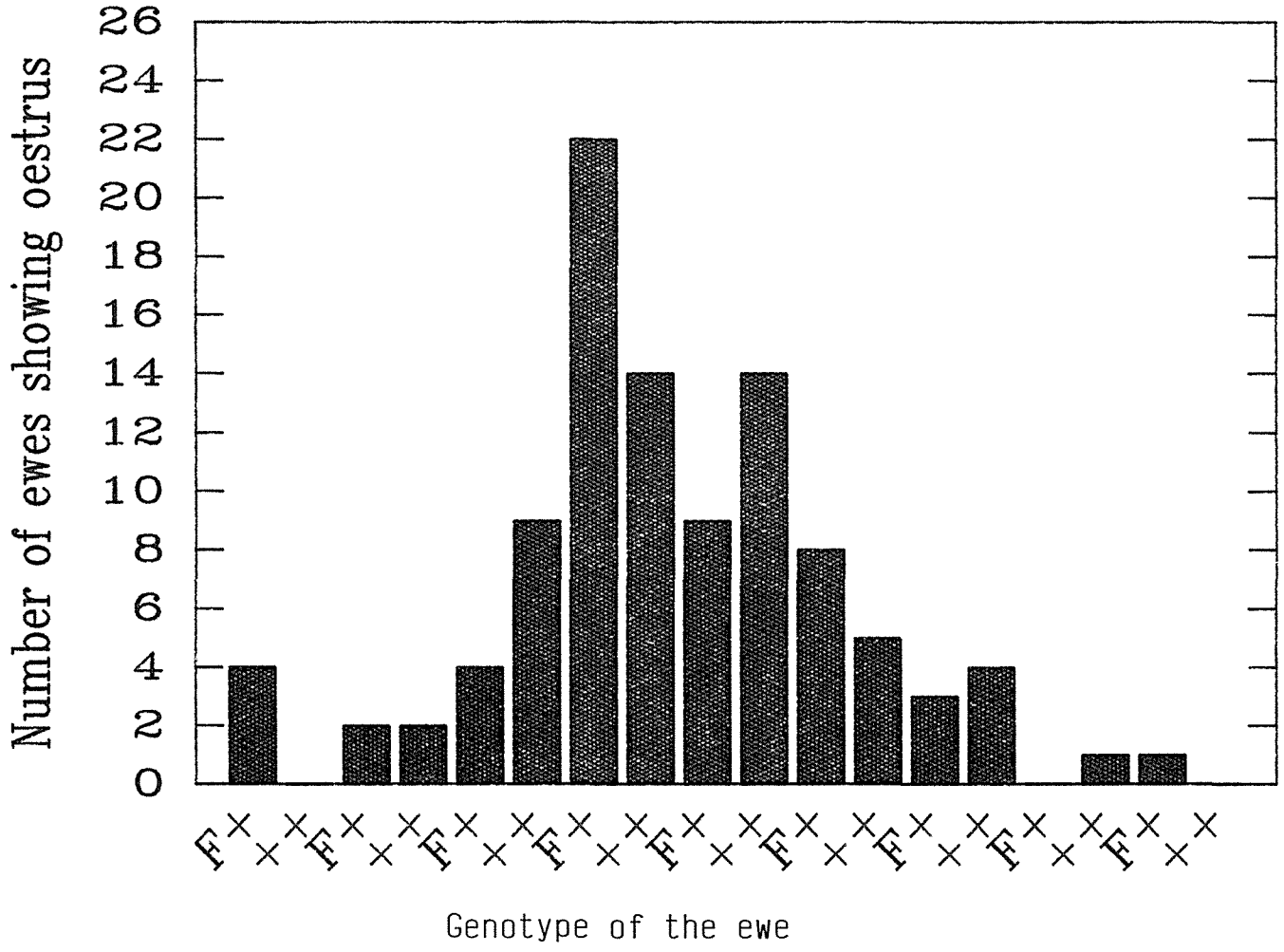


Table 4.9. Incidence of oestrus after PMSG treatment in ewe carriers (F+) and non carriers (++) of the high fecundity gene.

Genetic group	Hours from PMSG treatment: No (%) of ewes in oestrus									Total number of ewes
	48	60	72	84	96	108	120	132	166	
F+	4(8)	2(4)	4(8)	22(41)	9(17)	8(15)	3(5)	0(0)	1(2)	53
++	0(0)	2(4)	9(18)	14(29)	14(29)	5(10)	4(8)	1(2)	0(0)	49
	4	4	13	36	23	13	7	1	1	102

chi^2 = 11.484 N.S.

4.2.3.2. Effect of dose of PMSG on the incidence of oestrus.

The number and percentage of ewes showing heat after the injection of several doses of PMSG are shown in table 4.10. The statistical analysis of the data did not show any significant difference in the distribution of frequencies between treatments in the number of ewes detected in heat after PMSG treatment. If the PMSG treatments are considered independently, 80%, 79% and 90% of the ewes in treatment 2, 3 and 4 respectively showed oestrus on the days 3 and 4 after PMSG treatment; from the rest approximately 50% were detected in heat either before or after day 3 and 4.

Table 4.10. Effect of dose-level of PMSG treatment on the incidence of oestrus.

Dose of PMSG (i.u.)	Hours from PMSG injection									Total number of ewes
	48	60	72	84	96	108	120	132	166	
	No (%) of ewes in oestrus									
350	2(7)	1(3)	1(3)	16(53)	5(17)	2(7)	3(10)	0	0	30
700	1(3)	2(6)	4(12)	12(35)	6(17)	5(15)	4(12)	0	0	34
1050	1(3)	1(3)	8(21)	8(21)	12(31)	6(16)	0	1(3)	1(2)	38
	4	4	13	36	23	13	7	1	1	102

chi^2 = 21.5 N.S.

4.2.3.3. Effect of day of PMSG injection on the incidence of oestrus.

The number and percentage of ewes showing heat after PMSG treatment on days 11.5 and 12.5 of the cycle are shown in Table 4.11. Analysis of the data, revealed no significant difference between ewes treated on days 11.5 or 12.5, in the number of ewes detected in heat after PMSG treatment. Eighty and 87% of the ewes treated on days 11.5 and 12.5 respectively, showed oestrus 3 or 4 days after PMSG treatment. Among the rest injected on days 11.5 or 12.5 of the cycle, 6% and 9% showed oestrus on day 2 and 14% and 4% on day 5 or 6 after PMSG treatment, respectively.

Table 4.11. Effect of day of PMSG injection on the incidence of oestrus.

Day of PMSG inj.	Hours from PMSG injection									Total number of ewes
	48	60	72	84	96	108	120	132	166	
	No (%) of ewes in oestrus									
11.5	2(4)	1(2)	4(8)	17(35)	11(23)	7(14)	5(10)	1(2)	1(2)	49
12.5	2(4)	3(6)	9(17)	19(36)	12(22)	6(11)	2(4)	0	0	53
	4	4	13	36	23	13	7	1	1	102

$chi^2 = 1.49$ N.S.

4.3. Ovarian Response to PMSG Treatment, Embryo Recovery and Fertilisation Rate.

4.3.1. Ovulatory response.

Table 4.12 shows data on the ovulatory response and distribution between ovaries in 86 BMxP ewes classified according to the dose of PMSG. There was a significant difference ($P<0.05$) in the response to PMSG treatment between right and left ovaries (3.40 v. 2.82). In all groups the right ovary was more active than the left.

Table 4.12. Number of ovulations after PMSG Treatment and their distribution between ovaries.

Dose of PMSG (i.u.)	Number of Ewes	Number of Corpora Lutea Total	Right Ovary	Left Ovary
350	34	163	91	72
700	39	221	122	99
1050	13	82	52	30
	86	466(100)	265(57)	201(43)

4.3.2. Embryo recovery.

4.3.2.1. Efficiency of embryo recovery.

The efficiency of the technique of embryo recovery was evaluated for 79 BMxP ewes. The results on the basis of the number of corpora lutea recorded and number of embryos recovered relative to the treatment are indicated in Table 4.13. From a total of 450 corpora lutea recorded, 296 embryos were recovered which represents 66% of the total ovulatory response. Valid comparisons can be made only between treatments 3 and 4, since ewes included in treatment 5 were treated separately at the end of the experiment. The average embryo recovery from ewes in treatments 3 and 4 was 65% and this figure will be used in further calculations.

Examination of the data relative to individual ewes showed that embryo recovery was 63%, 64% and 78% for treatment 3, 4 and 5 respectively. The average embryo recovery rate was 68%. No significant difference in the percentage of embryos recovered between treatments 3 and 4 was found, when the percentage of embryo recovery was analysed on a per ewe basis. Analysis of variance was carried out after test for homogeneity of variances was confirmed.

Table 4.13. Efficiency of embryo recovery.

Dose of PMSG (i.u.)	Number of ewes	Number of ovulations	Number of embryos recovered	% embryos recovered
700	33	162	100	62
1050	36	217	145	67
1500	10	71	51	72
Total	79	450	296	66

4.3.3. Fertilisation rate.

4.3.3.1. Influence of ram on fertilisation rate.

Table 4.14 shows data on the incidence of fertilisation in 79 BMxP ewes from which ova and/or embryos were recovered. The results have been arranged to look at the effect of ram on the rate of fertilisation. From a total of 296 ova recovered, 241 were fertilised and classified as embryos.

If fertilisation rate is calculated on each ewe individually, fertilisation rate would be 84%, 71% and 79% for ram sires 1, 2 and 3 respectively. The average fertilisation rate was 78%. No significant difference in fertilisation rate between the three rams was found when fertilisation rate on each ewe was analised. The analysis of variance

was made after the test for homogeneity of variances was confirmed.

Table 4.14. Effect of ram on fertilisation rate.

Ram	Number of ewes	Number of ova/embryos recovered	Fertile embryos	% Fertilisation rate
1	24	87	80	92
2	27	127	88	69
3	28	82	73	89
Total	79	296	241	81

4.4. Reproductive Performance of Donor Ewes After Embryo Recovery.

4.4.1. Incidence of oestrus in donor ewes after embryo recovery.

The activity of the 91 donor ewes was recorded during the 21 days after flushing to attempt embryo recovery. The results regarding oestrus activity of the ewes within each of the treatments are shown in Table 4.15. In total 51 ewes (65%) were not detected on heat after flushing, while 28 ewes (35%) were detected on heat within 21 days after flushing.

