



COLLECTION, CULTURE AND PRONUCLEAR MICROINJECTION OF SHEEP ZYGOTES

BY

SIMON KERRY WALKER, M.Ag.Sc.

A thesis submitted to the University of Adelaide in fulfilment of the requirements for the degree of Doctor of Philosophy

Department of Obstetrics and Gynaecology,
The University of Adelaide.

May, 1989

awarded 6.11.89

TO
ALISON

TABLE OF CONTENTS

Abstract	i
Declaration	v
Acknowledgements	vi
Publications	vii
Abbreviations	ix
Definition of terms	x
 CHAPTER 1. LITERATURE REVIEW	 1
1.1 Introduction	1
1.2 Superovulation and embryo collection	1
1.2.1 Methodology of superovulation	1
1.2.1.1 Gonadotrophins	1
1.2.1.2 Factors influencing the response to gonadotrophins	3
1.2.1.2(a) Dosage	3
1.2.1.2(b) Season	4
1.2.1.2(c) Nutrition	4
1.2.1.2(d) Breed	5
1.2.1.2(e) Progestagen/progesterone treatment	5
1.2.1.2(f) Repeated gonadotrophin treatment	5
1.2.1.2(g) Time of cycle	6
1.2.1.2(h) FSH:LH ratios	7
1.2.1.2(i) Age of ewe	7
1.2.1.2(j) Anti-PMSG	7
1.2.1.2(k) Immunization against ovarian steroids and proteins	8
1.2.2 Time of ovulation following gonadotrophin treatment	8
1.2.3 Intra-uterine insemination	9
1.2.4 Methodology of embryo collection	10
1.3 Embryo transfer in the ewe	12
1.3.1 Regulation of the oestrous cycle	12
1.3.2 Factors affecting synchrony of oestrus	13
1.3.2.1 Progestagen/progesterone treatment	13
1.3.2.2 Dose of PMSG	14
1.3.2.3 Exposure to rams	14
1.3.2.4 Time of year	15
1.3.2.5 Breed	15
1.3.2.6 Day of cycle	16
1.3.3 Time of ovulation following synchrony of oestrus	16
1.3.4 Embryo transfer following synchrony of oestrus	17
1.3.5 Artificial insemination following synchrony of oestrus	17

1.3.6	Methodology of embryo transfer	18
1.3.7	Factors affecting the success of embryo transfer	19
1.3.7.1	Synchrony between donor and recipient	19
1.3.7.2	Age of embryo	20
1.3.7.3	Site of transfer	20
1.3.7.4	Number of embryos transferred	21
1.3.7.5	Ovulatory response of donor	21
1.3.7.6	Ovulatory response of recipient	21
1.3.7.7	Other factors	22
1.3.8	Application of embryo transfer	22
1.4	<u>In vitro</u> culture of sheep embryos	22
1.4.1	Introduction	22
1.4.2	Culture of embryos in simple media	23
1.4.2.1	Age of embryo	24
1.4.2.2	Media	25
1.4.2.3	Gas atmosphere	25
1.4.2.4	Energy substrates	25
1.4.2.5	Protein supplementation	26
1.4.2.6	Other factors	26
1.4.3	Co-culture of ovine embryos	27
1.4.4	Short term storage of embryos	29
1.4.5	Embryo viability after culture	29
1.5	Transgenesis in domestic animals	30
1.5.1	Introduction	30
1.5.2	Development of transgenic livestock	31
1.5.3	Potential application of recombinant DNA technology	32
1.5.4	Performance of transgenic livestock	34
1.5.5	Limitations to the production of transgenic livestock	34
1.5.5.1	Supply of zygotes	35
1.5.5.2	Visualization of pronuclei	35
1.5.5.3	Viability of zygotes after microinjection	36
1.5.5.4	Culture of microinjected zygotes	37
1.5.6	Alternative techniques for the transfer of genes	37
1.5.6.1	Retroviral vectors	38
1.5.6.2	Embryonic stem cells	38
1.5.6.3	Liposome carriers	38
1.5.6.4	Autonomous replicating vectors	39
1.6	Discussion	39
CHAPTER 2.	TIME OF OVULATION IN THE SUPEROVULATED EWE	42
2.1	Experiment 1. Time of multiple ovulations in the ewe after treatment with FSH or PMSG with and without GnRH	42
2.1.1	Introduction	42
2.1.2	Materials and Methods	43
2.1.2.1	Method of superovulation	43
2.1.2.2	Experimental procedure	43
2.1.2.3	Observations	44

2.1.2.4	Effects of stress associated with repeated laparoscopy	44
2.1.2.5	Statistical analyses	45
2.1.3	Results	45
2.1.3.1	FSH and PMSG treatments	45
2.1.3.2	FSH + GnRH and PMSG + GnRH treatments	50
2.1.3.3	Optimal time of GnRH administration	50
2.1.3.4	Effects of stress	51
2.1.4	Discussion	51
2.2	Experiment 2. Seasonal variation in the time of superovulation and its relevance to the collection of zygotes	53
2.2.1	Introduction	53
2.2.2	Materials and Methods	54
2.2.2.1	Sheep	54
2.2.2.2	Method of superovulation	54
2.2.2.3	Experimental procedure	55
2.2.2.4	Statistical analyses	56
2.2.3	Results	56
2.2.3.1	Variation through the year (part 1)	56
2.2.3.2	Ovular ewes versus previously anovular ewes (part 2)	58
2.2.3.3	Time of exposure to vasectomized rams (part 3)	58
2.2.3.4	PMSG versus FSH in August (part 4)	58
2.2.3.5	Accuracy of records	62
2.2.4	Discussion	62
CHAPTER 3.	TIME OF OVULATION IN THE NONSUPEROVULATED EWE FOLLOWING SYNCHRONY OF OESTRUS AND ITS RELEVANCE TO EMBRYO TRANSFER AND ARTIFICIAL INSEMINATION	65
3.1	Experiment 1. Variation in the time of ovulation within and between flocks	65
3.1.1	Introduction	65
3.1.2	Materials and methods	66
3.1.2.1	Sheep	66
3.1.2.2	Time of ovulation: variation within and between flocks	66
3.1.2.3	Effect of different progestagens or progesterone	67
3.1.2.4	Effect of different gonadotrophin preparations	67
3.1.2.5	Effect of stress	67
3.1.2.6	Statistical analyses	68
3.1.3	Results	68
3.1.3.1	Time of ovulation: variation within and between flocks	68
3.1.3.2	Effect of different progestagens or progesterone	71
3.1.3.3	Effect of different gonadotrophin preparations	71
3.1.3.4	Effects of stress	71
3.1.4	Discussion	72
3.2	Experiment 2. The effect of GnRH treatment on the time of ovulation in the nonsuperovulated	

ewe and its use in embryo transfer and artificial insemination programs	74
3.2.1 Introduction	74
3.2.2 Materials and methods	74
3.2.2.1 Sheep	75
3.2.2.2 Hormone treatment	75
3.2.2.3 Semen collection and insemination	75
3.2.2.4 Experimental procedure	76
3.2.2.4(a) GnRH and time of insemination	76
3.2.2.4(b) GnRH and embryo quality	76
3.2.2.4(c) GnRH and embryo transfer	77
3.2.2.4(d) GnRH and the insemination of frozen- thawed semen	78
3.2.2.5 Statistical analyses	78
3.2.3 Results	78
3.2.3.1 GnRH and time of ovulation	78
3.2.3.2 GnRH and embryo quality	80
3.2.3.3 GnRH and embryo transfer	80
3.2.3.4 GnRH and insemination of frozen-thawed semen	84
3.2.4 Discussion	84
CHAPTER 4. THE USE OF SYNTHETIC GONADOTROPHIN RELEASING HORMONE TREATMENT IN THE COLLECTION OF EMBRYOS FROM THE SUPEROVULATED EWE	87
4.1 Introduction	87
4.2 Materials and methods	88
4.2.1 Sheep	88
4.2.2 Superovulation and GnRH treatment	88
4.2.3 Semen preparation and insemination	88
4.2.4 Experimental procedure	89
4.2.4.1 GnRH and embryo quality	89
4.2.4.2 GnRH and time of insemination	89
4.2.4.3 GnRH and number of spermatozoa inseminated	90
4.2.4.4 GnRH and collection of two- and four-cell embryos	90
4.2.5 Statistical analyses	91
4.3 Results	91
4.3.1 GnRH and embryo quality	91
4.3.2 GnRH and time of insemination	92
4.3.3 GnRH and number of spermatozoa inseminated	92
4.3.4 GnRH and collection of two- to four-cell embryos	97
4.4 Discussion	97
CHAPTER 5. CULTURE OF SHEEP ZYGOTES IN SYNTHETIC OVIDUCT FLUID MEDIUM	101
5.1 Introduction	101
5.2 Materials and methods	102
5.2.1 Supply of zygotes	102
5.2.2 Culture media and conditions	103
5.2.3 Examination of components of SOFM	103
5.2.3.1 Protein supplementation	103
5.2.3.2 Storage age of HIHS	104
5.2.3.3 Concentration of HIHS	104
5.2.3.4 Osmolarity	104
5.2.3.5 Potassium concentrations	105

5.2.3.5(a)	Range 1 - 11 mM	105
5.2.3.5(b)	Range 1 - 35 mM	105
5.2.3.6	Calcium concentration	105
5.2.3.7	Concentration of sodium bicarbonate and HEPES	106
5.2.4	Number of nuclei per embryo	106
5.2.5	Fragmentation of cytoplasm	107
5.2.6	Viability of zygotes after culture	107
5.2.7	Statistical analyses	108
5.3	Results	108
5.3.1	Components of SOFM	108
5.3.1.1	Protein supplementation	108
5.3.1.2	Storage age of HIHS	109
5.3.1.3	Concentration of HIHS	109
5.3.1.4	Osmolarity	110
5.3.1.5	Potassium concentrations	110
5.3.1.6	Calcium concentrations	110
5.3.1.7	Concentration of sodium bicarbonate and HEPES	111
5.3.2	Fragmentation of cytoplasm	111
5.3.3	Number of nuclei per embryo	115
5.3.4	Viability of zygotes after culture	115
5.4	Discussion	116
CHAPTER 6. PRONUCLEAR MICROINJECTION OF SHEEP ZYGOTES		134
6.1	Introduction	134
6.2	Materials and methods	135
6.2.1	Supply of zygotes	135
6.2.2	Methodology of microinjection	135
6.2.2.1	Equipment	135
6.2.2.2	Gene constructs	136
6.2.2.3	Microinjection procedures	136
6.2.2.4	Assessment of DNA incorporation	137
6.2.3	Preparation of recipient ewes	137
6.2.4	Experimental procedures	137
6.2.4.1	Visualization of pronuclei	137
6.2.4.2	Viability of microinjected zygotes <u>in vivo</u>	138
6.2.4.3	Assessment of the viability of microinjected zygotes <u>in vitro</u>	139
6.2.5	Statistical analyses	139
6.3	Results	139
6.3.1	Visualization of pronuclei	139
6.3.2	Viability of microinjected zygotes <u>in vivo</u>	140
6.3.3	Assessment of the viability of microinjected zygotes <u>in vitro</u>	141
6.4	Discussion	146
CHAPTER 7. GENERAL DISCUSSION		150
CHAPTER 8. BIBLIOGRAPHY		155

ABSTRACT

Studies reported in this thesis examined aspects of the collection, in vitro culture and microinjection of zygotes with the aim of improving the efficacy of producing transgenic sheep.

In the first series of experiments, time of ovulation was examined in groups of ewes stimulated to superovulate using either PMSG or FSH. Ovulation occurred over approximately 24h, with ewes treated with PMSG ovulating significantly ($P < 0.05$) earlier than FSH-treated ewes; median times of 54 and 60h respectively after progestagen pessary removal were obtained. Ovulation in individual ewes was most often completed within six hours, irrespective of the gonadotrophin used. Administration of GnRH 24h after pessary removal synchronized the time of ovulation during the mid-breeding season; approximately 80% of ovulations occurred between 45 and 48h after pessary removal. However, during the late breeding and early non-breeding seasons (May and August), some ewes ovulated significantly earlier than at other times of the year, resulting in asynchrony in the time of ovulation. This phenomenon was more common in previously anovular ewes induced to superovulate compared with naturally cycling ewes and was not associated with the GnRH treatment, the gonadotrophin or the time of exposure of ewes to vasectomized rams.

A second series of experiments examined the time of ovulation in nonsuperovulated ewes following treatment for synchrony of oestrus. Ovulation in all ewes occurred over approximately 24h, with a median time of 63 - 69h after pessary removal. However, in some flocks induced to ovulate in the nonbreeding season (November and December), commencement of ovulation was delayed until 69h after pessary removal compared with 57h at other times of the year. This phenomenon was associated with a high proportion of ewes that failed to ovulate. GnRH treatment synchronized the time of ovulation, irrespective of when ovulation

occurred in ewes not treated with GnRH. However, in treated ewes, there was a reduced survival of zygotes after transfer and variable pregnancy rates in ewes following fixed-time insemination using a laparoscope. GnRH treatment was also associated with premature luteal regression, additional ovulations after those induced directly by the treatment and an increase in the proportion of ova that failed to fertilize. It is concluded that the extent to which the preovulatory LH surge can be advanced with GnRH treatment, without adverse effect, is limited. This limitation is likely to restrict the use of GnRH in the control of ovulation in both recipient ewes and ewes prepared for fixed-time insemination.

The third series of experiments evaluated the effect of GnRH treatment on the efficacy of embryo collection following superovulation. It was shown that GnRH treatment combined with appropriately timed intra-uterine insemination of frozen-thawed semen, significantly improved fertilization rates and the number of embryos collected per ewe. Fertilization rates were highest when insemination occurred approximately six hours before the expected median time of ovulation; insemination with 20×10^6 motile spermatozoa per uterine horn proved satisfactory. GnRH treatment also improved fertilization rates in ewes that were naturally mated but fewer embryos per ewe were recovered compared with intra-uterine insemination of frozen-thawed semen. The timing of GnRH treatment was also important with the proportion of unfertilized ova being significantly ($P < 0.05$) increased in FSH-treated ewes when GnRH was administered at 24 compared with 36h after pessary removal. Administration of GnRH at 24h had no adverse effect in ewes treated with PMSG. It is concluded that the use of GnRH, using appropriate treatment protocols, will increase the supply of zygotes for microinjection programs and also improve the reliability of commercial embryo collection procedures.

The fourth series of experiments examined the feasibility of developing an in vitro culture system capable of supporting the growth of the zygote to the blastocyst stage. The medium used was synthetic oviduct fluid medium (SOFM). Components of the medium that influenced zygote development were protein source, concentration of serum and the concentration of HEPES; potassium concentrations were unimportant except at unphysiological levels, while calcium concentrations, storage age of the serum and osmolarity of the medium were also unimportant. The preferred medium contained 20% heat inactivated human serum without HEPES. In this medium, 85.2% of zygotes developed to the blastocyst stage and 74.1% commenced hatching. However, most zygotes underwent mild anuclear fragmentation during the first 24h of culture and some blastocysts that developed were deficient in nuclei number (range 20 - 300). Viability of embryos cultured from zygotes was assessed following transfer to recipient ewes. A similar percentage of zygotes developed to the elongated blastodermic vesicle stage (range 88.5 - 94.1%) following either immediate transfer or in vitro culture for one or three days. Extending the culture period to five days resulted in a decline in viability with only 45.5 - 67.7% of zygotes developing into elongated conceptuses after transfer. It is concluded that the culture system described is suitable for the culture of zygotes for up to three days but that further refinement of the medium is required to prevent a decline in viability with extended periods of culture.

The fifth and final series of experiments examined factors likely to affect the success of gene transfer by pronuclear microinjection, including visibility of pronuclei, zygote viability after microinjection and the ability to culture microinjected zygotes in vitro. Time of collection and the gonadotrophin used for superovulation influenced the proportion of zygotes with visible pronuclei. Centrifugation was not worthwhile. Microinjection of zygotes with the porcine growth hormone

gene construct (pHMPG) resulted in 45.1% of the zygotes being nonviable within three days while 12.5% of the zygotes resulted in the production of lambs, none of which were transgenic. Studies on in vitro culture involved zygotes microinjected with the construct containing the Cys E and Cys M genes from Salmonella typhimurium (pGER003). There were no significant differences in the viability of microinjected zygotes cultured in SOFM for either one or three days compared with zygotes that were transferred immediately after microinjection; between 32.2 - 50.0% of zygotes developed into elongated conceptuses by day 14 of pregnancy. In vitro culture enabled (1) the identification of zygotes that lysed or otherwise failed to divide and (2) the identification of embryos more likely to develop after transfer. However, fragmentation of embryos during culture limited the assessment of embryo quality. Despite the need for further refinement of the culture medium, it is concluded that the viability of microinjected zygotes can be accurately assessed in the culture system described, thus enhancing the efficacy of transgenesis programs.

DECLARATION

The experimental work described in this thesis was conducted either in the Department of Obstetrics and Gynaecology, University of Adelaide or at Turretfield Research Centre, Department of Agriculture, South Australia. The data presented are from original studies by the author and any input from colleagues is duly acknowledged. This dissertation has not previously been submitted, wholly or in part, to any other University for any degree or diploma. I consent to this thesis being made available for photocopying and loan if accepted for the award of the degree.

Simon Kerry Walker

ACKNOWLEDGEMENTS

I express sincere gratitude to Dr R.F. Seamark for supporting the experimental work described in this thesis and to Professors J.S. Robinson and C.D. Matthews for their support during the second half of the study.

Many members of the Department of Obstetrics and Gynaecology, University of Adelaide assisted during the study. Dr R.J. Ashman conducted numerous laparotomies using the excellent facilities and support provided by Mr K. Porter and his staff. Drs G.M. Warnes and P. Quinn instilled an investigative enthusiasm for the problems of embryo collection and in vitro culture of embryos. Ms B.M. Godfrey assisted in the analysis of data and Dr S.P. Flaherty and Mr M. Guerin provided invaluable guidance on the photography of embryos and the use of computer graphics respectively. The studies on embryo culture were conducted in the IVF Laboratory, The Queen Elizabeth Hospital and the support and friendliness of the staff is valued. Similarly, the preparedness of the nursing staff of the Reproductive Medicine Unit to collect blood samples for the studies on embryo culture is acknowledged. Professor G.E. Rogers and Dr J.R.E. Wells and their staff of the Department of Biochemistry, University of Adelaide provided the gene constructs and conducted the DNA analysis while Mr K.J. McLaughlin and Mr P.J. Verma microinjected many zygotes.

This study would not have been possible without the assistance of the executive of the South Australian Department of Agriculture and the staff of Turretfield Research Centre. In particular, Mr D.H. Smith contributed significantly to study and Mr P. Ancell, Mr R.J. Lampe and Ms T. M. Heard were invaluable assistants. Many other people contributed in many ways. This thesis is dedicated to Alison who provided the greatest support.

PUBLICATIONS ARISING FROM INVESTIGATIONS REPORTED IN THIS THESIS

1. Walker, S.K., Smith, D.H. & Seamark, R.F. (1986). Timing of multiple ovulations in the ewe after treatment with FSH or PMSG with and without GnRH. J. Reprod. Fert. 77, 135-142.
2. Walker, S.K., Smith, D.H., Seamark, R.F. and Godfrey, B. (1987). Variation in the timing of multiple ovulations following gonadotropin releasing hormone treatment and its relevance to the collection of pronuclear embryos of sheep. Theriogenology 28, 129-137.
3. Walker, S.K., Seamark, R.F., Quinn, P., Warnes, G.M., Ashman, R.J., Smith, D.H. and Ancell, P. (1988). Culture of pronuclear embryos of sheep in a simple medium. Proceedings 11th International Congress Animal Reproduction and Artificial Insemination 4, 483-485, Dublin, Ireland.
4. Walker, S.K., Smith, D.H., Godfrey, B. and Seamark, R.F. (1989). Time of ovulation in the South Australian Merino ewe following synchronization of oestrus. 1. Variation within and between flocks. Theriogenology 31, 545-553.
5. Walker, S.K., Smith, D.H., Ancell, P. and Seamark, R.F. (1989). Time of ovulation in the South Australian Merino ewe following synchronization of oestrus. 2. Efficacy of GnRH treatment and its relevance to insemination programs utilizing frozen-thawed semen. Theriogenology 31, 555-564.
6. Walker, S.K., Smith, D.H., Frensham, A., Ashman, R.J. and Seamark, R.F. (1989). The use of synthetic gonadotropin releasing hormone in the collection of embryos of sheep. Theriogenology 31, 741-752.
7. Walker, S.K., Lampe, R.J. and Seamark, R.F. (1989). Culture of sheep zygotes in synthetic oviduct fluid medium with different concentrations of sodium bicarbonate and HEPES. Theriogenology (submitted).
8. Walker, S.K., Verma, P.J., Heard, T.M. and Seamark, R.F. (1989). Assessment of the viability of sheep zygotes in synthetic oviduct fluid medium following pronuclear microinjection. Biol. Reprod. (in preparation).
9. Walker, S.K., Quinn, P., Ashman, R.J. and Smith, D.H. (1985). Visualization of pronuclei and culture of one-cell embryos of sheep. Proc. Aust. Soc. Reprod. Biol. 17, 32 (Abst.).
10. Walker, S.K., Quinn, P., Ashman, R.J., Smith, D.H. and Seamark, R.F. (1986). Protein supplementation for the culture of one-cell embryos of sheep. Proc. Aust. Soc. Reprod. Biol. 18, 9 (Abst.).
11. Smith, D.H., Walker, S.K. and Seamark, R.F. (1986). Synchronization of timing of ovulation in the artificial insemination of sheep. Proc. Aust. Soc. Reprod. Biol. 18, 16 (Abst.).
12. Walker, S.K., Ashman, R.J., McLaughlin, K.J., Seamark, R.F., Smith, D.H., Vize, P. and Wells, J.R.E. (1987). Attempts to produce transgenic sheep using a proven gene construct. Proc. Aust. Soc. Reprod. Biol. 19, 52 (Abst.).

13. Walker, S.K., Smith, D.H. and Seamark, R.F. (1987). Timing of GnRH treatment and embryo quality in the Merino. Proc. Aust. Soc. Reprod. Biol. 19, 64 (Abst.).
14. Smith, D.H., Walker, S.K., Godfrey, B. and Seamark, R.F. (1987). Timing of ovulation following synchrony of oestrus in the nonsuperovulated Merino. Proc. Aust. Soc. Reprod. Biol. 19, 63 (Abst.).
15. Walker, S.K., Seamark, R.F., Quinn, P., Warnes, G.M., Ashman, R.J., Smith, D.H. and Ancell, P. (1988). Viability of pronuclear embryos of sheep after culture in vitro for one, three or five days. Proc. Aust. Soc. Reprod. Biol. 20, 49 (Abst.).
16. Walker, S.K., Verma, P.J., Heard, T.M., Matthews, C.D., Rogers, G.E. and Seamark, R.F. (1989). In vitro assessment of sheep zygotes following pronuclear microinjection. Proc. Aust. Soc. Reprod. Biol. 21, (in press).

ABBREVIATIONS

Chemicals

BSA	bovine serum albumin
CO ₂	carbon dioxide
DNA	deoxyribonucleic acid
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
N ₂	nitrogen
O ₂	oxygen
PBS	phosphate buffered saline

Hormones

FGA	flugestone acetate
FSH	follicle stimulating hormone
GH	growth hormone
GnRH	gonadotrophin releasing hormone
HAP	horse anterior pituitary extract
HCG	human chorionic gonadotrophin
LH	luteinizing hormone
LHRH	luteinizing hormone releasing hormone
MAP	medroxy-progesterone acetate
PMSG	pregnant mare serum gonadotrophin

Symbols and Units

C	centigrade
g	gram (s)
h	hour (s)
i.u.	international unit (s)
mg	milligram (s)
ml	millilitre (s)
mM	millimolar
mm	millimetre (s)
mOsm	milliosmolar
pl	picolitre (s)
ug	microgram (s)
ul	microlitre (s)
um	micrometre (s)

Others

B	early blastocyst
BMOc	Brinster's medium for ovum culture
CIDR	controlled internal drug release device
EB	expanded blastocyst
HB	hatching blastocyst
HF-10	Ham's F-10 culture medium
HIHS	heat inactivated human serum
HISS	heat inactivated sheep serum
i.m.	intra-muscular
IUI	intra-uterine insemination
i.v.	intra-venous
IVF	<u>in vitro</u> fertilization
MEM	minimum essential medium
N	number
NM	natural mating
pGER003	cysteine gene construct
pHMPG	porcine growth hormone gene construct

PN	pronuclei
s.e.m.	standard error of mean value
SOEM	synthetic oviduct fluid medium
TCM-199	tissue culture medium-199

DEFINITION OF TERMS

Oocyte (s) - (Gk. *oon*, egg; *kytos*, hollow). An ovum before formation of the first polar body.

Ovum (ova) - (L. *ovum*, egg). A female gamete.

Zygote (s) - (Gk. *zygotos*, yoked). The cell formed by the union of two gametes; a fertilized ovum containing a male and female pronucleus.

Embryo (s) - (Gk. *embryon*, embryo). The zygote after first cell division; contains two or more blastomeres.

Corpus luteum (corpora lutea). The glandular body developed from a Graafian follicle after extrusion of the ovum. For simplicity, corpus luteum is used synonymously with corpus haemorrhagicum (the body formed from the ruptured Graafian follicle and the blood clot).

CHAPTER 1

LITERATURE REVIEW



1.1 INTRODUCTION

The success of quantitative genetic selection of economically important traits in domestic animals is limited by the heritability of the particular trait. Heritability estimates for fleece weight in the Australian Merino average 0.3 (Mortimer, 1987), while estimates for fertility (ewes pregnant or lambing of ewes joined) and body weight in various breeds vary between 0.0 - 0.17 and 0.0 - 0.7 respectively (Turner and Young, 1969; Purvis, Atkins and Piper, 1987). Responses through genetic selection of up to 1 - 2% of the mean value per year are possible in some traits (Atkins, 1987).

The ability to alter genotype by transfer of cloned genes through pronuclear microinjection (Palmiter et al., 1982) offers a route to more rapid and feasibly much larger improvements in animal productivity. However, development of this technology has numerous limitations. This thesis examines constraints associated with the collection and handling of sheep zygotes and with the technique of pronuclear microinjection. The following topics are reviewed (1) superovulation and embryo collection, (2) embryo transfer in the ewe, (3) in vitro culture of sheep embryos and (4) transgenesis in domestic animals. The review is not exhaustive but aims to summarize significant developments in the relevant areas as well as being a useful reference of published information.

1.2 SUPEROVULATION AND EMBRYO COLLECTION

1.2.1 Methodology of Superovulation

1.2.1.1 Gonadotrophins

The two commonly used gonadotrophins for superovulation are pregnant mare serum gonadotrophin (PMSG) and follicle stimulating hormone (FSH) of pituitary origin. These gonadotrophins are used in conjunction with either a progestagen/progesterone or prostaglandin treatment for regulation of the oestrous cycle (see Gordon, 1983). PMSG is a glycoprotein of two dissimilar subunits and has the biological activity of both FSH and luteinizing hormone (LH; see Papkoff, 1978, 1981). FSH may be a partly purified preparation obtained from animal pituitaries or a crude extract from horse pituitaries (HAP; Moore and Shelton, 1964a; Boland and Gordon, 1977, 1982; Boland, Crosby and Gordon, 1983). The PMSG molecule contains a high level of sialic acid (10.8%; Papkoff, 1978) consistent with its long half-life of approximately 21h in the sheep (McIntosh, Moor and Allen, 1975), whereas ovine FSH has a half-life of approximately 2h (Akbar, Nett and Niswender, 1974). Plasma concentrations of PMSG reach peak values approximately 35h after injection (Meinecke-Tillman, Evers and Meincke, 1987).

PMSG treatment is associated with abnormal steroidogenic activity of follicles (Moor, Osborn and Crosby, 1985) and the development of follicles that fail to ovulate (Shelton and Moore, 1967; Quirke and Hanrahan, 1975; Boland and Gordon, 1977; Armstrong and Evans, 1983). Treatment has also been associated with abnormal embryo transport (Armstrong, Miller, Walton, Pfitzner and Warnes, 1982), low embryo survival (Wintenberger-Torres, 1967, cited by Torres, Cognie and Colas, 1987) and premature regression of corpora lutea (Armstrong et al., 1982). Field studies indicate that FSH is the preferred gonadotrophin (Shelton and Moore, 1967; Boland and Gordon, 1978; Armstrong and Evans, 1983; Evans, Holland, Nottle, Sharpe and Armstrong, 1984; Nottle, Armstrong, Holland and Sharpe, 1984; Nancarrow, Marshall, Wilmut, Hazelton and Murray, 1987a). In these studies, FSH treatment increased the number of

ovulations per ewe, the number of ova recovered relative to the number of corpora lutea and the number of embryos obtained per ewe compared with PMSG treatment. However, Nancarrow et al. (1987a) concluded that PMSG treatment was more cost effective than FSH treatment.

1.2.1.2 Factors Influencing the Response to Gonadotrophins

(a) Dosage

Determination of an optimal dose of gonadotrophin is difficult because of the influences of a variety of genetic and environmental factors. A dose-response relationship with PMSG has been obtained by Robinson (1951; dosage 500 - 2000 i.u. per ewe), Holst (1969; 250 - 1250 i.u. per ewe), Evans and Robinson (1980; 0 - 1600 i.u. per ewe), Gherardi and Lindsay (1980; 400 - 1000 i.u. per ewe) and Mutiga and Baker (1982; 1000 - 2000 i.u. per ewe). Gordon (1983) concluded that a dose-response relationship existed in cyclic ewes and that 2000 i.u. per ewe was the upper limit. Dose-responses have also been reported in Merino strains selected for prolificacy (Bindon, Ch'ang and Turner, 1971; Bindon and Piper, 1982). However, an increase in the dose of PMSG was associated with an increase in the number of unovulated follicles (Robinson, 1951; Van Rensberg, 1964; Shelton and Moore, 1967; Holst, 1969; Evans and Robinson, 1980; Mutiga and Baker, 1982). Bindon and Piper (1982) concluded that the administration of PMSG on a live weight basis was not worthwhile.

The effect of dose of FSH on ovulatory performance may vary with the injection regime (constant or decreasing dose per injection). Eppleston, Bilton and Moore (1984) found no effect of starting dose (4 v. 6 v. 9 mg), total dose (range 10 - 36 mg) or injection regime. Dose and the injection regime were significant factors in the study of Torres et al. (1987) with the high dose and decreasing regime preferred. Cognie, Chupin and Saumande (1985) obtained a superior ovulatory response with a

decreasing compared with a constant regime during the breeding season but found no effect in the nonbreeding season. Smith (1984) concluded that the optimal dose was between 19.5 - 26 mg per ewe.

(b) Season

Gherardi and Lindsay (1980), using 400 - 1000 i.u. of PMSG per ewe, reported significant variation in the ovulatory response during the year; the poorest response occurred in Spring (September) and the best response in Autumn (March). Holst (1969) examined responses to doses between 250 - 1250 i.u. per ewe and also concluded that time of season significantly influenced the ovulatory response. At low doses, the poorer responses were obtained during the anoestrous season compared with the breeding season whereas the reverse occurred at high doses. The findings of the studies of Braden, Lamond and Radford (1960) and Hulet and Foote (1967, 1969) were also indicative of seasonal variation in the ovulatory response to PMSG. With FSH, the ovulatory response was higher during the breeding season compared with the nonbreeding season (Armstrong and Evans, 1984a; Torres et al., 1987).

(c) Nutrition

A positive effect of nutrition on the ovulatory response to PMSG was demonstrated using doses of up to 1200 i.u. per ewe (Hedges and Reardon, 1973; Allison, 1975). In ewes treated with low doses of PMSG during the nonbreeding season, restrictions in feeding decreased the ovulatory response compared with unrestricted feeding (Keane, 1973, 1975). The nutritional status did not influence the ovulatory response to PMSG in other studies (Allen and Lamming, 1961a, 1961b; Bellows, Pope, Meyer, Chapman and Casida, 1963; Tait, 1971; Eastwood and McDonald, 1975).

(d) Breed

Robinson (1951) concluded that breed was not an important factor influencing ovulatory response to PMSG. However, significant breed effects have been obtained (Laster and Glimp, 1974; Eastwood and McDonald 1975). Superior responses to PMSG have occurred in fecund strains (Bindon et al., 1971; Trounson and Moore, 1972; Smith, 1976) and breeds (Bradford, Quirke and Hart, 1971) compared with less prolific sheep. Furthermore, Booroola Merinos with the F gene were more responsive than ewes without the gene (Piper, Bindon, Curtis, Cheers and Nethery, 1982; Kelly, Owens, Crosbie, McNatty and Hudson, 1983).

Breed differences in the response to FSH were reported by Torres et al. (1987) with the best response obtained in the highly prolific Romanov x Prealpes breed. Armstrong and Evans (1983) obtained a higher response in Merino ewes compared with Suffolk and Romney Marsh ewes; these authors also reported substantial differences in the ovulatory response between Suffolk flocks.

(e) Progestagen/Progesterone Treatment

Boland et al. (1983) compared the ovulatory response to PMSG following treatment with either 500 mg progesterone, 30 mg flugestone acetate (FGA) or 12% progesterone in Controlled Internal Drug Release Device (CIDR). Treatment significantly influenced ovulation rate with FGA being the preferred treatment. Results of studies using low doses of PMSG are summarized in Chapter 1.3.2.1.

(f) Repeated Gonadotrophin Treatment

The ovulatory response of sheep injected more than once with PMSG was decreased (Pigon, Clegg and Cole, 1960; Hulet and Foote, 1969; Clarke, 1973). This may have been due to the depletion of follicular reserves (Edwards and Fowler, 1960) or the production of antibodies or antigonadotrophic factors (Greenwald, 1963; Land and McLaren, 1967; Hulet and Foote, 1969). However, Gherardi and Martin (1978) and Gherardi and Lindsay (1980) found no refractoriness in sheep injected on up to five occasion. Lynch (1968, cited by Boland and Gordon, 1982) also obtained a satisfactory superovulation following treatment with PMSG three times within nine months.

Armstrong and Evans (1984a) concluded that the ovulatory response following repeated FSH treatment was highest at the second treatment compared with other treatments. In the study of Boland and Gordon (1982), the best response with HAP was obtained when four months elapsed between treatments. Moore and Shelton (1962) successfully induced superovulation in the same sheep at 12-month intervals using HAP.

(g) Time of Cycle

A similar number of follicles were stimulated by PMSG treatment when injected at different times of the luteal phase (Hay and Moor, 1975). Mutiga and Baker (1982) obtained a similar ovulatory response when PMSG was administered either during the luteal phase or after progestagen treatment. Injection of PMSG on days two or three of the cycle increased (Cahill and Dufour, 1979) or had no effect (Bindon and Piper, 1982) on the subsequent ovulatory response.

The time of commencement of FSH treatment in the study of Smith (1984) did not influence the ovulatory response. Administration of a priming dose of FSH preceding treatment for superovulation did not influence the ovulatory response (Ralph, Seamark and Geytenbeek, 1984;

Ware, Northey, Boland and First, 1988) although in the latter study, treatment was associated with an increase in the number of transferable embryos.

(h) FSH:LH Ratios

Commercial batches of FSH obtained from animal pituitaries varied widely in the FSH:LH ratio (range 0.20 - 4.42; Lindsell et al., 1986). Armstrong and Evans (1984a) reported that equal doses of FSH from preparations of different LH:FSH ratios (range 0.008 - 0.205) produced ovulatory responses inversely related to the LH activity. Furthermore, decreasing the FSH:LH ratio towards the end of the treatment period improved the ovulatory response compared with treatment with preparations containing equal amounts of FSH and LH (Cognie, Chupin and Saumande, 1986). Similar treatment in the nonbreeding season increased the number of ewes ovulating. Treatment with exogenous LH was of no benefit (Wright, Bondioli, Grammer, Kuzan and Menino, 1981).

(i) Age of Ewe

Torres et al. (1987) obtained a better ovulatory response to FSH treatment in six-year-old ewes compared with younger ewes. Superovulatory responses in prepubertal and pubertal lambs were variable (Trounson, Willadsen and Moor, 1976; Armstrong and Evans, 1983).

(j) Anti-PMSG

Treatment with antibodies to PMSG reduced the superovulatory response, depending on the interval between the injections of PMSG and anti-serum (Bindon and Piper, 1977). In cattle, treatment reduced the number of

large follicles and excessive oestrogen production (Kummer, Zrally, Veznik, Schlegelova and Hruska, 1980) but approximately doubled the ovulatory response in the study of Dieleman and Bevers (1987).