Table 4.15. Activity of ewes after flushing.

Ewes flushed	P M S G treatment			Total number of ewes
	T3	T4	T5	
Ewes returning to oestrus	11	15	2	28
Ewes not returning to oestrus	22	21	8	51
	33	36	10	79

4.4.2. Effect of efficiency of embryo recovery on the incidence of oestrus after flushing.

4.4.2.1. Ewes not returning to oestrus.

Results regarding the relationship between the number of embryos not recovered at the time of flushing and the effect of ovarian activity of the ewes are indicated in Table 4.16. From a total of 51 ewes that did not show oestrus within 21 days after flushing, there were 38 ewes (75%) from which at least one embryo was lost. On the other hand 13 ewes (25%) from which no embryos were found did not show oestrus within 21 days after flushing.

Table 4.16. Effect of embryo recovery on oestrus activity after flushing.

Ewes not returning to oestrus	P M S G treatment			Total number of ewes
	T3	T4	T5	
Ewes from which embryos were lost	18	14	6	38
Ewes from which no-embryos were lost.	4	7	2	13
	22	21	8	51

4.4.2.2. Ewes returning to oestrus.

Table 4.17 indicates the distribution between ewes showing oestrus after flushing and the number of ewes from which none or some embryos were lost at flushing. From a total of 28 ewes that returned to oestrus after flushing, in 20 of them (71%) at least one embryo was lost at the time of flushing, while in 8 ewes (29%) all the embryos were recovered.

Table 4.17. Influence of efficiency of embryo recovery on ewes returning to oestrus.

Ewes returning to oestrus	P M S G treatment			Total number of ewes
	T3	T4	T5	
Ewes from which embryos were lost	8	12	0	20
Ewes from which no embryos were lost	3	3	2	8
	11	15	2	28

4.4.3. Pregnancy rate in donor ewes.

From a total of 91 potential donors, only 79 ewes were flushed to attempt egg recovery. Eleven ewes were not flushed, 5 because they were not detected in heat after PMSG injection and 6 because of low ovulatory response to PMSG treatment. An additional ewe in which the uterus was damaged during surgery was not included in the calculations.

4.4.3.1. Pregnancy rate of flushed ewes.

Of 79 ewes subjected to egg recovery, 24 (30%) remained pregnant after flushing and produced lambs. Another 34 (43%) ewes were pregnant at subsequent cycles after PMSG treatment, while 21 ewes (27%) were not pregnant at the end of the experiment. From the ewes pregnant later on after PMSG treatment 5 ewes (15%) were pregnant within 9-13 days of the following cycle after PMSG treatment (short oestrous cycles), 19 ewes (56%) between 14 and 19 days (normal cycle) and 10 ewes (29%) 20 days or more after the oestrus when the PMSG was injected. In the last group, probably some ewes were pregnant for a short period of time and came into heat later after PMSG treatment or came into oestrus normally and thus were pregnant at the second or third oestrous cycle after PMSG treatment.

4.4.3.2. Pregnancy rate of non flushed ewes.

From the 5 ewes not flushed because they were not detected in heat

after PMSG treatment; 3 conceived to the PMSG treatment and produced lambs, one ewe became pregnant 21 days after the PMSG treatment and the other one remained dry. On the other hand, from the 6 ewes not flushed because of their low ovulatory response to PMSG treatment, 3 became pregnant at that time and produced lambs, one more ewe conceived after the interval of an oestrous cycle following PMSG treatment and two ewes remained dry.

4.4.4. Total reproductive rate.

In total from the 91 BMxP donor ewes included in this work, 95 lambs were obtained from the transfer of fresh embryos, 23 lambs from the transfer of frozen embryos and 10 more lambs were produced naturally by some of the ewes as a product of the superovulatory treatment; A total of 128 lambs were produced as a result of the PMSG injection. In addition to that 49 more lambs were obtained from pregnancies arising from the following cycles after superovulation. Thus in total 177 lambs were produced.

4.5. Factors Affecting the Ovulatory Response to PMSG treatment.

The results are presented in two sections; section 1, where the results were analysed including both genotypes and section 2 where the analysis was carried out by genotype.

4.5.1. Analysis including both genotypes.

Results from 157 BMxP ewes were used to evaluate the effect of dose of PMSG, genotype of ewe and age of the ewe on the ovulatory response to PMSG treatment. In the analysis of the effects the weight of the ewes was incorporated as a covariable. Results are presented as raw and transformed data in Appendix 2. The respective analysis of variance is shown in Table 4.18.

4.5.1.1. Effect of genotype.

The analysis of the results showed highly significant effect ($P<0.001$) of genetic group (ewe carriers or non-carriers of the fecundity gene) on the ovulatory response to PMSG treatment (Table 4.18). Ewe carriers of the fecundity gene (F+) recorded higher ovulatory response than non-carrier ewes. The least-squares means for ewe carriers (F+) and non-carriers (++) of the fecundity gene were 3.61 and 2.31 corpora lutea respectively.

4.5.1.2. Effect of dose-level of PMSG.

The analysis of the results showed a highly significant effect ($P<0.001$) of PMSG treatment on the ovulatory response (Table 4.18). No significant difference in the ovulation rate was found between treatment 1 (control) and treatment 2 (350 i.u. of PMSG) and between treatment 3 (700 i.u. of PMSG) and treatment 4 (1050 i.u. of PMSG).

However, significant difference was found when treatments 1 and 2 were compared with treatments 3 and 4. The higher ovulatory response was recorded in treatments 3 and 4. The least-squares means for treatments 1, 2, 3 and 4 were 2.10, 2.01, 3.74 and 4.37 corpora lutea respectively. These results were used to plot the effect of PMSG treatment on ovulation rate (Figure 4.3).

4.5.1.3. Effect of the interaction of genetic group by treatment on the ovulatory response.

The analysis of the data did not show any significant interaction between genotype of the ewe and treatment on the ovulatory response (Table 4.18). The least-squares means of the number of corpora lutea recorded are indicated in Table 4.19; and have been ploted in Figure 4.4.

Table 4.19. Least-squares means of ovulation rate in ewes classified as carriers (F+) or non-carriers (++) of the fecundity gene.

Dose level of PMSG (i.u.)	Genetic group	
	F+	++
0	2.47	1.80
350	2.65	1.52
700	4.70	2.97
1050	5.50	3.47

Figure 4.3. Effect of dose of PMSG on ovulation rate

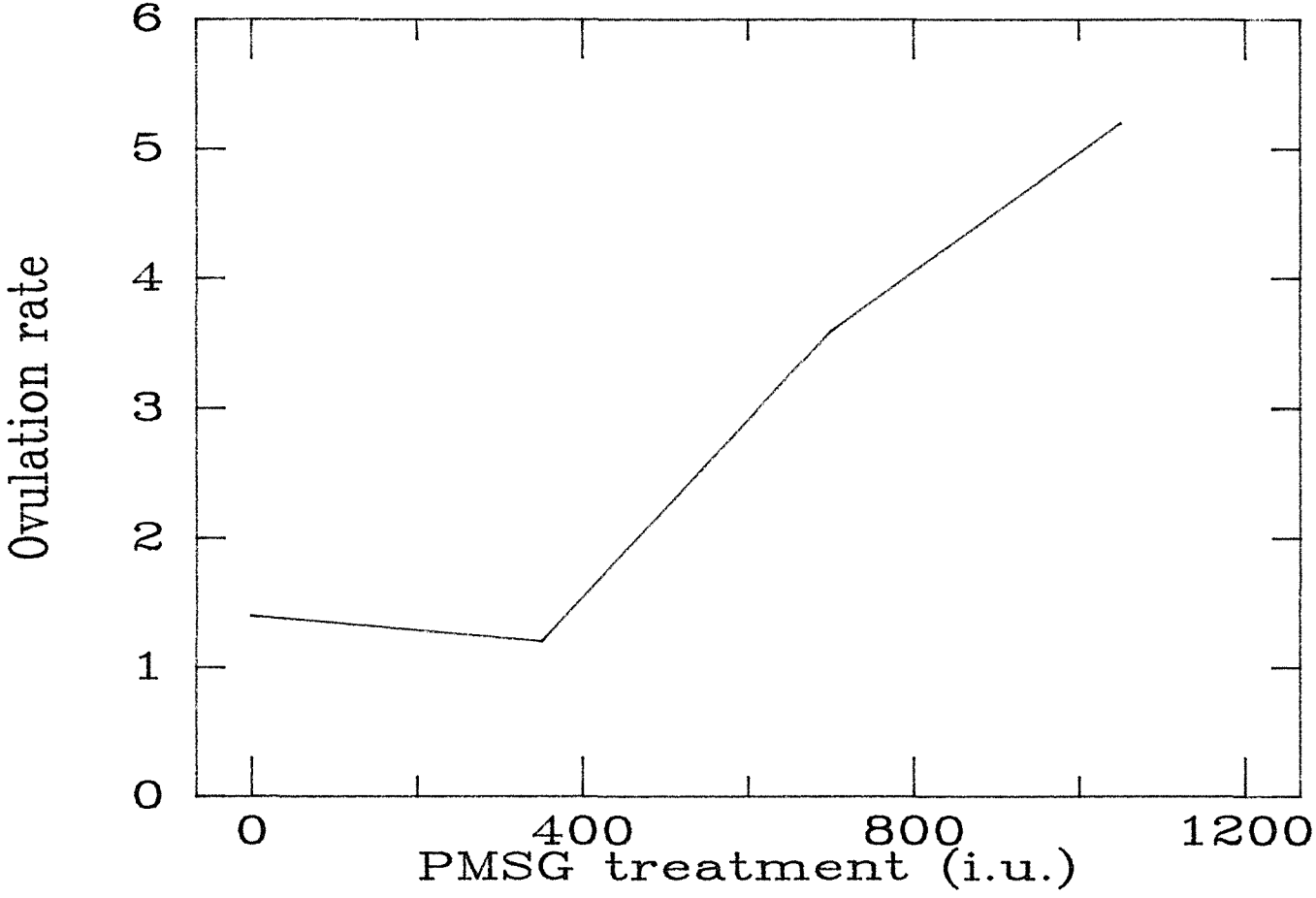
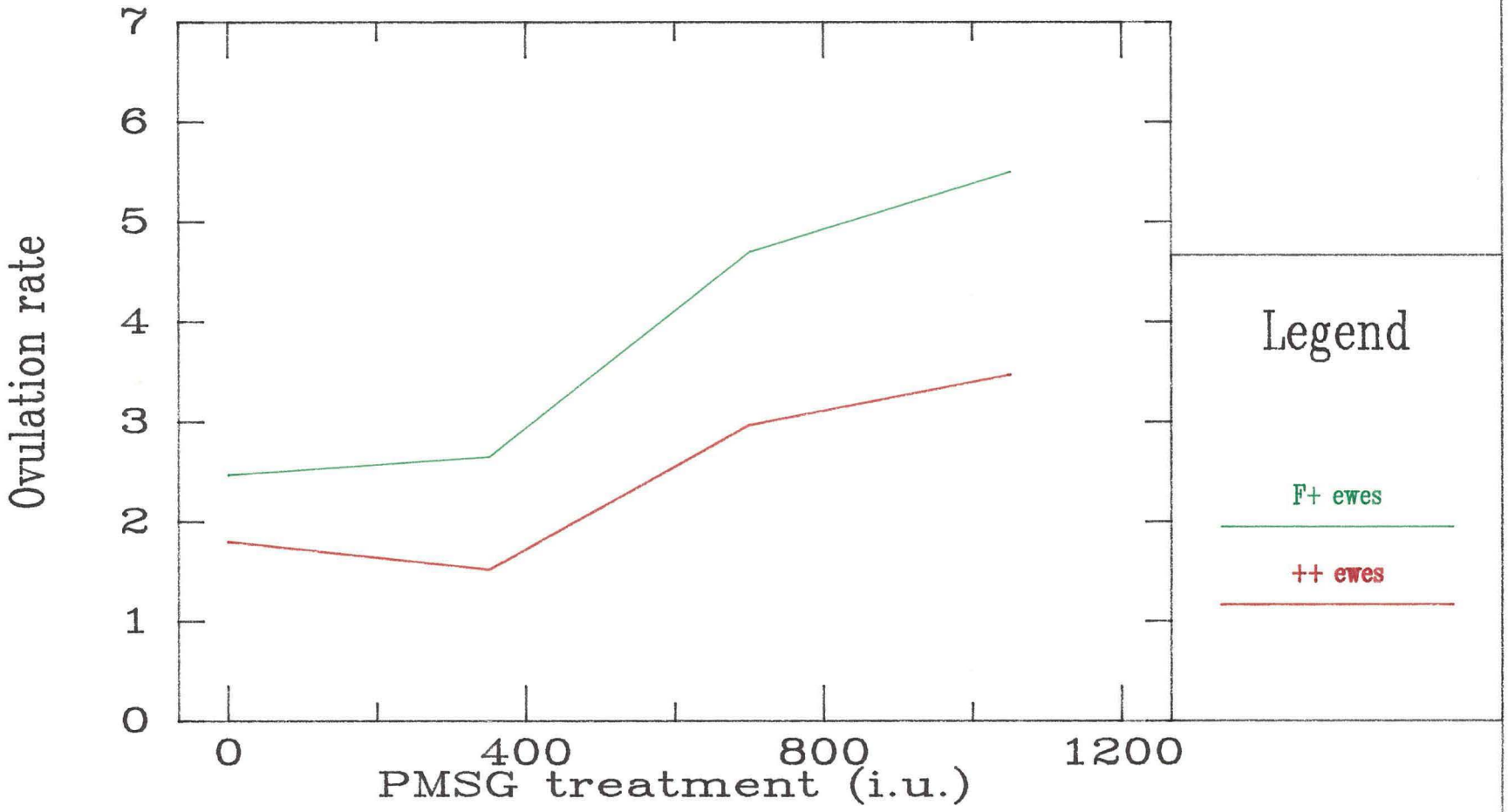