(k) Immunization Against Ovarian Steroids and Proteins

Immunization against ovarian steroids (Scaramuzzi, Davidson and Van Look, 1977) enhanced the ovulatory response to PMSG (Scaramuzzi and Hoskinson, 1984). Immunization against inhibin-enriched preparations from bovine follicular fluid increased ovulation rate without additional gonadotrophin treatment (O'Shea, Cummins, Bindon and Findlay, 1982; O'Shea et al., 1984; Henderson, Franchimont, Leconte-Yerna, Hudson and Ball, 1984). The ovulatory response to PMSG was enhanced when treatment was combined with the 'rebound' effect in FSH secretion after immunization (Cummins, 1983). Other protein hormones produced by the granulosa cells can inhibit the ovarian response to PMSG (Cahill, 1984); elimination of local effects by immunization may enhance the response to exogenous gonadotrophins.

1.2.2 Time of Ovulation Following Gonadotrophin Treatment

Treatment with either PMSG (Killeen and Moore, 1970) or FSH (Evans and Armstrong, 1984) advanced the time of ovulation compared with control ewes. Evans et al. (1984) reported that most ewes treated with FSH ovulated between 54 - 60h after pessary removal but comparable data for ewes treated with PMSG are not available.

Control of the time of ovulation using human chorionic gonadotrophin (HCG) was demonstrated by Ortavant, Thibault and Wintenberger (1949). The efficacy of the treatment in more recent studies is difficult to assess because of different treatment protocols (e.g. time of treatment relative to progestagen treatment or onset of oestrus). However,

evidence of control was reported by Braden et al. (1960), Lamond (1962), Dziuk, Hinds, Mansfield and Baker (1964), McGovern, Williams and Hancock (1969) and McGovern and Laing (1976). HCG treatment had no effect on the time of ovulation in the study of Killeen and Moore (1970) although it was associated with an increase in the proportion of follicles that ruptured. An improved ovulatory response following HCG treatment was also reported by Alwan, Boland and Gordon (1988) but Quirke and Hanrahan (1975) and Bindon (1975) obtained no evidence of such an effect. Onset of oestrus was not inhibited or delayed by HCG treatment (Braden et al., 1960; McGovern et al., 1969).

Time of ovulation was also controlled by treatment with luteinizing hormone releasing factor (LHRF; McGovern and Laing, 1976). Treatment with synthetic gonadotrophin releasing hormone (GnRH) improved embryo yield compared with control ewes (Nancarrow, Murray, Boland, Sutton and Hazelton, 1984a) but the effect of treatment on time of ovulation was not studied.

1.2.3 Intra-Uterine Insemination

Fertilization failure is a major physiological limitation to the collection of sheep embryos (Betteridge and Moore, 1977; Evans and Armstrong, 1984; Evans et al., 1984). This problem was attributed to ovarian hyperstimulation (Van Rensburg, 1964; Armstrong et al., 1982) associated with a reduction in the population of viable spermatozoa within the oviducts (Hawk, Cooper and Conley, 1987). Both the method of control of oestrus (progestagen v. prostaglandin) and the gonadotrophin used for superovulation (FSH v. PMSG) influenced the number of spermatozoa flushed from the reproductive tract (Evans and Armstrong, 1984). However, the effect of the superovulation treatment was not as severe as the effect of the treatment for regulation of oestrus (Hawk et al., 1987).

Intra-uterine insemination using the laparoscope was first assessed in sheep by Killeen and Caffrey (1982) and has since been evaluated in the superovulated ewe (Armstrong and Evans, 1984b; McKelvey, Robinson and Aitken, 1985). The technique is particularly relevant to the insemination of frozen-thawed semen; decreased fertility of frozen-thawed ram semen is well documented (Mattner, Entwistle and Martin, 1969; Lightfoot and Salamon, 1970; Langford et al., 1979). Low fertilization rates associated with either cervical insemination of frozen-thawed semen (Armstrong and Evans, 1984b) or the use of PMSG compared with FSH treatment (Evans et al., 1984) were partly or wholly overcome by intra-uterine insemination. However, there were no differences in fertilization rates following either cervical and intra-uterine insemination of fresh semen (Armstrong and Evans, 1984b; McKelvey et al., 1985).

Neither the minimum number of spermatozoa required for fertilization following intra-uterine insemination nor the optimal time of insemination have been satisfactorily determined in the superovulated ewe. Walker et al. (1984) compared the insemination of one million and ten million motile frozen-thawed spermatozoa per uterine horn and concluded that both doses were probably below that required for maximum fertilization rates. In the study of Davis, Kerton, McPhee, Grant and Cahill (1984), the insemination of 12.5 million fresh spermatozoa per ewe was as effective as 100 million. In the non-superovulated ewe following synchrony of oestrus, Maxwell (1986b) concluded that a minimum of 20 million frozen-thawed spermatozoa per ewe were required. Time of insemination (48 - 60h after pessary removal) did not significantly influence pregnancy rates (Maxwell, Butler and Wilson, 1984; Eppleston and Roberts, 1986). Site of insemination (body of uterus v. tip of horn) did not influence either ovum recovery or fertilization rate (Trounson and Moore, 1974a).

1.2.4 Methodology of Embryo Collection

Embryos located in the uterine lumen may be collected either by cannulation of the oviducts (Hunter, Adams and Rowson, 1955) or by cannulation of the uterine horn using a paediatric Foley catheter (Tervit and Havik, 1976; Armstrong, Pzitzner, Warnes and Seamark, 1983). Embryos in the oviducts may also be collected by cannulation.

The recovery of embryos, relative to the number of corpora lutea observed, has varied between 30.5 - 85.0% (Hunter et al., 1955; Hancock and Hovell, 1961; Tervit and Havik, 1976; Mutiga and Baker, 1982; Boland et al., 1983; Armstrong and Evans, 1984b; McKelvey et al., 1985; Torres et al., 1987). Intra-uterine insemination lowered the recovery rate compared with cervical insemination (Trounson and Moore, 1974a; Armstrong and Evans, 1984b; McKelvey et al., 1985). This may have resulted from either mechanical failure of the fimbriae or premature rupture of follicles (McKelvey et al., 1985) or from expulsion of ova from within the reproductive tract (Killeen and Moore, 1971). Boland, Hazelton, Murray, Sutton and Nancarrow (1984) concluded that disturbance of the reproductive tract during surgical insemination did not lower recovery rates. Embryo recovery was lower in ewes treated with PMSG compared with FSH (Nottle et al., 1984; Nancarrow et al., 1987a) and in ewes treated with either GnRH or HCG compared with control ewes (Quirke and Hanrahan, 1975). Low recoveries occurred in ewes with a longer than normal interval between pessary removal and the onset of oestrus (Torres et al., 1987).

The number of collections per animal was limited by the development of adhesions associated with surgery (Tervit and Havik, 1976; Elsdon, 1977). Attempts to overcome this problem have included implanting a permanent cannula in the reproductive tract (Mutiga, Baker and Jillella, 1983; Ball, Simkin and Foote, 1978), placing a catheter into the uterine lumen using the laparoscope (McKelvey and Robinson, 1984a; McKelvey,

Robinson, Aitken and Robertson, 1986) and passing a catheter through the cervix (Capehart, Bowen, Bassett, Shelton and Kraemer, 1984; Coonrod, Coren, McBride, Bowen and Kraemer, 1986). McKelvey and co-workers recovered ova from ewes on three consecutive occasions using the laparoscope (recovery rates of 35 - 76%) without the development of significant adhesions.

1.3 EMBRYO TRANSFER IN THE EWE

1.3.1 Regulation of the Oestrous Cycle

Oestrous cycles of donor and recipient ewes must be closely synchronized to maximize survival of transferred embryos (Hunter et al., 1955; Averill and Rowson, 1958; Moore, Rowson and Short, 1960; Hancock and Hovell, 1961; Moore and Shelton, 1964b; Rowson and Moor, 1966). This may be obtained by progestagen treatment combined with an injection of PMSG (e.g. Tervit and Havik 1976; Crosby, Boland, El-Kamali and Gordon, 1980), by appropriate selection of oestrous ewes from a cycling flock (e.g. Moore et al., 1960; Moore and Shelton, 1962; Moore and Shelton, 1964b) or by treatment with a synthetic prostaglandin analogue (e.g. Trounson et al., 1976; Acritopoulou, Haresign and Lamming, 1978).

Progestagen treatment using a polyurethane intravaginal pessary (Robinson, 1965) is the most common method of regulation of the oestrous cycle (see Gordon, 1983). The percentage of ewes induced into oestrus following treatment with FGA has varied between 65.7 - 100.0% (Robinson, Fraser and McHattie, 1975; Boland, Kelleher and Gordon, 1978; Quirke, Hanrahan and Gosling, 1981; Quirke and Gosling, 1981; Ainsworth and Wolynetz, 1982; Ainsworth and Shrestha, 1983; Ainsworth and Downey, 1986; Maxwell, 1986a, 1986b) and between 77.0 - 100.0% following treatment with 60 mg medroxy-progesterone acetate (MAP; Boland et al., 1978; Quirke, Jennings, Hanrahan and Gosling, 1979; Quirke and Gosling, 1981; Ainsworth

and Shrestha, 1983; Beck, Davies, Davies and Lees, 1987). Oestrus occurred approximately 36h after treatment (Signoret and Cognie, 1975; Boland et al., 1978; Quirke et al., 1979) and the preovulatory LH surge commenced between 34.5 - 45.5h after pessary removal (Signoret and Cognie, 1975; Quirke et al., 1981; Ainsworth, Lachance and Labrie, 1983). Ovulation was estimated to occur 27.4 ± 0.3 h after the onset of oestrus (Holst and Braden, 1972).

Trounson et al. (1976) reported that 84% of ewes were in oestrus within 29 - 48h of a single injection of cloprostenol; Haresign (1976) and Acritopoulou et al. (1978) obtained a mean interval from treatment to onset of oestrus of approximately 37h. Concurrent treatment with PMSG increased the proportion of ewes in oestrus within 36h (Trounson et al., 1976). Fairnie, Wales and Gherardi (1977) estimated that ovulation occurred within 67 - 96h of treatment; a mean figure of 73.1 ± 1.6 h was obtained by Acritopoulou et al. (1978). The combined use of progestagen pessaries and prostaglandin treatment has also been examined (Fitzgerald, Ruggles, Stellflug and Hansel, 1985; Allison and Kelly, 1978).

1.3.2 Factors Affecting Synchrony of Oestrus

1.3.2.1 Progestagen/Progesterone Treatment

FGA and MAP were equally effective in synchronizing oestrus in some studies (Gordon, 1975a; Smith, Boland and Gordon, 1978). In other studies, oestrus and ovulatory responses were lower in ewes treated with MAP compared with FGA (Boland et al., 1978; Alifakiotis, Michailidis and Gavrilidis, 1982). Pregnancy rates were similar in ewes treated with either progestagen (Gordon, 1975a). More pessaries containing MAP were lost during treatment compared with FGA-pessaries (Alifakiotis et al., 1982; Ainsworth and Shrestha, 1983). Progestagens have also been successfully administered in subcutaneous ear implants (Dziuk, Cook,

Kaltenbach and Niswender, 1966; Martin, Cognie, Espinosa and Juaristi, 1976; Boland et al., 1978; Ainsworth and Wolynetz, 1982; Ruttle et al., 1988).

Treatment with a CIDR containing 9 - 12% progesterone resulted in a highly synchronized oestrus (Welch, Andrewes, Barnes, Bremner and Harvey, 1984; Harvey, Johnson, Tervit and Welch, 1984; McMillan, 1987; Smith, Cruickshank, McGowan, Parr and Mortimer, 1988). Plasma progesterone concentrations reached maximum levels within four days of treatment and then gradually declined to day 13 (Hamra, Massri, Marcek and Wheaton, 1986; Barnes, 1987). The oestrus and pregnancy rates following CIDR treatment were similar to those following treatment with either FGA or MAP (Crosby, Boland and Gordon, 1983; Ainsworth and Downey, 1986; Maxwell and Barnes, 1986; Rhodes and Nathanielsz, 1988).

1.3.2.2 Dose of PMSG

Although the oestrous cycle can be regulated with progestagen/progesterone treatment alone (Gordon, 1975b; Quirke, 1979; Vipond and King, 1979; Spitzer and Carpenter, 1981), additional treatment with PMSG is preferred (Robinson, 1956, 1961; Gordon, 1971a). Significant improvements in the number of ewes in oestrus following combined treatment were obtained (Boland et al., 1978, 1981) but the effect of dose of PMSG was equivocal (Keane, 1975; Boland et al., 1978; Boland et al., 1981). Similarly, the effect of dose of PMSG on lambing performance was inconsistent (Gordon, 1971b; Eastwood and McDonald, 1975; Hackett, 1982; Ainsworth and Shrestha, 1985).

1.3.2.3 Exposure to Rams

The percentage of ewes conceiving was significantly greater in ewes first exposed to rams 48h after pessary removal compared with other times, even

though the time of onset of oestrus was unaffected (Boland and Gordon, 1979). Similar results were reported by Gordon (1971a), Joyce (1972) and Bryant and Tomkins (1976).

1.3.2.4 Time of Year

The interval between pessary removal and onset of oestrus was shorter in the breeding season compared with the nonbreeding season (Holst, 1969; Boshoff, van Niekerk and Morgenthal, 1973; Evans and Robinson, 1980; Faure, Boshoff and Burger, 1983; Smith et al., 1988). The ovulatory response following treatment also varied with time of year, with the lowest response in spring and the highest in autumn (Gherardi and Lindsay, 1980). Time of treatment within the breeding season appeared not to influence either the duration of oestrus or the time of ovulation (Van der Westhuysen, van Niekerk and Hunter, 1970).

Treatment during the anoestrous season resulted in low and variable lambing results (Gordon, 1963; Roberts and Edgar, 1966; Hunter, 1968; Hulet and Stormshak, 1972; Laster and Glimp, 1974; Christensen, 1976; Langford, Marcus and Batra, 1983). However, the percentage of ewes in oestrus was generally high following such treatment (Gordon, 1963; Moore and Holst, 1967; Lunstra and Christensen, 1981). Lunstra and Christensen (1981) concluded that anovulation was not a cause of reduced conception rates during the anoestrous season.

1.3.2.5 Breed

Significant differences among breeds in their response to treatment have been reported by Laster and Glimp (1974) and Shrestha, Ainsworth and Heaney (1983). The latter authors concluded that a large year-to-year variation existed in these differences. Quirke (1979) found that oestrus

was synchronized irrespective of breed but that lambing performance was variable and breed dependent.

1.3.2.6 Day of Cycle

Day of pessary insertion had no effect on synchrony of oestrus or ewe fertility but litter size was highest if treatment commenced between days 1 - 7 compared with days 7 - 14 of the cycle (Willemse, 1968). In the study of Woody et al. (1983), the percentage of ewes in oestrus was significantly lower if treatment commenced on day 13 compared with day 4. Roberts (1966) obtained a superior synchrony of oestrus following a 13-day treatment compared with either 15 or 17 days.

A reduced ovulatory and oestrous response was reported by Gordon (1969) when 500 i.u. PMSG was administered several days prior to pessary removal compared with at pessary removal. Robinson and Smith (1982) found no significant diurnal effect of time of PMSG treatment on onset of oestrus or subsequent fertility.

1.3.3 Time of Ovulation Following Synchrony of Oestrus

Quirke et al. (1979) estimated that ovulation in Galway ewes occurred between 48/58h and 78/88h after progestagen-PMSG treatment. In Karakul ewes, an average time of ovulation of 46.8 - 62.8h after treatment was obtained by Boshoff et al. (1973) and this interval decreased as the dose of PMSG increased. In the Australian Merino, a comparable time of 55.8h was obtained in ewes previously exposed to rams compared with 59.7h in ewes not previously exposed to rams (Maxwell, 1986a). In Merino ewes treated with progestagen alone, ovulation was observed to commence approximately 75h after pessary removal (van Niekerk and Belonje, 1970). Studies on the control of ovulation using either HCG or synthetic GnRH/LHRF have most often involved superovulated ewes (see Chapter

1.2.2.)). However, control of the time of ovulation with HCG has been reported in nonsuperovulated ewes following synchrony of oestrus (Braden et al., 1960; Roche and Crowley, 1971; Quirke et al., 1979).

1.3.4 Embryo Transfer Following Synchrony of Oestrus

Progestagen/progesterone-PMSG treatment is the standard method of regulation of the oestrous cycle in ewes prepared for either embryo transfer or fixed-time artificial insemination. In early embryo transfer studies, regulation of the cycle with daily injections of progesterone indicated that pregnancy and embryo survival were not adversely affected by the treatment (Hunter et al., 1955; Shelton and Moore, 1966). In recent studies, the percentage of embryos surviving after transfer to ewes treated with a progestagen and PMSG varied between 10.5 - 63.8% (Land and Wilmut, 1977; Crosby et al., 1980; Armstrong and Evans, 1983; Torres and Cognie, 1984; Torres et al., 1987). There appears to be no comparative information on embryo survival following synchrony of oestrus compared with naturally cycling ewes. However, rapid tubal transport of ova following progesterone treatment may occur (Robinson, 1962; Shelton, cited by Moore and Shelton, 1964b) indicating that embryo viability could be at greater risk in treated compared with untreated ewes.

1.3.5 Artificial Insemination Following Synchrony of Oestrus

The percentage of ewes pregnant following the insemination of fresh semen (either cervical, vaginal or intra-uterine) at the first synchronized oestrus has varied between 30 - 87% (Roberts, 1966; Hackett, Inskeep, Robertson, Shrestha and Wolynetz, 1979; Hackett and Wolynetz, 1981; Faure et al., 1983; Langford, 1982; Langford et al., 1983; Maxwell et al., 1984; Davis et al., 1984; Maxwell and Hewitt, 1986). In comparison, vaginal or cervical insemination of frozen-thawed semen resulted in

pregnancy rates of 17.6 - 55.0% (Maxwell, Curnock, Logue and Reed, 1980; Haresign, Read, Curnock and Reed, 1986; Maxwell and Hewitt, 1986) whilst between 8.8 - 76.8% of ewes became pregnant when frozen-thawed semen was inseminated into the uterus (Maxwell et al., 1984; Eppleston, Maxwell, Battye and Roberts, 1986; Eppleston and Roberts, 1986; Haresign et al., 1986; Maxwell, 1986a, 1986b; Maxwell and Hewitt, 1986; Hunton, Flecker and Maxwell, 1987). Cervical insemination of chilled semen resulted in pregnancy rates of 83 and 94.7% (Maxwell et al., 1980; Haresign et al., 1986).

Progestagen/progesterone-PMSG treatment has also been used successfully in natural mating programs; the percentage of ewes pregnant at the first synchronized oestrus varied between 33 - 86% (Eastwood and McDonald, 1975; Allison and Kelly, 1978; Hackett et al., 1979; Quirke, 1979; Velle and Helle, 1979; Boland et al., 1981; Lunstra and Christensen, 1981; Spitzer and Carpenter, 1981; Ainsworth and Wolynetz, 1982; Hackett, 1982; Hackett and Wolynetz, 1982; Steffan, Poissonnet and Thibier, 1982; Ainsworth and Shrestha, 1983; Shrestha et al., 1983; Ainsworth and Shrestha, 1985; Maxwell and Barnes, 1986).

1.3.6 Methodology of Embryo Transfer

Embryos may be transferred to either the oviducts or uterine lumen using techniques similar to those detailed by Hunter et al. (1955). Site of transfer depends on the age of the embryo, given that embryos enter the uterus between the third and fourth days after oestrus (Holst and Braden, 1972; Holst, 1974).

Conventional embryo transfer necessitates exteriorization of the reproductive tract at laparotomy. Several studies have examined the feasibility of transferring embryos by laparoscopy (McKelvey and Robinson, 1984b; Mutiga and Baker, 1984; Schiewe, Bush, Stuart and Wildt, 1984; Walker, Warnes, Quinn, Seamark and Smith, 1985). Between 40.6 -

75.0% of recipient ewes in these studies became pregnant. A limitation to transfer by laparoscopy has been the inability to transfer embryos to the oviducts. However, equipment has been designed (I.M.V., L'Aigle, France) to overcome this problem.

1.3.7 Factors Affecting the Success of Embryo Transfer

Factors likely to influence the success of embryo transfer have recently been reviewed by Wilmut, Sales and Ashworth (1985a, 1985b).

1.3.7.1 Synchrony Between Donor and Recipient

Both pregnancy rate and embryo survival were optimal if recipient ewes were in oestrus within 12h of donor ewes (Moore and Shelton, 1964b), although successful transfers occurred when the asynchrony was extended to two days. Rowson and Moor (1966) reported a pregnancy rate of 75% when the oestrous cycles were exactly synchronized and in the study of McKelvey et al. (1985), 94% of recipients became pregnant when the asynchrony was less than 8h. However, variation in the developmental age of embryos following superovulation was likely to contribute to embryo wastage following transfer (Betteridge, 1977).

The degree of chronological asynchrony and thus the hormone regimen of the recipient influenced the rate of embryo development after transfer (Wilmut and Sales, 1981; Lawson, Parr and Cahill, 1983; Wilmut et al., 1985a). Results of the above studies indicate that embryos retarded relative to the uterine environment develop quicker than those transferred to a synchronous environment. On the other hand, embryos relatively advanced compared with the reproductive tract experience a reduction in growth rate. Lawson and Cahill (1983) reported that asynchronously transferred embryos were able to continue growing for several weeks despite the inability to establish pregnancy. Furthermore,

the presence of a synchronously transferred embryo that was able to establish pregnancy was unable to protect an asynchronously transferred embryo (Wilmut and Sales, 1981).

Wilmut et al. (1985a) reviewed the potential ways in which synchrony between the hormone regimen of the recipient and the developmental stage of the embryo could be enhanced. These included 1) measurement of progesterone level in one cycle as an indicator of the level in the cycle of transfer, 2) assessment of progesterone levels in recipient and donor ewes on the day of transfer and 3) administration of progesterone to regulate the endocrine environment with appropriate adjustment in the time of embryo transfer.

1.3.7.2 Age of Embryo

Survival of transferred embryos increased with age, irrespective of whether transfers were to the oviducts or uterine horns (Moore and Shelton, 1964b). Rowson and Moor (1966) found no effect of age when embryos were transferred 5 - 9 days after the onset of oestrus. Armstrong and Evans (1983) obtained a higher embryo survival with the transfer of blastocysts compared with earlier stage embryos. Wilmut, Ashworth, Springbett and Sales (1988) concluded that the transfer into an advanced recipient was tolerated better by older embryos compared with younger embryos.

1.3.7.3 Site of Transfer

The significance of the site of transfer is believed to relate to the availability of stage specific proteins produced within the reproductive tract (Wilmut et al., 1985b). Moore et al. (1960) and Moore and Shelton (1962) reported a reduced survival when embryos were transferred to the oviducts compared with the uterine lumen. However, subsequent

observations indicated that the oviducts were the preferred site of transfer for embryos collected up to 3.5 days of age (Moore and Shelton, 1964b) while uterine transfers resulted in high survival rates with older embryos (70 - 75%; Rowson and Moor, 1966). However, Armstrong and Evans (1983) found no difference in survival rates when embryos of more than eight cells were transferred to either the oviducts or uterus.

1.3.7.4 Number of Embryos Transferred

In the study of Moore et al. (1960), a reduced embryo survival was obtained following the transfer of five compared with two embryos per ewe. However, there were no differences after the transfer of either one or two (Armstrong and Evans, 1983) or one to three embryos per ewe (Moore and Shelton, 1962).

1.3.7.5 Ovulatory Response of Donor

The level of superovulation did not influence the proportion of transferred embryos that developed normally after transfer in the studies of Moore et al. (1960) and Torres et al. (1987). However, Armstrong and Evans (1983) reported a reduction in the percentage of embryos that developed when embryos were transferred from high ovulatory responders compared with low responders.

1.3.7.6 Ovulatory Response of Recipient

Bilaterally ovulating ewes consistently experienced higher embryonic survival than ewes with multiple, unilateral ovulations (White, Rizzoli and Cumming, 1981). Pope (1988) concluded that embryos might be particularly sensitive to uterine asynchrony upon entry into the contralateral uterine horn. Pope suggested that these losses might occur

shortly after embryonic migration on days 14 - 15, given that embryo mortality was completed by days 17 - 18 (Moore et al., 1960).

1.3.7.7 Other Factors

Other factors that may influence the success of embryo transfer include time of the breeding season (Wilmut, Sales and Ashworth, 1986; Torres et al., 1987), nutrition of recipient (Edey, 1966), environmental stress (Griffiths, Gunn and Doney, 1970; Doney, Smith and Gunn, 1976), heterologous or homologous transfer (McDonald, 1969; Killeen, 1974), location of corpora lutea relative to the site of transfer and number of corpora lutea (Torres et al., 1987; Muwalla, Foote, Bunch and Call, 1988), time of storage of embryos (Torres and Cognie, 1984) and intrinsic factors (see King, 1985).

1.3.8 Applications of Embryo Transfer

Seidel and Seidel (1981) have reviewed the numerous applications of embryo transfer technology. Commercially, embryo transfer allowed faster rates of genetic progress in sheep due to lower generation intervals and/or increased selection among females (Smith, 1986). Smith concluded that if ten progeny per donor could be obtained (using six to eight-month-old ewes), then the rate of genetic change in economically important traits could be doubled compared with selection programs using normal rates of reproduction. Experimentally, embryo transfer is an integral part of the development of new technologies involving embryo manipulation and genetic engineering (Seidel and Seidel, 1981).

1.4 IN VITRO CULTURE OF SHEEP EMBRYOS

1.4.1 Introduction

Major interest in the culture of the preimplantation embryo was stimulated by the report that two-cell embryos of mice could be cultured to blastocysts in a simple medium (Whitten, 1957). Consequently, the physical and nutritional requirements of embryos were examined in several species including the mouse (Brinster, 1965a, 1965b; Chen and Hsu, 1982), rabbit (Maurer, Whitener and Foote, 1969; Kane and Foote, 1971; Ogawa, Satoh and Hashimoto, 1971; Kane, 1972), hamster (Bavister, Leibfried and Lieberman, 1983), ferret (Whittingham, 1975) and man (Edwards, Bavister and Steptoe, 1969; Edwards, Steptoe and Purdy, 1980).

Comparable studies in domestic species have been limited by costs of embryo collection (Kane, 1987). Culture facilities are required for two reasons. Firstly, there is a need for short term storage of embryos in transfer programs. Secondly, assessment of the viability of embryos after manipulation is desirable; this may include morulae after freezing or zygotes after pronuclear microinjection of DNA. However, despite the early culture of both ovine (Robinson, 1951) and bovine embryos (Pincus, 1951, cited by Wright and Bondioli, 1981; Brock and Rowson, 1952), in vitro culture techniques have not developed to the stage where embryos of various ages can be routinely cultured. Recent developments in the culture of embryos of domestic species have been reviewed by Wright and Bondioli (1981), Kane (1987) and Wright and O'Fallon (1987).

1.4.2 Culture of Embryos in Simple Media

Three main types of media have been used for culture of sheep embryos, (1) blood serum (Tervit and McDonald, 1969; Moore, 1970; Moor and Cragle, 1971; Moore and Spry, 1972; Trounson and Moor, 1974a), (2) complete media consisting of inorganic salts, carbohydrates, vitamins and amino acids (Moor and Cragle, 1971; Wright, Anderson, Cupps, Drost and Bradford, 1976b) and (3) simple media consisting of balanced inorganic salts and carbohydrates (Hancock, 1963; Kraemer, 1966; Moor and Cragle, 1971;

Tervit, Whittingham and Rowson, 1972; Tervit and Rowson, 1974; Wright et al., 1976b). Examples of complete media include Ham's F-10 (HF-10), Minimum Essential Medium (MEM) and Tissue Culture Medium-199 (TCM-199); simple media include Whitten's medium (Whitten, 1971), synthetic oviduct fluid medium (SOFM; Tervit et al., 1972) and Brinster's Medium for Ovum Culture (BMOC; Brinster, 1971). Details on the formulation of these media are provided in the review of Wright and O'Fallon (1987). Factors affecting the development of sheep embryos in vitro are poorly understood and the findings of relevant studies are summarized below.

1.4.2.1 Age of Embryo

Most attempts to culture ovine embryos have used either eight-cell or morulae stage embryos. Success rates, across various media and culture conditions, have varied from no development up to 26/35 embryos developing to early blastocysts (Moor and Cragle, 1971; Tervit et al., 1972; Tervit and Rowson, 1974; Peters, Anderson and Cupps, 1977; Tervit and Goold, 1978). Approximately 80% of two- to eight-cell embryos were reported to divide at least once in culture (Tervit and McDonald, 1969; Moore, 1970) while Wright et al. (1976b) reported up to an average of three divisions per embryo. In a study of one- to 16-cell embryos, Lindner, Dickey, Hill and Knickerbocker (1979) recorded development to the blastocyst stage only in embryos of six-cells or more. Similarly, in the study of Peters et al. (1977), a greater percentage of eight-cell embryos cleaved in vitro compared with earlier stage embryos.

Division of zygotes in simple media were reported by Moore and Spry (1972), Tervit et al. (1972), Tervit and Rowson (1974), Peters et al. (1977), Boone, Dickey, Luszcz, Dantzler and Hill (1978), Lindner et al. (1979) and Betterbed and Wright (1985). Development of zygotes to blastocysts was obtained by Tervit et al. (1972; one from six zygotes) and Peters et al. (1977; one from six zygotes).

1.4.2.2 Media

Wright et al. (1976b) reported that simple media (SOFM, BMOC-3 and Whitten's medium) were effective in promoting the development of eight-cell embryos whereas the complete media (MEM, TCM-199 and HF-10) failed to support the development of embryos past the 32-cell stage. The time of blastocyst formation was comparable with that obtained in vivo. Lindner et al. (1979) obtained no significant difference between BMOC-2 and Whitten's medium in the ability to support two cleavage divisions of one- to 16-cell embryos.

1.4.2.3 Gas Atmosphere

Tervit et al. (1972) and Tervit and Rowson (1974) reported that an oxygen atmosphere of 5% was optimal for the development of eight-cell embryos. However, Wright et al. (1976b) obtained similar embryo development in an atmosphere 5% CO₂ in air compared with 5% CO₂, 5% O₂ and 90% N₂. Similarly, Betterbed and Wright (1985) found no effect of oxygen tension (5 v. 20%) on zygote development. These authors suggested that contradictions in the significance of the gas atmosphere on in vitro development may be due to differences in culture techniques.

1.4.2.4 Energy Substrates

Pyruvate is the major energy source for the mouse embryo up to the eight-cell stage (Brinster, 1969) but extrapolation of this finding to embryos of domestic species should be viewed critically (Kane, 1987). Boone et al. (1978) concluded that sheep morulae can be cultured in defined medium containing either pyruvate or lactate but not glucose alone. Betterbed and Wright (1985) reported an enhanced development of zygotes when glucose was added to Brinster's Pyruvate Medium compared with medium

alone. Most simple media contain pyruvate, lactate and glucose (see Wright and O'Fallon, 1987).

1.4.2.5 Protein Supplementation

Common protein supplements are sheep serum (Moore, 1970; Moor and Cragle, 1971; Moore and Spry, 1972), fetal calf serum (Wright et al., 1976b; Gandolfi and Moor, 1987; Heyman et al., 1987), sheep serum mixed with fetal calf serum (Tervit and Goold, 1978) and bovine serum albumin (BSA; Moore, 1970; Tervit et al., 1972; Tervit and Rowson, 1974; Wright et al., 1976b; Peters et al., 1977; Lindner et al., 1979; Betterbed and Wright, 1985). Either 10 or 20% concentration of sera were used in these studies while the concentration of BSA varied between 0.1 - 32 mg/ml. The optimal concentration of sera has not been determined although Wright and Bondioli (1981) suggested that concentrations above 10% were unlikely to be of additional value. Recently, Batt and Miller (1988) concluded that the development of sheep embryos in vitro varied with the molecular weight fraction of serum used in the medium; the value of sera would thus depend on the concentrations of the relevant fractions. Lindner et al. (1979) obtained improved embryo development in media containing either 1.5 or 2.0% BSA compared with lower concentrations but Wright et al. (1976b) found no significant difference between media containing either 1 or 2 mg/ml.

1.4.2.6 Other Factors

The inorganic salt requirements for culture of ovine embryos have not been studied. Restall and Wales (1966) reported that the concentration of K ions in oviductal fluid of sheep was higher than in plasma. The effects of osmolarity and pH on in vitro development of embryos is also not well understood. However, embryos of laboratory animals developed in

media varying in osmolarity between 200 - 354 mOsm (Brinster, 1965a; Naglee, Maurer and Foote, 1969) and varying in pH between 6.0 - 7.8 (Brinster, 1965a; Kane, 1974). Moore and Spry (1972) reported development of one- to four-cell sheep embryos in phosphate buffered saline (PBS) varying in pH from 7.2 to 7.8, although growth beyond the eight-cell stage seldom occurred. Vitamins appeared not to be necessary for blastocyst formation in the sheep (Wright et al., 1976b) while the role of trace elements has not been studied. In vitro development of embryos from prepubertal ewes was lower than from adult ewes (Wright et al. 1976b), indicating the importance of the physiological status of the reproductive tract on embryo development.

1.4.3 Co-Culture of Ovine Embryos

Complete and simple media are unable to reproduce the tubal or uterine environments required for normal embryo development. Consequently, in vitro culture of early stage embryos may be associated with developmental blocks in several species including outbred strains of mice (Whittingham and Biggers, 1967), hamster (Whittingham and Bavister, 1974), cow (Thibault, 1966), pig (Polge, 1982) and sheep (Gandolfi and Moor, 1987). The oviduct may secrete nonspecific compounds (Heyman and Menezo, 1987) or be stimulated by the embryo to release specific substrates (Villalon, Ortiz, Aguayo, Munoz and Croxatto, 1982) required for normal embryo development.

Enhanced in vitro development of embryos co-cultured in the presence of another cell type was first reported with mouse embryos co-cultured with oviduct tissue (Biggers, Gwatkin and Brinster, 1962). Subsequently, the development of embryos of domestic species was examined in the presence of a variety of cell types including oviduct epithelium (Eyestone, Vignieri and First, 1987; Gandolfi and Moor, 1987; Rexroad and Powell, 1988a, 1988b; Sakkas, Batt, Cameron and Trounson, 1988; Eyestone

and First, 1989), trophoblastic vesicles (Heyman et al., 1987; Clarkson and Nancarrow, 1988; Rexroad and Powell, 1988a), fibroblasts (Kuzan and Wright, 1981; Gandolfi and Moor, 1987), endometrial cells (Allen and Wright, 1984; Voekle, Amborsk, Hill and Godke, 1985; Rexroad and Powell, 1988b) and kidney cell monolayers (Rexroad and Powell, 1988b). Heyman et al. (1987) reported that significantly more sheep zygotes co-cultured with trophoblastic vesicles developed to morulae over three days compared with embryos grown in medium alone. These authors concluded that cell-embryo contact, as occurred with porcine embryos on fibroblast monolayers (Kuzan and Wright, 1981), was not vital for development. Gandolfi and Moor (1987) concluded that the support provided by the feeder layer cells depended on both the type of cell and the developmental stage of the embryo. These authors found that oviduct cells and fibroblasts equally supported cleavage of sheep zygotes for the first three days. Thereafter, oviduct cells continued to support cleavage whereas embryo viability declined in the presence of fibroblasts alone; 42% of embryos co-cultured with oviduct cells developed into expanded blastocysts compared with 4.5% cultured on fibroblasts. Gandolfi and Moor (1988) subsequently reported the identification of an oviductal protein, thought to be required for embryonic development. This protein may be the same or similar to the glycoprotein identified in oviductal fluid by Sutton, Nancarrow, Wallace and Rigby (1984). Rexroad and Powell (1988a) reported that the survival of ovine embryos was enhanced when cultured in the presence of oviductal cells for 24h but not after 72h. These authors also suggested that the beneficial effects of co-culture may not be due to secreted products since embryos cultured in conditioned media showed no enhanced rate of development.

Enhanced development in co-culture has also been reported with embryos of the cow (Kuzan and Wright, 1982a, 1982b; Camous, Heyman, Meziou and Menezou, 1984; Heyman and Menezou, 1987; Eyestone and First,

1989), pig (Kuzan and Wright, 1981; Allen and Wright, 1984; Wright and O'Fallon, 1987) and goat (Sakkas et al., 1988).

1.4.4 Short Term Storage of Embryos

Short term storage is often a requirement in embryo transfer programs. PBS enriched with serum supported the growth of early stage embryos after various treatments (Moore and Bilton, 1973; Trounson and Moore, 1974b, 1974c; Bilton and Moore, 1976). PBS is also a proven medium for transport of embryos (Bunch, Foote, Call and Clark, 1984; Muwalla et al., 1988). However, extended periods of culture in PBS resulted in retarded embryo development compared with that obtained in SOFM (Tervit and Goold, 1978).