Figure 4.4. Effect of dose of PMSG on ovulation rate in ewes classified as carriers (F+) or non-carriers (++) of the fecundity gene.



4.5.1.4. Effect of age on the ovulatory response after PMSG treatment.

The analysis of the data showed a highly significant effect ($P<0.001$) of age of the ewes on their ovulatory response to PMSG treatment (Table 4.18). A significant difference in the response to PMSG treatment was found between 3 years old and 4-6 years old ewes. The ovulation rate was significantly higher in the older ewes (2.41 v. 3.44).

4.5.1.5. Effect of weight on the ovulatory respnse to PMSG treatment.

The analysis of the results showed a significant effect of weight of the ewe ($P<0.05$) on the ovulatory response to PMSG treatment (Table 4.18). However, of the total variation explained by the model (58%), only a small proportion (3.15%) was due to the effect of live weight.

Table 4.18. Factors affecting ovulation rate after PMSG treatment: Analysis of variance including both genotypes.

Source of variation	D.F.	S.S.(A)	
Genetic group	1	2.8990	**
Treatment	3	3.3639	**
GxT	3	0.0292	N.S.
Age	3	0.5449	**
Weight	1	0.2230	*
Error	145	5.2181	
Total	156	12.2782	

(A) = Transformed data.

N.S. = Not significant.

* P 0.05

** P 0.001

4.5.2. Analysis within genotype.

Data from 75 and 82 BMxP ewes classified as carriers (F+) and non-carriers (++) of the fecundity gene respectively, were used to evaluate the effect of PMSG treatment, Age and Weight on the number of corpora lutea recorded. Results are presented as raw and transformed data in Appendix 3. Table 4.20 and 4.21, show the analysis of variance for genotype 1 and 2 respectively.

4.5.2.1. Effect of age on the ovulatory response after PMSG treatment.

Analysis of the results showed a highly significant effect ($P<0.001$) of the age of the ewes from both genotypes on the ovulatory response to PMSG treatment (Table 8.3, 8.4). The least-squares means of the number of corpora lutea in 3 and 4-6 years old F+ genotype ewes were 2.70 and 4.36 respectively. The mean ovulation rate of the 2 ages of the non-carriers (++) ewes were 2.09 and 2.62 respectively.

4.5.2.2. Effect of PMSG treatment on the ovulatory response.

The analysis of the data showed a highly significant effect ($P<0.001$) of PMSG treatment in the ovulatory response to PMSG treatment in both genotypes (Table 4.20, 4.21). The least-squares means of ovulation rate in ewe carriers (F+) of the fecundity gene subjected to treatment 1, 2, 3 and 4 were respectively 2.31, 2.69, 4.72 and 4.71. In similar order the values recorded in ewe non-carriers (++) of the

fecundity gene were 1.83, 1.60, 2.95 and 3.48.

4.5.2.3. Effect of the interaction of age by treatment on the ovulatory response.

Analysis of the data did not show any significant interaction between the of age of the ewe and treatment in the ovulatory response to PMSG treatment in any of the genotypes (Table 4.20, 4.21).

4.5.2.4. Effect of weight on the ovulatory response to PMSG treatment.

The analysis of the data showed significant effect of weight ($P<0.05$) on the ovulatory response to PMSG treatment from ewe non-carriers (++) of the fecundity gene (Table 4.20). However, no such effect was found in ewe carriers (F+) of the fecundity gene (Table 4.21).

Table 4.20. Factors affecting ovulation rate after PMSG treatment: Analysis of variance of of data for F+ genotype ewes.

Source of variation	D.F.	S.S (A)	
Treatment	3	1.8828	**
Age	1	0.4413	**
Treatment x Age	3	0.1179	N.S.
Weight	1	0.0571	N.S.
Error	66	2.4429	
Total	74	4.9420	

(A) = Transformed data.

* P 0.05

** P 0.001

N.S. = Not significant.

Table 4.21. Factors affecting ovulation rate after PMSG treatment: Analysis of variance of data for ++ genotype ewes.

Source of variation	D.F.	S.S (A)	
Treatment	3	1.5104	**
Age	1	0.1843	*
Treatment x Age	3	0.1144	N.S.
Weight	1	0.1464	*
Error	73	2.4816	
Total	81	4.4371	

(A) = Transformed data.

* P 0.05

** P 0.001

N.S. = Not significant.

4.6. Factors Affecting the Success of Fresh Embryo Transfer.

The data from 49 BMxP ewes that were transplanted with 2 eggs each were examined to evaluate the effect of the fecundity gene, ovulation rate and hormonal treatment of the recipients on their ability to develop pregnancy. Embryo transfers were also made to 6 ewes given 1 embryo each and 8 ewes 3 embryos each, but these data have been excluded from the analysis.

4.6.1. Effect of genotype on pregnancy rate.

Table 4.22 shows the results for transfers classified according to the two genotypes of ewes. There was a higher pregnancy rate evident with the non-carrier ewes (96% v. 75%).

Table 4.22. Effect of genotype of the ewe on pregnancy rate after the transfer of two fresh embryos.

Genetic Group	Total Number of Ewes	No of Ewes Pregnant	No of Ewes Dry	% Pregnancy Rate
F+	24	18	6	75
++	25	24	1	96
Total	49	42	7	86

chi^2 = 4.410 * (P 0.05)

4.6.2. Effect of ovulation rate on pregnancy rate.

Table 4.23, indicates the results of ovulation rate on pregnancy rate. No significant difference in the ability to develop pregnancy was found between ewes with ovulation rate of 1, 2 or 3 corpora lutea.

Table 4.23. Effect of ovulation rate on pregnancy rate after the transfer of two fresh embryos.

Ovulation Rate	Total Number of ewes	No of Ewes Pregnant	No of Ewes Dry	% Pregnancy Rate
1	11	10	1	91
2	19	18	1	95
3	19	14	5	74
Total	49	42	7	86

$chi^2 = 3.751$ N.S.

4.6.3. Effect of PMSG treatment on pregnancy rate.

The results of the effect of hormonal treatment on the ability of the ewes to develop pregnancy are indicated in Table 4.24. No significant difference in the ability to develop pregnancy was found between ewes not injected with PMSG (control ewes) and ewes injected with 350 i.u. of P.M.S.G.

Table 4.24. Effect of hormonal treatment on pregnancy rate after the transfer of two fresh embryos.

Dose of PMSG (i.u.)	Total Number of Ewes	No of Ewes		% Pregnancy Rate
		Pregnant	Dry	
0	35	31	4	89
350	14	11	3	79
Total	49	42	7	86

chi^2 = 0.817 N.S.

4.6.4. Embryo survival.

The effect of number of embryos transferred on embryo survival is shown in Table 4.25. Although no statistical analysis was carried out, the survival rate after the transfer of 1 or 2 embryos was very similar, while the embryo survival when 3 embryos were transferred was considerably reduced. The quality of some of the sets of 3 embryos was considered to be poor when examined prior to transfer.

Table 4.25. Effect of number of embryos transferred on embryo survival.

Embryos Transferred	Number of Ewes	Total Embryos Transferred	Lambs Born	% Embryo Survival
1	6	6	5	83
2	49	98	79	81
3	8	24	11	46
	63	128	95	74

4.7. Factors Affecting the Success of Frozen Embryo Transfer.

Data from 43 Drysdale ewes was used to evaluate the effect of number of embryos transferred, ovulation rate and time from flushing to freezing on their ability to develop pregnancy. All of the embryos considered as normal on the basis of their morphology were transferred, but no distinction was made with regard to their quality.

4.7.1. Effect of number of embryos transferred on pregnancy rate.

The results of the effect of number of embryos transferred on the ability of the ewes to get pregnant are shown in Table 4.26. There was a significant difference ($P<0.05$) in the ability of the ewes to develop a pregnancy relative to the transfer of 1 or 2 embryos. When 2 embryos were transferred 43% pregnancy occurred but no ewes became pregnant

when only one embryo was transferred. It should be noted that only 8 single embryo transfers were attempted.

Table 4.26. Effect of number of embryos transferred on pregnancy rate.

Embryos transferred	Total Number of Ewes	No of Ewes Pregnant	No of Ewes Dry	% Pregnancy Rate
1	8	0	8	0
2	35	15	20	43
Total	43	15	28	35

chi^2 = 4.745 * (P 0.05)

4.7.2. Effect of ovulation rate on pregnancy rate.

Table 4.27, shows the results of the effect of ovulation rate on pregnancy rate. No significant difference was found between ewes with 1 or 2 corpora lutea in their ability to develop pregnancy.

Table 4.27. Effect of ovulation rate on pregnancy rate.

Ovulation Rate	Total Number of Ewes	No of Ewes Pregnant	No of Ewes Dry	% Pregnancy Rate
1	30	10	20	33
2	13	5	8	38
Total	43	15	28	35

$chi^2 = 0.296$ N.S.

4.7.3. Effect of time elapsing from flushing to freezing on pregnancy rate.

The influence of the interval elapsing from the time of flushing until the start of the freezing procedure on survival of embros are indicated in Table 4.28. No significant difference in pregnancy rate was found between embryos frozen 3 or 6 hours after their recovery. Prolonged delays prior to freezing were avoided as far as practicable because of possible reduced pregnancy rates.

Table 4.28. Effect of time elapsing from flushing until freezing on pregnancy rate.

Time from recovery to freezing(hrs)	Total Number of Ewes	No of Ewes		% Pregnancy Rate
		Pregnant	Dry	
3	32	11	21	34
6	11	4	7	36
Total	43	15	28	35

$chi^2 = 0.188$ N.S.

4.7.4. Embryo survival.

The effect of number of embryos transferred after freezing and thawing on embryo survival is shown in Table 4.29. No statistical analysis was carried out on these results. Nevertheless, it can be seen that none of the ewes in which only one embryo was transferred developed pregnancy. However, from 35 ewes each receiving 2 embryos 15 gave birth producing 23 lambs, giving a 33% embryo survival.

Table 4.29. Effect of number of embryos transferred on embryo survival.

Embryos transferred per ewe	Total embryos transferred	Lambs born	% embryo survival
1	8	0	0
2	70	23	33
Total	78	23	29

4.8. Observations after freezing procedure.

Some of the facts observed after the embryos were frozen and thawed are indicated in this section. In total 20 embryos were not considered suitable for transfer after thawing. The distribution was as follows:

- Embryos with broken zona pellucida.

Three embryos showed broken zona pellucida after thawing.

- Embryos with bent zona pellucida.

Two embryos did not show zona pellucida completely broken but only softened.

- Loss of embryos after thawing.

Five embryos were lost after thawing.

- Loss of the cell mass after thawing.

The cell mass of three embryos was split out and lost during the process of the removal of the cryoprotectant.

- Freezing empty zona pellucida.

The availability of four empty zona pellucida, made to look at the effect of the freezing procedure on them. All the empty zonas were apparently normal on the basis of their morphology after thawing.

- Appearance of dead embryos.

Three embryos apparently normal according to embryo morphology, were confirmed dead later on. They were one stage of development early than the embryos recovered from the same flushing; they were standing at the bottom of the petri dish and did not develpo any more after 12 hours of embryo culture.

CHAPTER 5

CHAPTER 5

DISCUSSION

5.1. General Discussion of Results.

Oestrus activity after hormonal treatment.

The oestrus synchronisation of the ewes was carried out by using intra-vaginal sponges charged with 40 mg of M.A.P. for 14 days.

Ninety three percent of the ewes included in the oestrus synchronisation programme showed oestrus and only 7% were not detected on heat. From the ewes showing oestrus 19% were detected in heat 56 hours after sponge removal, 65% from 70-80 hours and 16% from 94-104 hours after sponge removal. During the period when the ewes were expected to be in oestrus, observations for mating were made more frequently than once per day. No significant difference was found between ewes classified as carriers (F+) or non-carriers (++) of the fecundity gene, either in the number of ewes showing oestrus or in their distribution of the time of onset of oestrus. So it can be concluded that F+ ewes perform in a similar manner to ++ ewes, regarding the incidence of oestrus and its distribution of onset after oestrus synchronisation with M.A.P.

The efficiency of the oestrus synchronisation procedure used in this experiment is considered acceptable and agrees with most of the results from the literature. For example, similar efficiency of

sponges impregnated with progestagens to control oestrus in other breeds of sheep has been reported by Robinson (1964), Clarke et al. (1966), Larsen (1971) and Clarke (1973). Several attempts have been made to increase the efficiency of the oestrus synchronisation procedure in the breeding season, through the additional use of gonadotrophins (PMSG) at the end of the sponge treatment (Foord, 1966; Clarke, 1973), but unfortunately without a significant increase in the efficiency of the procedure. However, a significant increase in its efficiency has been reported when ewes were synchronised during the anoestrous period (Robinson and Smith, 1967) or when early breeding was attempted (Gordon, 1971).

Limited oestrous activity and a high incidence of silent oestrus during the late period of the non-breeding season has been reported by McKenzie and Terrill (1937) and Robinson (1950). In New Zealand, Cumming (1965) reported 32-38% of silent oestrus in ewes synchronised during February; and Larsen (1971) found 20% of the ewes experiencing silent oestrus when they were synchronised during March. Similar results were reported by Clarke (1973). In the present experiment only 7% of the ewes experienced silent oestrus; This may be partially explained because of seasonal differences, the low ram to ewes ratio used or probably an effect of breed of ewe. Bindon et al. (1980) reported that Booroola ewes experienced a longer reproductive season than less prolific breeds. Several researchers (Cumming, 1965; Larsen, 1971; Clarke, 1973) have indicated that some of the ewes not detected in heat after oestrus synchronisation, did in fact ovulate as revealed by laparotomy carried out a few days after the ewes were supposed to come into heat. The above results were confirmed in the

present experiment.

The oestrous activity after PMSG treatment was evaluated on 109 BMxP ewes. Ninety three percent of the ewes treated with PMSG showed oestrus. No significant effects of genotype, dose-level of PMSG or day of PMSG treatment was found either in the number of ewes showing oestrus after injection or in the distribution of the onset of oestrus.

From analysis of the results it can be concluded that F+ and ++ ewes showed similar oestrous activity after PMSG treatment. Wallace et al. (1954) suggested that the length of the cycle may be shortened by PMSG treatment but this has not been found in other studies of Robinson (1951), Wallace (1954), Cumming (1965), Tervit (1967), Larsen (1971) and Clarke (1973). The present results with BMxP ewes are in agreement with their observations.

The treatment of ewes with PMSG on day 11-13 of the cycle, generally has failed to show a significant effect on the length of the oestrous cycle (Wallace et al., 1954; Cumming, 1965; Tervit, 1967; Larsen, 1971; Clarke, 1973). In agreement with the above no significant difference in the incidence and distribution of oestrus was found between the ewes injected on days 11.5 or 12.5 of the cycle.

Ewe carriers of the fecundity gene.

The identification of ewe carriers (F+) or non-carriers (++) of the fecundity gene was attempted in 161 BMxP ewes, after all the ewes were laparoscoped 6-7 days after sponge removal. Ewes with an ovulation rate of 3 or higher were considered as carriers and ewes with less ovulations as non-carriers of the fecundity gene. This criterion of classification (Number of ovulations) has been used by all the researchers working with Booroola ewes and it's crosses to identify F+ and ++ ewes. In the present experiment, 76 (47%) animals were classified as carriers of the fecundity gene and 85 (53%) as non-carriers of the fecundity gene. In some ewes, it was not possible to record whether ovulation had occurred in one ovary (9 ewes) or both ovaries (2 ewes). In the first case, if the ewes had an ovulation rate of 3 or higher, they were classified as carriers of the fecundity gene (F+); and if ovulation rate was lower, information from previous lambing records was also considered in deciding on the genotype. In the second case, when ovulation rate was not recorded from any of the ovaries, the classification of the ewes as F+ or ++ animals was made on the basis of their previous lambing records. In this study only a few ewes experienced ovulation rate equal or above 5 corpora lutea (2 ewes), and these were classified as FF ewes. However, because of their low incidence, it was decided to include these animals within the F+ group of ewes. This situation was considered not to affect significantly the results.

The identification of F+ animals on the basis of ovulation rate through laparoscopy can be achieved easier and earlier than using

lambing data. Furthermore, Bindon (1975) and Bindon and Piper (1976) reported high repeatibility of ovulation rate in Booroola ewes. Most of the ewes in this study were identified for genotype on the basis of ovulation rate. The reliability of this criterion is not known for the present study, nevertheless it had to be applied to establish the two groups of trial ewes. More accurate criteria (such as blood groups or genetic indicators) need to be found to identify either F+ or ++ ewes and so generate more reliable data.

Ovarian activity.

The ovarian activity of the ewes was evaluated under two aspects: Ovulation rate after oestrus synchronisation and ovulation rate after PMSG treatment.