1.4.5 Embryo Viability After Culture

Transfer of cultured ovine embryos has generally resulted in low pregnancy rates. Buttle and Hancock (1964) obtained survival to term of 2/7 and 1/14 four- to twelve-cell embryos cultured in sheep serum for 24 and 48h respectively. Tervit and McDonald (1969) reported that 13/22 control embryos and 13/152 embryos cultured in sheep serum for either 24 or 48h were recovered as viable embryos 20 - 22 days after transfer. Tervit et al. (1972) recovered 1/4, 4/9 and 11/13 elongated conceptuses following the transfer of one-, four- and eight-cell embryos respectively that were cultured in SOFM. Subsequently, Tervit and Rowson (1974) obtained 10 lambs from 18 eight-cell embryos cultured in SOFM for 72h; however, none of 17 zygotes cultured for 144h developed after transfer. On the other hand, in the study of Moor and Cragle (1971), none of 15 eight-cell embryos cultured in TCM-199 for three days survived after transfer, while Schwartz and Ulberg (1972) cultured two- to eight-cell embryos in BMOC-2 for 24h and obtained a survival of between 0 - 60%.

Peters et al. (1977) cultured embryos in Whitten's medium for either 24 or 48h and obtained a survival of 38.8 and 0.0% respectively.

Gandolfi and Moor (1987) reported that 80 and 30% of zygotes were viable following co-culture with oviduct cells for three and six days respectively. Heyman et al. (1987) used a three-day in vivo incubation period to assess viability and reported that 40% of embryos developed to the expanded or hatched blastocyst stage after three days of co-culture. In the study of Rexroad and Powell (1988a), one- to eight-cell embryos cultured in the presence of oviductal cells for 24h had a cleavage index 77% of that of control embryos when recovered from recipient ewes, eight days after oestrus.

1.5 TRANSGENESIS IN DOMESTIC ANIMALS

1.5.1 Introduction

The ability to introduce foreign genes into the germ line of farm animals (Hammer et al., 1985) has increased the potential for rapid genetic improvement in economically important traits. Large increases in growth rate were obtained following the incorporation of a foreign growth hormone (GH) gene construct in the mouse (Palmiter et al., 1982, 1983). Steps involved in the production of transgenic animals include (1) identification and isolation of the relevant DNA, (2) the addition, if required, of regulatory sequences to control expression, (3) multiplication of the construct in a prokaryotic vector (e.g. Escherichia coli), (4) microinjection of the construct into the pronucleus and (5) subsequent transfer of the microinjected zygote into a recipient female. The appropriate methodology and results of recent studies have been reviewed by several authors (Brinster and Palmiter, 1982; Church, Schaefe and Meckling, 1985; Palmiter and Brinster, 1986; Wagner, 1985).

Artificial manipulation of the genotype was first obtained with the production of chimeric mice (Tarkowski, 1961; Mintz, 1962; Papaioannou et al., 1975). Subsequently, viral DNA was incorporated into the genome of the early mouse embryo following microinjection into the blastocoele (Jaenisch and Mintz, 1974). The pronuclear microinjection technique was based on the observation that foreign DNA could be injected into somatic cells (Graessmann et al., 1979; Capecchi, 1980). Soon after, incorporation of foreign genes into the mouse genome was reported by Gordon et al. (1980), Brinster et al. (1981), Constantini and Lacy (1981), Wagner, Stewart and Mintz (1981), Palmiter et al. (1982, 1983), Gordon and Ruddle (1983) and McKnight, Hammer, Kuenzel and Brinster (1983). Expression of injected gene sequences was subsequently reported by Wagner E. et al. (1981), Wagner T. et al. (1981), Brinster et al. (1981), Palmiter et al. (1982, 1983), Constantini et al. (1983) and McKnight et al. (1983). There are now mouse lines that contain cloned transgenes coding for B-globin from several species, Herpes simplex virus thymidine kinase, GH from several species, chicken transferrin, SV40 viral t-antigen, immunoglobins, rat elastase, the cellular oncogene myc-product, rat myosin light chain, mouse alphafetoprotein, human hypoxanthine phosphoribosyltransferase, somatostatin and GH releasing factors (see Wagner, Chen and Hayes, 1986). Transgenic mice can be produced with reasonable frequency, with up to 25% of manipulated embryos being transgenic (Brinster, Chen, Trumbauer, Yagle and Palmiter, 1985).

1.5.2 Development of Transgenic Livestock

Studies have resulted in the production of transgenic pigs (Hammer et al., 1985; Brem et al., 1985; Pinkert, Pursel, Miller, Palmiter and Brinster, 1987; Ebert et al., 1988; Pursel et al., 1987, 1988; Vize et al., 1988), sheep (Hammer et al., 1985; Nancarrow, Marshall, Murray, Hazelton and Ward, 1987b; Pinkert et al. 1987; Pursel et al., 1987;

Simons et al., 1988), goats (Fabricant et al., 1987) and cows (Biery, Bondioli and De Mayo, 1988). Expression of transgenes has been reported in the pig (Hammer et al., 1985; Ebert et al., 1988; Pursel et al., 1987, 1988; Vize et al., 1988), sheep (Pinkert et al., 1987; Pursel et al., 1987; Simons et al., 1988) and cow (Biery et al., 1988). Transgenic pigs (Pursel et al., 1987, 1988) and sheep (Rexroad and Pursel, 1988; Simons et al., 1988) have transmitted integrated genes to their offspring.

The efficiency of producing transgenic livestock by pronuclear microinjection is low. Between 0.00 - 2.07% of injected zygotes developed into transgenic animals (see Rexroad and Pursel, 1988). In the sheep, 6.2% of lambs born in all studies were transgenic (see Rexroad and Pursel, 1988; Simons et al., 1988). There is insufficient data to assess if the gene construct was important in determining integration efficiency. Brinster et al. (1985) proposed that randomly generated chromosomal breaks were the rate limiting step for gene integration. The number of copies of the integrated gene varied widely between animals (Pursel et al., 1987; Vize et al., 1988). Furthermore, in pigs transgenic with the MThGH construct, there was no correlation between the number of integrated copies and the serum concentration of hGH (Pursel et al., 1987). The genomic site of integration may alter the ability of the gene to express and thus remove any correlation between number of gene copies per cell and level of expression (Rexroad and Pursel, 1988). However, the metallothionein promoter may provide tissue-specific expression of the transgenes (Rexroad and Pursel, 1988), although the ability to induce the promoter with Zn appears equivocal given that hGH was present in MThGH transgenic pigs at birth (Pursel et al., 1987).

1.5.3 Potential Application of Recombinant DNA Technology

Various aspects of the application of recombinant DNA technology in livestock industries have been reviewed or discussed by Ward, Murray,

Nancarrow, Sutton and Boland (1982), Wagner, Murray, Minhas, and Kraemer (1984), Church et al. (1985), Wagner (1985), Kraublich (1986), Petters (1986), McConnell (1986), Wagner and Jochle (1986), Land and Wilmut (1987), Smith, Meuwissen and Gibson (1987), Renard and Babinet (1987), Rexroad and Pursel (1988) and Murray, Nancarrow and Ward (1988).

Genes that are potentially useful to animal industries fit one of three categories. Firstly, those that regulate or influence traits such as lactation and reproduction. These traits are controlled by a large number of genes whose products interact to influence performance. Secondly, those that regulate or influence immune responses and resistance to diseases (e.g. genes of the major histocompatibility complex). Thirdly, single genes or a small number of genes with major effect. These genes include those associated with wool growth (Ward, 1982), GH production (Woychik et al., 1982), litter size (Piper and Bindon, 1985; Jonmundsson and Adalsteinson, 1985; Hanrahan and Owens, 1985) and milk quality (Lathe et al., 1986; Simons, McClenaghan and Clark, 1987). Other examples of single genes affecting normally polygenetic traits are the halothane sensitivity-stress susceptibility gene in pigs and the double muscling gene in cattle. Land and Wilmut (1987) concluded that the ability of the molecular biologist to identify single genes with favourable effects for transgenesis was severely limited.

Cloning of the GH gene (Woychik et al., 1982) offered the means of increasing the endogenous supply of GH. The effect of exogenous GH on animal growth was first examined by Evans and Simpson (1931). Rate of liveweight gain and feed conversion efficiency of treated animals were significantly increased by GH treatment (e.g. Machlin, 1972). Similarly, administration of GH to dairy cows increases milk production (Machlin, 1972; Peel, Bauman, Gorewit and Sniffen, 1981; Peel, Fronk, Bauman and Gorewit, 1982). Recombinant DNA technology also offers the potential of

introducing novel metabolic pathways into cells to alter metabolism. Of particular interest is the potential for the sheep to synthesize cysteine rather than rely on microbial production (Ward, 1982). Two enzymes that synthesize cysteine in micro-organisms (serine transacetylase and o-acetylserine sulphydrase) are absent from higher animals. Progress towards the isolation of genes encoding these enzymes in bacteria and yeast is progressing (Bawden et al., 1987).

1.5.4 Performance of Transgenic Livestock

Incorporation and expression of genes does not necessarily result in the production of superior genotypes. Pursel et al. (1987) reported that pigs transgenic with the MThGH construct and with high concentrations of serum hGH did not grow quicker than control pigs. Similarly, the growth rate of MTsGH9 transgenic lambs was not increased despite high levels of GH (Nancarrow, Ward and Murray, 1988). Although MThGH and MTpGH transgenic pigs may grow more efficiently than control pigs (Ebert et al., 1988; Vize et al., 1988), high concentrations of GH can be associated with health problems. A greater incidence of arthritis and skeletal malformation has been observed in expressing transgenic animals compared with control and nonexpressing transgenic animals (Pursel et al., 1987). Expression may also be associated with lack of libido, anoestrus and anovulation (Pursel et al., 1987).

The future role of recombinant DNA technology in livestock industries may depend on the ability of researchers to control both the site and number of genes incorporated and also to regulate the expression of these genes.

1.5.5 Limitations to the Production of Transgenic Livestock

Several technical problems, in addition to the development of suitable gene constructs, limit the rate at which transgenic farm animals can be produced.

1.5.5.1 Supply of Zygotes

Low and variable responses to gonadotrophin treatment and the cost of treatment severely limit the supply of zygotes for microinjection. An additional problem is that embryo transfer technology is based on the collection of cleaved embryos rather than zygotes. Nancarrow et al. (1984a) included treatment with GnRH in their embryo collection protocol and reported an increase in the number of cumulus free zygotes collected per ewe. There is little information available on the optimal time to collect zygotes for pronuclear microinjection. However, the following findings are relevant (1) first cell division occurred approximately 19 - 24h after ovulation (Dziuk, 1965), (2) cumulus cells were lost from the ovum within four to six hours of ovulation (Dziuk, 1965; Thompson and Wales, 1987) and (3) the male pronucleus formed approximately three to nine hours after ovulation (Dziuk, 1965). These data highlight the importance of a synchronized time of ovulation preceding the collection of zygotes for microinjection.

1.5.5.2 Visualization of Pronuclei

The cytoplasm of zygotes of farm animals is optically dense compared with those of the mouse (Wagner et al., 1984), thus reducing the visibility of pronuclei. Centrifugation (e.g. 15,000 g for three minutes) improved the visibility of nuclear structures in pig (Wall, Pursel, Hammer and Brinster, 1985) and cow zygotes (Lohse, Robl and First, 1985; Hammer et al., 1986). Centrifugation did not impair subsequent development of the zygote in vivo (Wall et al., 1985; Wall and Hawk, 1988). Pronuclei can

be observed in 80 - 90% of sheep zygotes using differential interference contrast microscopy (Hammer et al., 1985; Murray et al., 1988; Simons et al., 1988). Centrifugation did not improve the proportion of sheep zygotes with visible pronuclei (Nancarrow, Murray, Boland, Hazelton and Sutton, 1984b).

Other procedures used to improve the visibility of pronuclei include staining with the DNA fluorescent stain 4'-6'-diamidino-2-phenylindole (Minhas et al., 1984) and treatment with cytochalasin B followed by centrifugation (Ward et al., 1986). Neither of these two treatments influenced the viability of the zygote. Nancarrow et al. (1987a) reported a significant increase in the proportion of zygotes with visible pronuclei following treatment with FSH for superovulation compared with PMSG.

1.5.5.3 Viability of Zygotes After Microinjection

Microinjection significantly reduced the survival of zygotes, with the reduction being greater in sheep than in pigs (Hammer et al., 1986). Rexroad and Wall (1987) showed a progressive reduction in the viability of sheep zygotes during the microinjection procedure. Immediate transfer resulted in 86% of zygotes developing to blastocysts, injection of zygotes with buffer resulted in 42 and 28% developing to blastocysts in two studies while injection with DNA resulted in only 18% developing to blastocysts. In the study of Walton, Murray, Marshall and Nancarrow (1987), zygote viability after microinjection was affected by pipette size, taper of bore and the time of injection relative to first cell division. Ward et al. (1986) reported that 51% of injected sheep zygotes survived the injection procedure as assessed by failure to lyse within one hour. The percentage of injected zygotes of domestic animals that developed into offspring was generally less than 20% (see Rexroad and Pursel, 1987). In the sheep, figures of 7.1% (Hammer et al., 1985), 6.4%

(Pursel et al., 1988), 6.2% (Nancarrow et al., 1987b), 14.5% (Pinkert et al., 1987), 7.5% (Murray et al., 1988) and 18.0% (Simons et al., 1988) have been obtained.

1.5.5.4 Culture of Microinjected Zygotes

The inability to assess zygote viability after microinjection contributes to the cost of producing transgenic livestock. Studies on the culture of sheep zygotes have been reviewed in Chapter 1.4. Hammer et al. (1986) cultured microinjected zygotes in vitro for 12h and found that 10 and 23% of sheep and pig zygotes respectively developed into blastocysts in recipient ewes. However, only 26% of uninjected sheep zygotes and 52% of centrifuged uninjected pig zygotes survived to blastocysts, indicating deficiencies within the culture system. Intermediate hosts have been used to reduce costs of transfer and/or to assess viability of zygotes after microinjection (Biery et al., 1988; Rexroad and Pursel, 1988). The development of a reliable in vitro culture system for microinjected zygotes is of high priority (Kane, 1987; Murray et al., 1988).

1.5.6 Alternative Techniques for the Transfer of Genes

Although pronuclear microinjection is anticipated to be the principle route for the production of transgenic animals for the next five to ten years (Murray et al., 1988), the technique has two major limitations. Firstly, the incorporation rate is low and little is known of the processes likely to improve this rate (Palmiter and Brinster, 1986). The second limitation is the tendency for transgenes to integrate in tandem arrays, with up to several hundred copies being incorporated. Murray et al. (1988) reviewed alternative procedures for the incorporation of foreign genes and these are summarized below.

1.5.6.1 Retroviral Vectors

Both intact viruses (Jaenisch, 1976) and genetically engineered retroviruses (Jahner, Haase, Mulligan and Jaenisch, 1985; van der Putten et al., 1985) were used to infect cells of mouse embryos, resulting in the production of transgenic offspring. Genes can be transferred into embryos either by an injection of infectious virus into the blastocoele cavity or by placing blastomeres on cultured cells that are shedding viral particles. Integration of gene sequences was highly efficient and genes were incorporated as single copies rather than as tandem arrays (Stewart, Stuhlman, Jahner and Jaenisch, 1982). Retroviral vectors were recently used to produce transgenic ovine and porcine fetuses (Hettle, Harvey, Cameron, Johnston and Onions, 1989; Petters, Johnson and Shuman, 1989).

1.5.6.2 Embryonic Stem Cells

Foreign DNA can be transferred into pluripotent cells by calcium phosphate precipitation (Wagner and Mintz, 1982) or by retroviral vectors (Evans, Bradley, Kuehn and Robertson, 1985; Stewart, Vanek and Wagner, 1985; Kuehn, Bradley, Robertson and Evans, 1987). A chimera produced by combining a blastocyst and genetically engineered pluripotent cells is mosaic for both cell types. However, if the embryonic stem cells contribute to the germ line, progeny are produced that contain the genetically engineered genome of the stem cell. Germ-line transmission of genes transferred into mice using embryonic stem cells has been reported (Gossler, Doetschman, Korn, Serfling and Kemler, 1986; Robertson, Bradley, Kuehn and Evans, 1986). This technique is limited in domestic species due to the lack of stable stem cell lines.

1.5.6.3 Liposome Carriers

DNA encapsulated in liposomes was used to transform L-cells in culture (Schaefer-Ridder, Wang and Hofschneider, 1982). Germ line transformation was unsuccessful with liposomes, but gene transfer into somatic cells of animals has occurred (Nicolau, Le Pape, Soriano, Fargette and Juhel, 1983; Soriano et al., 1983).

1.5.6.4 Autonomous Replicating Vectors

Rassoulzadegan, Leopold, Vailly and Cuzin (1986) demonstrated that a bacterial plasmid based vector, containing the origin of replication and large T antigen of polyoma virus, produced 100% transformation of mice following pronuclear microinjection. Founder mice and subsequent generations transmitted the vector DNA to 80 - 100% of their progeny. The vector was considerably rearranged and did not express the polyoma large T gene. The use of autonomously replicating viruses, including the bovine papilloma virus (Gilbert and Cohen, 1987), may be of value in future gene transfer studies.

1.6 DISCUSSION

The ability to collect, microinject and transfer zygotes is an integral part of studies on the development and application of recombinant DNA technology to genetic improvement programs. However, these procedures, in the sheep, have several limitations:

(1) Embryo collection is inefficient, characterized by variable responses to gonadotrophin treatment and fertilization failure. The latter problem is attributed to a reduced population of spermatozoa within the reproductive tract (Hawk et al., 1987). Elimination of fertilization failure in the Merino would improve embryo yields from prohibitively low values to values where microinjection programs could be successfully implemented. The inability to eliminate fertilization

failure using intra-uterine insemination (Evans et al., 1984) indicates that asynchrony in the time of ovulation contributes to the problem. Asynchrony in the time of ovulation also influences the proportion of embryos collected at the pronuclear stage (Moore, 1970; Rexroad and Powell, 1988). Evidence indicates that microinjection of two- and four-cell embryos is a less efficient means of producing transgenic sheep compared with the microinjection of zygotes (Simons et al., 1988). Synchronizing the time of superovulation for the production of zygotes is thus desirable. Consequently, studies in Chapters 2 and 4 evaluate GnRH treatment in embryo collection programs, with emphasis on the time of ovulation.

(2) The methodology of embryo transfer, like embryo collection, is based on studies with cleaved embryos rather than zygotes. The review indicates that numerous factors are likely to influence the success of embryo transfer, particularly synchrony between donor and recipient. The survival of transferred embryos increases with age (Moore and Shelton, 1964b), indicating that zygotes may be particularly susceptible to handling and transfer procedures. Studies in Chapter 3 firstly examine the natural variation in time of ovulation in the nonsuperovulated ewe following synchrony of oestrus. Secondly, the effect of GnRH treatment on time of ovulation is examined as a means of synchronizing the time of ovulation between donor and recipient. The latter study is extended to evaluate GnRH treatment in both embryo transfer and artificial insemination programs.

(3) A reliable in vitro culture system for sheep zygotes has not been developed. Studies on the culture of sheep embryos have been characterized by a general lack of definitive data, the lack of progress during the last two decades and the lack of experience in the culture of zygotes. The ability to assess zygote viability after pronuclear microinjection would enhance the efficiency of microinjection programs,

with nonviable zygotes being excluded from the transfer procedure. The development of a reliable in vitro culture system is also central to future progress, including the likely developments of embryo biopsy, embryo splitting after microinjection and rapid assessment of gene incorporation and expression.

(4) Pronuclear microinjection is a relatively new experimental procedure and its application to sheep zygotes presents several technical problems. Problems of immediate concern include the ability to visualize pronuclei and the extent of zygote wastage after microinjection. These problems, together with the in vitro assessment of microinjected zygotes, are examined in the final experimental chapter.

Although several transgenic sheep have been produced by various research groups since these studies commenced, there remain numerous deficiencies in the technology of collecting, handling and microinjecting sheep zygotes. Studies reported in this thesis address some of these problems.

CHAPTER 2

TIME OF OVULATION IN THE SUPEROVULATED EWE

2.1 EXPERIMENT 1. TIME OF MULTIPLE OVULATIONS IN THE EWE AFTER TREATMENT WITH FSH OR PMSG WITH AND WITHOUT GnRH

2.1.1 INTRODUCTION

The efficient collection of sheep zygotes is a limitation to studies on micromanipulation and gene injection. Although gonadotrophin treatment for multiple ovulations is frequently being re-assessed (Ryan, Bilton, Hunton and Maxwell, 1984; Evans et al., 1984), little recent research has been conducted to improve the efficacy of embryo collection programs. An exception has been the development of the technique whereby semen is deposited directly into the uterus using a laparoscope (Killeen and Caffrey, 1982). Improved fertilization rates have been obtained following the intra-uterine insemination of frozen-thawed semen (Armstrong and Evans, 1984b). The collection of zygotes is particularly inefficient because some embryos have usually cleaved at the time of collection (Moore, 1970). Synchronizing the time of ovulation should overcome this problem and also improve the proportion of ova that are fertilized.

Quirke et al. (1979) reported that GnRH treatment synchronized the time of ovulation in the nonsuperovulated sheep; when 50 ug per ewe were given 24h after progestagen pessary removal, ovulations occurred in 44 - 46% of ewes within 24h and in all ewes within 34h. Nancarrow et al. (1984a) administered 50 ug GnRH per ewe after treatment with 1500 i.u. PMSG and found an increase in ovulation rate, fertilization rate and synchrony of ovulation.

The aims of this study were (1) to examine the time of multiple ovulations after treatment with FSH or PMSG, (2) to examine the effect of GnRH administration on the synchrony of ovulation after gonadotrophin treatment and (3) to assess the potential usefulness of using GnRH in the collection of zygotes.

2.1.2 MATERIAL AND METHODS

Observations were conducted during the mid-breeding season (March) of 1985, using five-year-old South Australian Merino ewes located at Turretfield Research Centre, South Australia (138° 44'E, 34° 38'S).

2.1.2.1 Method of Superovulation

Ewes were treated for 12 days with 60 mg MAP (Upjohn Pty Ltd, Sydney, New South Wales) and either 15 mg FSH (Burns Biotech, Omaha, Nebraska) or 1200 i.u. PMSG (Heriot Agvet Pty Ltd, Melbourne, Victoria). FSH was administered (i.m.) as six injections, about 12h apart, with the first given 48h before pessary removal.

GnRH was obtained from Ayerst Laboratories Pty Ltd (Parramatta, New South Wales; Gonadorelin) and Bachem Ag (Bubendorf, Switzerland).

2.1.2.2 Experimental Procedure

The time of ovulation was examined after treatment with either FSH or PMSG with and without GnRH. Treatments were (1) FSH (N = 20), (2) FSH + GnRH (25 or 100 ug i.v., N = 30), (3) PMSG (N = 20) or (4) PMSG + GnRH (100 ug i.v., N = 20). GnRH was administered 24h after pessary removal. The optimal time of GnRH administration to synchronize time of ovulation was examined in a second study. In this study, all ewes received FSH and

50 ug GnRH administered (i.v.) at 16 (Treatment 1, N = 12), 20 (Treatment 2, N = 13) or 24h (Treatment 3, N = 12) after pessary removal.

2.1.2.3 Observations

Ovaries were examined by repeated laparoscopy and the number of corpora lutea recorded. Observations began at either 24 or 30h after pessary removal and were then made every 3h in ewes treated with GnRH and every 6h in other ewes until all preovulatory follicles had ovulated. Laparoscopy ceased 54h after pessary removal in the GnRH treatments and by 72h in the non-GnRH treatments. A random sample of ewes from each treatment was re-examined at 144h to check accuracy of records.

The time of GnRH treatment was such that (1) the interval from administration to initial laparoscopy and (2) the interval between successive observations were similar for all sheep. Ewes given GnRH were sedated with 0.2 ml 2% xylazine (i.m.; Bayer Australia Ltd, Botany, New South Wales) whilst other ewes remained unsedated. Preliminary observations indicated that xylazine administration did not affect time of ovulation when GnRH was used but may delay or prevent ovulation in the absence of GnRH.

2.1.2.4 Effects of Stress Associated with Repeated Laparoscopy

The effects of stress were assessed by replicating the PMSG and PMSG + GnRH treatments in an additional group of ewes; ewes in these replicates were returned to the paddock after treatment and not subjected to repeated laparoscopy. Ovaries were examined 72 and 144h after pessary removal. The number of ovulations per ewe at these two times were determined and compared with data from ewes that underwent repeated laparoscopy.

2.1.2.5 Statistical Analyses

Superovulation was defined as three or more ovulations. Comparisons were made between FSH and PMSG treatments (data collected on a six-hour basis) and between FSH + GnRH and PMSG + GnRH treatments (data collected on a three-hour basis). Medians were compared using the Mann-Whitney and Kolmogorov-Smirnov tests (Siegal, 1956). Medians in the second study were compared using the Kruskal-Wallis test (Siegal, 1956). The effect of treatment on the mean number of ovulations per ewe was tested for significance using the statistical package for social science (Nie, Hull, Jenkins, Steinbrenner and Bent, 1975).

2.1.3 RESULTS

Many ewes ovulated one or occasionally two follicles up to 24h before the characteristic superovulatory response; these ovulations are referred to as premature ovulations. No new corpora lutea were observed at 144h compared with the penultimate observation. Three ewes were eliminated from the study, one in the FSH treatment due to loss of pessary and one in each of the PMSG and PMSG + GnRH treatments due to failure to ovulate. Data are presented in Tables 1 and 2 and Figures 1 and 2.

2.1.3.1 FSH and PMSG Treatments

There were no significant differences between treatments in ovulation rate, median time to first ovulation or in median time from first to last ovulation (excluding premature ovulations in the latter trait) (Table 1). However, the median time of all ovulations was significantly less with PMSG than with FSH (54 and 60h respectively, $P < 0.05$). Superovulation

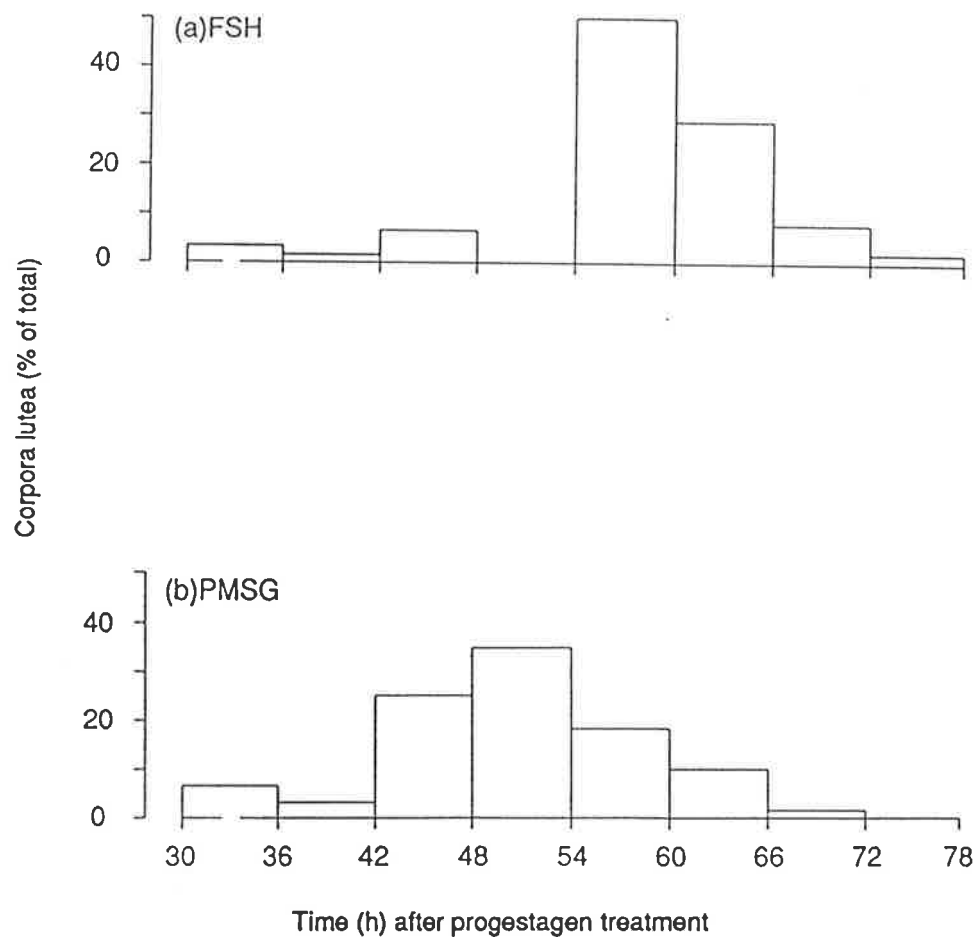


Figure 1. Number of new corpora lutea observed in successive 6-h periods after removal of progestagen pessary in ewes treated with (a) FSH or (b) PMSG.

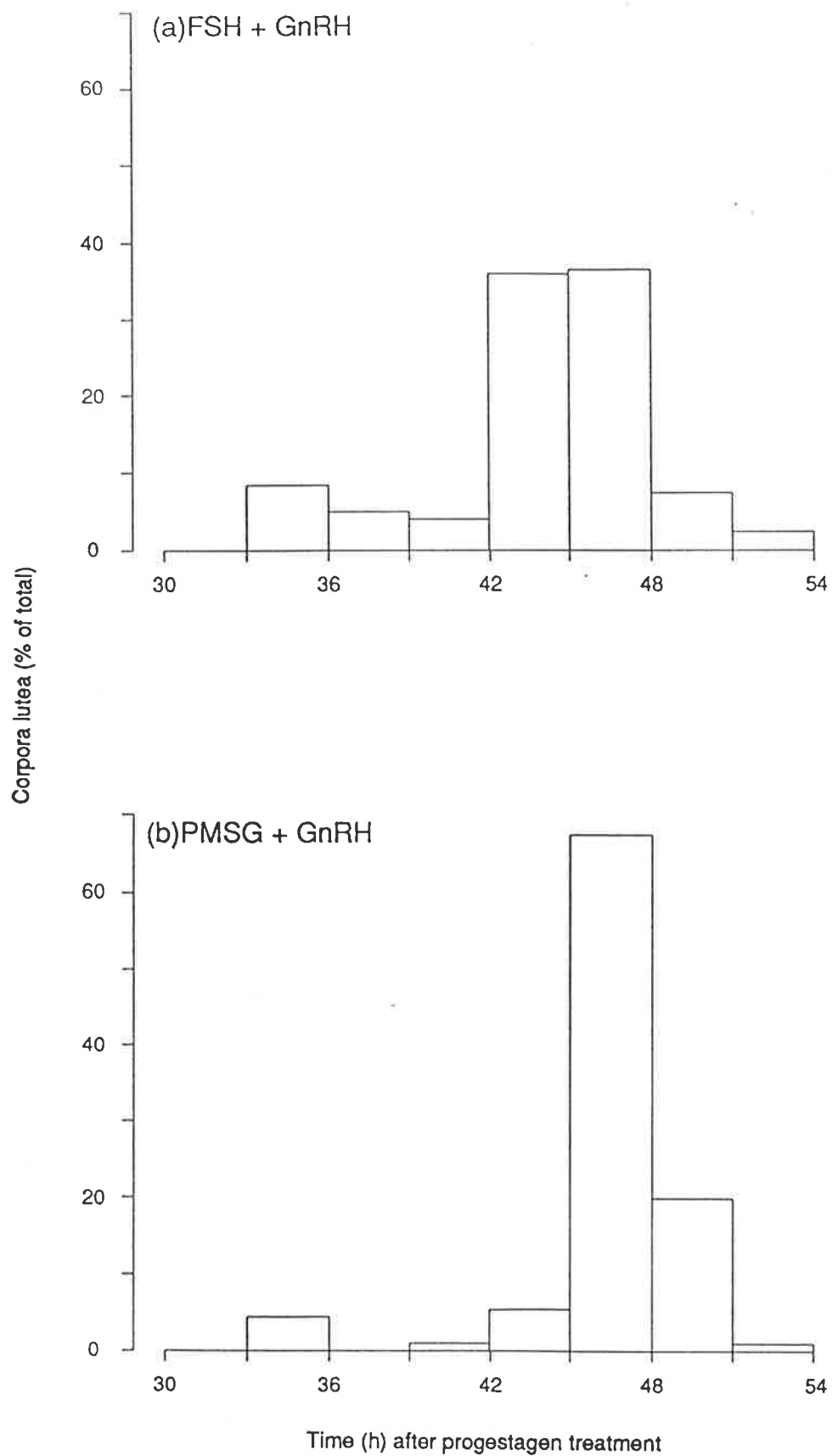


Figure 2. Number of new corpora lutea observed in successive 3-h periods after removal of progestagen pessary in ewes treated with (a) FSH + GnRH or (b) PMSG + GnRH.

Table 1. Ovulatory performance, as estimated by repeated laparoscopy, of ewes treated with FSH $\pm$ GnRH or PMSG $\pm$ GnRH. Observations were made every six hours in ewes treated with either FSH or PMSG and every three hours in ewes treated with either FSH + GnRH or PMSG + GnRH.

Treatment (N)	Time of GnRH injection* (h)	Proportion of ewes superovulated	Mean ovulation rate ($\pm$ s.e.m.)	Median (range) time (h)		
				First ovulation	All ovulations	First-last ^{**} ovulation
Exp. 1 (March)						
FSH(19)	—	17/19	8.4 $\pm$ 0.81 ^a	60 (<36–72)	60 (<36–78) ^a	6 (6–18)
PMSG(19)	—	14/19	7.3 $\pm$ 1.21 ^{ab}	48 (<36–60)	54 (<36–72) ^b	6 (6–24)
FSH+GnRH(30)	24	24/30	7.4 $\pm$ 0.78 ^{ab}	45 (<36–48)	45 (<36–54) ^a	6 (3–12)
PMSG+GnRH(19)	24	16/19	5.5 $\pm$ 0.55 ^b	45 (<36–51)	48 (<36–54) ^b	6 (3–9)
Exp. 2 (March)						
FSH+GnRH(12)	16	10/12	5.3 $\pm$ 0.97	35 (<33–39) ^a	39 (<33–39) ^a	9 (6–15) ^a
FSH+GnRH(13)	20	12/13	6.6 $\pm$ 0.87	42 (<33–42) ^b	42 (<33–48) ^b	6 (3–12) ^b
FSH+GnRH(12)	24	10/12	4.5 $\pm$ 0.73	45 (<33–48) ^c	45 (<33–48) ^c	6 (3–6) ^b

*Relative to time of progestagen pessary removal.

**Excludes premature ovulations and is a measure of time taken for superovulation.

Within each column and between rows, means with different superscripts are significantly different, $P < 0.05$.

Table 2. Number of ewes assessed to ovulate for the first time and in parentheses the number of ewes assessed as having begun to superovulate at different times after progestagen pessary removal (a, 6-h interval; b, 3-h interval).

	Time (h) after progestagen pessary removal											
	30	33	36	39	42	45	48	51	54	60	66	72
(a) FSH (Exp. 1)	0	—	6	—	1	—	1(1)	—	0	6(9)	4(6)	1(1)
PMSG (Exp. 1)	0	—	2(1)	—	3	—	5(3)	—	5(5)	4(3)	0(2)	0
(b) FSH+GnRH (Exp. 1)	0	0	7(3)	2(1)	1(1)	17(12)	3(7)	0				
PMSG+GnRH (Exp. 1)	0	0	5	0	2	3(1)	8(14)	2(1)				
FSH+GnRH (Exp. 2)												
Treatment 1	1(1)	1	6(3)	4(6)	0	0	0	0				
Treatment 2	2	0	0	3(5)	8(7)	0	0	0				
Treatment 3	2(1)	0	1	0	1(1)	7(6)	7(1)	1				

also occurred over a longer period in PMSG-treated ewes; 78% of the ovulations occurred between 42 and 60h after pessary removal whereas 79% of the FSH-stimulated ovulations occurred between 54 and 66h (Figure 1).

Premature ovulations were observed in 7/17 and 4/14 ewes that superovulated in the FSH and PMSG treatments respectively. However, these ovulations accounted for only 4.9 and 5.7% of the total ovulations of the respective groups (Figure 1).

2.1.3.2 FSH + GnRH and PMSG + GnRH Treatments

There was no significant difference in the ovulatory response of FSH-treated ewes administered 25 or 100 ug GnRH. The results have therefore been combined.

There were no significant differences between treatments in ovulation rate, median time to first ovulation or in median time from first to last ovulation (Table 1). However, median time of all ovulations was significantly less with FSH + GnRH than with PMSG + GnRH (45 and 48h respectively, $P < 0.05$). A high degree of synchrony in the time of ovulation was obtained in both treatments (Figure 2).

Premature ovulations were observed in 6/24 and 6/16 ewes superovulated in the FSH + GnRH and PMSG + GnRH treatments respectively and these ovulations accounted for 18.3 and 11.5% of the total ovulations of the respective groups.

2.1.3.3 Optimal Time of GnRH Administration

There were significant ($P < 0.05$) effects of treatment on median time to first ovulation, median time of all ovulations and on the median time from first to last ovulation (Table 1). Median time to first ovulation and of all ovulations increased as the time between pessary removal and

GnRH administration increased. The median time from first to last ovulation was longer in ewes treated with GnRH at 16h after pessary removal compared with treatment at 20 and 24h.