In the analysis of ovulation rate after oestrus synchronisation, the effects of genotype, age and weight of the ewes were evaluated. The ovulation rate was significantly affected by genotype and age but not by the weight of the ewes.

F+ ewes recorded a significantly higher ovulation rate (3.1) than ++ ewes (1.8). Highly significant differences in ovulation rate between Booroola ewes and other breeds (Merino, Romney, Border-Leicester, Dorset-Horn) have been widely reported (Robertson, 1976; Allison et al., 1977; Piper et al., 1979; Allison et al., 1980; Beetson, 1980; Bindon et al., 1980; McGuirk et al., 1980; Piper and Bindon, 1980; Ponzoni et al., 1980; Robertson, 1980). Some of the above researchers have also indicated a highly significant

difference in ovulation rate between Booroola cross ewes and the ovulation rate of some other breeds, such as Romney and Perendale.

The ovulation rate in the 4-6 years old ewes was significantly higher than the ovulation rate of the 3 years old ewes. In agreement with the present results Montgomery et al. (1985) reported a significant increase in ovulation rate of F1 and F2 BMxR (Booroola-Merino x Romney) ewes from 1.5 to 2.5 years of age. However, no significant difference in ovulation rate was found in ewes 3.5 to 5.5 years old. On the other hand Bindon et al. (1980) reported 2.49, 3.00 and 2.88 ovulations in Booroola ewes of 2, 3-5 and 6-10 years old respectively. It seems that the expression of the fecundity gene is partially influenced by age, since higher ovulation rate has been reported to be more consistent in older than in younger ewes. Such a situation is quite important, because of the necessity to identify as early as possible ewe carriers of the fecundity gene.

In the present trial the weight of the ewes did not show any significant effect on ovulation rate. Similar results were reported by Allison and Kelly (1978) working with Booroola cross ewes. A positive relationship between live weight and ovulation rate in ewes has been reported by Allison and Kelly (1978) and Morley et al., (1978). The results from several studies including several breeds have shown an increase of 0.0265 corpora lutea per kg of increase in live weight. More recently Montgomery et al., (1983) working with F+ and ++ animals indicated that an increase in live weight was associated with an increase in ovulation rate in both groups of animals. The incidence of ewes ovulating 1 or 2 corpora lutea was decreased in heavy animals, but

increased in the lighter ones. In another study carried out by the same authors it was indicated that the proportion of animals with three or more ovulations classified as carriers (F+) of the fecundity gene decreased from 0.75 to 0.49 when mean live weight differed by 8 kg (51.8 v. 43.7 kg respectively).

Ovulation rate after PMSG treatment was evaluated including the effects of genotype, dose-level of PMSG, age and weight of the ewes together with possible interactions. Significant effects of genotype, dose-level of PMSG, age and weight were found. However, there was no interaction of genotype by treatment on ovulation rate.

The ovulation rate in the F+ ewes was significantly higher than in the ++ ewes (3.61 v. 2.31). Variation between breeds in the response to PMSG treatment has been reported in several studies. Higher ovulation rate after PMSG injection from F+ ewes was also reported by Bindon and Piper (1982), Piper et al., (1982) and Kelly et al. (1983/84). Similar response from strains with high normal ovulation rate was indicated by Bindon et al. (1971) and Trounson and Moore (1972); and Eastwood and McDonald (1975) found a similar tendency when crossbred ewes were compared with straight breeds (Romney v. Border-Romney). Higher sensitivity to PMSG treatment from mice (Bindon and Pennycuik, 1974) and cattle (Thimonier et al., 1979; Snyder, 1986) selected for high fecundity indicates the possibility that indeed the high sensitivity to PMSG treatment is a particular characteristic in animals of high prolificacy. However, in contrast Newton et al. (1970), found that a higher dose of PMSG was required to induce similar ovulation rate in ewes with a high mean litter size.

In this experiment significant effect of dose-level of PMSG was found in the ovulation rate of the ewes. Comparisons of the results of treatments (dose) did not show a significant difference between treatments 1 and 2 and between treatments 3 and 4, but the ovulatory response in the last two treatments was significantly higher than the response in the former ones. However, because of the remaining heterogeneity of variances between PMSG treatments after the data were transformed, these results should be taken with caution. Further analysis within each genotype was then carried out. Homogeneity of variance was found in the data for the F+ ewes, but not in that from ++ ewes. Similar conclusions were drawn after the analysis by genotype, although in the case of ++ ewes the results should be taken with caution once the heterogeneity of variance between treatments remained. These results confirm the findings from the literature that PMSG treatment affect significantly the ovulation rate of the ewes, and that superovulation can be induced with 700 or 1050 i.u. of PMSG. However, the net ovulatory response to 700 or 1050 i.u. of PMSG (2.1 and 2.5 respectively) obtained in the present experiment was not as big as has been reported in other studies. The above can be explained on the basis of the high natural ovulation rate of the flock (2.3 corpora lutea). In comparison with the present results Kelly et al. (1983/84) working with F+ and ++ Booroola ewes with an average ovulation rate of 2.16 found a net response of 1.7 corpora lutea to 700 i.u. of PMSG and a significant increase to 4.84 ovulations following 1000 i.u. of PMSG, i.e. a steeper dose response relationship than in the present work.

Only one batch of PMSG was used in the present trial, and the ovulatory response to 700 or 1050 i.u. of PMSG was considerably lower than the average response that might have been anticipated from data supplied by the commercial company (4.7 and 9.3 corpora lutea respectively). In this study most of the ewes (75% approximately) were injected with fresh PMSG immediately after dilution and the rest of the animals with hormone that had been frozen for no more than three days. Such conditions have been indicated not to reduce the potency of PMSG (Lamond, 1957, 1964). On this basis it is suggested that the low ovulatory response obtained in this work was at least partly due to low potency of the batch of PMSG used. This suggestion is supporetd by Gonzalez-Mencio et al. (1978) who found variation in the composition and biological activity of PMSG isolated from different mares, within the same mare but at different stages of gestation and between procedures of isolation. Values of 0.9 to 1.3 in the ratio of FSH:LH from several batches of PMSG were reported in a review carried out by Betteridge (1977). However, because such variation was not significant it was concluded that the variation between animals in the response to PMSG treatment was unlikely to be due to differences in the FSH:LH ratio of the preparations used.

Hyperstimulation of the ovaries (20 corpora lutea) and high incidence of large persistent follicles as has been reported in other studies (Moore and Shelton, 1962; Tervit, 1967; Clark, 1973; Boland and Gordon, 1977; Mutiga and Baker, 1982; Ryan et al., 1982; Armstrong and Evans, 1983) was not found in the present work. This can be explained on the basis of the low to moderate doses of PMSG used in

this experiment, although the high natural ovulation rate could be involved.

There was no interaction between the genotype of the ewe and PMSG for the ovulatory response, which means that both genotypes responded in a similar manner to the PMSG treatment.

Significant differences in the ovulatory response to PMSG treatment was found between 3 and 4-6 years old ewes. The ovulatory response was significantly higher in 4-6 years old ewes than in the younger ewes (3 years old). Similar results were found when the effect of age was evaluated in each genotype. These results are in agreement with those reported by Averill (1958) who indicated a consistent higher ovulatory response from mature ewes compared to younger ewes given PMSG treatment; and Torres et al. (1986) inducing superovulation with FSH.

Small significant effect of weight on the ovulatory response to PMSG treatment was found in the present experiment, when the analysis included both genotypes. However, the analysis of the results by genotype, showed a small significant effect of weight in the ovulatory response to PMSG treatment in ++ ewes but not in F+ ewes. Overall, the variation in ovulation response owing to differences in live weight was small, even in the ++ ewes.

Factors affecting pregnancy rate.

Effects of genotype, ovulation rate and hormonal treatment were included to evaluate differences in pregnancy rate after the transfer of 2 fresh embryos into each recipient ewe. In total 86% of 49 transfers were successful and resulted in lambs born. The pregnancy rate was significantly affected by genotype, but not by ovulation rate of the recipient ewes and hormonal treatment.

The ability of ewes non-carriers (++) of the fecundity gene to develop pregnancy after the transfer of 2 fresh embryos (96% pregnancy rate), was significantly higher ($P< 0.05$) than the ability of ewes carriers (F+) of the fecundity gene (75% pregnancy rate). No clear explanation can be given for such results. Although because of the low number of transfers carried out, further research is suggested on this regard.

No significant difference in the ability to develop pregnancy was found between recipient ewes with 1, 2 or 3 corpora lutea. Moore et al. (1960), Cumming (1965), Cumming and McDonald (1970) and Bradford et al. (1974) have all reported no relationship between the number of corpora lutea in recipient ewes and the survival of transferred ova; and Averill and Rowson (1958) reported a consistent, but non significant increase in the ability of ova to develop pregnancy as ovulation rate was increased from 1 to 3. However, a decrease in pregnancy rate when the ovulatory response of the recipient ewes increased from 1 to 3 was reported by Tervit (1967). Although in his study only 7 transfers were carried out.

No significant difference in the ability to develop pregnancy was found between recipient ewes not injected with any hormone at all (control ewes) and animals injected with 350 i.u. of PMSG. Tervit (1967) reported the possibility of using ewes stimulated with 1000 i.u. of PMSG or above as recipients. Results obtained in this work suggest the possibility of using low doses of PMSG (350 i.u.) in recipient ewes during the breeding season without any negative effect on their ability to develop pregnancy. This situation could be helpful especially if potential recipient animals were treated with PMSG to increase the occurrence of oestrus during the anoestrous season.

In general the pregnancy rate obtained in the present experiment (86%) is particularly high. For example embryo transfers carried out under similar conditions by Moore and Shelton (1962), Moore (1968), Crosby et al. (1980) and Killeen (1980) the last two authors cited by Killeen (1981) reported a pregnancy rate of 74%, 75%, 64% and 79% respectively. The above results include some studies in which only one embryo was transferred, but comparisons can be made with confidence since Killeen (1981) mentioned that differences in pregnancy rate after the transfer of one and two embryos are usually small and not statistically significant.

Pregnancy rate after the transfer of frozen embryos was evaluated including the effects of number of embryos transferred, ovulation rate of the recipient ewes and time elapsing from flushing to freezing. In total 35% of 43 transfers were successful. The pregnancy rate was significantly affected by the number of embryos transferred but not by

the number of corpora lutea in the recipient ewes nor by the time elapsing from fushing to freezing of the embryos.

Forty three percent of 35 ewes receiving 2 frozen-thawed embryos developed pregnancy and produced lambs. However none of the ewes receiving only one embryo were pregnant. The small number of ewes involved make it difficult to suggest reasons for the difference in conception rate but aspects relevant to embryo quality are discussed later.

Results from this study indicate no difference in pregnancy rate when embryos were frozen starting 3 or 6 hours after flushing. Opposite results were reported by Pettit, Jr. (1985) and Wright (1985). In their studies a decrease in pregnancy rate was found when freezing of the embryos was delayed from 3 to 4 hours after flushing. The small number of transfers conducted in the present work precludes reliable conclusions being made, however, it is suggested that embryos should be frozen within 3 hours after flushing to avoid the possibility of any reduction in their ability to develop pregnancy when later transplanted.

In general the pregnancy rate obtained with the previously frozen embryos (35%) is within the range reported from the literature. Early reports indicated pregnancy rates from 33 to 40% (Willadsen et al., 1976; Willadsen, 1977), but nowadays pregnancy rates close to 50% are frequently reported for frozen-thawed embryos. Lehn-Jensen (1981) noted wide variation (17-60%) in pregnancy rate. This might be anticipated as Schneider and Mazur (1986) have pointed out that unless

all the steps in the embryo transfer programme are controlled closely, the pregnancy rate from frozen-thawed embryos may vary considerably and the extent of variation can be a significant factor in the effectiveness of the embryo transfer programme.

Embryo recovery.

Embryo recovery was carried out using the technique described by Tervit and Havik (1976). The influence of the dose-level of PMSG on the percentages of embryos collected for the donors was examined. No significant difference in embryo recovery was found between treatments 3 and 4 (63 and 64% respectively). Comparisons were not strictly comparable between treatments 3, 4 and 5 as the ewes included in the latter treatment were examined as a separate group. The efficiency of the embryo recovery procedure was considerably increased in treatment 5 (78%). Such increase in the efficiency is likely to be due to an improvement in the skill of flushing ewes gained with experience. However, it should be borne in mind that only 10 ewes were flushed in treatment 5. The efficiency of the technique of embryo recovery including all the treatments and on a per ewe basis was 68%. These results are in agreement with those reported from the literature. For example although they are lower than the 83% embryo recovery reported by Tervit and Havik (1976), and the range (75 to 86%) reported by Torres et al. (1986); they were higher than the 59% reported by Tervit et al. (1976) and very close to the 69% embryo recovery indicated by Torres and Cognie (1984). It can be seen from the literature that the efficiency of embryo recovery achieved in the

present experiment could be improved. This is supported by the relatively high recovery rate achieved in treatment 5 (78%). In an attempt to increase the percentage of embryos recovered, it was considered worthwhile to use up to 30 ml of fluid for flushing each horn, especially in ewes with long uterine horns. It would also be worthwhile to re-flush ewes with high ovulatory responses ($\leq$5) from which only 50% or less of the embryos were recovered. Both refinements in method were attempted in the present experiment showing positive results, although it was done only in a few ewes. If uterine flushings can yield close to 80% of the embryos, such results will be comparable with those obtained after flushing oviducts, but resulting in a lower incidence and severity of reproductive tract adhesions (Tervit and Havik, 1976).

Fertilisation.

Three entire Booroola rams were used to serve the donor ewes. No significant difference between rams was found when the fertilisation rate was evaluated on a per ewe basis. On average 78% fertility occurred.

Failure of fertilisation after superovulatory treatments has been indicated as the main factor limiting embryo transfer in sheep (Betteridge and Moore, 1977; Armstrong and Evans, 1983); and has been attributed to a decrease in the transport of spermatozoa through the cervix (Trounson and Moore, 1974; Evans and Armstrong, 1984). Although the fertilisation rate obtained in the present experiment

would be considered reasonable, it is likely that it cuold be increased. Reports by Killeen and Moore (1971) and Trounson and Moore (1974) indicated that a fertilisation rate over 90% occurred when ewes were "served" by intrauterine insemination. However, Trounson and Moore (1974) noted that the advantage of getting a high fertilisation rate after intrauterine insemination by laparotomy was somewhat reduced because of a decline in the percentage of embryos recovered. The subsequent development of techniques for intrauterine insemination by laparoscopy has overcome this problem (Evans and Armstrong, 1984).

Embryo survival.

The incidence of embryos surviving after the transfer of 1, 2 or 3 fresh embryos was 83%, 81% and 46% respectively. No statistical comparison of these numbers was made because of the low number of transfers carried out with 1 and 3 embryos. However, it can be seen that there was no considerable difference in the survival rate when 1 or 2 embryos were transferred. Embryo survival after the transfer of 3 embryos was considerbly reduced. This is partially explained by the fact that most of the sets of three embryos were of poor quality. It is interesting to mention that one of the ewes receiving 3 embryos gave birth to triplets. Such results confirm the fact that some of the morphologically poor quality embryos, really are able to develop pregnancy and produce lambs.

The incidence of embryos surviving after the transfer of 1 or 2 frozen embryos was 0% and 33%. The number of transfers carried out

were too small to allow statistical analysis, however it can be seen that increasing the number of embryos transferred from one to two, the embryo survival was considerably increased. It is difficult to explain the unsuccessful results after the transfer of 1 frozen embryo, but it is likely that those embryos were of low quality before freezing and were more affected during the freezing and thawing procedure, so that their ability to develop pregnancy was completely stopped. Effect of embryo quality assessed morphologically before and after freezing and thawing on their ability to develop pregnancy has been reported by several researchers. For instance Wright (1985) reported pregnancy rates of 43% and 23% when embryos of good and poor quality respectively were transferred. Similar findings reported by Pettit. Jr. (1985) and Elsden et al (1982) indicated a significant effect of embryo quality before freezing on pregnancy rate. Studies of embryo development in vitro after freezing and thawing carried out by Bui-Xuan-Nguyen et al. (1984) have confirmed the above results. In the present work most of the embryos transferred were morulas and there were only a few blastocysts available. Such situation was considered not to affect significantly the results. No significant differences in pregnancy rate after the transfer of early morula, morula, early blastocyst or blastocyst have been reported by Elsden et al. (1982), Pettit. Jr. (1985) and Wright (1985).

Fertility of the ewes after flushing.

The reproductive performance of the donor ewes which were flushed, was evaluated at least during the following 21 days. Thirty five

percent of 79 flushed ewes returned to oestrus. Of the 51 ewes not showing overt oestrus, 75% were animals from which at least one embryo was lost. The high incidence of ewes not showing oestrus after flushing, is explained on the basis that one or more embryos were not recovered. Probably some of these animals were pregnant only for a short period of time beyond 21 days while others were able to develop full pregnancy and produce lambs. Unfortunately the first possibility was not determined in this work; the second possibility is discussed elswhere.

The presence of an embryo in the uterus about day 12 or 13 of the oestrous cycle, prevents regression of the corpus luteum and as a consequence the length of the cycle is extended. If the embryo does not die, the ewe will develop full pregnancy and produce lambs. However, if the embryo dies after day 12 or 13 of the cycle, the length of the next cycle will be extended (Moor and Rowson, 1964, 1966; Edey, 1967; Chapman, 1980). In this work from the 65% of the ewes not showing overt oestrus after flushing, 39% were able to develop full pregnancy and produce lambs. These results confirm the fact that embryos not recovered at flushing can develop pregnancy and produce normal lambs. If such a situation is considered undesirable or if the ewe is going to be superovulated once again, luteolysis of the corpora lutea should be induced. This can be achieved by giving an injection of prostaglandin on day 7-12 after superovulation (Willadsen, 1979).

Of the 28 ewes showing oestrus after flushing, in 71% of them at least one embryo was lost and from the rest of the ewes all the embryos were recovered. These results suggest that ewes in which embryos were

lost and came on heat at the next cycle after flushing, experienced early embryo mortality. This would be because the ovum or embryo already was nonviable at the time of flushing or because it failed to survive and did not implant. These results are in support to those indicated by Edey (1966, 1967) in which it was indicated that the death of embryos before day 12 or 13 did not extend the length of the next oestrous cycle. These results show that some ewes are able to experience normal oestrus after flushing, despite one or more embryos not being recovered at surgery. The fact that 86% of the ewes experiencing oestrus after flushing became pregnant at the next mating, confirms the normality of the cycle. This situation is worthy of note, since ewes that have been used as donors, can be served the next cycle after flushing and many should produce extra lambs to that mating.

The twenty five percent of ewes not showing overt oestrus after flushing even when no embryos were lost, (on the basis of number of corpora lutea recorded and the number of embryos recovered) suggests a disturbance of the ovarian activity in the donor ewes after flushing. Probably the corpora lutea after PMSG stimulation were not destroyed at the normal time for regression of the oestrous cycle, therefore extending the length of the cycle. Unfortunately this was not evaluated in this study. On the other hand, it can be assumed also that some of these ewes would experience silent oestrus, as has been already pointed out. This assumption could be enhanced by the fact that the last group of animals were treated at the end of the normal breeding season, and therefore would be less likely to show overt estrus. Silent oestrus has been reported in ewes at such time.

5.2. Feasibility of Embryo Transfer and Storage of Embryos from Selected Sheep.

The sequence of events of the embryo transfer programme carried out during the present experiment are shown in Figure 5.1. Also shown are two examples of the degree of success of an embryo transfer programme. The first example, outlines the results of the present work and example 2 illustrates the possible results if several assumptions were applied in the programme.

5.2.1. Example 1.

The results from the present experiment shows that if 100 donor ewes were inserted with sponges for oestrus synchronisation, only 93 ewes (93%) showed oestrus. From the 7% not showing oestrus, 2.5% lost the sponges and 4.5% were not detected in heat by the teaser rams. All the ewes detected in heat were injected with PMSG, on day 11-13 of the oestrous cycle and 93% of the ewes showed oestrus after PMSG injection. Thus only 86 ewes were detected on heat. Among the ewes showing oestrus after the injection of 1050 i.u. of PMSG there were 4.37 corpora lutea per ewe, which gives a total of 376 corpora lutea. The efficiency of the technique for embryo recovery on a per ewe basis was 65%, which means that only 244 ova and embryos were recovered. The observations on the fertilisation rate calculated on a per ewe basis and taking into account that 3 rams were used for mating as single sires, shows that only 78% of the total number of ova or embryos recovered were fertilised. Thus there was a yield of 190 embryos which were able either to be transferred or used for freezing.