Premature ovulations were observed in 1/10, 1/10 and 2/10 ewes that superovulated in Treatments 1, 2 and 3 respectively. These ovulations accounted for only 3.2, 3.5 and 3.6% of the total ovulations in the respective treatments.

2.1.3.4 Effects of Stress

There was no significant effect of repeated laparoscopy (2 - 9 observations per sheep) on ovulation rate. PMSG-treated ewes that underwent repeated laparoscopy had a mean ovulation rate of 7.3 ± 1.21 (N = 19) compared with 8.9 ± 1.03 (N = 12) in control ewes that underwent laparoscopy on only two occasions. Comparable figures for ewes treated with PMSG + GnRH were 5.5 ± 0.55 (N = 19) and 4.8 ± 1.09 (N = 11). However, 12.6% (13/103) of the ovulations in the control ewes occurred later than 72h after pessary removal whereas no ovulations occurred in this period in ewes that underwent repeated laparoscopy ($P < 0.001$).

2.1.4 DISCUSSION

The time of ovulation in relation to pessary withdrawal is known to be advanced by treatment with both PMSG (Killeen and Moore, 1970) and FSH (Evans and Armstrong, 1984). In the present study, ewes treated with PMSG commenced ovulation earlier than those treated with FSH (Table 1) but the time of ovulation was less synchronous in the former treatment (Figure 1). In FSH-treated ewes, most ovulations occurred between 54 and 60h after pessary removal, confirming similar data of Evans et al. (1984).

Several factors contributed to the spread in the time of ovulation. Firstly, premature ovulations were observed in all treatments although their contribution to total ovulations was small (11.1 and 10.7% in FSH and PMSG treatments respectively). Secondly, some ewes required two or more six-hour periods to complete superovulation. A total of 4/17 and 5/14 ewes in the FSH and PMSG treatments respectively fitted this category. Thirdly, there was a lack of synchrony in the time when ewes began to superovulate. This was greater in the PMSG treatment (36 - 66h) than in the FSH treatment (48 - 72h) (Table 2).

Administration of GnRH to ewes synchronized the time of ovulation; 19/24 and 15/16 ewes in Experiment 1 treated with FSH + GnRH and PMSG + GnRH respectively commenced ovulation at either 45 or 48h after pessary removal. The synchrony was due to a reduction in both the time from first to last ovulation and in the time when superovulation began (Table 2). A similar degree of synchrony was obtained at 20 compared with 24h after pessary removal. Premature ovulations were also observed in ewes treated with GnRH.

These findings are relevant to the time of collection of zygotes following gonadotrophin treatment. Attempts to collect zygotes following superovulation have resulted in the collection of embryos at various stages of development (Moore, 1970; Rexroad and Powell, 1988a). The ability of GnRH treatment to induce approximately 80% of ovulations within six hours will, with correct timing of collection, ensure that most cells are collected before first cell division. Some cleaved embryos may still be obtained because of the occurrence of premature ovulations (assuming ova produced from those ovulations are viable).

These findings are also relevant to the time of insemination in the superovulated ewe. Time of intra-uterine insemination has varied between 24 - 60h after pessary removal (Armstrong and Evans, 1984b; Evans and Armstrong, 1984; Evans et al., 1984; Ryan et al., 1984). However, the

ability to synchronize the time of ovulation using GnRH treatment necessitates a reassessment of the optimal time of insemination and also the minimum number of spermatozoa required for maximum fertilization rates.

It is postulated that the spread in the time of ovulation following either FSH or PMSG treatment contributes to fertilization failure. However, the likelihood of fertilization failure occurring after GnRH treatment is low assuming that ova produced are viable and insemination is correctly timed. Nancarrow et al. (1984a) reported that, in ewes treated with PMSG and inseminated into the uterus with fresh semen, 75.9% of the ova were fertilized when GnRH was used compared with 49.8% without GnRH. There was an associated increase in embryo yield (from 1.9 to 4.2 per ewe) although this increase may have been due in part to an increase in ovulation rate.

In summary, results of this study indicate that time of ovulation after gonadotrophin treatment is imprecise unless ewes are treated with GnRH. This synchrony may lead to an improvement in embryo yield and also the production of embryos of a very similar age. An assessment of the use of GnRH treatment in embryo collection programs is reported in Chapter 4.

2.2 EXPERIMENT 2. SEASONAL VARIATION IN THE TIME OF SUPEROVULATION AND ITS RELEVANCE TO THE COLLECTION OF ZYGOTES

2.2.1 INTRODUCTION

Collecting zygotes throughout the year is desirable for the continuity of microinjection programs. In Southern Australia, this is possible in Merino ewes since they ovulate naturally or can be induced to ovulate in

any month (Cutten, 1980). Results of the previous experiment indicate that the time of ovulation is imprecise unless GnRH is included in the treatment protocol. Experiment 1 was conducted during the breeding season and no information is available on the effect of the treatment at other times of the year.

Thus, the aims of this study were (1) to examine the time of multiple ovulations in Merino ewes following treatment with either PMSG + GnRH or FSH + GnRH at four times of the year, (2) to identify factors affecting the synchrony of time of ovulation following GnRH treatment, and (3) to assess the relevance of variation in the time of ovulation on the collection of zygotes.

2.2.2 MATERIALS AND METHODS

2.2.2.1 Sheep

Observations were made in two South Australian Merino strains designated Flock 1 (Collinsville strain) and Flock 2 (Koonoona strain). Both flocks consisted of mixed-age ewes located at Turretfield Research Centre. Collinsville and Koonoona are parent studs within the strong-wool sheep population of Australia; a parent stud is defined (Roberts, Jackson and Phillips, 1975) as one "closed" for at least 20 successive years with a maximum of one introduced ram during that period.

2.2.2.2 Method of Superovulation

The methods used are described in Chapter 2.1.2.1. In this study, synthetic GnRH was obtained from Intervet (Australia) Pty Ltd (Artamon, New South Wales); it was administered 24h after pessary removal in doses of 100 ug Gonadorelin per ewe (i.v.).

2.2.2.3 Experimental Procedure

In Part 1, the time of superovulation was observed in both flocks in May (late breeding season), August (early anoestrous season), November (late anoestrous season), and February (midbreeding season) of 1985/86. In each month, the ovulatory status of 30 ewes in each flock was determined by laparoscopy. These ewes were allocated to either FSH + GnRH or PMSG + GnRH treatment groups so that each group contained the same proportion of ovular and anovular ewes. These animals were not used in subsequent observations.

Part 2 was conducted in August 1985 to determine if the time of superovulation in naturally cycling ewes was similar to that in anoestrous ewes induced to ovulate using the ram effect. Fifteen ovular and 15 anovular ewes were identified in Flock 2 by laparoscopy and treated with PMSG + GnRH. In both Parts 1 and 2, ewes were exposed to vasectomized rams from the time of pessary removal until laparoscopy.

In Part 3, the ovulatory pattern of ewes exposed to vasectomized rams at the time of GnRH administration was compared with that observed in ewes exposed at the time of pessary removal. Thirty-two ewes in Flock 2 were examined by laparoscopy, and ovular and anovular ewes were equally allocated to either PMSG + GnRH or FSH + GnRH treatments. This study was conducted concurrently with the November observation of Part 1 in which ewes were exposed to vasectomized rams at the time of pessary removal.

Part 4 was conducted in August 1986 to examine the time of superovulation in ewes treated with either PMSG or FSH without GnRH. August was the month in which greatest variability in the time of ovulation was observed in ewes treated with GnRH (see results of Part 1). In this experiment, 40 ewes from Flock 2 were randomly allocated to each gonadotrophin treatment. Ewes were exposed to vasectomized rams at the time of pessary removal.

Ovaries were examined by laparoscopy and the number of corpora lutea recorded. Observations on each ewe were made hourly in Parts 1 and 3, every 2h in Part 2 and every 6h in Part 4. In Part 1, the time of first observation was initially 45h after pessary removal (May) but was subsequently reduced to 42h (August) and then to 38h (November and February) as variation in the synchrony of superovulation became apparent. In Parts 2 and 3, ewes were first observed 38h after pessary removal while in Part 4 observation commenced at 36h. Observations were completed by 51h in Parts 1 and 3, by 52h in Part 2 and by 78h in Part 4. Ewes in the May and July observations of Part 1 and all ewes in Part 2 were re-examined seven days after observation to assess accuracy of records.

2.2.2.4 Statistical Analyses

Early superovulation was defined as that occurring before 45h after pessary removal in Parts 1 to 3 and before 42h in Part 4. Data analysed were time to first ovulation, time of all ovulations and time from first to last ovulation. Medians were compared using the Mann-Whitney test; the Kruskal-Wallis test was used for one-way analysis of variance (Siegal, 1956). The Chi-square test was used to compare numbers of ewes ovulating. Premature ovulations (see Chapter 2.1.3) were excluded from the analysis.

2.2.3 RESULTS

2.2.3.1 Variation Through the Year (Part 1).

The mean numbers ($\pm$ s.e.m.) of corpora lutea recorded per ewe in Flock 1 were 4.4 ± 1.02 and 7.2 ± 0.68 for the PMSG + GnRH and FSH + GnRH

treatments, respectively. Comparable figures for Flock 2 were 6.2 ± 1.02 and 8.1 ± 1.07 . Means did not differ significantly with month of observation.

The median time of all ovulations in Flock 1 varied between 47 - 49h after pessary removal (Table 3). Between 74.5 - 87.5% and 69.7 - 87.7% of the ovulations occurred within three hours for the PMSG + GnRH and FSH + GnRH treatments, respectively. Significant differences occurred between months in some traits; time to first ovulation and time of all ovulations were significantly ($P < 0.001$) longer in May compared with other months. Significant differences between gonadotrophin treatments were recorded in some months for the time of all ovulations and for the time from first to last ovulation.

In contrast, there was marked asynchrony in the time of ovulation in Flock 2 during the year (Figures 3a and 3b); 3/29, 16/27, 1/30 and 2/30 ewes in May, August, November and February respectively, commenced superovulation within 45h of pessary removal (Table 4). These figures differed significantly between months ($P < 0.001$). Ewes that ovulated early accounted for 16.5, 65.6, 9.2, and 7.5% of all ovulations recorded in the respective months. This phenomenon was observed in both gonadotrophin treatments.

Analysing the pattern of superovulation in Flock 2 is difficult because of the asynchrony. However, if the early ovulations are analysed as one category, then there was a significant effect of month on time to first ovulation ($P < 0.001$), time of all ovulations ($P < 0.001$), and time from first to last ovulation ($P < 0.05$) in both gonadotrophin treatments. Similarly, significant ($P < 0.05$) differences between gonadotrophin treatments occurred in the time to first ovulation and time to all ovulations; median times were one hour longer with FSH compared with PMSG treatment.

2.2.3.2 Ovular Ewes Versus Previously Anovular Ewes (Part 2).

The mean numbers ($\pm$ s.e.m.) of corpora lutea recorded per ewe were 4.5 ± 0.70 and 4.8 ± 0.45 for the ovular and previously anovular groups respectively. Superovulation commenced early (before 45h) in 1/15 ewes in the ovular group compared with 4/15 in the previously anovular group ($P > 0.05$); these ovulations accounted for 8.4 and 46.2% of the total ovulations recorded for the respective groups. In the previously anovular group, three of the four ewes that ovulated early did so within 38h of pessary removal whilst the one ewe in the ovular group commenced ovulation 39 to 41h after pessary removal.

2.2.3.3 Time of Exposure to Vasectomized Rams (Part 3).

The mean numbers ($\pm$ s.e.m.) of corpora lutea recorded per ewe were 5.1 ± 0.79 and 7.30 ± 0.90 for the PMSG + GnRH and FSH + GnRH treatments respectively. Early superovulation (before 45h) was observed in 4/16 ewes treated with FSH, even though the introduction of rams was delayed to coincide with the administration of GnRH. All four ewes commenced ovulation within 38h of pessary removal and these ewes produced 32% of all ovulations recorded for that treatment.

Early superovulation was not observed in 16 ewes treated with PMSG + GnRH.

2.2.3.4 PMSG Versus FSH in August (Part 4).

The mean numbers ($\pm$ s.e.m.) of corpora lutea recorded per ewe were 6.6 ± 1.04 and 9.0 ± 1.50 for the PMSG and FSH treatments, respectively; 1/20 and 5/20 ewes in the respective treatments failed to ovulate. Superovulation before 42h after pessary removal occurred in 5/19 and 4/15

Table 3. Time of superovulation in Flock 1 ewes following treatment with either PMSG + GnRH or FSH + GnRH (Part 1). Data on first ovulation and all ovulations are presented as median times (h) after progestagen pessary removal.

		Month of observation			
		Gonadotrophin	May	Aug	Nov
First ovulation	PMSG	48	47	47	47
	FSH	48	48	47	48
All ovulations	PMSG	48 ^a	48	47 ^a	47 ^a
	FSH	49 ^b	48	48 ^b	48 ^b
First-last ovulation	PMSG	1.5	2	2 ^a	1 ^a
	FSH	2	3	3 ^b	2 ^b

Within each column, medians with different superscripts differ significantly ($P < 0.05$).

Table 4. Proportion of ewes in Part 1 that commenced superovulation within 45h of progestagen pessary removal.

Flock		Gonadotrophin	Time of year			
			May	Aug	Nov	Feb
1	PMSG		0/14	0/12	0/14	0/13
	FSH		0/14	0/13	1/14	0/15
2	PMSG		3/18	8/13	1/15	0/15
	FSH		0/11	8/14	0/15	2/15

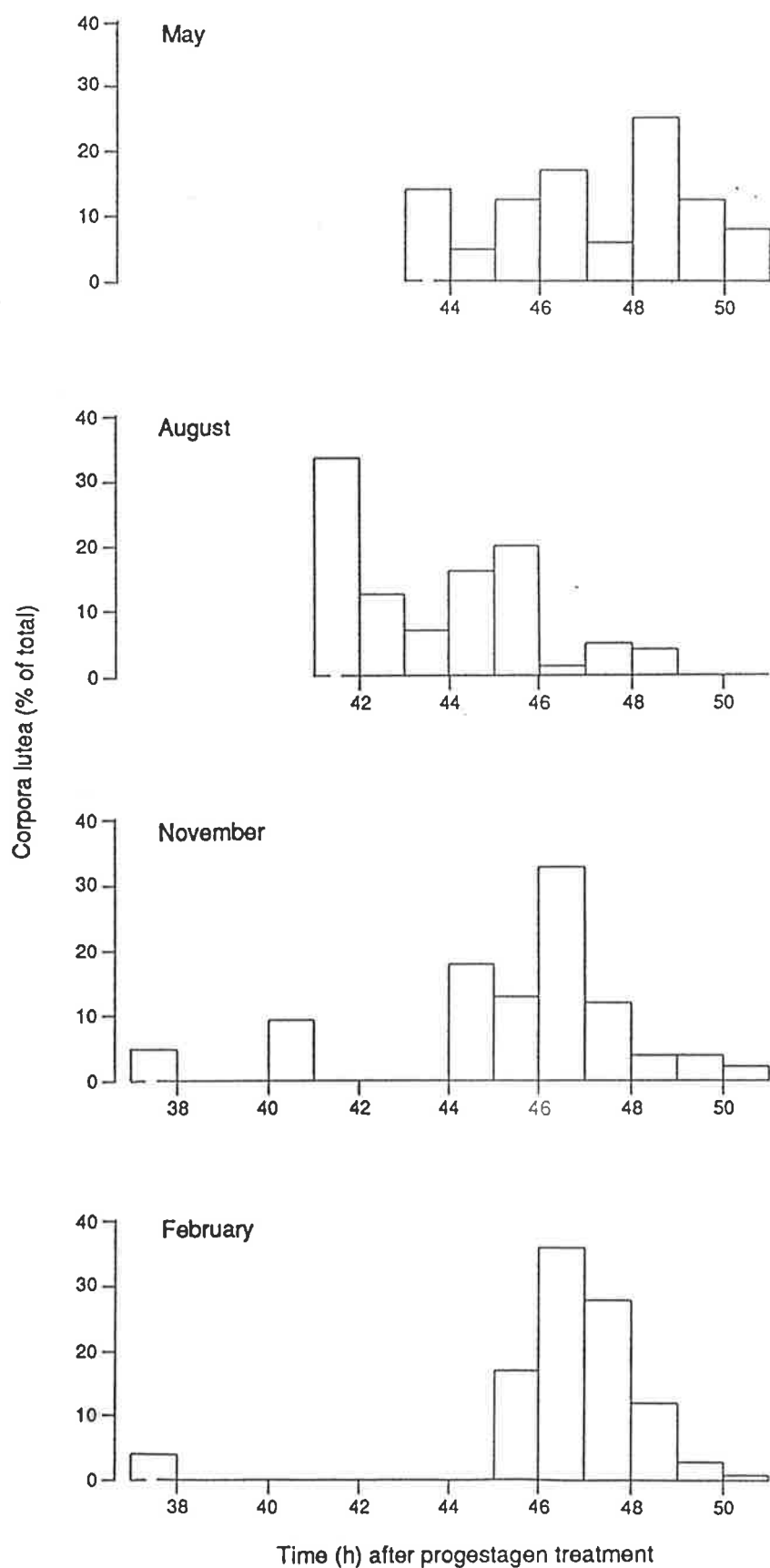


Figure 3a. Number of new corpora lutea recorded in successive 1-h periods in Flock 2 ewes treated with PMSG + GnRH at four times of the year (Part 1).

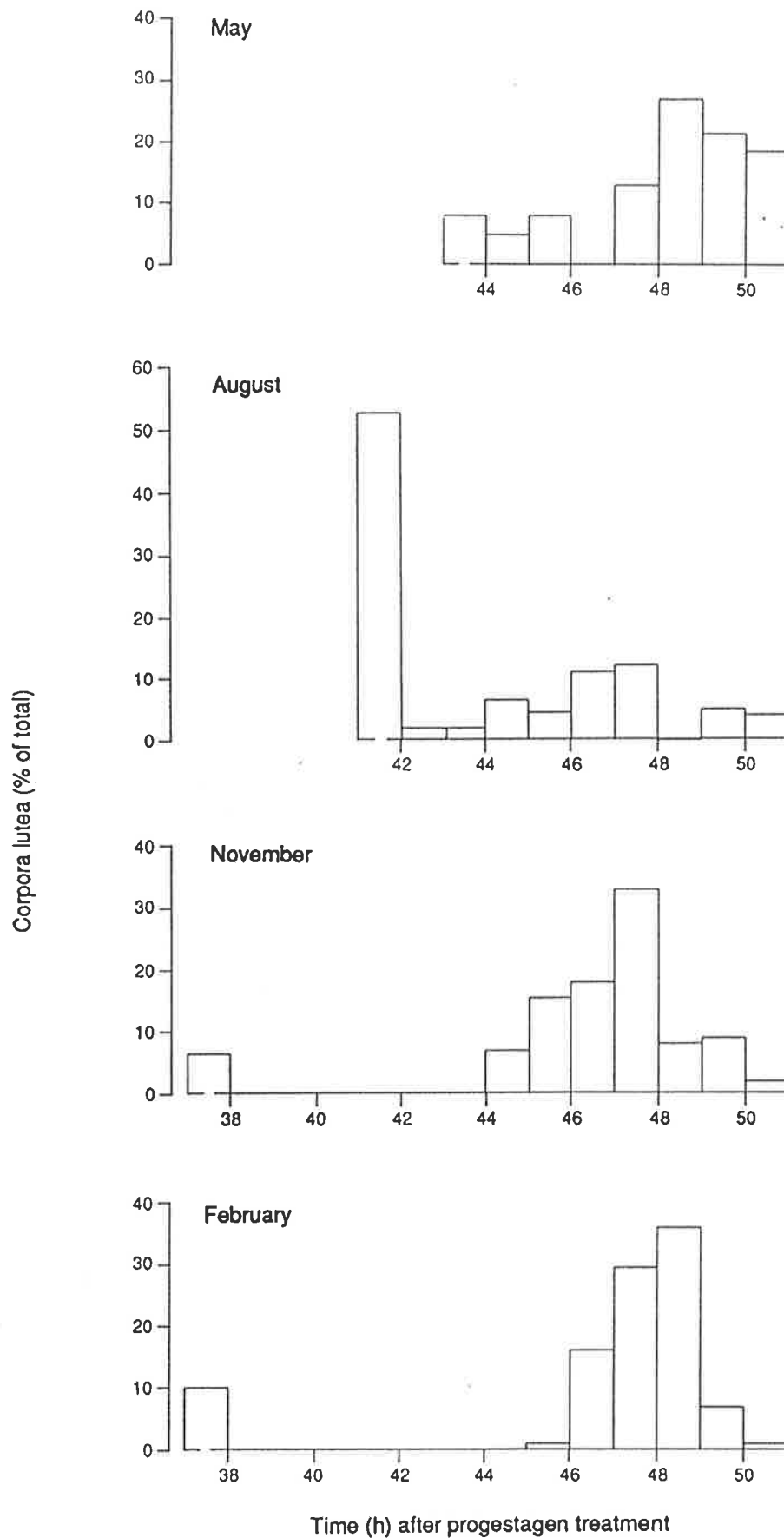


Figure 3b. Number of new corpora lutea recorded in successive 1-h periods in Flock 2 ewes treated with FSH + GnRH at four times of the year. (Part 1).

ewes that ovulated in the PMSG and FSH treatments respectively. These ewes produced 31.9 and 23.0% of the ovulations recorded in the respective groups.

2.2.3.5 Accuracy of Records

Additional corpora lutea were recorded in ewes re-examined seven days after the initial observation. An extra 50 corpora lutea were observed in 106 ewes in Part 1 while an additional five corpora lutea were observed in the 30 ewes of Part 2. The discrepancy in Part 1 appeared to be associated with premature regression and reovulation within seven days of the initial observation. In Part 2, the additional ovulations were in ewes removed from the study presumably before all ovulations had occurred.

2.2.4 DISCUSSION

The GnRH-induced synchrony in the time of superovulation obtained in Experiment 1 was also obtained in this study. The synchrony occurred in Flock 1 irrespective of the stage of the breeding season and in Flock 2 during the late anoestrous and midbreeding season (Figures 3a and 3b). Features of the synchrony were (1) approximately 80% of all ovulations were recorded within three hours, (2) the time of the median ovulation varied from 47 to 49h after pessary removal, (3) superovulation was a relatively brief event taking on average one to two hours per ewe and (4) only slight differences occurred in the time of ovulation between ewes treated with either FSH + GnRH or PMSG + GnRH.

During the late breeding and early anoestrous seasons, superovulation commenced in some ewes in Flock 2, ten or more hours before the medians obtained during the late anoestrous and midbreeding seasons. It

is unlikely that GnRH treatment caused these ovulations given that nine ewes in Part 4 superovulated early despite not being treated with GnRH. The preovulatory LH surge in sheep occurs approximately 22 to 26h before ovulation (Cumming et al., 1973), indicating that an LH surge occurred before the administration of GnRH. Ram introduction at the time of pessary removal was unlikely to be responsible, given that delaying ram introduction for 24h did not prevent the occurrence of early superovulation.

Early superovulation was more common in previously anovular ewes compared with naturally cycling ewes (Part 2). Although this difference was not significant, several other factors indicated that the phenomenon may be associated with time of the breeding season. Firstly, the number of ewes superovulating early in Flock 2 differed significantly with month of observation ($P < 0.001$, Part 1); early superovulation was most prevalent in the early anoestrous period (Figures 3a and 3b). Secondly, early superovulation was observed in ewes treated with either PMSG or FSH in August (Part 4) but was not obtained in Experiment 1 which was conducted during the breeding season. Furthermore, early superovulation was uncommon in Flock 1 and most ewes (81 to 87%) in this flock remained cyclic throughout the year. In contrast, 61 and 29% of ewes in Flock 2 were cycling naturally in August and November respectively; these figures are typical for Merino ewes in Southern Australia (Cutten, 1980). The observation that more ewes superovulated early in Flock 2 in August (16/27) than in November (1/30) indicates that the phenomenon may be associated with the transition between the breeding and nonbreeding seasons.

Any asynchrony in the time of superovulation is likely to affect fertilization rates (particularly if fixed-time artificial insemination is used) and also embryo age. Microinjection programs are dependent on the availability of zygotes and are thus likely to be adversely affected

by the occurrence of early superovulation. Early superovulation has been observed in additional flocks, including the Collinsville strain, since the completion of this study. These observations were also made during either the late breeding or anoestrous seasons and all were associated with a marked increase in the proportion of two- and four-cell embryos in flushings of the reproductive tract. Results of a preliminary investigation (unpublished data) indicate that a ram-induced ovulation preceding progestagen treatment will eliminate early onset of superovulation in previously anoestrous ewes.

In conclusion, it is postulated that a relatively early preovulatory LH surge occurs in some Merino ewes induced to superovulate and that this surge pre-empts that otherwise induced by GnRH treatment. This phenomenon is associated with the time of the breeding season and is likely to restrict the year-round supply of zygotes for microinjection. The severity of the problem will, however, vary between flocks.

CHAPTER 3

TIME OF OVULATION IN THE NONSUPEROVULATED EWE FOLLOWING SYNCHRONY OF OESTRUS AND ITS RELEVANCE TO EMBRYO TRANSFER AND ARTIFICIAL INSEMINATION

3.1 EXPERIMENT 1. VARIATION IN THE TIME OF OVULATION WITHIN AND BETWEEN FLOCKS.

3.1.1 INTRODUCTION

Although various aspects of embryo transfer in the sheep have been researched (see Wilmut et al., 1985a, 1985b), little is known of the optimal conditions for the transfer of zygotes. Factors known to influence the success of embryo transfer include synchrony of oestrus between donor and recipient (Moore and Shelton, 1964b; Rowson and Moor, 1966; Wilmut and Sales, 1981), age of embryos (Moore and Shelton, 1964b; Rowson and Moor, 1966) and site of transfer (Moore and Shelton, 1964b). Moore and Shelton (1964b) reported a significant increase in the number of ewes that lambed as age of the transferred embryos increased (range two-cell to greater than eight-cell), indicating specific requirements for the transfer of early stage embryos.

The most appropriate criterion for synchrony between donor and recipient is time of ovulation. There are, however, few direct estimates of the time of ovulation in nonsuperovulated ewes following synchrony of oestrus. In Galway ewes, ovulation was estimated to occur between 48/58h and 78/88h after pessary removal (Quirke et al., 1979), while in Karakul ewes an average time of ovulation of 46.8 - 62.8h was obtained (Boshoff et al., 1973) depending on the dose of PMSG. In the Australian Merino, a median time of ovulation of 55.8h (range 52/56 - 60/64h) was reported in

ewes previously exposed to rams compared with 59.7h (range < 52h to > 64h) in ewes not previously exposed (Maxwell, 1986a).

The aim of this study was to determine the within and between flock variation in the time of ovulation and to assess the likely significance of this variation in embryo transfer programs, particularly those involving zygotes. The effects of the type of progestagen and the source of PMSG on the time of ovulation were also studied in an attempt to identify the likely causes of the variation observed.

3.1.2 MATERIALS AND METHODS

3.1.2.1 Sheep

Seven flocks of South Australian Merino ewes were purchased from the commercial market over two years. Six flocks (numbered 1 - 6 inclusively) were of multiparous ewes and one flock (number 7) was of two-year-old nulliparous ewes. The flocks were located at Turretfield Research Centre. Observations described below were made within two months of purchase.

3.1.2.2 Time of Ovulation: Variation Within and Between Flocks

The time of ovulation was determined in a random sample of sheep (N = 10 - 34) from each of the seven flocks. The first flock was observed in November 1985 and the last flock in January 1988. There were three observations of Flocks 1 and 2, two observations of Flock 3 and one observation of the remaining four flocks.

Ewes were treated with intravaginal pessaries containing 60 mg MAP (Upjohn Pty Ltd, Sydney, New South Wales) for 12 days and PMSG (Pregnenol, 400 i.u. per ewe; Heriot Agvet Pty Ltd, Melbourne, Victoria)

at the time of pessary removal. Time of ovulation was assessed by repeated observations of the ovaries using the laparoscope. Observations commenced 57h after pessary removal and continued every six hours for a maximum of 24h unless ovulation was assessed as being completed.

3.1.2.3 Effect of Different Progestagens or Progesterone

Ewes in Flock 3 were treated for 12 days with intravaginal pessaries containing either 60 mg MAP (N = 18), 40 mg FGA (Intervet (Australia) Pty Ltd, Artamon, New South Wales; N = 17) or CIDR-progesterone (AHI Plastic Moulding Co., Hamilton, New Zealand; N = 17). All ewes were administered Pregnenol (400 i.u. per ewe) at the time of pessary removal. Observations with the laparoscope commenced 45h after pessary removal in ewes treated with progesterone and 57h in other ewes. This study was conducted in April 1987.

3.1.2.4 Effect of Different Gonadotrophin Preparations

Time of ovulation was examined in ewes treated with 60 mg MAP for 12 days and 400 i.u. per ewe of either Pregnenol or Folligon (Intervet (Australia) Pty Ltd, Artamon, New South Wales). Observations were made in Flock 4 in February 1987 (14 ewes per treatment) and in Flock 6 in January 1988 (18 and 19 ewes per treatment).

3.1.2.5 Effect of Stress

The effect of stress associated with laparoscopy on the time of ovulation was examined in three groups of ewes (N = 14 - 16 per group) from Flock 2. Two groups were examined once, either at 57 or 81h after pessary removal and the other underwent repeated laparoscopy as described above. The effect of stress was assessed by comparing, between groups, the

number of ewes that had ovulated at either 57 or 81h and the number of ovulations per ewe. In all studies, ewes were exposed to vasectomized rams at the time of pessary removal.

3.1.2.6 Statistical Analyses

Data analysed included time to first ovulation and number of ovulations per ewe. Several ewes had multiple ovulations but an assessment of the time from first to last ovulation was not made because most ovulations occurred within one observation period. Medians were compared using the Mann-Whitney test and the Kruskal-Wallis test was used for one-way analysis of variance (Siegal, 1956) except where a multiple range test was required, in which case a parametric one-way analysis of variance was used.

3.1.3 RESULTS

3.1.3.1 Time of Ovulation: Variation Within and Between Flocks

Ovulation commenced within 57h of pessary removal in eight of twelve observations and was assessed as being completed in all observations by 81h (Table 5). However, in one of three observations of Flock 1 and in two of three observations of Flock 2, corpora lutea were not observed until 69h after pessary removal. Consequently, there were significant differences in the median time of ovulation between observations within Flocks 1 and 2 (63 v. 72 v. 63h, $P < 0.01$, Flock 1; 75 v. 72 v. 69h, $P < 0.01$, Flock 2). Delayed onset of ovulation was associated with a high proportion of anovular ewes (Table 5).

Ovulation commenced relatively early in Flock 7 (two-year-old ewes) with corpora lutea observed in 18/39 ewes within 57h of pessary removal.

Table 5. Number of ewes in seven South Australian Merino flocks observed to commence ovulation at different times following treatment with MAP-PMSG.

Flock no.	Month	No. ewes ovulated	Ovulations* per ewe ($\pm$ s.e.m.)	Time (h) after pessary removal					Median** time (h)
				57	63	69	75	81	
1	Nov.	12/15	2.1 ± 0.22^a	1	6	4	1	0	63^a
	Dec.	16/30		0	0	8	7	1	72^b
	Feb.	13/15		2	7	3	1	0	63^a
2	Nov.	8/14	2.2 ± 0.15^a	0	0	2	4	2	75^a
	Dec.	8/11		0	0	4	4	0	72^{ab}
	April	30/34		2	9	13	3	3	69^b
3	Jan.	10/10	3.0 ± 0.31^{bc}	1	6	2	1	0	63^a
	April	17/18		1	7	4	3	2	69^a
4	Feb.	14/14	3.2 ± 0.76^c	1	2	6	3	2	69
5	April	16/20	2.1 ± 0.22^a	3	3	6	0	4	69
6	Jan.	16/19	2.7 ± 0.27^b	0	3	9	3	1	69
7	Jan.	39/39	1.9 ± 0.25^a	18	17	3	1	0	63

*Means within flocks not significantly different (data combined); values with different superscripts differ significantly ($P < 0.05$).

**Values within flocks with different superscripts differ significantly ($P < 0.05$); comparisons between flocks made within the text.

Table 6. Number of ewes observed to commence ovulation at different times following treatment with either MAP, FGA or CIDR-progesterone. Each ewe was administered 400 i.u. Pregnecol at pessary removal.

Treatment	No. ewes ovulated	Ovulations per ewe ($\pm$ s.e.m.)	Time (h) after pessary removal							Median time(h)
			45	51	57	63	69	75	81	
MAP	17/18	3.1 $\pm$ 0.46	*	*	1	7	4	3	2	69 ^a
FGA	17/17	2.7 $\pm$ 0.48	*	*	2	7	5	3	0	63 ^a
CIDR	17/17	1.8 $\pm$ 0.33	3	7	5	0	2	*	*	51 ^b

*Not examined. Values within columns with different superscripts differ significantly ($P < 0.05$).

Table 7. Number of ewes observed to commence ovulation at different times following treatment with either Pregnecol or Folligon (400 i.u. per ewe). Ewes were pre-treated with MAP for 12-days.

Treatment	No. ewes ovulated	Ovulations per ewe ($\pm$ s.e.m.)	Time (h) after pessary removal					Median time (h)
			57	63	69	75	81	
Pregnecol	30/33	2.9 $\pm$ 0.30	1	5	15	6	3	69
Folligon	31/34	2.9 $\pm$ 0.31	2	10	13	6	0	69

The median time of ovulation (63h) was significantly ($P < 0.01$) different from those of all other observations with the exception of the November and February observations in Flock 1 and the January observation in Flock 3 (Table 5).

There were no significant differences between observations within flocks in the number of corpora lutea recorded per ewe, but there were significant differences between flocks (range $1.9 \pm 0.25 - 3.2 \pm 0.76$) (Table 5).

3.1.3.2 Effect of Different Progestagens or Progesterone

Ewes treated with CIDR-progesterone commenced ovulation significantly ($P < 0.001$) earlier than ewes treated with either MAP or FGA (Table 6); the median times were 51, 69 and 63h respectively. Progesterone treatment resulted in a similar synchrony to that obtained with the other treatments. There were no significant differences between treatments in the number of corpora lutea recorded per ewe.

3.1.3.3 Effect of Different Gonadotrophin Preparations

There were no significant differences in the time of ovulation following treatment with either Pregnenol or Folligon in either Flock 4 or 7. Consequently the data are combined and summarized in Table 7. The number of corpora lutea per ewe did not differ significantly between the two treatments.

3.1.3.4 Effects of Stress

There were no significant differences in either the number of ewes ovulating or the number of ovulations per ewe following repeated laparoscopy compared with ewes observed at either 57 or 81h.

3.1.4 DISCUSSION

In the most common ovulatory pattern, ovulation commenced within 57h of pessary removal and continued until 81h, with the median ovulation occurring at either 63 or 69h. These medians were similar to that reported in Galway ewes (Quirke et al., 1979) but were later than those reported for Karakul (Boshoff et al., 1973) and Merino ewes (Maxwell, 1986a). Neither the progestagen nor PMSG treatment significantly influenced time of ovulation. However, treatment with CIDR-progesterone significantly advanced the time of ovulation, even though a previous study (Maxwell and Barnes, 1986) indicated that oestrus occurred at a similar time to that in ewes treated with MAP.

The most significant finding of this study was the identification of the variation that existed within and between flocks in the time of ovulation. Delayed onset of ovulation (69h after pessary removal) was observed in one or more observations in both Flocks 1 and 2 while a significantly earlier onset of ovulation was observed in Flock 7. The effects of several factors on the time of ovulation have been studied, including parity (no effect, Boshoff et al., 1973), exposure of ewes to rams (accelerated ovulation, Maxwell, 1986a; Lindsay, Cognie, Pelletier and Signoret, 1975) and time of breeding season (equivocal results, Boshoff et al., 1973; Van der Westhuysen et al., 1970). Time of breeding season has been reported to significantly influence the time of onset of oestrus in Merino (Lamond and Bindon, 1962), Coopworth (Smith et al., 1988) and Border Leicester x Merino ewes (Holst, 1969). In the latter study, onset of the induced oestrus during the non-breeding season

occurred 36 - 48h after pessary removal compared with 24 - 36h during the breeding season. In the current experiment, the delay in the commencement of ovulation was only observed in November and December (Table 5), a time when most Merino ewes would be expected to be in anoestrus (Cutten, 1980). It is most likely that seasonal refractoriness to PMSG was responsible for the delayed onset of ovulation. However, comparisons between flocks are limited by the numerous environmental and genotype differences. There was no detectable association between ovulation rate and time of ovulation nor was variability between batches of Pregnecol likely to be responsible because the phenomenon has also been observed following treatment with Folligon (unpublished observations).