If the decision was to transfer the embryos within 6 hours after flushing and in each recipient two embryos were deposited, then 95 recipient ewes would be required. The recipient ewes should be at the same stage of the cycle to that in the donor ewes, or within ±12 hours. A pregnancy rate of 86 percent after the transfer of two fresh embryos, would mean that 82 ewes could conceive and produce lambs. Finally, if an embryo survival of 74% is considered, then from the 190 embryos transferred, 141 lambs would be obtained. An average number of lambs obtained per ewe of 1.41 arising from a potential of 2.0 after embryo transfer shows that a significant component of prenatal mortality exists.

If the decision is then made to conserve the embryos for a long period of time, they need to be frozen. The freezing procedure used in the present experiment showed the following results: A pregnancy rate of 43% after the transfer of 2 embryos per recipient ewe, without making a strict selection of embryos for quality after freezing and thawing; and an embryo survival of 33%. This means that if 100 recipient ewes were transferred with 2 embryos each, 43 would develop pregnancy and 66 lambs would be born. The embryos in the present experiment, were frozen using a programmable freezing machine. Once the embryos were frozen, they were maintained in liquid nitrogen, contained in a special tank (20 litres capacity). The tank was refilled when it was necessary, to keep the level of nitrogen always above one third of its capacity.

5.2.2. Example 2.

Example 2 in Figure 5.1, illustrates the possible results following the implementation of several assumptions which are as follows.

Assumption 1.

The treatment of the ewes should be commenced after the start of the breeding season. Provided the embryo transfer programme is started after the ewes begin to cycle, then of 100 ewes that are treated with sponges at least 90 should be able to be mated and programmed for embryo recovery. Ewes mated were considered to be those which were marked on their rump. It was also evident that some of the ewes which were not marked by the teasers after oestrus synchronisation, were recorded with corpora lutea at the time of laparoscopy (5-7 days after sponge removal). A similar situation was found after PMSG treatment at the succeeding ovarian cycle. Subsequently it was found that some of the ewes which were not marked by the entire rams and apparently not mated, were recorded to be pregnant and produced lambs at normal time.

Assumption 2.

The ovulatory response in the present experiment was 4.37 corpora lutea per ewe after the injection of 1050 i.u. of PMSG. Increased ovulation rate could be achieved by two methods; 1. by injecting the ewes with a higher dose of PMSG and, 2. the injection of pituitary F.S.H., to induce superovulation. For example the injection of 1500 i.u. of PMSG has yielded 7 ovulations or above (Bindon et al., 1971; Allison, 1973). Also more consistent ovulatory response has been reported when superovulation of ewes has been attempted with FSH, 8 or

more ovulations has been reported by Torres and Cognie (1984).

Provided the superovulation treatment is carried out using approximately 1500 i.u. of PMSG or adequate doses of FSH, then 8 ovulations per donor should be achieved. On this basis a total of 720 corpora lutea could be obtained.

Assumption 3.

The efficiency of the technique of embryo recovery on a per ewe basis in this study was 65%. However, the recovery rate of embryos can be increased by at least 5%, and yield 70% of the potential embryos. This assumption is made because in some ewes with ovulation rates over 5, few embryos were recovered, but in several cases when these ewes were reflushed, 1 or 2 additional embryos were obtained. It did appear that attempts to reflush ewes can be justified in ewes with ovulation rates of 6 or over, from which only 50% of the embryos or less were recovered. If a 70% rate of embryo recovery is assumed, it means that from the total ovulatory response (720 corpora lutea), some 504 embryos could be obtained.

Assumption 4.

The average fertilisation rate on a per ewe basis was 78%. It is likely that the fertilisation rate could be increased to 85%, through a strict selection of high fertility ewes and rams. The assumption of 85% fertilisation rate, means that from the 504 embryos recovered, 428 embryos would be fertilised and probably be able to be transferred or conserved as frozen embryos.

Assumption 5.

The pregnancy rate after the transfer of 2 fresh embryos per ewe was 86%. It is believed that with the procedure developed in this work, a similar pregnancy rate could be achieved, but it would be unwise to expect much higher values consistently.

The availability of 428 embryos means therefore that 214 ewes will be required as recipients. If an 86% pregnancy rate is assumed, then 184 ewes should carry their pregnancy and produce lambs.

Assumption 6.

The embryo survival rate after the transfer of 2 fresh embryos per ewe was 74%. It is believed that this figure can be consistently obtained and probably increased, provided a strict selection in the quality of the embryos before transfer is made.

Assuming an embryo survival rate of 74%, it means that out of 428 embryos transferred, 316 embryos will be able to develop until parturition. Therefore each ewe would be able to produce on average 3.16 lambs.

Assumption 7.

In the present work, some of the ewes from which some embryos were not recovered at flushing, remained pregnant and produced lambs. Also, some of the ewes in heat after surgery conceived. Unfortunately, in this study not all the ewes had the opportunity to be mated after flushing, so no valid figure can be quoted regarding the percentage of ewes getting pregnant after flushing. Nevertheless, it can be assumed

that at least 1 extra lamb can be obtained from each donor ewe after flushing. A similar assumption has been suggested by Allison (1973) also.

Taking into account the last assumption, it is predicted that at the end of the transfer programme a total of 4.16 lambs per donor ewe would be obtained.

Assumption 8.

Under farm conditions a natural reproductive rate of 150% (number of lambs born over 100 ewes mated) is frequently obtained. After the use of embryo transfer the number of lambs can be increased considerably, from 1.50 lambs per ewe to 4.16 lambs per ewe.

Assumption 9.

In this experiment, the pregnancy rate after the transfer of 2 frozen-thawed embryos per ewe was 43%, and with an embryo survival of 33%. It is believed that, with more experience and refinement of the freezing procedure used, the pregnancy rate could be increased to about 50%; and embryo survival increased to about 40%. This could be achieved, provided a strict selection of quality embryos was carried out after freezing and thawing, and before their transfer.

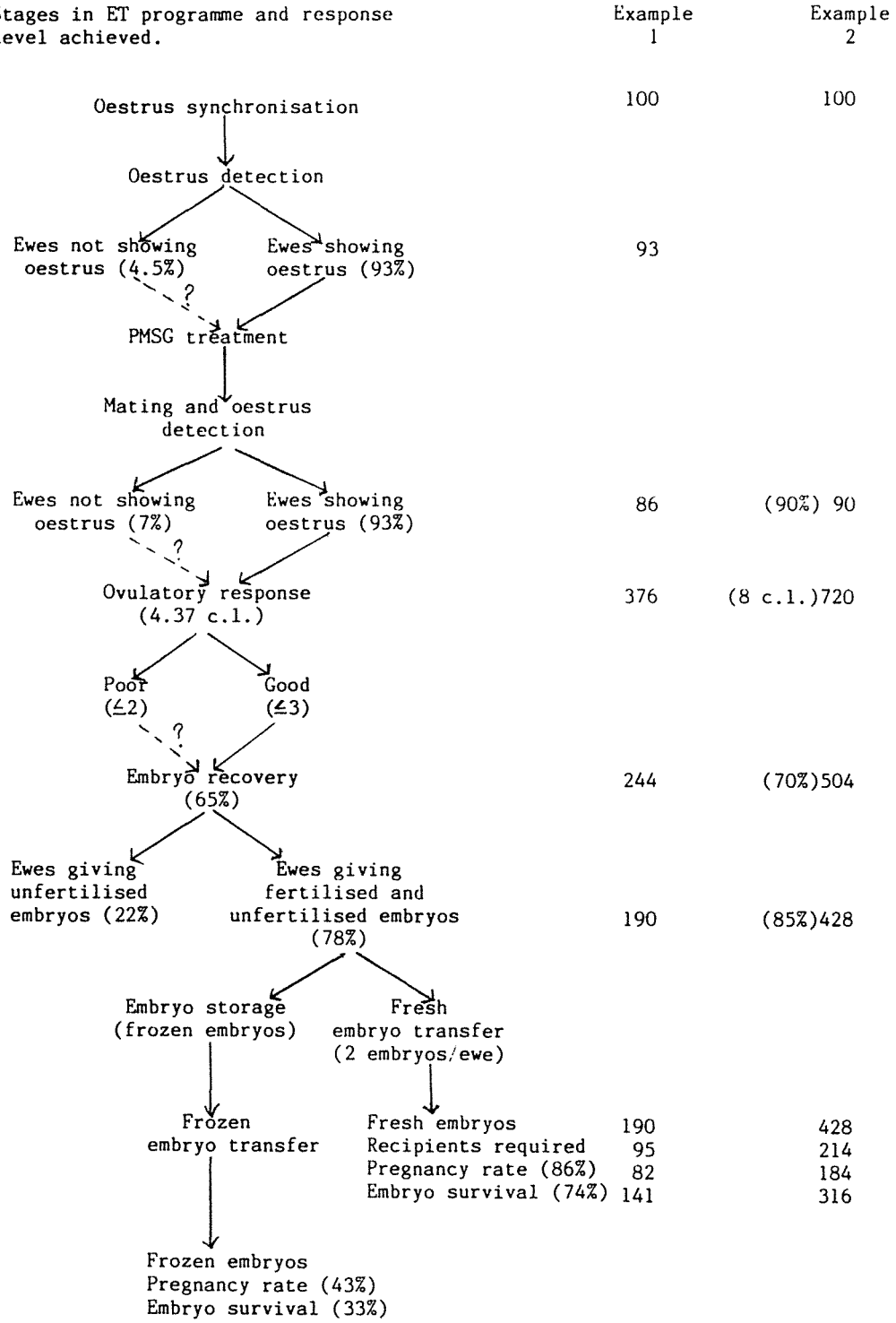
If the above assumptions were met, it means that from 100 recipient ewes given 2 frozen embryos each, 50 ewes would be able to develop pregnancies and produce 80 lambs.

In the present study, once the embryos were frozen, they were conserved in liquid nitrogen, in a small special container. This was

reflected in the fact that the container had to be refilled with liquid nitrogen several times. Such a situation could be avoided, if the embryos after being frozen were taken to a storage bank such as The Livestock Improvement Association, until their transplantation into ewes was imminent.

Figure 5.1. Sequence of events in an embryo transfer programme and results achieved.