Variation in the time of ovulation is likely to affect the efficacy of both embryo transfer and fixed-time artificial insemination. In embryo transfer, survival of embryos is optimized if the time of onset of oestrus between donor and recipient is within 12h (Moore and Shelton, 1964b). Variation in the time of ovulation of the magnitude observed within and between flocks in this study is likely to result in significant asynchrony in some transfers. This may be more significant with the transfer of zygotes compared with embryos. On the other hand, the time of insemination is chosen so that it occurs shortly before ovulation but based on the present data, spermatozoa would need to remain viable for up to 24h if all sheep were to conceive. However, the viability of frozen-thawed semen can begin to decline within 6 - 12h of insemination (see Chapter 4.3.3) and the use of a standard time of insemination across all flocks could result in poor conception rates, particularly in those flocks in which the onset of ovulation is delayed.

Comparable studies have not been conducted in other strains of Merino nor in other breeds of sheep. Observations, within flocks, are

continuing in the hope of gaining further knowledge of the factors involved.

3.2 EXPERIMENT 2. THE EFFECT OF GnRH TREATMENT ON THE TIME OF OVULATION IN THE NONSUPEROVULATED EWE AND ITS USE IN EMBRYO TRANSFER AND ARTIFICIAL INSEMINATION PROGRAMS

3.2.1 INTRODUCTION

Results of the previous experiment indicate that significant variation in the time of ovulation occurs both within and between flocks of South Australian Merino ewes following synchrony of oestrus. It is postulated that this variation contributes to embryo wastage after transfer due to asynchrony between some donors and recipients. Similarly, inappropriate timing of insemination relative to the time of ovulation could affect the efficacy of artificial insemination programs, particularly when frozen-thawed semen is used.

Treatment with GnRH is potentially useful in controlled breeding programs because of the ability to synchronize the time of ovulation (Quirke et al., 1979). The aims of this study were thus (1) to examine the effect of GnRH treatment on the variability in the time of ovulation in the nonsuperovulated ewe, (2) to examine the effect of GnRH treatment on the quality of embryos produced following the synchronized ovulation, (3) to determine if GnRH treatment in recipient ewes improves the survival of embryos after transfer and (4) to determine if GnRH treatment improves conception rates following the insemination of frozen-thawed semen.

3.2.2 MATERIALS AND METHODS

3.2.2.1 Sheep

Eight flocks of South Australian Merino ewes were studied. Flocks 1 - 4 and 7 - 8 were ewes of mature age located at Turretfield Research Centre, Flock 5 was a mixed-age flock located at Mt. Bryan ($138^{\circ} 57'E$, $33^{\circ} 40'S$) and Flock 6 consisted of two and three-year-old ewes located at Wanbi Research Centre, South Australia ($140^{\circ} 19'E$, $34^{\circ} 46'S$).

3.2.2.2 Hormone Treatment

Recipient ewes and ewes to be inseminated were treated for 12 days with 60 mg MAP (Upjohn Pty Ltd, Sydney, New South Wales) and PMSG (400 i.u. per ewe; Heriot Agvet Pty Ltd, Melbourne, Victoria) was given at the time of pessary removal. Donor ewes were treated with 60 mg MAP and PMSG (1200 i.u. per ewe) was administered 36h before pessary removal. Synthetic GnRH (50 ug per ewe; Intervet (Australia) Pty Ltd, Artamon, New South Wales) was given (i.m.) to donor ewes 24h after pessary removal; details on GnRH treatment in nonsuperovulated ewes is provided below. Vasectomized rams were run with ewes from the time of pessary removal until either laparoscopy or laparotomy.

3.2.2.3 Semen Collection and Insemination

Ewes were inseminated with either fresh or frozen semen. Semen was collected by electro-ejaculation and frozen in pellets (Visser and Salamon, 1973). The pellets were stored in liquid nitrogen until thawed at $37^{\circ}C$ on the day of use. Only ejaculates containing at least 10×10^6 motile spermatozoa per 20 ul after thawing were used. Fresh semen was collected by electro-ejaculation and diluted 1:2 with PBS (Flow Laboratories, North Ryde, New South Wales). Ejaculates containing less

than 3×10^9 spermatozoa per ml were discarded. Semen used was from pooled ejaculates.

The technique for insemination with the laparoscope was similar to that detailed by Killeen and Caffrey (1982). Ewes were fasted for 24h before insemination and local anaesthesia was used at the site of abdominal puncture. Both uterine horns were inseminated. The number of spermatozoa inseminated varied with the study (see below).

3.2.2.4 Experimental Procedures

(a) GnRH and Time of Ovulation

To examine the effect of synthetic GnRH treatment on the time of ovulation following synchrony of oestrus, ewes in Flock 1 were randomly allocated to be treated with GnRH (100 ug per ewe, i.m.) at either 24 (N = 31) or 36h (N = 31) after pessary removal or to receive no further treatment (control group, N = 25). Occurrence of ovulation was determined by laparoscopic inspection of the ovaries every three hours. Observations commenced 45h after pessary removal in the 24h group and 54h after pessary removal in the control and 36h groups.

To examine the effect of dose of GnRH, ewes from Flock 2 were treated with either 0, 6.25, 25 or 100 ug per ewe administered (i.m.) 24h after pessary removal (N = 16, 16, 14 and 15 respectively). Observations were similar to those described above.

(b) GnRH and Embryo Quality

The effect of time of GnRH treatment on the proportion of ova fertilized, the quality of embryos obtained and the percentage of ewes pregnant was examined. Ewes from Flock 3 were randomly allocated to three groups - a

control group, a group to be treated with GnRH (25 ug per ewe) 24h after pessary removal and a group to be similarly treated at 36h. Ewes were naturally mated (one ram per five ewes) and also inseminated 8 - 12h before the expected median time of ovulation with approximately 100×10^6 fresh spermatozoa per uterine horn. Ewes were slaughtered four days after ovulation and ova and embryos were collected by flushing the reproductive tracts with saline. A second group of ewes was similarly treated and litter size was determined by ultrasonography 50 days after insemination. The numbers of ewes involved are shown in Tables 8 and 9.

After an initial assessment (40x magnification), embryos were fixed and then stained with orcein and the number of nuclei counted using phase contrast optics. An embryo was classified as being retarded if it contained less than 16 nuclei.

(c) GnRH and Embryo Transfer

The effect of GnRH treatment on survival of embryos after transfer was examined. Recipient ewes (from Flocks 7 and 8) were prepared as described in Chapter 3.2.2.2 and then treated with GnRH (25 ug per ewe) at 24 or 36h after pessary removal; control ewes received no GnRH. Two- and four-cell embryos were collected from donor ewes (see Chapter 5.2.1 for details on method of collection); between 2 - 10 morphologically normal embryos were then transferred to each recipient ewe within 30 minutes of collection. These ewes (see Table 10 for numbers per treatment) were slaughtered on day 14 of pregnancy and the reproductive tracts flushed with saline solution to recover elongated conceptuses and undeveloped embryos. Embryo development was assessed under 40x magnification. The number of corpora lutea per ovary was recorded at the time of transfer and also after slaughter.

The median time of ovulation in control ewes was assessed in preliminary observations so that the synchrony between donor and recipient was optimized.

(d) GnRH and the Insemination of Frozen-Thawed Semen

An examination was made of the effect of GnRH treatment (25 ug per ewe) given (i.m.) either 24 or 36h after pessary removal on ewe fertility following fixed-time insemination of frozen-thawed semen. Ewes were inseminated (20×10^6 motile spermatozoa per uterine horn) either 0 - 4h (Flock 4) or 8 - 12h (Flocks 1, 5 and 6) before the median time of ovulation which was determined in preliminary observations. Litter size was determined by ultrasonography 50 days after insemination.

3.2.2.5 Statistical Analyses

The median times of ovulation were compared using the Mann-Whitney test (Siegal, 1956). The number of ovulations, fertilized and unfertilized ova and fetuses per ewe were compared by one-way analysis of variance. The proportion of embryos of different quality and the proportion of ewes pregnant were compared using the Chi-square test.

3.2.3 RESULTS

3.2.3.1 GnRH and Time of Ovulation

The time of ovulation in control ewes and in ewes following treatment with GnRH at 24 and 36h is shown in Figure 4; the median times were 63, 48 and 60h respectively ($P < 0.001$). Treatment reduced the time required for ovulation; the number of three-hour periods during which ovulation

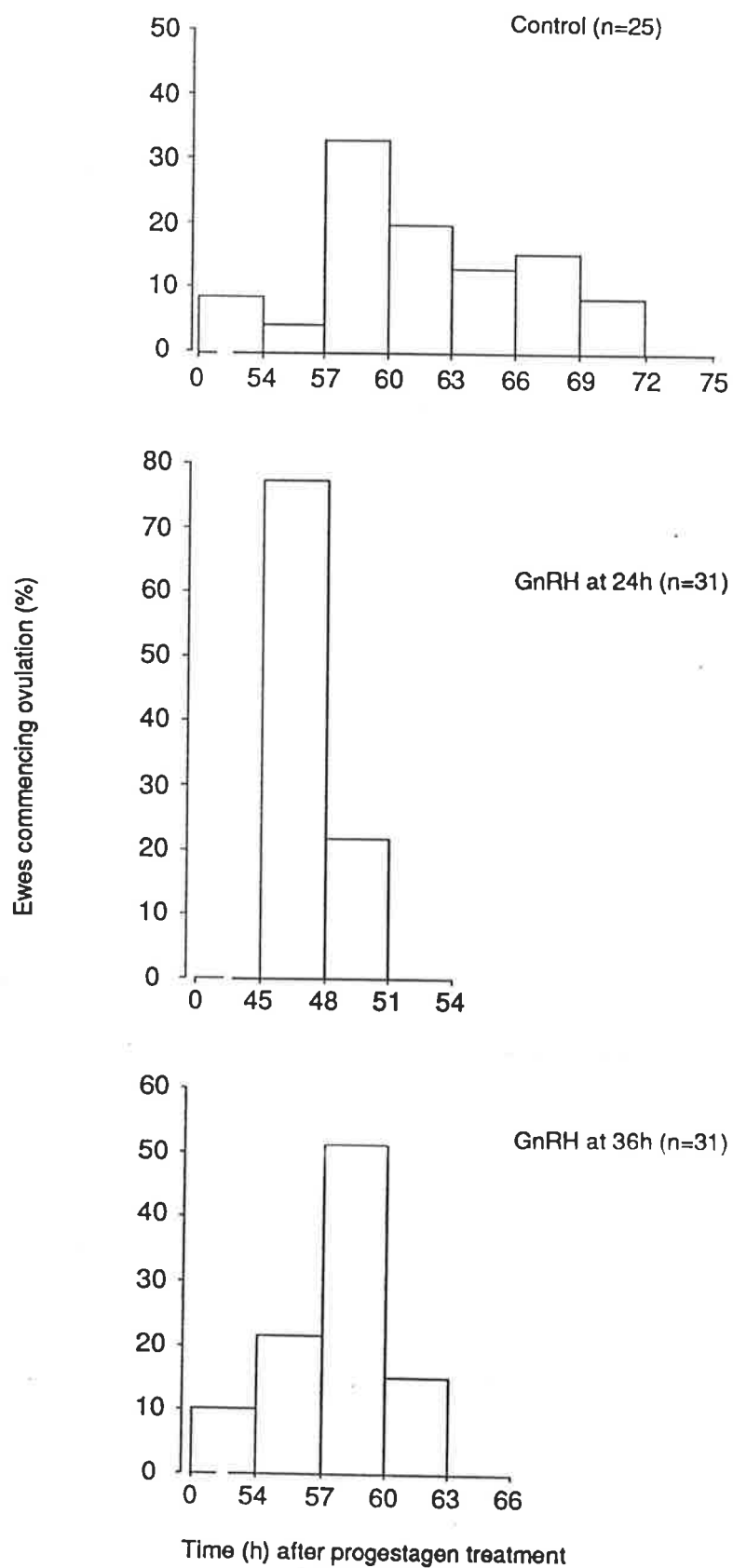


Figure 4. Percentage of ewes observed to commence ovulation in successive 3-h periods after progestagen-PMSG treatment. Ewes were administered GnRH (100 ug per ewe) at either 24 or 36h after treatment or received no further treatment.

occurred was 2, 4 and 7 for ewes treated at 24 and 36h and for control ewes respectively.

Time of ovulation was similar in ewes treated with either 6.25, 25 or 100 ug GnRH per ewe with a median time of 48h for each of the treatment groups compared with 72h for control ewes ($P < 0.01$).

3.2.3.2 GnRH and Embryo Quality

Treatment at 24h significantly reduced the number of ova and embryos collected per ewe ($P < 0.01$), the percentage of ova that were fertilized ($P < 0.01$) and the percentage of embryos that were classified as being normally developed ($P < 0.001$) compared with control ewes and ewes treated at 36h (Table 8). There were no significant differences in these characteristics between control ewes and ewes treated with GnRH at 36h.

The percentage of pregnant ewes was significantly ($P < 0.05$) lower in ewes treated with GnRH at 24h compared with other groups (Table 9) although there were no significant differences in the number of fetuses per ewe. Again there were no differences between control ewes and ewes treated with GnRH at 36h.

3.2.3.3 GnRH and Embryo Transfer

The percentage of embryos recovered on day 14 was significantly ($P < 0.05$) reduced in ewes treated with GnRH at either 24 or 36h compared with control ewes; this figure was lowest (65.3%) when GnRH was administered at 24h (Table 10). In both GnRH treatments, several ewes had additional corpora lutea at slaughter compared with the time of transfer. In these ewes, only 62.5% (35/56) of embryos transferred were recovered. Another two ewes in the 24h treatment group had regressed

Table 8. The effect of time of GnRH treatment (relative to removal of progestagen pessary) on the number of ovulations per ewe, number of ova collected per ewe and classification of embryo quality. Ewes were naturally mated and also artificially inseminated with 100×10^6 fresh spermatozoa per uterine horn.

Treatment (N)	Mean $\pm$ s.e.m. per ewe			Classification of embryos (%)		
	Ovulations	Ova	Fertilized ova	Normal	Retarded*	Unfertilized
Control (33)	2.9 ± 0.40^a	2.1 ± 0.34^a	1.94 ± 0.34^a	88.1 ^a (59/67)	4.5 ^a (3/67)	7.5 ^a (5/67)
GnRH at 24h (35)	2.10 ± 0.24^a	0.9 ± 0.21^b	0.62 ± 0.14^b	53.3 ^b (16/30)	16.7 ^b (5/30)	30.0 ^b (9/30)
GnRH at 36h (67)	3.0 ± 0.31^a	1.8 ± 0.22^a	1.67 ± 0.21^a	85.0 ^a (102/120)	5.8 ^a (7/120)	9.2 ^a (11/120)

*Less than 16 cells four days after ovulation.

Values within columns with different superscripts differ significantly ($P < 0.05$).

Table 9. The effect of time of GnRH treatment (relative to removal of progestagen pessary) on the number of ovulations per ewe, percentage of pregnant ewes and number of fetuses per ewe. Ewes were naturally mated and also artificially inseminated with 100×10^6 fresh spermatozoa per uterine horn.

Treatment (N)	Ovulations per ewe ($\pm$ s.e.m.)	% Pregnant ewes	Fetuses per ewe ($\pm$ s.e.m.)
Control (45)	3.2 ± 0.34^a	61.9^a (26/42)	1.6 ± 0.11^a
GnRH at 24h (41)	4.3 ± 0.40^a	37.1^b (13/35)	1.5 ± 0.14^a
GnRH at 36h (88)	4.3 ± 0.39^a	65.9^a (54/82)	1.8 ± 0.09^a

Values within columns with different superscripts differ significantly ($P < 0.05$).

Table 10. The effect of GnRH treatment in recipient ewes on the recovery and development of transferred two- and four-cell embryos (embryos recovered on day 14 of pregnancy).

	Control ewes	Ewes treated with GnRH*	
		24h	36h
No. ewes	10	12	13
Embryos transferred	75	95	98
Embryos recovered (%)	71/75 (94.7)	62/95 (65.3)	77/98 (78.6)
Elongated conceptuses (% of recovered)	67/71 (94.4)	57/62 (91.9)	64/77 (83.1)
Ewes with extra** ovulations	0	3	3
Ewes with regressed corpora lutea	0	2	0

*Time relative to removal of progestagen pessary.

**Extra ovulations occurred within 13 days of transfer.

Table 11. The effect of GnRH treatment on the percentage of pregnant ewes in four South Australian Merino flocks. Ewes were inseminated with approximately 20×10^6 motile frozen-thawed spermatozoa per uterine horn either 8 - 12h (Flocks 1, 5 and 6) or 0 - 4h (Flock 4) before the median time of ovulation.

Treatment*	Flock 1	Flock 4	Flock 5	Flock 6	
	(aged ewes)	(aged ewes)	(mixed age)	(2-yr-old)	(3-yr-old)
Control	47.8 ^a (22/46)	47.1 ^a (16/34)	49.0 ^a (72/147)	84.1 ^a (37/44)	71.2 ^a (42/59)
GnRH at 24h	23.5 ^b (12/51)	45.7 ^a (16/35)	—	—	—
GnRH at 36h	55.1 ^a (27/49)	32.4 ^a (11/34)	54.7 ^a (82/150)	67.5 ^a (27/40)	77.4 ^a (41/53)

*Time relative to removal of progestagen pessary. Values within columns with different superscripts differ significantly ($P < 0.05$).

corpora lutea and none of 18 embryos transferred to those ewes were recovered.

There were no significant differences between treatments in the percentage of recovered embryos that had commenced elongation.

3.2.3.4 GnRH and Insemination of Frozen-Thawed Semen

Data are summarized in Table 11. In flocks inseminated 8 - 12h before the median time of ovulation, GnRH at 36h increased the percentage of pregnant ewes, with the exception of the two-year-old ewes in Flock 6. However, these increases (range 5.7 - 7.3%) were not significant. In Flock 4 (inseminated 0 - 4h before the median time of ovulation), treatment at 36h resulted in a non-significant decrease in the percentage of pregnant ewes. Treatment at 24h significantly ($P < 0.01$) reduced the percentage of ewes pregnant in Flock 1 but had no effect in Flock 4 (this treatment was not studied in Flocks 5 and 6). GnRH treatment did not significantly influence the number of fetuses per ewe in any flock (range 1.4 - 1.8).

3.2.4 DISCUSSION

The purpose of Experiment 2 was to determine if GnRH treatment could reduce the variation in the time of ovulation following synchrony of oestrus and improve pregnancy rates following either embryo transfer or the insemination of frozen-thawed semen. Control of the time of ovulation in the ewe following synchrony of oestrus has been obtained with varying success using either GnRH or HCG (Braden et al., 1960; Dziuk et al., 1964; Roche and Crowley, 1971; Quirke et al., 1979). In the present study, treatment with GnRH synchronized the time of ovulation

irrespective of the time of administration, dosage and when control ewes commenced ovulation.

Although the study on embryo transfer was preliminary, it is evident that GnRH treatment in recipient ewes was associated with a reduction in the proportion of transferred embryos recollected from the reproductive tract on day 14 compared with control ewes. This reduction occurred irrespective of the time of GnRH treatment and was associated with the occurrence of additional ovulations between the time of transfer and day 14. Luteal regression was also observed in a small number of ewes treated with GnRH at 24h but not in ewes treated at 36h. Additional ovulations following treatment with HCG have been observed by Radford, Avenell and Szell (1984).

GnRH treatment also failed to significantly improve the percentage of pregnant ewes following fixed-time insemination. In ewes treated with GnRH at 24h, there was a significant increase in the percentage of unfertilized ova and retarded embryos (Table 8). On the other hand, treatment at 36h produced embryos of a similar quality to those obtained from control ewes. Nevertheless, the percentage of pregnant ewes was not significantly increased by this treatment presumably due to the occurrence of additional ovulations as identified in the study on embryo transfer. A reduced fertility was obtained in two-year-old ewes in Flock 6 (Table 11) and limited evidence (see Chapter 3.1.3.1) indicates that ewes of this age may ovulate earlier than older ewes; these ewes would probably have been inseminated at approximately the time of ovulation.

The extent to which the preovulatory LH surge can be manipulated without adversely affecting ovarian function and embryo quality appears limited. The LH surge following synchrony of oestrus has been estimated to occur 34.5 - 45.5h after pessary removal (Signoret and Cognie, 1975; Quirke et al., 1981; Ainsworth et al., 1983). However, it is likely that rises later than 45.5h occur given the within flock variation in the time

of ovulation observed in Experiment 1. It might therefore be expected that treatment with GnRH at 24h would initiate an LH rise at least 10 - 21h earlier than normal. This interval would be considerably greater in flocks with a delay in the onset of ovulation. The effect of a prematurely induced LH surge on ovarian function in the sheep has not been documented but the results of this study indicate that significant disturbances can occur.

It is concluded that GnRH treatment at either 24 or 36h after progestagen treatment resulted in a synchronized time of ovulation and that when administered at 36h, embryos of normal quality were produced. However, treatment appeared to adversely affect ovarian function in some ewes, resulting in additional ovulations or premature luteal regression. Consequently, embryo survival was reduced in recipient ewes treated with GnRH whilst only small increases in pregnancy rates were obtained when artificial insemination followed treatment. GnRH treatment is thus unlikely to be of value in embryo transfer programs involving zygotes or in artificial insemination programs, at least when the protocols used in these studies are employed.

CHAPTER 4

THE USE OF SYNTHETIC GONADOTROPHIN RELEASING HORMONE TREATMENT IN THE COLLECTION OF EMBRYOS FROM THE SUPEROVULATED EWE

4.1 INTRODUCTION

Pronuclear microinjection programs are dependent on the supply of zygotes. However, embryo yields frequently obtained in South Australian Merino ewes are low (eg. 0.7 - 2.3 per ewe; Walker et al., 1984). Several factors are implicated, but fertilization failure, due to an impaired migration of spermatozoa through the cervix, is recognized as a major physiological limitation (Evans and Armstrong, 1984; Evans et al., 1984). This problem is overcome, at least partly, if either fresh (Trounson and Moore, 1974a) or frozen-thawed semen (Armstrong and Evans, 1984b) is inseminated directly into the uterus.

Results reported in Chapter 2 indicate that time of superovulation, within and between ewes, is asynchronous. It is postulated that asynchrony contributes to fertilization failure and that GnRH treatment together with properly timed insemination will alleviate the problem. An improved embryo yield has been obtained in the sheep following GnRH treatment (Nancarrow et al., 1984a) but a thorough assessment of the treatment has not been reported.

Experiments in this Chapter examine the efficacy of GnRH treatment in embryo collection programs using frozen-thawed semen. The aims were (1) to determine the effect of time of GnRH treatment on embryo quality, (2) to determine the optimal time, relative to the median time of ovulation, of inseminating frozen-thawed semen into the uterus, (3) to determine the number of motile frozen-thawed spermatozoa required for maximal fertilization rates following GnRH treatment and (4) to assess

the effect of treatment on the collection of two- to four-cell embryos following either intra-uterine insemination of frozen-thawed semen or natural mating.

4.2 MATERIAL AND METHODS

4.2.1 Sheep

South Australian Merino ewes of mixed age located at Turretfield Research Centre were used in the studies. Observations were conducted during the breeding season (January - May).

4.2.2 Superovulation and GnRH Treatment

Superovulation was induced using either FSH or PMSG; the methodology was similar to that outlined in Chapter 2.1.2.1. Either entire or vasectomized rams were introduced to the ewes at the time of pessary removal.

Preliminary observations were conducted to determine the optimal dose of synthetic GnRH (Intervet (Australia) Pty Ltd, Artamon, New South Wales). Doses of 6.25, 12.5, 25 and 100 ug per ewe, administered (i.m.) 24h after pessary removal, were compared following treatment with 1200 i.u. of PMSG per ewe. Time of ovulation was synchronized irrespective of dosage; in each treatment, the median time of ovulation occurred 48h after pessary removal. However, for convenience, a standard dose of 25 ug per ewe was used in all experiments.

4.2.3 Semen Preparation and Insemination

The techniques used were similar to those outlined in Chapter 3.2.2.3. The number of spermatozoa inseminated per ewe varied with the experiment.

4.2.4 Experimental Procedure

4.2.4.1 GnRH and Embryo Quality

This study was similar to that conducted in the nonsuperovulated ewe (see Chapter 3.2.2.4 (b)) and aimed to determine the effect of time of GnRH treatment (relative to time of pessary removal) on the number of ova fertilized and the quality of embryos obtained. Ewes were randomly allocated to be treated with either PMSG or FSH and one of three GnRH treatments (no GnRH, GnRH at 24h or GnRH at 36h). Ewes were paddocked with entire rams (one ram per five ewes) and also inseminated with approximately 100×10^6 fresh spermatozoa per uterine horn. Data were collected on 21 - 27 ewes per treatment in the PMSG group and 12 - 16 ewes per treatment in the FSH group.

The reproductive tracts were collected four days after ovulation and flushed with saline solution. Embryos were assessed morphologically (40x magnification), fixed and stained with orcein and the number of nuclei counted using phase contrast optics. An embryo was classified as being retarded if it contained fewer than 16 nuclei. Data from ewes with regressed corpora lutea were eliminated from the study.

4.2.4.2 GnRH and Time of Insemination

This study investigated the effect of varying the time of insemination of frozen-thawed semen on embryo yield following GnRH treatment. Two hundred and ten ewes were treated with PMSG and randomly allocated to a 2 x 5 factorial design to examine the effect of GnRH treatment (no

treatment or GnRH administered 24h after pessary removal) and five insemination treatments (intra-uterine insemination at either 0, 6, 12 or 18h before the median time of ovulation or natural mating using one ram per five ewes). Ewes that were artificially inseminated received 20×10^6 motile frozen-thawed spermatozoa per uterine horn. Embryo collection procedures and method of embryo assessment were similar to those outlined above.

4.2.4.3 GnRH and Number of Spermatozoa Inseminated

The aim of this study was to determine the approximate number of frozen-thawed spermatozoa required for fertilization following GnRH treatment. Fifty-four ewes were treated with PMSG and GnRH (24h after pessary removal) and randomly allocated to a 2×3 factorial design to examine the effect of two concentrations of motile spermatozoa (1×10^6 or 20×10^6 per uterine horn) and three times of insemination (0, 6 or 12h before the median time of ovulation). Embryos were collected by mid-ventral laparotomy between 3 - 5 days after ovulation and assessed morphologically (40x magnification).

4.2.4.4 GnRH and Collection of Two- to Four-Cell Embryos

Observations were made to assess the usefulness of GnRH treatment in the collection of two- to four-cell embryos. Ideally, this study should have been conducted with zygotes but the difficulty of identifying fertilized ova under field conditions necessitated the collection of cleaved embryos. Eighty ewes (treated with PMSG) were randomly allocated to a 2×2 design to examine the effect of GnRH treatment (no treatment or GnRH administered 24h after pessary removal) and the method of insemination (intra-uterine insemination or natural mating). Ewes that

were artificially inseminated received 20×10^6 motile frozen-thawed spermatozoa per uterine horn six hours before the median time of ovulation. Ewes that were naturally mated were paddocked with rams in the ratio of one ram per two ewes. Embryos were collected by mid-ventral laparotomy 2 days after ovulation and assessed morphologically (40x magnification).

4.2.5 Statistical Analyses

The analysis of variance and log-linear approaches were used to investigate treatment main effects and interactions. The number of ovulations per ewe, number of fertilized and unfertilized ova per ewe and the percentage of ova recovered from all ovulations recorded were analysed using analysis of variance. The number of ovulations was included as a covariate. The assumption of normality and variance homogeneity were checked and if found to be invalid, the variable concerned was log-transformed and re-analysed. The percentage of fertilized ova was analysed by log-linear model procedures.

4.3 RESULTS

4.3.1 GnRH and Embryo Quality

In ewes treated with PMSG, neither embryo quality (normal v. retarded development) nor the proportion of unfertilized ova were significantly influenced by the time of GnRH treatment (Table 12). However, in ewes treated with FSH, there was a significant ($P < 0.05$) increase in the percentage of unfertilized ova when GnRH was administered 24h after pessary removal compared with 36h (Table 13); there was also an associated decrease in the number of normal embryos obtained ($P < 0.1$).

GnRH treatment did not significantly influence the number of ovulations per ewe or the number of ova recovered (fertilized and unfertilized) per ewe in either gonadotrophin treatment.

4.3.2 GnRH and Time of Insemination

Results are summarized in Table 14. GnRH treatment significantly ($P < 0.001$) increased the percentage of recovered ova that were fertilized irrespective of the method and time of insemination (overall 71.7 v. 51.5%) but it did not significantly influence any other character (the number of fertilized ova obtained per ewe approached significance, $P < 0.1$). Time of insemination significantly influenced the percentage of recovered ova that were fertilized ($P < 0.001$); fertilization rates were highest when insemination occurred 6h before the median time of ovulation, irrespective of GnRH treatment. Time of insemination also significantly ($P < 0.05$) influenced the number of ova recovered per ewe; recovery was lowest when insemination occurred at the time of ovulation (0^a v. 6^{ab} v. 12^b v. $18h^b$; different superscripts indicate significant differences, $P < 0.05$).

Ewes that were artificially inseminated differed significantly from naturally mated ewes in the percentage of recovered ova that were fertilized (overall 64.1 v. 49.6% respectively, $P < 0.001$). The number of ova recovered per ewe after natural mating was significantly ($P < 0.05$) higher than when ewes were artificially inseminated at 0h but there were no significant differences at other times.

There was no significant interaction between GnRH treatment and time of insemination nor between GnRH treatment and method of insemination (intra-uterine insemination v. natural mating).

4.3.3 GnRH and Number of Spermatozoa Inseminated

Table 12. The effect of time of GnRH treatment (relative to time of removal of progestagen pessary) on number of ovulations per ewe, number of ova collected per ewe and embryo quality in PMSG-treated ewes. Ewes were naturally mated and also artificially inseminated (intra-uterine) with fresh semen. Embryos were collected four days after ovulation.

Treatment* (N)	Mean $\pm$ s.e.m. per ewe			Classification of embryos (%)		
	Ovulations	Ova	Fertilized ova	Normal	Retarded**	Unfertilized
Control (24)	10.5 $\pm$ 1.74	4.0 $\pm$ 1.16	3.5 $\pm$ 1.15	85.0 (51/60)	5.0 (3/60)	10.0 (6/60)
GnRH at 24h (27)	7.8 $\pm$ 1.12	3.7 $\pm$ 0.72	3.2 $\pm$ 0.68	83.6 (61/73)	4.1 (3/73)	12.3 (12/73)
GnRH at 36h (21)	11.8 $\pm$ 1.29	5.3 $\pm$ 0.93	4.9 $\pm$ 1.03	90.6 (58/64)	1.6 (1/64)	7.8 (5.64)

*There were nine, seven and nine ewes with regressed corpora lutea in the control, 24 and 36h groups, respectively; data from these ewes discarded.

**Retarded embryos were those with fewer than 16 nuclei.

Table 13. The effect of time of GnRH treatment (relative to time of removal of progestagen pessary) on number of ovulations per ewe, number of ova collected per ewe and embryo quality in FSH-treated ewes. Ewes were naturally mated and also artificially inseminated (intra-uterine) with fresh semen. Embryos were collected four days after ovulation.

Treatment* (N)	Mean $\pm$ s.e.m. per ewe			Classification of embryos (%)		
	Ovulations	Ova	Fertilized ova	Normal	Retarded**	Unfertilized
Control (15)	9.9 $\pm$ 1.80	6.6 $\pm$ 1.67	6.4 $\pm$ 1.69	87.2 (75/86)	9.3 (8/86)	3.5 ^a (3/86)
GnRH at 24h (12)	12.4 $\pm$ 1.22	9.0 $\pm$ 1.10	7.4 $\pm$ 1.11	75.8 (75/99)	6.1 (6/99)	18.2 ^b (18/99)
GnRH at 36h (16)	13.3 $\pm$ 1.28	10.1 $\pm$ 1.10	9.8 $\pm$ 1.10	84.7 (105/124)	9.7 (12/124)	5.6 ^a (7/124)

*There were two, one and four ewes with regressed corpora lutea in the control, 24 and 36h groups, respectively; data from these ewes discarded.

**Retarded embryos were those with fewer than 16 nuclei.

Values within column with different superscripts differ significantly ($P < 0.05$).

Table 14. The effect of GnRH treatment on the collection of day-4 embryos following either (1) the insemination of 20×10^6 motile frozen-thawed spermatozoa per uterine horn at different times before the median time of ovulation or (2) natural mating. Relevant statistical comparisons are made within the text.

(a) -GnRH ewes					
	Time of insemination				Natural mating
	0h	6h	12h	18h	
No. ewes	20	19	22	22	20
Ovulations/ewe ($\pm$ s.e.m.)	9.8 $\pm$ 1.3	10.0 $\pm$ 1.1	9.2 $\pm$ 1.1	9.0 $\pm$ 1.1	10.8 $\pm$ 1.6
Ova recovered/ewe ($\pm$ s.e.m.)	4.7 $\pm$ 1.0	5.6 $\pm$ 0.9	5.5 $\pm$ 0.8	5.0 $\pm$ 0.8	5.4 $\pm$ 1.3
Fertilized ova/ewe ($\pm$ s.e.m.)	2.6 $\pm$ 0.8	3.8 $\pm$ 0.8	3.2 $\pm$ 0.7	2.2 $\pm$ 0.6	2.2 $\pm$ 0.6
% ova recovered/ovulations ($\pm$ s.e.m.)	50.0 $\pm$ 6.6	57.0 $\pm$ 7.0	61.3 $\pm$ 5.9	51.8 $\pm$ 6.7	53.9 $\pm$ 6.9
% recovered ova fertilized	55.3 (52/94)	68.9 (73/106)	57.9 (70/121)	44.0 (48/109)	34.4 (43/125)
(b) +GnRH ewes					
	Time of insemination				Natural mating
	0h	6h	12h	18h	
No. ewes	21	19	22	22	21
Ovulations/ewe ($\pm$ s.e.m.)	8.5 $\pm$ 0.8	8.3 $\pm$ 1.2	10.4 $\pm$ 1.2	10.0 $\pm$ 1.2	8.0 $\pm$ 0.8
Ova recovered/ewe ($\pm$ s.e.m.)	3.0 $\pm$ 0.4	3.7 $\pm$ 0.5	4.2 $\pm$ 0.6	5.9 $\pm$ 0.8	5.3 $\pm$ 0.8
Fertilized ova/ewe ($\pm$ s.e.m.)	2.4 $\pm$ 0.4	3.1 $\pm$ 0.6	3.0 $\pm$ 0.5	3.9 $\pm$ 0.9	3.5 $\pm$ 0.9
% ova recovered/ovulations ($\pm$ s.e.m.)	37.0 $\pm$ 5.1	51.8 $\pm$ 5.9	47.3 $\pm$ 6.2	62.8 $\pm$ 6.2	62.7 $\pm$ 5.8
% recovered ova fertilized	80.6 (50/62)	83.1 (59/71)	71.7 (66/92)	65.4 (85/130)	66.7 (74/111)

Table 15. Percentage of fertilized ova obtained following the insemination into the uterus of a high or a low dose of frozen-thawed spermatozoa at different times before the median time of ovulation. Ewes were treated with PMSG (1200 i.u. per ewe) and GnRH (25 ug per ewe).

No. motile sperm per uterine horn	Time of insemination		
	0h	6h	12h
20 x 10 ⁶	86.7 (26/30)	97.4 (37/38)	58.1 (18/31)
1 x 10 ⁶	35.3 (6/17)	51.5 (17/33)	25.0 (5/20)

Table 16. The effect of GnRH treatment and method of insemination (intra-uterine insemination of frozen-thawed semen, IUI) or natural mating (NM) on the collection of two- to four-cell embryos. Relevant statistical comparisons are made within the text.

	IUI+GnRH	IUI-GnRH	NM+GnRH	NM-GnRH
No. ewes	19	19	19	19
Ovulations/ewe ($\pm$ s.e.m.)	7.0 $\pm$ 0.24	7.1 $\pm$ 1.30	8.7 $\pm$ 1.47	6.1 $\pm$ 0.97
Ova recovered/ ewe ($\pm$ s.e.m.)	5.2 $\pm$ 0.85	4.6 $\pm$ 0.98	6.3 $\pm$ 1.29	3.3 $\pm$ 0.64
Fertilized ova/ ewe ($\pm$ s.e.m.)	4.9 $\pm$ 0.85	1.2 $\pm$ 0.48	4.0 $\pm$ 1.32	1.9 $\pm$ 0.50
% ova recovered/ ovulations ($\pm$ s.e.m.)	75.8 $\pm$ 6.21	59.2 $\pm$ 7.2	68.5 $\pm$ 6.78	56.7 $\pm$ 7.19
% recovered ova fertilized	94.9 (93/98)	24.7 (18/73)	57.1 (68/119)	16.9 (10/59)

In sheep treated with GnRH, the insemination of 20×10^6 motile frozen-thawed spermatozoa per uterine horn resulted in the collection of significantly ($P < 0.001$) more fertilized ova than the insemination of 1×10^6 spermatozoa (Table 15). There was a significant ($P < 0.001$) reduction in the number of fertilized ova when insemination occurred 12h before the median time of ovulation compared with 0 and 6h.

4.3.4 GnRH and Collection of Two- to Four-Cell Embryos

GnRH treatment significantly improved the percentage of ova that were fertilized ($P < 0.001$), the number of fertilized ova per ewe ($P < 0.01$) and the percentage of ova recovered of ovulations recorded ($P < 0.05$) (Table 16). Intra-uterine insemination of frozen-thawed semen significantly ($P < 0.001$) improved the percentage of ova that were fertilized compared with natural mating but there were no significant differences in any other character.

Significant interactions between the main effects were obtained in the number of fertilized ova per ewe ($P < 0.05$) and the percentage of recovered ova that were fertilized ($P < 0.001$). However, these interactions are considered unimportant.

4.4 DISCUSSION

The most significant feature of this study was the increase in the percentage of recovered ova that were fertilized following GnRH treatment. Associated increases in the number of embryos collected per ewe approached significance ($P < 0.1$) when day-4 embryos were collected and were highly significant when two- to four-cell embryos were collected. This finding is similar to that of a comparable study of Nancarrow et al. (1984a), in which the yield of zygotes was increased

from 1.9 to 4.2 per ewe following GnRH treatment and the intra-uterine insemination of fresh semen. The results of studies in this Chapter particularly highlight the efficacy of GnRH treatment because the increases in embryo yield were obtained with the insemination of frozen-thawed semen. The use of frozen semen in embryo collection programs is convenient but fertility is compromised. However, the use of GnRH treatment, using protocols similar to those outlined in this study, overcomes this problem.

Results from Chapter 2 indicate that GnRH administered 24h after pessary removal synchronizes the time of ovulation, irrespective of the gonadotrophin used for superovulation. However, in ewes treated with FSH, GnRH given at 24h significantly increased the percentage of unfertilized ova compared with control ewes and ewes given GnRH at 36h (Table 13). Treatment at this time would pre-empt the endogenous LH rise by about 12h (Cumming et al., 1973) and comparable studies in the nonsuperovulated ewe indicate that premature induction of the LH surge is associated with a significant increase in the percentage of unfertilized ova (see Chapter 3.2.3.2). Delaying GnRH treatment until 30 - 36h after pessary removal in FSH-treated ewes appears appropriate, even though this would result in a less synchronized time of ovulation. On the other hand, ewes treated with PMSG have a median time of ovulation 54h after pessary removal (6h earlier than FSH-treated ewes) and GnRH administered at 24h produced embryos of normal quality (Table 12).

The percentage of fertilized ova obtained following GnRH treatment was highest when insemination occurred 6h before the median time of ovulation. This figure declined when insemination occurred earlier than 6h, although the decline was not significant in one of the two observations. Different batches of frozen-thawed semen were used, indicating that the relevance of time of insemination may vary with the quality of semen used. Insemination at the time of ovulation in GnRH-

treated ewes is not recommended, as recovery of embryos was lowest at this time, possibly due to physical disturbance of the reproductive tract.

The number of spermatozoa required for fertilization following intra-uterine insemination is equivocal (Armstrong and Evans, 1984b; Walker et al., 1984). Synchronizing the time of ovulation is likely to reduce the number required, provided insemination is properly timed. In this study, the insemination of 20×10^6 motile frozen-thawed spermatozoa per uterine horn resulted in a similar percentage of fertilized ova to that obtained when sheep were naturally mated and also inseminated (into the uterus) with fresh semen. Insemination of a higher number of spermatozoa is therefore unlikely to be of any additional value. The ability to obtain high fertilization rates reliably with the insemination of 20×10^6 motile spermatozoa per uterine horn following GnRH treatment ensures more efficient use of semen compared with that obtainable with other treatment protocols and insemination techniques. Presumably, some potential exists to reduce the number of spermatozoa used without adversely affecting fertilization rates but insemination of 1×10^6 per uterine horn was clearly too low (Table 15).

GnRH treatment also significantly improved fertilization rates in ewes that were naturally mated (Table 16). Progestagen-gonadotrophin treatment reduces the number of spermatozoa in the oviducts, uterus and anterior cervix (Evans and Armstrong, 1984; Hawk et al., 1987) associated with an increase in the proportion of motile, dead and damaged spermatozoa. The improvement in embryo yield following GnRH treatment in naturally mated sheep indicates that the period over which viable spermatozoa are available in the oviducts is limited. This may be due to both an impaired viability of spermatozoa and the inability of the anterior cervix to replenish oviductal populations during the course of superovulation. Despite the improvement in embryo yield with GnRH

treatment, fewer embryos were recovered per ewe after natural mating compared with intra-uterine insemination of frozen-thawed semen.

GnRH treatment had no effect on embryo yield in ewes that were inseminated into the uterus with fresh semen and also naturally mated (Tables 12 and 13). The improved longevity of fresh spermatozoa compared with frozen-thawed spermatozoa is likely to reduce the significance of GnRH treatment in ewes inseminated into the uterus with fresh semen. However, an improved embryo yield has been obtained following GnRH treatment plus the insemination of fresh semen (Nancarrow et al., 1984a). It is likely that GnRH treatment is relevant to embryo collection programs using fresh semen when (1) a low number of spermatozoa is inseminated, (2) spermatozoa of impaired viability are inseminated or (3) the viability of spermatozoa in the oviducts is adversely affected by the progestagen-gonadotrophin treatment.

The cost of GnRH treatment is insignificant, given that dosages as low as 6.25 ug per ewe synchronized the time of ovulation. The inclusion of this treatment in embryo collection programs is thus recommended at least when either frozen-thawed semen or natural mating is used. It is concluded that proper timing of both the GnRH treatment and intra-uterine insemination will enhance the supply of zygotes for microinjection programs.



CHAPTER 5

CULTURE OF SHEEP ZYGOTES IN SYNTHETIC OVIDUCT FLUID MEDIUM.

5.1 INTRODUCTION

Studies on embryo manipulation and embryogenesis are limited by the inability of in vitro culture systems to support embryo growth without jeopardizing viability. The development of techniques for the pronuclear microinjection of foreign DNA has increased the need for a culture system in which zygote viability can be assessed. Microinjection is known to damage plasma membranes resulting in reduced viability (Walton et al., 1987). Rexroad and Wall (1987) reported that only 19% of embryos were viable after microinjection and in Chapter 6.3.2, 45.1% of embryos were degenerate or severely retarded within three days of microinjection.

Most studies on the culture of sheep embryos have used simple media. However, only limited success has been obtained, particularly with the culture of zygotes (see Wright and Bondioli, 1981). Tervit and Rowson (1974) reported the birth of one lamb following the transfer of 23 zygotes cultured for 3 - 6 days in SOFM. Peters et al. (1977) obtained one blastocyst from six zygotes cultured for up to 238h in Whitten's medium and Betterbed and Wright (1985) obtained two blastocysts from 104 zygotes cultured in Brinster's Pyruvate Medium. Development was enhanced when embryos were co-cultured in the presence of either oviduct cells (Gandolfi and Moor, 1987; Rexroad and Powell, 1988a, 1988b) or trophoblastic vesicles (Heyman et al., 1987; Rexroad and Powell, 1988a). Gandolfi and Moor (1987) reported that 80% of sheep zygotes cleaved regularly when cultured for three days in the presence of oviduct cells while after six days, 42% developed into expanded blastocysts. These authors concluded that oviduct cells were required to support embryo growth through the fourth cell cycle.

The need for an in vitro system to assess zygote development has been stated by several authors (Gandolfi and Moor, 1987; Heyman et al., 1987; Kane, 1987; Rexroad and Powell, 1988a). Experiments reported in this Chapter aimed to develop a culture system capable of supporting the growth of the sheep zygote to the blastocyst stage.

5.2 MATERIALS AND METHODS

5.2.1 Supply of Zygotes

Zygotes were obtained from South Australian Merino ewes following treatment with PMSG (Heriot Agvet Pty Ltd, Melbourne, Victoria; see Chapter 2.1.2.1 for details of treatment). Synthetic GnRH (25 ug per ewe; Intervet (Australia) Pty Ltd, Artamon, New South Wales) was administered 24h after progestagen treatment. Ewes were paddocked with entire rams (one ram per ewe) at the time of pessary removal. Forty-two hours later (six hours before the expected median time of ovulation), ewes were inseminated with a minimum of 20×10^6 motile frozen-thawed spermatozoa per uterine horn using the laparoscope. A brief inspection of the ovaries was conducted at the time of insemination.

Zygotes were collected by mid-ventral laparotomy approximately 12 - 17h after the expected median time of ovulation. A Tom Cat Catheter (Sherwood Medical, St. Louis, Missouri) was inserted into the fimbriated end of the oviduct and 10 ml of medium flushed through each oviduct after puncturing the uterine wall distal to the utero-tubular junction. The medium was PBS (Flow Laboratories, North Ryde, New South Wales) supplemented with 5% heat-inactivated human serum (HIHS). Zygotes were recovered from the flushings within five minutes of collection, washed twice in culture medium and then placed in culture droplets.

5.2.2 Culture Medium and Conditions

The basic medium used was SOFM as formulated by Tervit et al. (1972). HEPES (12.5 mM; N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) was included in the medium in most studies to minimize changes in pH following the addition of serum. When HEPES was not included, the concentration of sodium bicarbonate before the addition of serum was 25 mM. Details on stock solutions, mixing procedures and final composition of the media are provided in Tables 17 and 18. The medium was prepared either one or two days before embryo collection and then placed in the incubator for gas equilibration.

Embryos were cultured in 50 μ l droplets under paraffin oil (BDH Chemical Ltd, Poole, England). The atmosphere was 5% CO₂, 5% O₂ and 90% N₂. Temperature was 38°C and humidity 93%. The incubator used in most studies was the Forma Scientific model 3029 (Marietta, Ohio).

5.2.3 Examination of Components of SOFM.

Studies were conducted to assess the importance of various components of SOFM on the in vitro development of zygotes. In each study, zygotes were cultured for nine days.

5.2.3.1 Protein Supplementation

SOFM was supplemented with one of the following (1) 10% HIHS (v/v), (2) 10% heat inactivated sheep serum (v/v, HISS), (3) 10% ovine lymph (v/v), (4) 10% bovine lymph (v/v), (5) 10% Nu-serum (v/v; Collaborative Research, Inc., Lexington, Massachusetts) and (6) 32 mg/ml bovine serum albumin (BSA, Fraction 5, Grand Island Biological Company, New York). HIHS was randomly selected from samples available from the IVF

Laboratory, The Queen Elizabeth Hospital, Adelaide while both sources of lymph were obtained from the Australian National University. Zygotes were collected over a seven-week period and randomly allocated, within ewes, to the treatment groups. There were 19 - 31 zygotes per treatment.

5.2.3.2 Storage Age of HIHS

In this study, HIHS was stored for either two, seven or 12 days before use. To standardize the quality of serum used in this and all subsequent comparisons, blood was collected from the author after a 24h fasting period. The sample was centrifuged twice after clotting (2000 r.p.m. for 20 minutes), heat inactivated at 56°C for 30 minutes and then stored at 4°C. Zygotes were collected over a five-week period and randomly allocated, within ewes, to the treatment groups; media contained 10% HIHS (v/v). There were 35 - 36 zygotes per treatment group.

5.2.3.3 Concentration of HIHS

Zygotes were cultured in SOFM containing either 0, 10, 20 or 30% HIHS (v/v). Zygotes were collected over a four-week period and randomly allocated, within ewes, to the treatment groups. There were 31 - 39 zygotes per treatment group.

5.2.3.4 Osmolarity

Zygotes were cultured in SOFM of either 260, 270, 280, 290 or 300 mOsm; media contained 20% HIHS (v/v). Osmolarity was adjusted by the addition of either Stock A (see Table 17) or water from the Milli-Q reagent water system (Millipore Corporation, Bedford, Massachusetts). Minor changes in the composition of the medium brought about by these additions were

considered unimportant. Zygotes were collected over a five-week period and randomly allocated, within ewes, to treatment groups. There were 26 - 31 zygotes per treatment group.

5.2.3.5 Potassium Concentrations

(a) Range 1 - 11 mM

Media were prepared in which the K concentrations, before the addition of 20% HIHS (v/v), were targetted to be 0, 3, 5, 7, 9 and 11 mM. Actual concentrations were determined after the addition of serum; the concentration of NaCl was adjusted so that the combined concentration of K and Na approximated 140 mM. Zygotes were collected over a six-week period and randomly allocated, within ewes, to treatment groups. There were 45 - 63 zygotes per treatment group.

(b) Range 1 - 35 mM

This study was similar to (a) except the K concentrations, before the addition of 20% HIHS (v/v), were targetted to be 1, 7, 15, 25 and 35 mM. Zygotes were collected over a three-week period and randomly allocated, within ewes, to treatment groups. There were 31 - 41 zygotes per treatment group.

5.2.3.6 Calcium Concentrations

Media were prepared in which the concentrations of Ca before the addition of 20% HIHS (v/v) were targetted to be 0, 1, 2, 3, 4 and 5 mM; actual concentrations were determined after the addition of serum. Zygotes were

collected over a four-week period and randomly allocated, within ewes, to treatment groups. There were 15 - 25 zygotes per treatment group.

Concentrations of Na, K and Ca in the above studies were determined by staff of the Department of Clinical Chemistry, The Queen Elizabeth Hospital.

5.2.3.7 Concentration of Sodium Bicarbonate and HEPES

Media were prepared in which the concentrations of sodium bicarbonate and HEPES, before the addition of 20% HIHS (v/v), were targetted to be:

- (a) 25.00 mM NaHCO_3 : 0.00 mM HEPES
- (b) 18.75 mM NaHCO_3 : 6.25 mM HEPES
- (c) 12.50 mM NaHCO_3 : 12.50 mM HEPES
- (d) 6.25 mM NaHCO_3 : 18.75 mM HEPES
- (e) 0.00 mM NaHCO_3 : 25.00 mM HEPES

The actual concentrations of total bicarbonates, after the addition of serum, were determined using the phosphoenolpyruvate carboxylase kit method (Trace Scientific Pty Ltd, Sydney, New South Wales). The pH of media (a), (c) and (e) were monitored every second day during the culture period. Zygotes were collected over a six-week period and randomly allocated, within ewes, to treatment groups. There were 53 - 57 zygotes per treatment group.

5.2.4 Number of Nuclei per Embryo

An assessment was made in the above studies of the number of nuclei per embryo following culture. Embryos were fixed and stained with orcein and the number of nuclei counted using phase contrast and Nomarski differential interference contrast optics (40x magnification). There was a loss of cells from some hatching blastocysts during this procedure.

Data on the number of nuclei per embryo were collated in all studies in which 20% HIHS was used but excluded any treatment in which blastocyst formation was significantly influenced by the treatment. Observations were also made on the association between time required for blastocyst formation and the number of nuclei per blastocyst. In these studies, zygotes were cultured in separate droplets.

5.2.5 Fragmentation of Cytoplasm

The degree of fragmentation of the cytoplasm was assessed approximately 24h after the commencement of culture. The following scoring system was used: 1 = no significant fragmentation, 2 = mild fragmentation (approximately 2 - 4 anuclear fragments per embryo), 3 = moderate fragmentation (approximately 5 - 8 anuclear fragments per embryo) and 4 = severe fragmentation (approximately 9 or more anuclear fragments per embryo). An assessment was made of the association between fragmentation score and subsequent development.

Embryos with different fragmentation scores are shown in Plate 1.

5.2.6 Viability of Zygotes After Culture

Two studies were conducted. In the first, zygotes were cultured in SOFM containing sodium bicarbonate (12.5 mM before the addition of serum) plus 12.5 mM HEPES for either one (N = 42), three (N = 51) or five (N = 36) days. After culture, all embryos were transferred to appropriately synchronized recipient ewes (N = 5 - 10 per ewe). Control zygotes (N = 36) were transferred within 30 minutes of collection. Control zygotes and embryos cultured for either one or three days were transferred to the oviducts while those cultured for five days were transferred to the uterine horn adjacent the utero-tubular junction. Embryos were

transferred irrespective of their morphological appearance. Recipient ewes were slaughtered on day 14 of pregnancy and the reproductive tracts collected. Each uterine horn was flushed with 20 ml saline solution and the flushings examined for elongated conceptuses and undeveloped embryos.

In the second study, embryos were cultured for either three or five days in SOFM with sodium bicarbonate (12.5 mM before the addition of serum) plus 12.5 mM HEPES or in medium free of HEPES (N = 31 - 33 per treatment). After culture, the embryos were transferred to recipient ewes in groups of 5 - 10 per ewe. Control zygotes (N = 26) were transferred within 30 minutes of collection. Embryos were subsequently recovered at slaughter on day 14 and the number of elongated conceptuses determined as described above. The length of each conceptus was measured and the presence or absence of an embryonic disc recorded.

5.2.7 Statistical Analyses

The percentages of zygotes that developed into blastocysts were analysed using the Chi-square test. Data were summarized into (1) zygotes that developed to either the early blastocyst, expanded blastocyst or hatching blastocyst stage, (2) zygotes that developed to the 9 - 16 cell stage, (3) zygotes that developed to the 2 - 8 cell stage and (4) zygotes that failed to divide.

5.3 RESULTS

5.3.1 Components of SOFM

5.3.1.1 Protein Supplementation

The protein source significantly ($P < 0.05$) influenced the percentage of zygotes that developed into blastocysts (Table 19). Relatively more blastocysts developed in media supplemented with either HIHS or HISS compared with other protein sources. Blastocyst formation occurred significantly ($P < 0.05$) earlier in media supplemented with HIHS compared with HISS (Table 19).

Similar percentages of zygotes developed into blastocysts in media supplemented with either BSA or Nu-serum; there was a significant ($P < 0.05$) delay in the time required for blastocyst formation in the former treatment (Table 19). A feature of BSA supplementation was the significantly ($P < 0.05$) higher percentage of zygotes (39.3%) that developed to the 9 - 16 cell stage but failed to develop further compared with other treatments. Supplementation with either ovine or bovine lymph resulted in significantly ($P < 0.05$) fewer zygotes developing to blastocysts compared with all other protein sources.

5.3.1.2 Storage Age of HIHS

Storage age of serum did not significantly influence the percentage of zygotes that developed to blastocysts or the time required for blastocyst formation (Table 20).

5.3.1.3 Concentration of HIHS

Supplementation of media with 20% HIHS significantly ($P < 0.05$) improved the percentage of zygotes (87.1%) that developed to blastocysts compared with other concentrations (Table 21). All zygotes that developed to the fourth cell cycle in media supplemented with either 20 or 30% serum continued growing to the blastocyst stage. However, significantly ($P < 0.05$) more embryos commenced hatching in the former group (53.8 v.

37.8% respectively). In the absence of serum, 29.0% of the zygotes developed to the 9 - 16 cell stage. Time required for blastocyst formation was not influenced by the concentration of serum (Table 21).

5.3.1.4 Osmolarity

Osmolarity (range 260 - 300 mOsm) did not significantly influence the percentage of zygotes that developed to blastocysts or the time required for blastocyst formation (Table 22).

5.3.1.5 Potassium Concentrations

Actual K concentrations in the media varied between 2.5 ± 0.02 - 10.4 ± 0.06 in the first study and between 2.5 ± 0.02 - 27.8 ± 0.75 in the second study. Varying the K concentration between 2.5 - 10.4 mM, with compensatory adjustments in the Na concentration, did not significantly influence zygote development (Table 23). However, in the second study, an increase in the concentration above 13.4 mM, resulted in a significant ($P < 0.05$) reduction in the percentage of zygotes that developed to blastocysts (Table 24). There was also an associated delay in the time required for blastocyst formation at the higher concentrations.

5.3.1.6 Calcium Concentrations

Actual Ca concentrations in the media varied between 1.29 ± 0.002 - 5.00 ± 0.009 . Varying the concentration did not significantly influence either the percentage of zygotes that developed to blastocysts or the time required for blastocyst formation (Table 25). A feature of this study was the high percentage of zygotes that commenced hatching (range 60.0 - 75.0%).

5.3.1.7 Concentration of Sodium Bicarbonate and HEPES

Actual total bicarbonate concentrations of 11.5 ± 0.50 , 16.0 ± 0.51 , 19.7 ± 0.67 , 26.5 ± 0.42 and 32.2 ± 0.37 were obtained for media containing 0, 6.25, 12.5, 18.75 and 25 mM sodium bicarbonate respectively, before the addition of serum. Replacing sodium bicarbonate with HEPES increased the acidity of the medium (Table 26); without HEPES, readings as high as 7.88 were recorded.

In the absence of HEPES, 85.2% of zygotes developed into blastocysts (Table 27). This figure declined as sodium bicarbonate was replaced with HEPES; the decline was significant ($P < 0.05$) at concentrations of HEPES of 18.75 and 25 mM. The percentage of zygotes that commenced hatching (range 47.2 - 74.1%) was significantly ($P < 0.05$) reduced and the time required for blastocyst formation (range four to seven days) was significantly ($P < 0.05$) increased in medium with 25 mM HEPES compared with other media (Table 27).

Developmental stages of embryos cultured from the zygote stage are shown in Plates 2 and 3.

5.3.2 Fragmentation of Cytoplasm

There were no significant differences in fragmentation score between treatments. Fragmentation score was associated with the subsequent development of the embryo. The percentages of embryos with fragmentation scores of 1, 2, 3, and 4 that developed to blastocysts were 71.7^{ab} , 74.0^a , 60.4^{bc} and $49.4\%^c$ respectively (figures with different superscripts differ significantly, $P < 0.05$).

PLATE 1

Two- and four-cell embryos with different fragmentation scores. Zygotes for culture were collected approximately 12-17h after the median time of ovulation and photographed approximately 24h after the commencement of culture.

Photograph 1. Two-cell embryo collected from the oviducts.

Photograph 2. Cultured embryo, fragmentation score = 1 (no significant fragmentation).

Photograph 3. Cultured embryo, fragmentation score = 2 (mild fragmentation).

Photograph 4. Cultured embryo, fragmentation score = 3 (moderate fragmentation).

Photograph 5. Cultured embryo, fragmentation score = 4 (severe fragmentation).

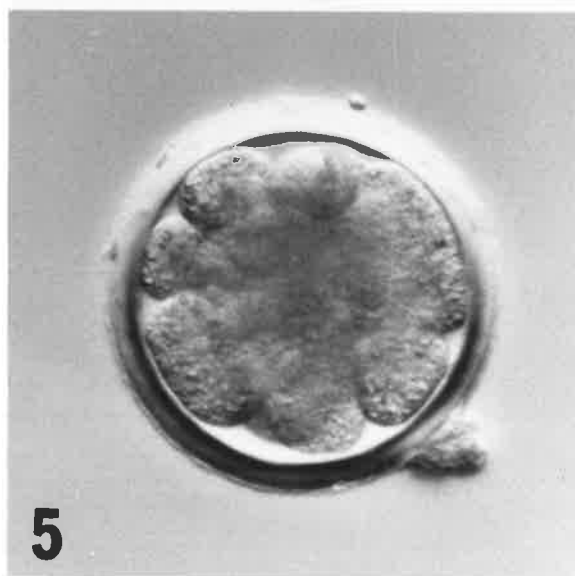
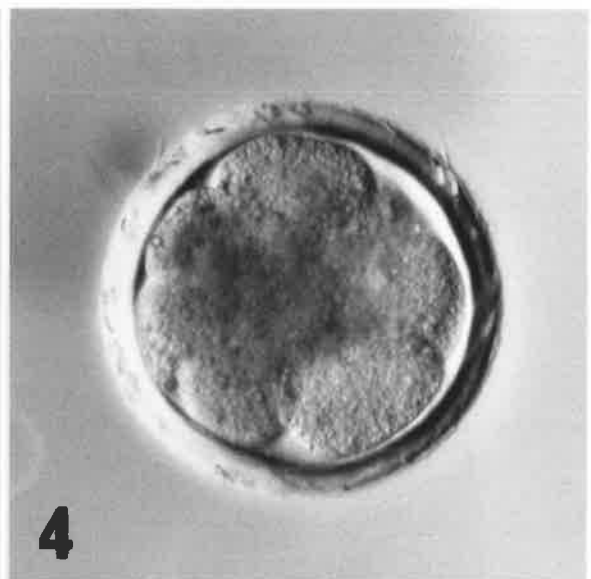
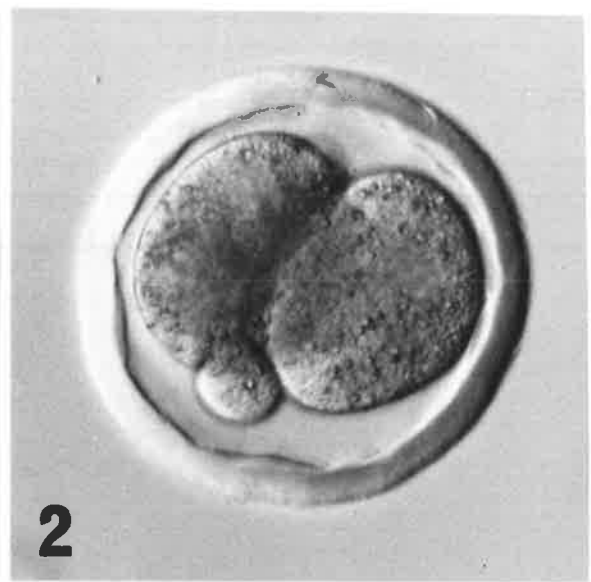
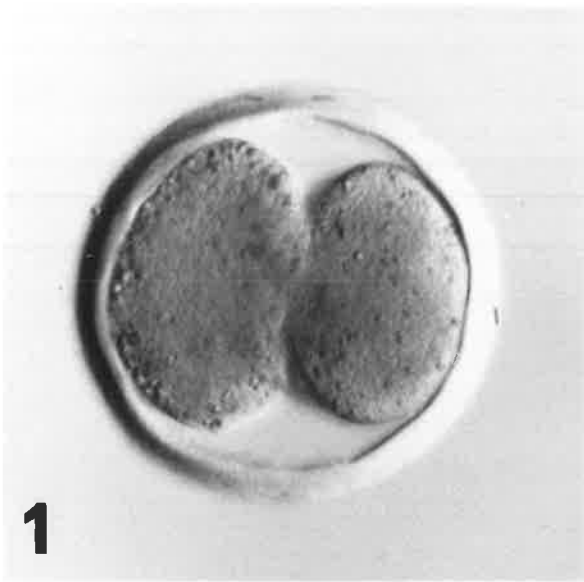


PLATE 2

Typical developmental stages of embryos cultured in SOFM from the zygote stage (zygotes collected approximately 12-17h after the median time of ovulation).

Photograph 1. Freshly collected zygote.

Photograph 2. Four-cell embryo with fragments (day 1 of culture).

Photograph 3. Morula (day 3 of culture).

Photograph 4. Early blastocyst (day 4 of culture).

Photograph 5. Expanded blastocyst (day 5 of culture).

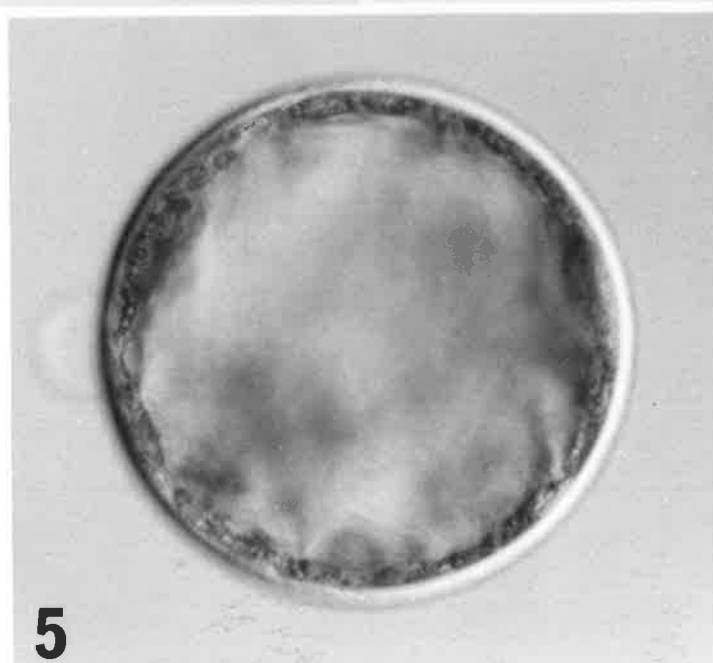
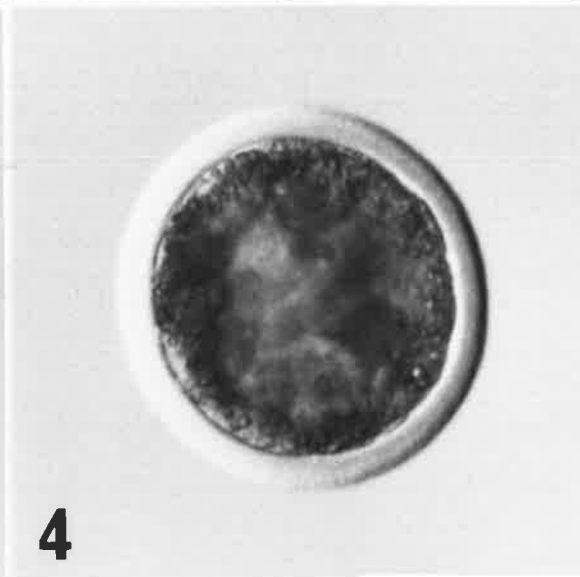
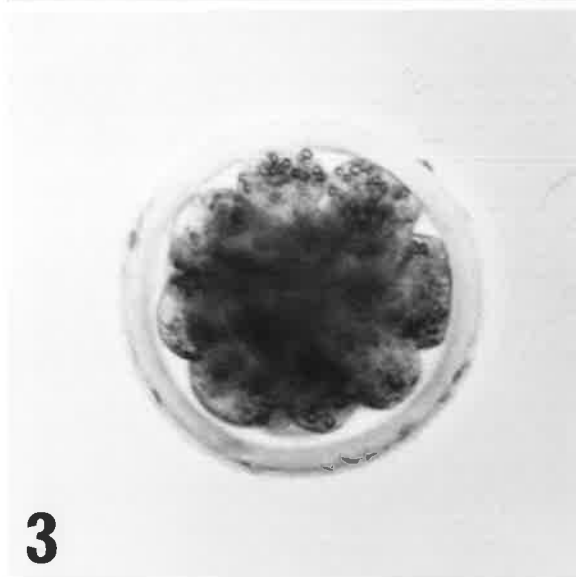
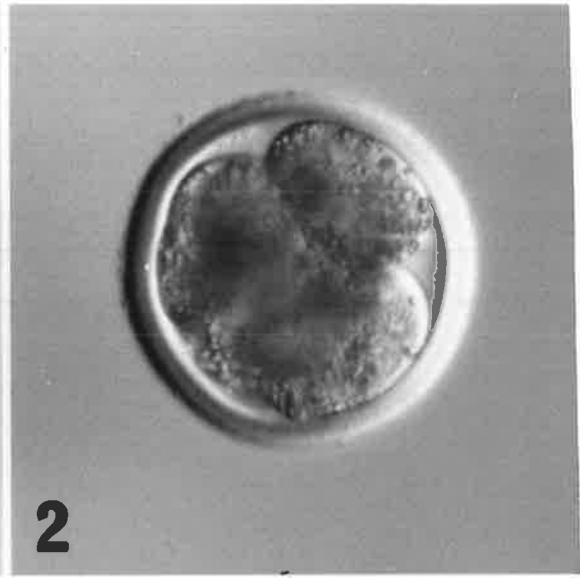
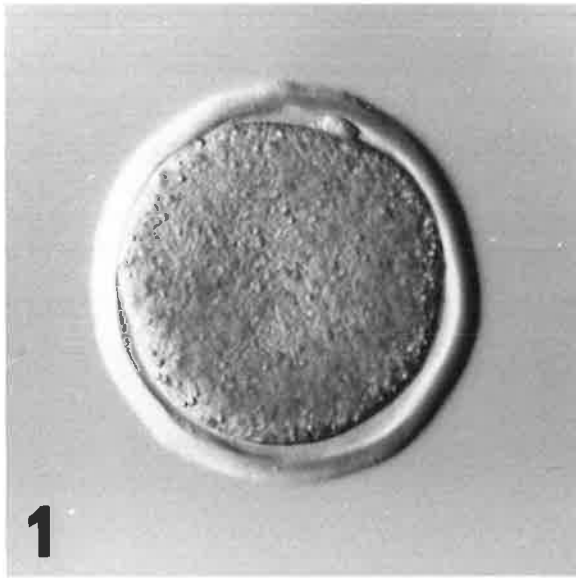
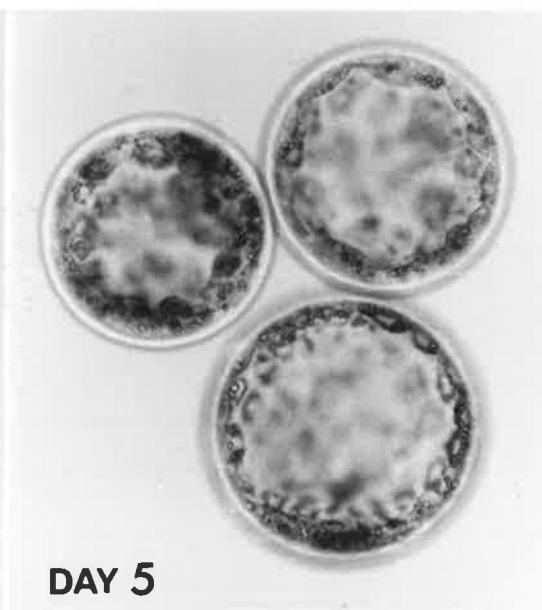
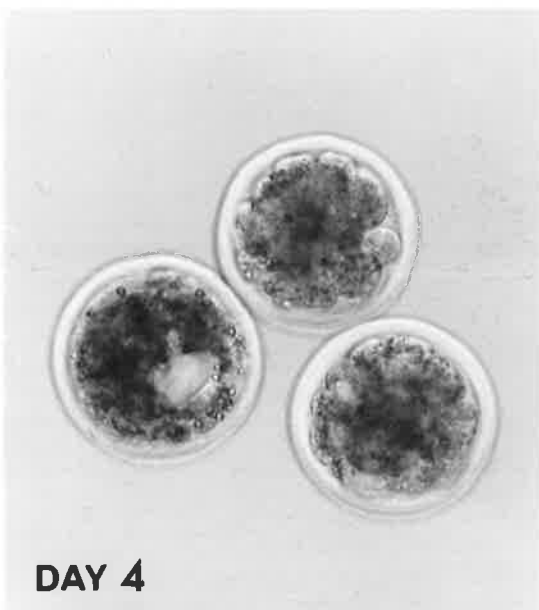
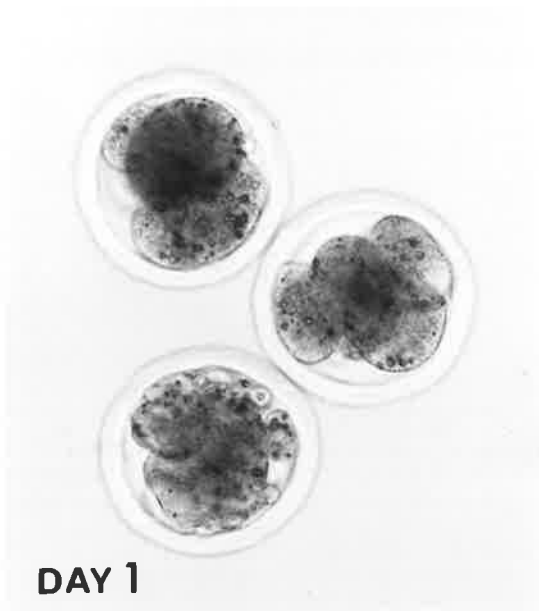


PLATE 3

Sequential development of embryos cultured in SOFM from the zygote stage (zygotes collected approximately 12-17h after the median time of ovulation; day 0 = day of commencement of culture).



5.3.3 Number of Nuclei per Embryo

The number of nuclei per embryo was assessed in 1273 embryos. Blastocysts that commenced hatching contained between 100 - 300 nuclei each while blastocysts that failed to hatch contained between 20 - 100 nuclei. Blastocysts with fewer than 40 nuclei each (11.5% of total) most often developed after the fifth day of culture.

Of the zygotes that failed to develop to blastocysts, 16.9% (93/551) ceased development between the 9 - 16 cell stage, 73.5% (405/551) ceased development at or before the eight-cell stage and 9.6% (53/551) did not divide.