1. Data from present study.
2. Predicted results after modification of techniques (see text).



5.2.3. Problems found through the experiment.

Several problems that affect the successful outcome of the work have become apparent.

1. Oestrus detection.

After treatment to induce sinchronise oestrus not all animals showed heat. Those failing to do so in most cases would have ovulated (silent oestrus) at about the same time as for those showing oestrus coincident with ovulation. Because the injection of PMSG needs to be given about 12 days after oestrus, then an assumption must be made that the silent oestrus ewes had ovulated as expected. A further possibility might be to laparoscope the silent oestrus animals to confirme the occurrence and approximate time of ovulation.

2. Ovulatory response.

There was a great variation in the number of ovulations to standard doses of PMSG. The variation in the response was greater as the dose of PMSG was increased. The low ovulatory response recorded in this experiment, could be attributed to the low doses of PMSG used (350, 700 and 1050 i.u.).

Because one of the main objectives of an embryo transfer programme is to get as many embryos as possible for transfer, it is suggested that increased doses of PMSG should be used but not to a level when excessive non-ovulatory stimulation occurs. Also the use of FSH for superovulation should be considered, although such material can be expensive relative to PMSG treatment.

3. Fertilisation rate.

Considerable variation in fertilisation rates was found between rams. An increase in the total number of fertilised embryos can be achieved, only if rams of high fertility are used in the embryo transfer programme.

4. Embryo recovery.

Although the efficiency of the technique of embryo recovery was acceptable (65%), there is evidence to suggest that it can be increased. It is suggested that ewes be reflushed when more than 5 corpora lutea occur and less than 50% of the embryos are recovered after the initial flushing.

5. Number of ewes flushed.

Under the conditions of this experiment, with 4 persons each day involved in the surgical and embryo manipulation work each, then it is not advisable to flush more than 10 donors a day, especially when the possibility to freeze some of the embryos is considered.

6. Embryo preservation.

Preservation of embryos for a long period of time, implies first that they need to be processed quickly for successful freezing and second that they need to be preserved in a special container with liquid nitrogen. In this experiment the embryos were preserved in a small container (20 l. capacity), purchased for this purpose. The tank had to be refilled with liquid nitrogen several times, to maintain the liquid nitrogen at an adequate level. This method of storage

especially for a long period might be improved through the storage of the embryos in a Central Bank such as maintained by The Livestock Improvement Association. The costs of storage should be reduced if this was adopted.

5.2.4. Potential application of the programme at Massey.

The development of procedures and possible results that can be achieved through the technique of embryo transfer, have already been indicated. The possibility of embryo preservation for a long period of time has been mentioned as well.

Undoubtedly, the use of embryo transfer in animal production has a number of applications; and definitely one of the most important is to increase rapidly the number of offspring from a selected group of animals. The availability of several flocks at Massey University with important productive and reproductive characteristics is an objective for which an embryo transfer programme could be developed.

The reasons for wanting to store embryos include being able to sample the population at times to measure genetic progress, as well as being able to have offspring of selected individuals conveniently on hand. In this way a bigger number of animals could be available for experimental and productive purposes. On the other hand, if there is interest to preserve for a long period of time certain genetic material, it can be conserved indefinitely as frozen embryos and evaluated when required.

Another important point is that frozen embryos can be transported anywhere and their transfer can be done at any time. Hence embryos produced and frozen at the University can be transplanted in any place within or outside the country and at any time of the year. To satisfy Animal Health requirements special systems to process the embryos may have to be developed such as the requirement to wash embryos several times more than is adopted normally.

5.3. Areas Needing Further Research.

The results from the present experiment have contributed to a better understanding of some of the factors influencing the success of an embryo transfer programme. However, to achieve improvements in the efficiency of the current techniques used, further research is required in some areas such as:

1. Superovulation.

The use of high doses of PMSG (over 1500 i.u.) or FSH to induce superovulation in ewes carriers and non-carriers of the high fecundity gene, under New Zealand conditions needs to be evaluated. Data on the ovulatory response to FSH from most of the sheep breeds under New Zealand conditions are scarce.

2. The use of artificial insemination.

Poor fertilisation rates frecuently occur when donor ewes are excessively stimulated with gonadotrophins. The possibility that laparoscopic insemination might be adopted to overcome the failure of

natural mating to give good fertility should be investigated.

3. Production of flushing solutions.

While most of the flushing media can be obtained from commercial sources, there may be good reasons to manufacture these modified preparations within the University. This would allow experimentation involving new preparations and also the possibility to reduce their costs.

5. Freezing procedure.

The freezing procedure used in this experiment, needs to be refined in an atttempt to increase its efficiency. The high price of the equipment actually used for embryo freezing, represents a big handicap to its intensive use. Research is required to develop less expensive technology for embryo freezing, keeping in mind the basic principles of the freezing procedure.

6. Embryo micromanipulation.

The technology for embryo micromanipulation and particularly embryo splitting, has been developed recently. It is a technique which can be used, to reproduce in a faster way the offspring from animals with desirable characteristics from the productive and reproductive points of view. The use of this technique within an embryo transfer programme with sheep, requires further investigation.

5.4. Conclusions.

Arising from work done to test embryo transplantation procedures that might be applied to a selected flock of sheep the following conclusions are made.

1. BMxP ewes in which the fecundity gene was present (F+) or absent (++) showed a similar incidence and onset of oestrus after progestagen-synchronisation treatment, and also after the injection of PMSG (0, 350, 700, 1050 i.u.) given in the succeeding ovarian cycle.

2. The ovarian response to PMSG injection was greater in F+ ewes than in ++ ewes (3.61 v. 2.31 ovulations). There was no significant genotype by treatment interaction.

3. Variation in the dose of PMSG did not significantly affect the percentages of embryos recovered from the donor ewes when subjected to uterine flushing on day 5-6 after mating.

4. The incidence of fertilised eggs was not affected by the ovarian response to PMSG. No effects on fertility were evident between the 3 rams used.

5. Transfer of 2 freshly collected embryos to the recipient ewes resulted in 85% pregnancy rate and 75% of the potential embryos survived to lambing.

6. Transfer of 2 embryos after freezing-thawing procedure resulted in 43% of the ewes becaming pregnant.

7. While freshly collected embryos have a higher survival rate than frozen-thawed embryos, tha availability of freezing procedures dose offer considerable scope for Embryo Transfer Programmes to be developed for a wide range of uses.

CHAPTER 6

CHAPTER 6

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Appendix 1. Bartlett's test for homogeneity of variance of ovulation rate data: Normal ovulation rate.

Age.

a. Raw data (A).

Age	Variance s^2
3 years	0.6475
4-6 years	0.8203

Chi^2 = 1.075 ** p 0.05, with 1 d.f.

The variance approaches homogeneity.

Bartlett's test for homogeneity of variance of ovulation rate data.

Genotype.

a. Raw data (A).

Genotype	Variance s^2
F+	0.4433
++	0.2067

Chi^2 = 11.539 ** p 0.01, with 1 d.f.

The variance is significantly heterogeneous.

(cont. Appendix 1)

b. Transformed data (B).

Genotype	Variance s^2
F+	0.0137
++	0.0116

Chi^2 = 0.408 ** p 0.01, with 1 d.f.

The variance approaches homogeneity.

(B) Data transformed to log (y).

Appendix 2. Bartlett's test for homogeneity of variance of ovulation rate data: Ovulation rate after PMSG treatment in both genotypes.

Age.

a. Raw data (A).

Age	Variance s^2
3 years	9.2173
4-6 years	3.1063

Chi^2 = 19.948 ** P 0.01, with 1 d.f.

The variance is significantly heterogeneous.

b. Transformed data (B).

Age	Variance s^2
3 years	0.0151
4-6 years	0.0095

Chi^2 = 2.943 ** P 0.05, with 1 d.f.

The variance approaches homogeneity.

(B) Data transformed to log (y).

(cont. Appendix 2)

Bartlett's test for homogeneity of variance of ovulation rate data.

Genotype.

a. Raw data(A).

Genotype	Variance s^2
F+	10.6706
++	2.5392

Chi^2 = 40.823 ** P 0.01, with 1 d.f.

The variance is significantly heterogeneous.

b. Transformed data (B).

Genotype	Variance s^2
F+	0.0146
++	0.0079

Chi^2 = 6.427 ** P 0.01, with 1 d.f.

The variance approaches homogeneity.

(B) Data transformed to log (y).

(cont. Appendix 2)

Bartlett's test for homogeneity of variance of ovulation rate data.

PMSG treatment.

a. Raw data (A).

PMSG treatment	Variance s^2
0	0.8087
350	0.9768
700	7.1988
1050	14.4730

Chi^2 = 121.367 ** P 0.01, with 3 d.f.

The variance is significantly heterogeneous.

b. Transformed data (B).

PMSG treatment	Variance s^2
0	0.0030
350	0.0039
700	0.0131
1050	0.0182

Chi^2 = 39.989 ** P 0.01, with 3 d.f.

The variance is significantly heterogeneous.

Appendix 3. Bartlett's test for homogeneity of variance of ovulation rate data: Ovulation rate after PMSG treatment within genotype.

Genotype 1 (F+).

PMSG treatment.

a. Raw data (A).

PMSG treatment	Variance s^2
0	1.2056
350	0.7059
700	7.9379
1050	17.0905

Chi^2 = 53.178 ** P 0.01, with 3 d.f.

The variance is significantly heterogeneous.

b. Transformed data (B).

PMSG treatment	Variance s^2
0	0.0039
350	0.0040
700	0.0113
1050	0.0121

Chi^2 = 9.374 ** P 0.01, with 3 d.f.

The variance approaches homogeneity.

(B) Data transformed to log (y).

(cont. Appendix 3)

Bartlett's test for homogeneity of variance of ovulaton rate data.

PMSG treatment.

Genotype 2 (++).

a. Raw data (A).

PMSG treatment	Variance s^2
0	0.4075
350	0.4667
700	2.7833
1050	5.6000

Chi^2 = 53.977 ** P 0.01, with 3 d.f.

The variance is significantly heterogeneous.

b. Transformed data (B).

PMSG treatment	Variance s^2
0	0.0011
350	0.0013
700	0.0088
1050	0.0148

Chi^2 = 38.649 ** P 0.01, with 3 d.f.

The variance is significantly heterogeneous.

(B) Data transformed to log (y).