5.3.4 Viability of Zygotes After Culture

In the first study, 97.6% of the embryos transferred were recovered (Table 28). There were no significant differences in the percentage of elongated conceptuses obtained from control zygotes (94.4%) or zygotes cultured for either one (92.9%) or three days (94.1%). In comparison, there was a significant reduction when zygotes were cultured for five days (52.8%; $P < 0.001$); elongated conceptuses were only obtained from embryos that were blastocysts at the time of transfer (19/22 v. 0/13 for all other embryos).

In the second study, 96.8% of the zygotes transferred were recovered (Table 29). The percentage of elongated conceptuses obtained after a three-day culture period was similar for zygotes cultured in media with 12.5 mM HEPES compared with zygotes cultured in media without HEPES. After five days, significantly ($P < 0.05$) more elongated conceptuses were obtained from zygotes cultured in the former medium. However, this difference resulted from the failure to collect elongated conceptuses from only one ewe in the latter treatment (embryos were collected at the

hatched blastocyst stage). There was no significant difference when this ewe was eliminated from the analysis. The percentage of conceptuses with visible embryonic discs and the mean length of the trophoblast did not differ between treatments. Irrespective of the culture medium, there was a significant ($P < 0.05$) decline in the percentage of elongated conceptuses obtained when the culture period was extended from three to five days (Table 29).

5.4 DISCUSSION

The results of these studies clearly indicate that SOFM is a suitable medium for the culture of sheep zygotes to the blastocyst stage. The preferred medium contained 25 mM sodium bicarbonate (before the addition of serum) and 20% HIHS; in this medium, 85.2% of zygotes developed into blastocysts, 74.1% commenced hatching and 93.9% were viable when transferred to recipient ewes after a three-day culture period. These figures are superior to those reported elsewhere although comparisons with other studies are limited by different experimental conditions.

(1) Components of SOFM

Protein supplementation, concentration of serum and the concentration of sodium bicarbonate and HEPES in the medium significantly influenced zygote development. In the studies on K and Ca concentrations, embryo growth occurred over a wide range of concentrations, supporting similar findings with mouse embryos (Wales, 1970; Whitten, 1971; Ducibella and Anderson, 1975). Embryo development was retarded only at concentrations of K greater than those normally used in culture media.

Similar percentages of zygotes developed into blastocysts when cultured in the presence of either HIHS or HISS. However, HIHS was the preferred protein source because blastocyst formation occurred significantly ($P < 0.05$) earlier than when HISS was used. Concentration of HIHS was also important, with significantly more zygotes developing into blastocysts in media supplemented with 20% serum compared with 10 and 30%; the former concentration may reflect a compromise between levels of growth factors and other factors that are detrimental to embryo growth. In comparison, Wright and Bondioli (1981) concluded that sera supplements of greater than 10% offered little additional advantage in the culture of embryos of domestic species.

HIHS has seldom been used in embryo culture media despite limited evidence that its inclusion enhances embryo development. Stone, Quinn and Seamark (1984) reported that the development of four- to eight-cell porcine embryos was enhanced in media containing HIHS compared with other protein sources. However, most attempts to culture sheep zygotes have used media supplemented with BSA (Tervit et al., 1972; Tervit and Rowson, 1974; Peters et al., 1977; Betterbed and Wright, 1985). In the current study, SOFM supplemented with BSA, albeit at high concentrations, did not support zygote growth as well as medium supplemented with serum (only 35.7% of zygotes cultured in BSA-medium developed into blastocysts). Several other studies have reported inferior embryo growth in media supplemented with BSA compared with sera, including that of Wright, Anderson, Cupps and Drost (1976a) with one- to eight-cell bovine embryos and that of Peters et al. (1977) with one- to four-cell ovine embryos. However, neither BSA nor sera are chemically defined media (Sirard and Lambert, 1985; Kane, 1987) and the results of studies using different batches of either supplement are thus not directly comparable.

The inclusion of HEPES lowered the pH of the medium but its presence was associated with reduced zygote development. Media containing HEPES

are traditionally used for short term storage of embryos and little data are available on the effect of HEPES on in vitro development. Normal development of two-cell mouse embryos was observed in Whittingham's modified Tyrode's medium containing both HEPES and sodium bicarbonate (Mahadevan, Fleetman, Church and Taylor, 1986). However, Carnevale, Squires and McKinnon (1987) obtained significantly fewer pregnancies when equine embryos were transferred after storage for 24h in Ham's F10 with HEPES compared with medium without HEPES. The decline in zygote development obtained in the current study may have occurred for one or more of the following reasons. Firstly, replacing sodium bicarbonate with HEPES had a direct toxic effect on viability; HEPES is known to increase the production of cytotoxic substances during exposure of the medium to light (Zigler, Lepe-Zuniga, Vistica and Gery, 1985). Secondly, replacing sodium bicarbonate with HEPES resulted in inadequate bicarbonate ions in the medium. Thirdly, changes in the pH of the HEPES-medium during the culture period approximated less closely those occurring in vivo compared with medium without HEPES. The effect of pH on the development of embryos of farm animals is unknown, although both mouse (Brinster, 1965a) and rabbit (Kane, 1974) embryos have developed over a wide range of values (6.0 to 7.8). On the other hand, the recent study of Ganz, Boyarsky, Sterzel and Borou (1989) highlighted the role of bicarbonate in cellular growth and development, and in particular the homeostatic mechanisms maintaining intracellular pH.

Formulation of SOFM is based on the inorganic composition of oviduct fluid as assessed by Restall and Wales (1966). Other studies have indicated several pertinent features of the biochemical composition of sheep oviduct fluid and tissue. These include (1) amino acids at concentrations up to four to five times those obtained in other species, with glycine being the most concentrated (Perkins and Goode, 1965; Warnes, 1976), (2) cyclic variation in the concentration of hypotaurine,

at oestrus (Van der Horst and Brand, 1969) and (3) cyclic variation in the concentration of myo-inositol, peaking during pro-oestrus (Van der Horst and Brand, 1969; Warnes, 1976). Relevant studies on the significance of these findings to in vitro development of zygotes may be worthwhile. One component of SOFM not examined was the concentration of energy substrates. Published data on the energy requirements of the sheep zygote are equivocal (Boone et al., 1978; Betterbed and Wright, 1985) and further refinement of the concentration of appropriate substrates may be possible.

(2) Fragmentation of Cytoplasm

Fragmentation of the cytoplasm was observed in the majority of embryos within 24h of the commencement of culture. Mild fragmentation did not affect subsequent embryo development but development declined significantly as the degree of fragmentation increased. The occurrence and severity of fragmentation were not influenced by any treatment. Gandolfi and Moor (1987) reported fragmentation and irregular cleaving in zygotes grown in TCM-199 and it was also observed in up to 24.2% of freshly collected two- and four-cell sheep embryos (Killeen and Moore, 1970).

Fragmentation is most likely caused by an imbalance or a deficiency in the medium. Recent studies with rabbit and hamster embryos indicate that embryos require specific vitamins for development, most notably inositol (Kane, 1988; Kane and Bavister, 1988). Zygotes are also known to sequester long chain fatty acids from the culture medium (Waterman and Wall, 1988). Requirements for these factors by sheep zygotes are not known. Studies also indicate that oil used to cover microdrops may absorb compounds from the medium (Miller and Pursel, 1987; Waterman and Wall, 1988). In recent observations (unpublished data), the degree of

fragmentation was reduced when zygotes were cultured in medium in the absence of oil.

(3) Number of Nuclei per Embryo

There is little information on the number of nuclear divisions that occur when sheep embryos are cultured in vitro. Peters et al. (1977) reported that eight-cell embryos contained between 105 - 130 nuclei at hatching and blastocysts that failed to hatch contained between 50 and 94 nuclei. In the current study, hatching blastocysts contained between 100 - 300 nuclei each, whilst blastocysts that failed to hatch contained less than 100 nuclei each. A severe reduction in the number of nuclei per blastocyst (less than 40) was associated with an increase in the time required for blastocyst formation. It is deduced from the study on zygote viability (Table 28) that these embryos are not viable when transferred to recipient ewes.

Data from zygotes that failed to develop to blastocysts indicate that most development ceased at or before the fourth cell cycle thus supporting the suggestion (Gandolfi and Moor, 1987) that an 8 - 16 cell "block" exists in the development of sheep embryos in vitro.

(4) Viability of Zygotes After Culture

There are few estimates of the viability of sheep zygotes after in vitro culture. One of four and none of 17 zygotes cultured for six days in SOFM were viable after transfer to recipient ewes (Tervit et al., 1972; Tervit and Rowson, 1974). It is evident, using elongation of the trophoblast as a criterion of embryo viability, that zygotes may be cultured in SOFM for three days without significant loss of viability. However, viability was significantly reduced when the culture period was

extended from three to five days. This study did not examine the natural wastage of embryos between days three and five; however, in Chapter 4.4.1 the development of up to 9.3% of embryos collected was retarded within four days of ovulation. The decline in viability supports the conclusion of Gandolfi and Moor (1987) that secretory products of oviduct cells are required by embryos for normal development. These authors reported that 42.0 and 4.5% of embryos cultured from the zygote stage in the presence of either oviduct cells or fibroblasts respectively, developed into expanded blastocysts after six days. The secreted products of oviduct cells may be similar to or interchangeable with growth factors present in HIHS given the percentage of zygotes that elongated in the current study following five days of culture (range 45.5 - 67.7%).

The loss of viability in some embryos cultured for five days was associated with a delay in the time of blastocyst formation. This delay would increase the likelihood of asynchrony occurring between the developmental age of the embryo and the physiological status of the reproductive tract. The viability of embryos cultured for five days may be improved if retarded embryos are transferred to recipients less advanced in the reproductive cycle.

(5) General Comments

Limited evidence indicates that SOFM is a superior medium for embryo growth compared with several other media. Wright and Bondioli (1981) reported that Tervit et al. (1972), in preliminary studies, obtained better development of both ovine and bovine embryos when cultured in SOFM compared with either TCM-199 or Whittingham's Medium (Whittingham, 1971). Wright et al. (1976b) reported that ovine embryos developed into blastocysts when cultured in either BMOC-3, SOFM or Whitten's Medium but not when cultured in MEM, TCM-199 or Ham's F10. It was initially

intended, in the current studies, to examine the development of zygotes in the presence of somatic cells. Enhanced development of zygotes has occurred in the presence of either oviduct cells (Gandolfi and Moor, 1987; Rexroad and Powell, 1988a, 1988b) or trophoblastic vesicles (Heyman et al., 1987). However, the percentage of zygotes that developed into blastocysts and the viability of embryos after culture in SOFM were higher than comparable figures reported in the above studies. Nevertheless, culture of zygotes in SOFM may be improved with the judicious inclusion of somatic cell support, particularly as a means of arresting the decline in viability when the culture period is extended past three days.

It is concluded that the culture medium described is able to support the growth of zygotes for up to three days without loss of viability. As such, the medium may be suitable for the assessment of zygote viability after pronuclear microinjection; this topic is examined in the following Chapter.

Table 17. Stock solutions used in the preparation of SOFM

<u>Stock</u>	<u>Component</u>	<u>g/100 ml</u>	<u>mM (in mixed medium)</u>
A	NaCl	6.29	107.7
	KCl	0.534	7.16
	KH ₂ PO ₄	0.162	1.19
	MgSO ₄ ·7H ₂ O	0.182	0.74
	Na.lactate	0.6ml	3.3
	glucose	0.27	1.5
	penicillin	0.06	-
		<u>g/10 ml</u>	
B	NaHCO ₃	0.210	12.5*
	phenol red	0.001	-
C	Na.pyruvate	0.051	0.33
D	CaCl ₂ ·2H ₂ O	0.262	1.78
E	HEPES	0.651	12.5*

*The concentration of NaHCO₃ was increased to 25 mM when HEPES was not included in the medium.

Table 18. Mixing of stock solutions in the preparation of SOFM

<u>Stock</u>	<u>Culture medium</u>	<u>Holding medium</u>
H ₂ O*	7.83 ml	7.83 ml
A ² *	1.0	1.0
B	0.5	0.16
C	0.07	0.07
D	0.1	0.1
E	0.5	0.84

*From Milli-Q reagent water system (Millipore Corporation, Bedford, MA).

Notes on mixing:

- (1) Refrigerated shelf-life of stocks: A-3 months; B-2 weeks, C-2 weeks, D-3 months and E-3 months.
- (2) Expected osmolarity after mixing approximately 270 mOsm.
- (3) The pH of stock E adjusted to 7.45 with sterile 1N HCl.
- (4) Medium filtered (0.2 um Acrodisc, Gelman Sciences Inc., Ann Arbor, Michigan) after the addition of serum or before the addition of BSA.

Table 19. Development of sheep zygotes in SOFM supplemented with different sources of protein. Values within columns with different superscripts differ significantly ($P < 0.05$).

(a) Percentage of zygotes at different stages of development after 9 days

Source of* protein	Final no. nuclei/developmental stage**				
	1	2-8	9-16	B+EB+HB	(HB)
Sheep serum (n=27)	3.7 ^{ac}	29.6 ^a	14.8 ^a	51.9 ^a	(11.1) ^a
Human serum (n=28)	28.6 ^b	10.7 ^b	10.7 ^a	50.0 ^a	(10.7) ^a
Ovine lymph (n=19)	10.5 ^{ab}	68.4 ^c	10.5 ^a	10.5 ^b	(0.0) ^a
Bovine lymph (n=31)	12.9 ^{ab}	54.8 ^c	16.1 ^a	16.1 ^b	(0.0) ^a
Nu-serum (n=28)	0.0 ^c	53.6 ^c	14.3 ^a	32.1 ^{ac}	(7.1) ^a
BSA (n=28)	10.7 ^{ab}	14.3 ^{ab}	39.3 ^b	35.7 ^{ac}	(0.0) ^a

(b) Number of new blastocysts observed per day as a percentage of total blastocysts

Source of protein	Days of culture***					
	4	5	6	7	8	9
Sheep serum (n=14)	0.0	0.0	14.3 ^a	42.9 ^a	35.7 ^{ab}	7.1 ^{ac}
Human serum (n=14)	0.0	0.0	50.0 ^b	35.7 ^a	14.3 ^a	0.0 ^a
Ovine lymph (n=2)	0.0	0.0	0.0 ^{ab}	0.0 ^a	0.0 ^{ab}	100.0 ^b
Bovine lymph (n=5)	0.0	0.0	0.0 ^{ab}	40.0 ^a	0.0 ^{ab}	60.0 ^b
Nu-serum (n=9)	0.0	0.0	11.1 ^{ab}	22.2 ^a	55.5 ^b	11.1 ^{ac}
BSA (n=10)	0.0	0.0	0.0 ^a	30.0 ^a	30.0 ^{ab}	40.0 ^b

*Sera and lymph concentrations = 10% (v/v); BSA concentration = 32 mg/ml.

**B = early blastocyst; EB = expanded blastocyst; HB = hatching blastocyst. Figures in brackets are the percentages of all zygotes that commenced hatching.

***Zygotes placed in culture approximately 12 - 17h after ovulation.

Table 20. Development of sheep zygotes in SOFM supplemented with 20% HHS (v/v) of different storage age. There are no significant differences between values within columns.

(a) Percentage of zygotes at different stages of development after 9 days

Age of serum (days)	Final no. nuclei/developmental stage*				
	1	2-8	9-16	B+EB+HB	(HB)
2 (n=36)	5.6	36.0	13.9	44.4	(19.4)
7 (n=36)	0.0	25.0	13.9	61.1	(33.3)
12 (n=35)	8.6	25.7	5.7	60.0	(31.4)

(b) Number of new blastocysts observed per day as a percentage of total blastocysts

Age of serum (days)	Day of culture**					
	4	5	6	7	8	9
2 (n=16)	25.0	25.0	50.0	0.0	0.0	0.0
7 (n=22)	13.6	40.9	40.9	4.5	0.0	0.0
12 (n=21)	4.8	28.6	52.3	14.3	0.0	0.0

*B = early blastocyst; EB = expanded blastocyst; HB = hatching blastocyst. Figures in brackets are the percentages of all zygotes that commenced hatching.

**Zygotes placed in culture approximately 12 - 17h after ovulation.

Table 21. Development of sheep zygotes in SOFM supplemented with different concentrations (v/v) of HIHS. Values within columns with different superscripts differ significantly ($P < 0.05$).

(a) Percentage of zygotes at different stages of development after 9 days

Concentration of serum	Final no. nuclei/developmental stage*				
	1	2-8	9-16	B+EB+HB	(HB)
0% (n=31)	12.9 ^a	58.1 ^a	29.0 ^a	0.0 ^a	(0.0) ^a
10% (n=39)	7.7 ^a	25.6 ^b	7.7 ^b	59.0 ^b	(30.8) ^b
20% (n=39)	2.6 ^a	10.3 ^b	0.0 ^b	87.1 ^c	(53.8) ^c
30% (n=37)	10.8 ^a	21.7 ^b	0.0 ^b	67.5 ^b	(37.8) ^b

(b) Number of new blastocysts observed per day as a percentage of total blastocysts

Concentration of serum	Day of culture**					
	4	5	6	7	8	9
0% (n=0)	-	-	-	-	-	-
10% (n=23)	13.0	39.1	43.5	4.3	0.0	0.0
20% (n=34)	2.9	35.3	50.0	11.8	0.0	0.0
30% (n=25)	8.0	36.0	52.0	4.0	0.0	0.0

*B = early blastocyst; EB = expanded blastocyst; HB = hatching blastocyst. Figures in brackets are the percentages of all zygotes that commenced hatching.

**Zygotes placed in culture approximately 12 - 17h after ovulation.

Table 22. Development of sheep zygotes in SOFM of different osmolarity. There were no significant differences between values within columns.

(a) Percentage of zygotes at different stages of development after 9 days

Osmolarity (mOsm)	Final no. nuclei/developmental stage*				
	1	2-8	9-16	B+EB+HB	(HB)
260 (n=28)	3.6	46.4	3.6	46.4	(14.3)
270 (n=31)	3.2	41.9	6.5	48.4	(19.4)
280 (n=29)	10.3	27.6	13.8	48.3	(13.8)
290 (n=29)	0.0	41.5	10.3	48.3	(24.1)
300 (n=26)	7.7	57.7	7.7	26.9	(7.7)

(b) Number of new blastocysts observed per day as a percentage of total blastocysts

Osmolarity (mOsm)	Day of culture**					
	4	5	6	7	8	9
260 (n=13)	0.0	23.1	61.5	15.4	0.0	0.0
270 (n=15)	0.0	20.0	66.7	13.3	0.0	0.0
280 (n=14)	0.0	14.3	50.0	28.6	7.1	0.0
290 (n=14)	0.0	14.3	42.9	21.4	21.4	0.0
300 (n=7)	0.0	28.6	42.9	14.3	14.3	0.0

*B = early blastocyst; EB = expanded blastocyst; HB = hatching blastocyst. Figures in brackets are the percentages of all zygotes that commenced hatching.

**Zygotes placed in culture approximately 12 - 17h after ovulation.

Table 23. Development of sheep zygotes in SOFM in which the concentration of K varied in the range 2.5 - 10.4 mM. There were no significant differences between values within columns.

(a) Percentage of zygotes at different stages of development after 9 days

Mean K in medium (mM)	Final no. nuclei/developmental stage*				
	1	2-8	9-16	B+EB+HB	(HB)
2.5±0.02 (n=45)	6.7	33.3	13.3	46.6	(13.3)
4.0±0.03 (n=46)	4.3	37.0	10.9	47.8	(10.9)
5.6±0.03 (n=51)	3.9	45.1	9.8	41.2	(15.7)
7.3±0.02 (n=56)	1.8	42.9	14.3	41.1	(14.3)
8.4±0.03 (n=57)	1.8	35.1	8.6	54.4	(15.8)
10.4±0.06 (n=63)	4.8	27.0	19.0	49.2	(14.3)

(b) Number of new blastocysts observed per day as a percentage of total blastocysts

Mean K in medium (mM)	Day of culture**					
	4	5	6	7	8	9
2.5±0.02 (n=21)	4.8	14.3	66.7	9.5	4.8	0.0
4.0±0.03 (n=22)	4.5	22.7	59.1	9.1	4.5	0.0
5.6±0.03 (n=21)	4.8	33.3	38.1	14.3	9.5	0.0
7.3±0.02 (n=23)	8.7	34.8	43.5	8.7	4.3	0.0
8.4±0.03 (n=31)	6.5	22.6	48.4	12.9	9.7	0.0
10.4±0.06 (n=31)	6.5	25.9	61.3	3.2	3.2	0.0

*B = early blastocyst; EB = expanded blastocyst; HB = hatching blastocyst. Figures in brackets are the percentages of all zygotes that commenced hatching.

**Zygotes placed in culture approximately 12 - 17h after ovulation.

Table 24. Development of sheep zygotes in SOFM in which the concentration of K varied between 2.5 - 27.8 mM. Values within columns with different superscripts differ significantly ($P < 0.05$).

(a) Percentage of zygotes at different stages of development after 9 days

Mean K in medium (mM)	Final no. nuclei/developmental stage*				
	1	2-8	9-16	B+EB+HB	(HB)
2.5±0.02 (n=41)	9.8 ^a	31.7 ^a	9.8 ^a	48.8 ^a	(14.6) ^{ab}
7.3±0.02 (n=31)	0.0 ^a	45.2 ^a	6.5 ^a	48.4 ^a	(32.3) ^a
13.4±0.18 (n=36)	16.7 ^{ab}	47.2 ^a	8.3 ^a	27.8 ^{ab}	(16.7) ^{ab}
20.6±0.42 (n=38)	15.8 ^{ab}	55.3 ^a	13.2 ^a	15.8 ^b	(10.5) ^b
27.8±0.75 (n=32)	31.3 ^b	40.6 ^a	3.1 ^a	25.0 ^b	(3.1) ^b

(b) Number of new blastocysts observed per day as a percentage of total blastocysts

Mean K in medium (mM)	Day of culture**					
	4	5	6	7	8	9
2.5±0.02 (n=20)	10.0 ^a	25.0 ^a	60.0 ^a	5.0 ^a	0.0 ^a	0.0 ^a
7.3±0.02 (n=15)	6.7 ^a	26.7 ^a	66.7 ^a	0.0 ^a	0.0 ^a	0.0 ^a
13.4±0.18 (n=10)	20.0 ^a	10.0 ^a	60.0 ^a	10.0 ^a	0.0 ^{ab}	0.0 ^a
20.6±0.42 (n=6)	0.0 ^a	0.0 ^a	66.7 ^a	16.7 ^a	16.7 ^b	0.0 ^a
27.8±0.75 (n=8)	0.0 ^a	0.0 ^a	0.0 ^b	62.5 ^b	25.0 ^b	12.5 ^a

*B = early blastocyst; EB = expanded blastocyst; HB = hatching blastocyst. Figures in brackets are the percentages of all zygotes that commenced hatching.

**Zygotes placed in culture approximately 12 - 17h after ovulation.

Table 25. Development of sheep zygotes in SOFM in which the concentration of Ca varied between 1.29 - 5.00 mM. There were no significant differences between values within columns.

(a) Percentage of zygotes at different stages of development after 9 days

Mean Ca in medium (mM)	Final no. nuclei/developmental stage*				
	1	2-8	9-16	B+EB+HB	(HB)
1.29±0.002 (n=25)	0.0	24.0	4.0	72.0	(60.0)
2.00±0.003 (n=24)	0.0	12.5	4.2	83.3	(75.0)
2.76±0.018 (n=20)	0.0	20.0	5.0	75.0	(65.0)
3.62±0.009 (n=16)	6.3	6.3	6.3	81.3	(75.0)
4.25±0.005 (n=18)	0.0	27.8	0.0	72.2	(55.5)
5.00±0.009 (n=15)	0.0	26.7	0.0	73.3	(66.7)

(b) Number of new blastocysts observed per day as a percentage of total blastocysts

Mean Ca in medium (mM)	Day of culture**					
	4	5	6	7	8	9
1.29±0.002 (n=18)	33.3	50.0	11.1	5.6	0.0	0.0
2.00±0.003 (n=20)	30.0	55.0	15.0	0.0	0.0	0.0
2.76±0.018 (n=15)	20.0	53.3	20.0	6.7	0.0	0.0
3.62±0.009 (n=13)	15.4	76.9	7.7	0.0	0.0	0.0
4.25±0.005 (n=13)	23.1	46.2	23.1	7.7	0.0	0.0
5.00±0.009 (n=11)	36.4	54.5	9.1	0.0	0.0	0.0

*B = early blastocyst; EB = expanded blastocyst; HB = hatching blastocyst. Figures in brackets are the percentages of all zygotes that commenced hatching.

**Zygotes placed in culture approximately 12 - 17h after ovulation.

Table 26. Mean ($\pm$ s.e.m.) pH values (range in brackets) of SOFM with different concentrations of sodium bicarbonate and HEPES (day 0 = day of media preparation). Concentrations of sodium bicarbonate are before the addition of 20% HHS; actual values are given in text.

Concentration (mM)		Day			
HEPES	NaHCO ₃	1	3	5	7
25.0	0.0	7.13 $\pm$ 0.03 (7.03-7.26)	7.10 $\pm$ 0.03 (7.00-7.22)	7.04 $\pm$ 0.02 (6.96-7.10)	7.03 $\pm$ 0.02 (6.90-7.11)
12.5	12.5	7.38 $\pm$ 0.03 (7.30-7.60)	7.36 $\pm$ 0.03 (7.29-7.52)	7.33 $\pm$ 0.01 (7.30-7.38)	7.32 $\pm$ 0.02 (7.26-7.40)
0.0	25.0	7.59 $\pm$ 0.05 (7.50-7.88)	7.53 $\pm$ 0.04 (7.44-7.75)	7.48 $\pm$ 0.02 (7.44-7.56)	7.47 $\pm$ 0.02 (7.40-7.57)

Table 27. Development of sheep zygotes in SOFM with different concentrations of NaHCO_3 and HEPES. Concentrations of NaHCO_3 are before the addition of 20% HIHS; actual values are given in text. Values within columns with different superscripts differ significantly ($P < 0.05$).

(a) Percentage of zygotes at different stages of development after 9 days

Concentration (mM)		Final no. nuclei/developmental stage*				
NaHCO_3	HEPES	1	2-8	9-16	B+EB+HB	(HB)
25.00 (n=54)	0.00	0.0 ^a	13.0 ^a	1.9 ^a	85.2 ^a	(74.1) ^a
18.75 (n=57)	6.25	0.0 ^a	21.1 ^a	3.5 ^a	75.4 ^{ab}	(59.6) ^a
12.50 (n=56)	12.50	0.0 ^a	25.0 ^a	1.8 ^a	73.2 ^{ab}	(71.4) ^a
6.25 (n=56)	18.75	0.0 ^a	26.8 ^a	3.6 ^a	69.6 ^b	(58.9) ^a
0.00 (n=53)	25.00	0.0 ^a	37.7 ^a	1.9 ^a	60.4 ^b	(47.2) ^b

(b) Number of new blastocysts observed per day as a percentage of total blastocysts

Concentration (mM)		Days of culture**					
NaHCO_3	HEPES	4	5	6	7	8	9
25.00 (n=46)	0.00	19.6 ^a	65.2 ^{ab}	15.2 ^a	0.0 ^a	0.0	0.0
18.75 (n=43)	6.25	14.0 ^{ab}	65.1 ^{ab}	18.6 ^a	2.3 ^a	0.0	0.0
12.50 (n=41)	12.50	22.0 ^a	68.3 ^{ab}	9.8 ^a	0.0 ^a	0.0	0.0
6.25 (n=39)	18.75	20.5 ^a	59.0 ^b	17.9 ^a	2.6 ^a	0.0	0.0
0.00 (n=32)	25.00	3.1 ^b	81.3 ^a	12.5 ^a	0.0 ^a	0.0	0.0

*B = early blastocyst; EB = expanded blastocyst; HB = hatching blastocyst. Figures in brackets are the percentages of all zygotes that commenced hatching.

**Zygotes placed in culture approximately 12 - 17h after ovulation.

Table 28. Number of zygotes that developed into elongated conceptuses (day 14 of pregnancy) after culture in SOFM containing 12.5 mM NaHCO_3 plus 12.5 mM HEPES for either one, three or five days (concentrations before the addition of 20% HIHS). Control zygotes were transferred within 30 minutes of collection.

	Control	Days in culture		
		1	3	5
No. zygotes transferred	36	42	51	36
No. recovered	35	40	51	35
No. elongated (% of total)	34 (94.4)	39 (92.9)	48 (94.1)	19* (52.8)

*Elongated conceptuses only obtained from embryos that were early blastocysts at the time of transfer (19/22 v. 0/13).

Table 29. Number of zygotes that developed into elongated conceptuses (day 14 of pregnancy) following three or five days culture in SOFM containing either 25 mM NaHCO_3 or 12.5 mM NaHCO_3 plus 12.5 mM HEPES (concentrations before the addition of 20% HIHS). Control zygotes were transferred within 30 minutes of collection.

	Control	3 Days		5 Days	
		NaHCO_3	NaHCO_3 +HEPES	NaHCO_3	NaHCO_3 +HEPES
No. zygotes transferred	26	33	31	33	31
No. recovered	23	33	30	33	30
No. elongated (% of total)	23 (88.5)	31 (93.9)	28 (90.3)	15 (45.5)	21 (67.7)
No. visible embryonic discs (%)	22 (95.7)	29 (93.5)	27 (96.4)	15 (100.0)	20 (95.2)
Length (mm) of conceptus (mean $\pm$ s.e.m.)	7.9 $\pm$ 1.6	9.0 $\pm$ 0.9	8.9 $\pm$ 1.6	12.4 $\pm$ 2.3	7.0 $\pm$ 1.2

CHAPTER 6

PRONUCLEAR MICROINJECTION OF SHEEP ZYGOTES

6.1 INTRODUCTION

The production of transgenic mice (Gordon et al., 1980; Palmiter et al., 1982, 1983), sheep and pigs (Hammer et al., 1985) by gene injection has markedly increased the potential for rapid genetic improvement in animal breeding. Several genes have been identified that are of potential value to the sheep industry including the growth hormone gene, wool keratin genes and genes associated with the control of ovulation (see Ward, Murray, Nancarrow, Boland and Sutton, 1984; Church et al., 1985; Wagner, 1985, 1986; Simons and Land, 1987). Gene transfer technology also offers the potential of introducing novel metabolic pathways into the animal; the microbial pathway for biosynthesis of cysteine from serine is most relevant in the sheep (Ward et al., 1984).

Studies reported in this thesis were stimulated by the lack of information available on the methodology of collecting, handling and microinjecting sheep zygotes. In this Chapter, problems associated with microinjection are examined, including difficulty in visualizing pronuclei and the viability of zygotes after microinjection. Nancarrow et al. (1984a) reported that as few as 18% of zygotes had visible pronuclei due to opacity of the cytoplasm. However, higher percentages have since been reported with the use of differential interference contrast optics (range 58.5 - 90%; Hammer et al., 1985; Ward et al., 1986; Murray et al., 1988; Simons et al., 1988). Microinjection programs are limited by many zygotes failing to develop after microinjection, due in part to membrane damage (Walton et al., 1987). Ward et al. (1986) reported that 51% of zygotes survived the injection procedure and 24% of

recipient ewes became pregnant when one to six injected zygotes were transferred per ewe. Rexroad and Wall (1987) found that following the injection of either buffer or DNA, 28 and 18% respectively of the zygotes developed to blastocysts in vivo. These authors concluded from a review of published data, that less than 20% of microinjected embryos of domestic species result in the birth of offspring.

The aims of experiments reported in this Chapter were (1) to identify the optimal conditions for visualization of pronuclei, (2) to determine the proportion of zygotes that fail to develop after microinjection and (3) to assess zygote viability after microinjection using the in vitro culture system described in Chapter 5.

6.2 MATERIALS AND METHODS

6.2.1 Supply of Zygotes

Zygotes were obtained following treatment with either PMSG or FSH; details on the relevant protocols are outlined in Chapter 5.2.1.

6.2.2 Methodology of Microinjection

Procedures and equipment for microinjection were similar to those previously reported in this laboratory for the injection of mouse and pig zygotes (Michalska, 1988). Brief details are provided below.

6.2.2.1 Equipment

A Zeiss inverted microscope, incorporating Nomarski differential interference contrast optics, was used. Holding pipettes and microinjection pipettes were connected to Leitz micromanipulators. The

instrument collar of the holding pipette was connected to a 0.5 ml Hamilton syringe (Alltech Pty Ltd, Australia) and the instrument collar of the microinjection pipette was connected by teflon tubing to a nitrogen cylinder. Microinjection was conducted using a 40x objective.

Holding pipettes were 20 - 40 μ m internal diameter and the microinjection pipettes approximately 1 μ m outer diameter. Pipettes were mechanically pulled in a Brown-Flaming horizontal pipette puller (Sutter Instruments Co., California) and the holding pipette was fire polished in a DeFonbrune microforge.

6.2.2.2 Gene Constructs

The porcine growth hormone gene (pHMPG) was constructed using the human metallothionein IIA promoter (Vize et al., 1988). This construct was successfully incorporated into the mouse genome before this study (Michalska, 1988) and was subsequently incorporated into the pig genome (Vize et al., 1988). The cysteine gene construct was a 6.4 kilobase fragment isolated from the plasmid pGER003; the fragment contained the Cys E and Cys M genes from Salmonella typhimurium (Rogers, Sivaprasad, D'Andrea, Bawden and Kuczek, 1989). This construct was also incorporated into the mouse genome with high efficiency prior to this study.

DNA was diluted in PBS (Flow Laboratories, North Ryde, New South Wales) to a concentration of 2 - 5 μ g per ml, frozen and then subsequently centrifuged at 11,600 g for 30 - 60 seconds immediately prior to use.

6.2.2.3 Microinjection Procedures

Zygotes were washed twice in culture medium and then placed in a microinjection chamber identical to that described by Michalska (1988).

The medium used was SOFM containing 25 mM HEPES (Table 18); 50 μ l droplets were prepared within the chamber and covered with paraffin oil (BDH Chemical Ltd, Poole, England). Pronuclei were microinjected with approximately 1 - 2 μ l of the DNA solution. Zygotes were then either transferred immediately to recipient ewes or cultured in SOFM (see below). Mr K.J. McLaughlin and Mr P.J. Verma conducted the microinjection.

6.2.2.4 Assessment of DNA Incorporation

Incorporation of the pHMPG construct was determined in tail tissue by Southern blot (Southern, 1975) and dot and slot-blot analyses. Incorporation of the pGER003 construct was assessed by polymerase chain reaction techniques (Mullis and Faloona, 1987) in tissue from elongated conceptuses obtained on day 14 of pregnancy; however, results were not available at the time of writing. These determinations were conducted by staff of the Department of Biochemistry, University of Adelaide.

6.2.3 Preparation of Recipient Ewes

Donor and recipient ewes were synchronized on the basis of median times of ovulation of 54 and 63h respectively. During the course of the study, it became apparent that recipient ewes in some flocks were significantly late in the time of onset of ovulation (see Chapter 3.1.3.1). Consequently, time of ovulation was assessed in recipient ewes from all flocks; those with delayed ovulation were excluded from the program.

6.2.4 Experimental Procedures

6.2.4.1 Visualization of Pronuclei

The aim of this study was to determine the optimal time to visualize pronuclei. Zygotes were collected at three-hour intervals between either 2 - 10h or 14 - 22h inclusively after the estimated median time of ovulation. If pronuclei were not visible using differential interference contrast optics, the zygotes were centrifuged at 7000 g for 90 seconds and re-examined. Zygotes in which pronuclei were not observed were subsequently stained (see Chapter 5.2.4) and examined for evidence of fertilization.

Zygotes obtained following treatment of ewes with either FSH + GnRH or PMSG + GnRH were observed to determine the effect of gonadotrophin treatment on the visibility of pronuclei. Zygotes were collected between 12 - 17h after the median time of ovulation. Twelve replicated observations were conducted.

6.2.4.2 Viability of Microinjection Zygotes In Vivo

Zygotes were collected between 12 - 17h after the median time of ovulation and those with visible pronuclei microinjected with the pHMPG construct. The zygotes were held in SOFM for 1 - 6h before transfer to recipient ewes. A second group of uninjected zygotes, also held in SOFM for 1 - 6h, were transferred to recipients. Each recipient received between 5 - 10 zygotes.

Three days after transfer, embryos were recovered from the reproductive tracts. Each embryo was examined (40x magnification) and the number of blastomeres recorded. The number of degenerate and fragmented embryos and the number of empty zonae pellucidae were also recorded. Embryos with healthy blastomeres were then transferred, in groups of 1 - 3, to recipient ewes. The number of fetuses per ewe was subsequently determined by ultrasonography approximately 50 days later.

6.2.4.3 Assessment of the Viability of Microinjected Zygotes In Vitro

Zygotes were microinjected with the pGER003 construct and then randomly allocated to be cultured either in vitro or in vivo (control). Zygotes were cultured in vitro in 50 ul droplets of SOFM without HEPES (see Chapter 5.2.2) for either one or three days. Zygotes cultured for three days were divided into two quality grades on the basis of number and definition of blastomeres and the degree of fragmentation. All embryos, irrespective of their morphological appearance, were subsequently transferred to synchronized recipient ewes in groups of 4 - 12 per ewe.

Zygotes for in vivo development were transferred to the oviducts of recipient ewes within four hours of microinjection. These ewes were then divided into two groups. In one group, embryos were recovered either one or three days after transfer and the developmental stages assessed; viable embryos were subsequently retransferred to other recipient ewes. In the other group, zygotes were allowed to develop without interference. All zygotes/embryos were transferred in groups of 4 - 12 per ewe.

Recipients were slaughtered on day 14 of pregnancy and elongated conceptuses and undeveloped embryos recovered from the reproductive tract (see Chapter 5.2.6). This study was conducted as a series of six replicates.

6.2.5 Statistical Analyses

Data were analysed using either the Student's t test or Chi-square test.

6.3 RESULTS

6.3.1 Visualization of Pronuclei

The optimal time to visualize pronuclei was between 14 - 19h after the expected median time of ovulation (Table 30). Attempts to visualize pronuclei within 10h of ovulation was limited by many ova not being fertilized (as assessed by the presence of pronuclei after staining). Nevertheless, the percentage of fertilized ova with visible pronuclei in this period was less than in the 14 - 22h period. Centrifugation of zygotes in which pronuclei were not immediately visible resulted in a small improvement in the overall visibility; this improvement was greater in zygotes collected between 2 - 10h than between 14 - 22h.

The percentage of zygotes with visible pronuclei was significantly ($P < 0.01$) higher when superovulation was induced with FSH compared with PMSG (Table 31). The number of ovulations per ewe was not a confounding factor. Treatment with FSH also significantly ($P < 0.01$) increased the number of ova collected as a proportion of ovulations recorded.

6.3.2 Viability of Microinjected Zygotes In Vivo

Overall, significantly ($P < 0.001$) more microinjected zygotes failed to develop to the eight-cell stage after transfer compared with control zygotes (45.1 versus 13.5% respectively, excluding fragmented embryos; Table 32). Most nonviable zygotes in the former group were those that failed to divide after microinjection. A significantly ($P < 0.05$) higher percentage of control zygotes fragmented compared with microinjected zygotes. Fragmentation was only observed when zygotes were transferred to recipients with a delay in the onset of ovulation (see Chapter 3.1.3.1)

More ewes became pregnant following the transfer of control compared with microinjected zygotes (72.2 v. 48.7% respectively, $P < 0.1$; Table 33). The percentage of zygotes that developed into fetuses was also significantly higher in the former group (38.1 v. 20.2%, $P < 0.05$). More

lambs born from the microinjected zygotes were dead at birth compared with control zygotes (7/43 v. 0/16, $P > 0.05$). None of the 43 lambs born following microinjection were proven to have incorporated the pHMPG construct.

6.3.3 Assessment of the Viability of Microinjected Zygotes In Vitro

More zygotes were assessed as viable, on the basis of blastomere number and definition, after in vitro compared with in vivo culture (Tables 34 and 35). After one day of culture, 83.3 and 76.7% respectively of the embryos were assessed as viable ($P > 0.05$), whereas comparable figures after three days were 70.7 and 50.0% ($P < 0.01$).

Between 92.1 and 100% of the zygotes and embryos transferred were recovered on day 14 (Tables 34 and 35). The number of elongated conceptuses obtained as a percentage of the original number of zygotes did not differ significantly between control zygotes (i.e. those transferred immediately after microinjection and not re-assessed) and zygotes cultured either in vitro or in vivo for one or three days. Overall, between 32.6 - 50.0% of transferred zygotes/embryos developed into elongated conceptuses; this was increased to 42.4 - 65.8% when embryos assessed as nonviable after culture were excluded from the analysis.

The sequential development of microinjected zygotes in vitro is shown in Plate 4.

Table 30. The effect of time after the median time of ovulation on the percentage of fertilized ova and the percentage of zygotes with visible pronuclei (PN) before and after centrifugation. Relevant statistical comparisons are made within the text.

	Time (h) after median time of ovulation					
	2-4	5-7	8-10	14-16	17-19	20-22
No. ova	37	33	45	30	29	38*
Fertilized ova(%)**	32.4	51.5	70.0	89.3	86.2	88.9
Zygotes with visible PN (%)						
(a) before centrif.	9.1	23.5	3.6	57.7	72.0	23.5
(b) after centrif.	18.2	58.8	21.4	69.2	76.0	47.1

*Includes 17 two-cell embryos.

**Assessed by presence of two pronuclei after staining.

Table 31. Collection of zygotes and the visibility of pronuclei following treatment with either PMSG + GnRH or FSH + GnRH.

	PMSG + GnRH	FSH + GnRH	P Value
No. ewes	138	79	
Ovulations/ewe ($\pm$ s.e.m.)	9.6 $\pm$ 0.45	9.0 $\pm$ 0.68	ns
Ova recovered/ ewe ($\pm$ s.e.m.)	5.3 $\pm$ 0.32	6.3 $\pm$ 0.54	ns
Zygotes/ewe ($\pm$ s.e.m.)	4.4 $\pm$ 0.30	5.1 $\pm$ 0.46	ns
% ova recovered/ ovulations	54.8 (726/1326)	69.5 (495/712)	< 0.01
% recovered ova fertilized	83.6 (607/726)	81.2 (402/495)	ns
% zygotes with visible pronuclei	45.6 (254/557)	63.0 (247/392)	< 0.01

Table 32. Percentage of microinjected (pHMPG construct) and control zygotes at different stages of development, three days after transfer to synchronized recipient ewes. Values within columns with different superscripts differ significantly ($P < 0.05$).

	No. blastomeres						
	F*	Degen.*	2	4	6	8-16	>16
Microinjected zygotes (N=343)	1.2% ^a	25.2% ^a	7.0% ^a	3.5% ^a	8.2% ^a	50.2% ^a	4.7% ^a
Control zygotes (N=111)	24.4% ^b	7.2% ^b	2.7% ^a	0.0% ^a	3.6% ^a	48.6% ^a	13.5% ^b

*F = fragmented; degen. = degenerate or lysed cells or empty zonae.

Table 33. Pregnancy and lambing results following the transfer of microinjected (pHMPG construct) and control zygotes. Zygotes were cultured in oviducts for three days and lysed and degenerate embryos eliminated before retransfer. Relevant statistical comparisons are made within the text.

	Microinjected zygotes	Control zygotes
No. zygotes retransferred	233*	42
No. pregnant ewes (%)	37/76 (48.7%)	13/18 (72.2%)
No. fetuses/embryos** retransferred (%)	47/233 (20.2%)	16/42 (38.1%)
No. lambs	43	16
(a) alive	36	16
(b) dead	7	0

*189 embryos were of eight blastomeres or more while 44 contained fewer than eight blastomeres.

**Number of fetuses determined by ultrasonography 50 days after transfer.

Table 34. Number of microinjected zygotes (pGER003 construct) that developed into elongated conceptuses (day 14 of pregnancy) in recipient ewes after either in vivo (oviduct) or in vitro culture for one day. Control zygotes were transferred within four hours of microinjection. Relevant statistical comparisons are made within the text.

	Control	<u>In vivo</u> culture	<u>In vitro</u> culture
No. zygotes	42	43	42
Viable embryos* after culture (%)	-	33 (76.7)	35 (83.3)
No. recovered (%)	40 (95.2)	32 (97.0)	35 (100.0)
No. elongated	15	14	21
(1) % of total	35.7	32.6	50.0
(2) % of viable embryos after culture	-	42.4	60.0

*Viability assessed on number and definition of blastomeres. Viable embryos were transferred after culture to synchronized recipient ewes.

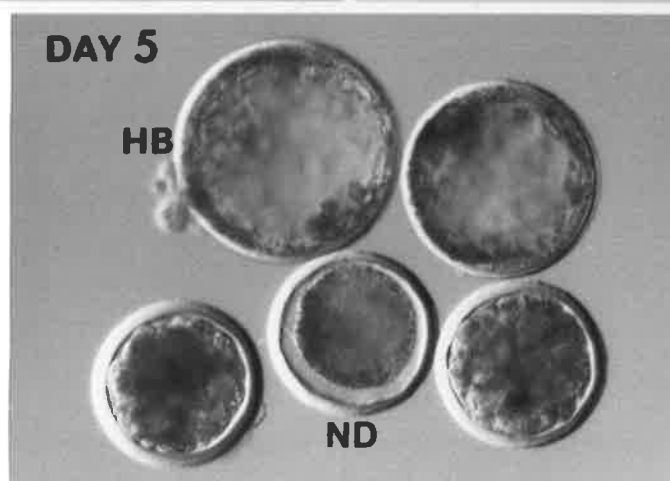
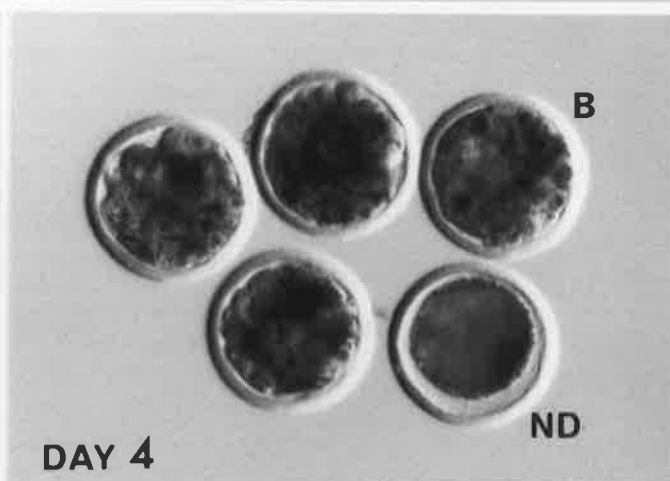
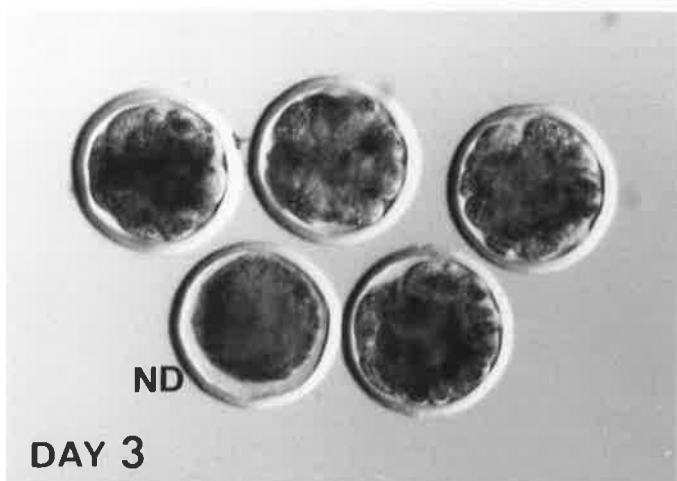
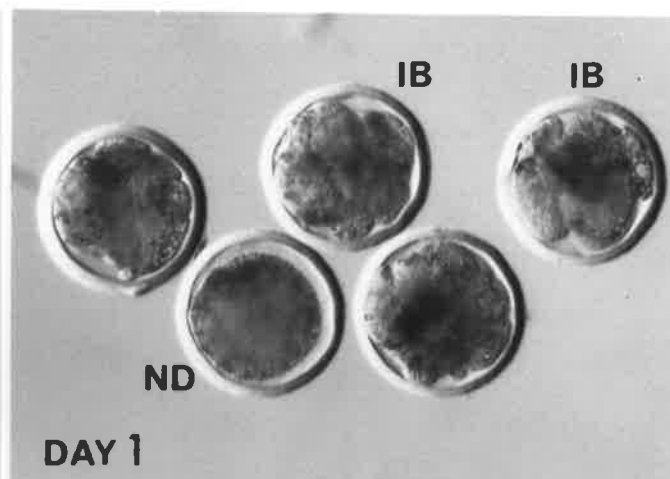
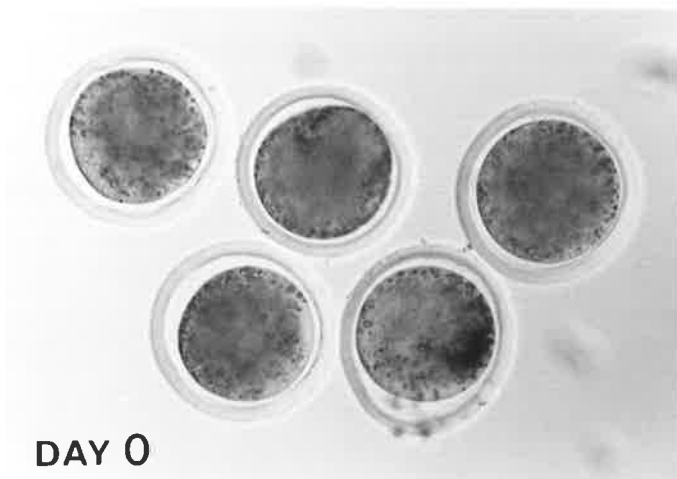
Table 35. Number of microinjected zygotes (pGER003 construct) that developed into elongated conceptuses (day 14 of pregnancy) in recipient ewes after either in vivo (oviduct) or in vitro culture for three days. Control zygotes were transferred within four hours of microinjection. Relevant statistical comparisons are made within the text.

	Control	<u>In vivo</u> culture	<u>In vitro</u> culture
No. zygotes	67	76	75
Viable embryos* after culture (%)	-	38 (50.0)	53 (70.7)
No. recovered (%)	66 (98.5)	35 (92.1)	49 (92.5)
No. elongated	30	25	26
(1) % of total	44.8	32.9	34.7
(2) % of viable embryos after culture	-	65.8	49.1

*Viability assessed on number and definition of blastomeres. Viable embryos were transferred after culture to synchronized recipient ewes.

PLATE 4

Sequential development in SOFM of zygotes microinjected into the pronucleus with the pGER003 construct (see Chapter 6.2.4.3). One zygote did not divide (ND) and irregular blastomeres (IB) and high fragmentation scores were observed on day 1. Two blastocysts developed, including one on day 4 (B) which subsequently commenced hatching (HB) on day 5.



6.4 DISCUSSION

Visualization of pronuclei was a major problem in early studies on pronuclear microinjection of zygotes of domestic species. Nancarrow et al. (1984a) reported that as few as 18% of sheep zygotes had visible pronuclei but with the use of differential interference contrast optics, pronuclei have been seen in 80 - 90% of zygotes (Hammer et al., 1985; Murray et al., 1988; Simons et al., 1988). The results of the current study indicate that visualization is enhanced if zygotes are collected 17 - 19h after the expected median time of ovulation. Dziuk (1965) estimated that the male pronucleus formed approximately 3 - 9h after ovulation and the lifespan of the pronucleus is thought to be about 10 - 15h (McLaren, 1980). Pronuclei migrate from the cortex towards the centre of the ovum during which time they enlarge (Austin, 1951, 1961). The time sequences indicate that it is during this change that pronuclei are most easily observed. A feature of this study was the high proportion of zygotes collected compared with cleaved embryos; two-cell embryos were only obtained when laparotomy was conducted between 20 - 22h after the median time of ovulation. In other studies, lack of precise control of the time of ovulation resulted in the collection of two- and four-cell embryos as well as zygotes (Moore, 1970; Rexroad and Powell, 1988a; Simons et al., 1988). Evidence indicates that the microinjection of cleaved embryos is a less efficient means of producing transgenic offspring compared with zygotes (Brinster et al., 1985; Simons et al., 1988).

Centrifugation improved the visibility in zygotes of the pig (Wall et al., 1985) and cow (Lohse et al., 1985) but its effect in the current study was minimal. Nancarrow et al. (1984b) also reported that centrifugation failed to improve the visibility of pronuclei in sheep zygotes. An interesting observation was the significantly higher

percentage of zygotes with visible pronuclei following FSH treatment compared with PMSG. The former treatment produced zygotes that contained fewer lipid droplets in the cytoplasm. Nancarrow et al. (1987a) reported a similar observation but concluded that PMSG treatment was more cost effective than FSH in the production of zygotes for microinjection.

The study on the viability of microinjected zygotes in recipient ewes indicated that approximately half the zygotes ceased development within three days of microinjection (Table 32). Most nonviable zygotes lysed or failed to divide while others divided but were retarded. A higher than expected percentage (24.4%) of control zygotes fragmented after transfer (Table 32), presumably due to asynchrony between donor and recipient ewes in some replicates. Recipient ewes in those replicates were obtained from one flock in which the commencement of ovulation was delayed (see Chapter 3.1.3.1). Why a comparable percentage of microinjected zygotes did not fragment is not known. Of the microinjected zygotes, 12.5% resulted in the production of lambs and this figure is comparable with published data (range 5.5 - 18.0%; Hammer et al., 1985; Murray et al., 1988; Nancarrow et al., 1987b; Pinkert et al., 1987; Pursel et al., 1988; Simons et al., 1988). Although the differences were not significant, more fetuses from microinjected zygotes were lost after 50 days of pregnancy compared with control zygotes and more lambs in the former group were dead at birth. The latter could only have resulted from chance or from a disturbance of the fetal-placental relationship brought about by the microinjection procedure as none of the lambs had incorporated the gene construct.

Studies of factors likely to influence zygote viability after microinjection implicate pipette size and taper (Walton et al., 1987), time of microinjection (Walton et al., 1987) and the injection of fluid into the pronucleus, particularly DNA solution (Rexroad and Wall, 1987). Dimensions of the pipette are most likely to influence cell lysis after

microinjection; up to 49% of injected zygotes have lysed within one hour (Ward et al., 1986). On the other hand, the injection of DNA may induce direct toxic effects or unfavourably change the genome after incorporation, resulting in death of the embryo or fetus. Hammer et al. (1985) concluded that sheep zygotes were less likely to survive microinjection than pig zygotes.

The most significant aspect of this series of studies was the demonstration that microinjected zygotes could be cultured in SOFM for three days without viability being adversely affected (Table 35). Culture enabled (1) the identification of zygotes that lysed or failed to divide and (2) assessment of embryo quality based on the number and definition of blastomeres and the degree of fragmentation. In the three-day culture study, 63.6% (21/33) of embryos with good blastomere definition developed into elongated conceptuses compared with 25.0% (5/20) for poorer quality embryos. The ability to accurately assess embryo viability was limited by the occurrence of fragmentation (see Chapter 5.3.2). To improve the efficiency of microinjected programs, more poor quality embryos could be transferred per ewe compared with better quality embryos.

Fewer microinjected zygotes lysed or degenerated in vitro compared with in vivo (Tables 34 and 35). Various functions have been ascribed to the zona pellucida which could explain this difference, including protection from leucocytes, bacteria and macromolecules (see Nichols and Gordon, 1989). These authors also concluded that the zona may protect the blastomeres from pressure exerted from the oviduct musculature; they reported that splitting the zona of two-cell mouse embryos did not affect development in vitro but retarded development in vivo. Despite the differences obtained in early zygote development in the current study, the percentage of zygotes that elongated did not differ significantly between in vivo and in vitro culture treatments.

In conclusion, these studies highlight the importance to microinjection programs of the time of zygote collection and the reduced viability after microinjection. More importantly, the ability to assess the viability of microinjected zygotes in vitro has been demonstrated.

CHAPTER 7

GENERAL DISCUSSION

The production of transgenic animals requires the integration of expertise in both molecular and reproductive biology. Potential limitations to progress are numerous but a major problem, common to most domestic species, is the lack of knowledge on the collection and handling of zygotes. The problem is more pronounced in the sheep, where superovulation and embryo collection have both been highly unreliable procedures. The aim of studies reported in this thesis was thus to examine factors likely to influence the efficacy of microinjection programs, with emphasis on the collection and handling of zygotes.

Studies on the time of ovulation in both donor and recipient ewes established a basis for the reliable collection and transfer of zygotes. Particularly important was the effect of GnRH treatment in synchronizing the time of ovulation in donor ewes. The ability to induce approximately 80% of ovulations within three hours increased the proportion of ova that were fertilized and the proportion of fertilized ova collected at the pronuclear stage. Ewes treated with either PMSG or FSH ovulated at significantly different times (median times of 54 and 60h respectively after pessary removal) and adjustment in the time of GnRH treatment was necessary to produce ova of normal quality. These studies also identified several previously unreported phenomena, the most relevant to the collection of zygotes being the relatively early onset of superovulation in some ewes induced to ovulate late in the breeding season or during seasonal anoestrus. Early superovulation was a major hindrance to the year-round collection of zygotes, with two- and four-cell embryos being frequently collected. The cause of early

superovulation is not known and relevant studies on the underlying endocrinology are warranted.

Studies on the use of GnRH in nonsuperovulated ewes following synchrony of oestrus were aimed at maximizing the chronological synchrony between donor and recipient. Although GnRH treatment synchronized the time of ovulation in the nonsuperovulated ewe, it was associated with reduced zygote development after transfer. Treatment also resulted in a nonsignificant increase in conception rates following fixed time insemination using the laparoscope. These results, together with comparable studies in donor ewes, indicate that the extent to which the LH surge can be advanced, without untoward effect, is limited. In nonsuperovulated ewes, treatment with GnRH was associated with luteal regression, the occurrence of additional ovulations after those induced directly by the treatment and an increase in the proportion of unfertilized ova.

The within and between flock variation in the time of ovulation in recipient ewes highlighted the potential that exists for asynchronous transfer of zygotes. To minimize the likelihood of asynchrony occurring, the time of pessary removal should be adjusted so that the median time of ovulation in both donors and recipients occurs at approximately the same time. However, even after this adjustment, natural variation in the time of ovulation within flocks could result in an asynchrony of up to 18h. Optimizing the time of transfer was further complicated by the tendency of some flocks to commence ovulation significantly later in November and December compared with other times of the year (69 compared with 57h after pessary removal). A delay in the onset of ovulation of this magnitude is likely to be detrimental to both fixed time insemination and embryo transfer programs. This phenomenon was most likely a consequence of seasonal refractoriness to PMSG treatment. Synchronizing the oestrous cycle throughout the year using a standard progestagen-PMSG treatment

thus has its limitations in the Merino and further studies (e.g. dose-response relationships) may be worthwhile. The identification of factors that influence the rate of follicular development (see Driancourt, 1987) may ultimately lead to a treatment capable of inducing a more synchronous time of ovulation.

GnRH treatment in donor ewes was associated with a marked increase in fertilization rates and a concurrent improvement in embryo yield. Fertilization failure had previously been a major limitation to the collection of sheep embryos, due primarily to impaired migration and reduced longevity of spermatozoa within the reproductive tract. It is assumed that the enhanced fertilization rates obtained with GnRH treatment resulted from a reduction in the time during which ovulation occurred. The ability to synchronize the time of ovulation necessitated a reassessment of the optimal time to inseminate ewes and also the number of spermatozoa required for maximum fertilization rates. Results of studies in Chapter 4 indicated that the insemination of 20×10^6 motile frozen-thawed spermatozoa per uterine horn, six hours before the expected median time of ovulation, was optimal. Previously published information on these topics has been inconclusive, possibly due to differences between studies in the time of insemination, the time of ovulation or the number and viability of spermatozoa inseminated. The current findings are not only relevant to the collection of zygotes but also to commercial embryo collection programs. Embryos collected four days after the GnRH-induced ovulation were of normal quality and the percentage of ova (fertilized and unfertilized) recovered of ovulations recorded was not significantly affected by the treatment. The use of GnRH, using treatment protocols outlined in these studies, is thus recommended in commercial programs.

The studies on in vitro culture of zygotes examined, in stepwise progression, the effect of various components of SOFM on zygote

development. Development was unaffected by large variations in the majority of these components, except at unphysiological levels. The preferred medium was similar to that described by Tervit et al. (1972), with the exception that 20% HIHS was used as the protein source instead of BSA. In this medium, approximately 85% of zygotes developed to the blastocyst stage and time of blastocyst formation was commensurate with that obtained in vivo. Despite the high rate of development, there remain several problems with the culture system. Firstly, mild fragmentation occurred in most embryos within 24h of the commencement of culture and this hindered assessment of embryo quality. Secondly, some blastocysts were deficient in nuclei number. Thirdly and most importantly, there was a significant decline in viability when the culture period was extended from three to five days. The latter two problems were related; embryos that failed to develop to blastocysts by the fifth day of culture had fewer than normal number of nuclei and were nonviable when transferred to recipient ewes. The decline in viability after three days supports the suggestion of Gandolfi and Moor (1987) that oviduct cell support is required for embryo development through the fourth cell cycle. However, proportionately more embryos cultured from zygotes in the current studies were viable after five days compared with zygotes cultured for a comparable period in the presence of oviduct cells (Gandolfi and Moor, 1987). It is postulated that HIHS may contain growth factors similar to or interchangeable with secreted products of oviduct cells.

The most significant aspect of the final experimental chapter was the demonstration that microinjected zygotes could be cultured in SOFM for up to three days without loss of viability. This is particularly relevant to studies on transgenesis, given the high loss of zygotes following microinjection (see Chapter 6.3.2). Culture not only enabled the identification of zygotes that failed to divide but also facilitated

an assessment of embryo quality. However, this assessment was limited by the tendency of most zygotes to undergo mild fragmentation when placed in the culture medium. Further refinement of the medium may be possible with the addition of selected vitamins or growth factors or the incorporation of co-culture with somatic cells. Although microinjected zygotes can be cultured to the blastocyst stage in vitro (Plate 4), the expected loss of viability between days three and five makes extended culture unattractive. The availability of a refined medium, in which microinjected zygotes can be cultured to blastocysts at a rate similar to that obtained in vivo, is pivotal to future developments in studies on transgenesis. These developments include embryo biopsy and embryo splitting with the concurrent assessment of DNA incorporation using polymerase chain reaction techniques.

Conclusions on zygote viability after in vitro culture and/or microinjection were based on elongation of the trophoblast by day 14 of pregnancy. However, embryo mortality in the sheep is thought to be maximal between days 14 - 21 of gestation, during embryo migration (Pope, 1988) and allantoic placentation (Jainudeen and Hafez, 1980). The conclusions from the relevant studies in Chapters 5 and 6 are thus limited by the possibility that additional embryo wastage occurred after day 14. Appropriate data on the production of lambs following in vitro culture of zygotes are required.

In conclusion, studies reported in this thesis have improved the understanding of factors likely to influence the collection, culture and microinjection of sheep zygotes.

BIBLIOGRAPHY

- ACRITOPOULOU, S., HARESIGN, W. & LAMMING, G.E. (1978). Time of ovulation in ewes after treatment with a prostaglandin F-2 alpha analogue. J. Reprod. Fert. 54, 189-191.
- AINSWORTH, L. & WOLYNETZ, M.S. (1982). Synchronization of estrus and reproductive performance of ewes treated with synthetic progestagens administered by subcutaneous ear implant or by intravaginal sponge pessary. J. Anim. Sci. 54, 1120-1127.
- AINSWORTH, L., LACHANCE, R. & LABRIE, F. (1983). Effects of progestagen treatments and PMSG on the induction of the preovulatory LH discharge in ewes. Anim. Reprod. Sci. 5, 281-286.
- AINSWORTH, L. & SHRESTHA, J.N.B. (1983). Effect of type of intravaginal progestagen treatment on estrous response and reproductive performance of ewes. Theriogenology 19, 869-875.
- AINSWORTH, L. & SHRESTHA, J.N.B. (1985). Effect of PMSG dosage on the reproductive performance of adult ewes and ewe lambs bred at a progestagen-PMSG synchronized estrus. Theriogenology 24, 479-487.
- AINSWORTH, L. & DOWNEY, B.R. (1986). A controlled internal drug release dispenser containing progesterone for control of the estrous cycle of ewes. Theriogenology 26, 847-856.
- AKBAR, A.M., NETT, R.M. & NISWENDER, G.D. (1974). Metabolic clearance and secretion rates of gonadotrophins at different stages of the estrous cycle in ewes. Endocrinology 94, 1318-1324.
- ALIFAKIOTIS, T., MICHAILIDIS, I. & GAVRILIDIS, G. (1982). Induced breeding in anestrus milking ewes of dairy breeds: comparison of norgestomet, medroxyprogesterone and fluorogestone in two regimes of PMSG. Theriogenology 17, 603-610.
- ALLEN, D.M. & LAMMING, G.E. (1961a). Nutrition and reproduction in the ewe. J. Agric. Sci., Camb. 56, 69-79.
- ALLEN, D.M. & LAMMING, G.E. (1961b). Some effects of nutrition on the growth and sexual development of ewe lambs. J. Agric. Sci., Camb. 57, 87-95.
- ALLEN, R.L. & WRIGHT, R.W. (1984). In vitro development of porcine embryos in coculture with endometrial cell monolayers or culture supernatants. J. Anim. Sci. 59, 1657-1661.
- ALLISON, A.J. (1975). Effect of nutritionally induced live-weight differences on the ovarian response and fertility in ewes treated with pregnant mare's serum gonadotrophin. N. Z. J. Agric. Res. 18, 101-107.
- ALLISON, A.J. & KELLY, R.W. (1978). Synchronisation of oestrus and fertility in sheep treated with progestagen-impregnated implants, and prostaglandin with or without intravaginal sponges and subcutaneous pregnant mare's serum. N. Z. J. Agric. Res. 21, 389-393.

- ALWAN, S.F., BOLAND, M.P. & GORDON, I. (1988). Superovulation and oocyte recovery in the ewe. Theriogenology 29, 1143-1147.
- ARMSTRONG, D.T., MILLER, B.G., WALTON, E.A., PFITZNER, A.P. & WARNES, G.M. (1982). Endocrine responses and factors which limit the response of follicles to PMSG and FSH. In: Embryo Transfer in Cattle, Sheep and Goats. Eds J. Shelton, A.O. Trounson, N.W. Moore and J.W. James, pp 8-15. Australian Society of Reproductive Biology, Sydney.
- ARMSTRONG, D.T. & EVANS, G. (1983). Factors influencing success of embryo transfer in sheep and goats. Theriogenology 19, 31-42.
- ARMSTRONG, D.T., PFITZNER, A.P., WARNES, G.M. & SEAMARK, R.F. (1983). Superovulation treatments and embryo transfer in Angora goats. J. Reprod. Fert. 67, 403-410.
- ARMSTRONG, D.T. & EVANS, G. (1984a). Hormonal regulation of reproduction: induction of ovulation in sheep and goats with FSH preparations. Proc. 10th Int. Cong. Anim. Reprod. A.I. 8, 8-15.
- ARMSTRONG, D.T. & EVANS, G. (1984b). Intrauterine insemination enhances fertility of frozen semen in superovulated ewes. J. Reprod. Fert. 71, 89-94.
- ATKINS, K.D. (1987). Potential responses to selection in Merino sheep given current industry structure and selection practices. In: Merino Improvement Programs in Australia, pp 299-312. Ed. B.J. McGuirk. Australian Wool Corporation, Melbourne.
- AUSTIN, C.R. (1951). The formation, growth and conjunction of the pronuclei in the rat egg. J. Roy. Microsc. Soc. 71, 295-306.
- AUSTIN, C.R. (1961). The Mammalian Egg. C.C. Thomas Publ., Springfield, Ill.
- AVERILL, R.L. & ROWSON, L.E.A. (1958). Ovum transfer in the sheep. J. Endocr. 16, 326-336.
- BALL, P.H.J., SIMKIN, M.E. & FOOTE, R.H. (1978). Effects of cannulae in the ampulla of the oviduct and in the utero-tubular junction on reproductive phenomena in sheep. Theriogenology 12, 389-398.
- BARNES, D.R. (1987). CIDR dispensers - ewe and hogget plasma progesterone levels during the insertion period. Proc. 4th AAAP Animal Science Congress, p. 228, Hamilton, New Zealand.
- BATT, P.A. & MILLER, B.G. (1988). Development of sheep embryos in vitro in a medium supplemented with different serum fractions. Aust. J. Biol. Sci. 41, 189-199.
- BAVISTER, B.D., LEIBFRIED, M.L. & LIEBERMAN, G. (1983). Development of preimplantation embryos of the golden hamster in a defined culture medium. Biol. Reprod. 28, 235-237.
- BAWDEN, C.S., CAM, G.R., D'ANDREA, R.J., KUCZEK, E.S., POWELL, B.C., ROGERS, G.E. & SIVAPRASAD, A.V. (1987). A review of the genes for wool keratin and for cysteine biosynthesis: their significance for studies directed towards modified wool production. In: Merino

Improvement Programs in Australia, pp 511-518. Ed. B.J. McGuirk. Australian Wool Corporation, Melbourne.

- BECK, N.F.G., DAVIES, M.C.G., DAVIES, B. & LEES, J.L. (1987). Oestrus synchronization and fertility in ewes: a comparison of three methods. Anim. Prod. 44, 251-254.
- BELLOWS, R.A., POPE, A.L., MEYER, R.K., CHAPMAN, A.B. & CASIDA, L.E. (1963). Physiological mechanisms in nutritionally induced differences in ovarian activity of mature ewes. J. Anim. Sci. 22, 93-100.
- BETTERBED, B. & WRIGHT, R.W. (1985). Development of one-cell ovine embryos in two culture media under two gas atmospheres. Theriogenology 23, 547-553.
- BETTERIDGE, K.J. (1977). Embryo Transfer in Farm Animals, Agriculture Canada, Ottawa.
- BETTERIDGE, K.J. & MOORE, N.W. (1977). Techniques and results in sheep and goats. Superovulation. In: Embryo Transfer in Farm Animals, pp 37-38. Ed. K.J. Betteridge. Agriculture Canada, Ottawa.
- BIERY, K.A., BONDIOLI, K.R. & DE MAYO, F.J. (1988). Gene transfer by pronuclear injection in the bovine. Theriogenology 29, 224 (Abst.).
- BIGGERS, J.D., GWATKIN, R.B.L. & BRINSTER, R.L. (1962). Development of mouse embryos in organ culture of fallopian tubes on a chemically defined medium. Nature 194, 747-749.
- BINDON, B.M. (1975). Ovulation in ewes selected for fecundity: effect of synthetic Gn-RH on the day of oestrus. J. Reprod. Fert. 44, 325-328.
- BILTON, R.J. & MOORE, N.W. (1976). Effects of ice seeding and of freezing and thawing rate on the development of sheep embryos stored at -196°C. Theriogenology 6, 635 (Abst.).
- BINDON, B.M. & PIPER, L.R. (1977). Induction of ovulation in sheep and cattle by injection of PMSG and ovine anti-PMSG immune serum. Theriogenology 8, 171 (Abst.).
- BINDON, B.M., CH'ANG, T.S. & TURNER, H.N. (1971). Ovarian response to gonadotrophin by Merino ewes selected for fecundity. Aust. J. Agric. Res. 22, 809-820.
- BINDON, B.M. & PIPER, L.R. (1982). Physiological basis of the ovarian response to PMSG in sheep and cattle. In: Embryo Transfer in Cattle, Sheep and Goats, pp 1-5. Eds J.N. Shelton, A.O. Trounson, N.W. Moore and J.W. James. Australian Society of Reproductive Biology, Sydney.
- BOLAND, M.P. & GORDON, I. (1977). Superovulation in the anoestrous Galway ewe following PMSG and HAP. J. Irish Dept. Agric. 74, 81-83.
- BOLAND, M.P. & GORDON, I. (1978). Recovery and fertilization of eggs following natural service and uterine insemination in the Galway ewe. Irish Vet. J. 32, 123-125.

- BOLAND, M.P., KELLEHER, D. & GORDON, I. (1978). Comparison of control of oestrus and ovulation in sheep by an ear implant (SC 21009) or by intravaginal sponge (cronolone or MAP). Anim. Reprod. Sci. 1, 275-281.
- BOLAND, M.P. & GORDON, I. (1979). Effect of timing of ram introduction on fertility in progestagen-PMSG treated anoestrous ewes. J. Agric. Sci., Camb. 92, 247-249.
- BOLAND, M.P., CROSBY, T.F. & GORDON, I. (1981). Effect of mating management and PMSG dose on lambing outcome in ewes bred in anoestrus. J. Agric. Sci., Camb. 97, 445-447.
- BOLAND, M.P. & GORDON, I. (1982). Effect of repeated horse anterior pituitary extract treatment on ovulatory response in the ewe. Vet. Rec. 111, 391-392.
- BOLAND, M.P., CROSBY, T.F. & GORDON, I. (1983). Ovarian response in ewes following horse anterior pituitary extract and progestagen treatment. Anim. Reprod. Sci. 6, 119-127.
- BOLAND, M.P., HAZELTON, I.G., MURRAY, J.D., SUTTON, R. & NANCARROW, C.D. (1984). Towards gene transfer into ruminant embryos: factors affecting the supply of embryos. Theriogenology 21, 222 (Abst.).
- BOSHOF, D.A., van NIEKERK, C.H. & MORGENTHAU, J.C. (1973). Time of ovulations in the Karakul ewe following synchronization of oestrus. S. Afr. J. Anim. Sci. 3, 13-17.
- BOONE, W.R., DICKEY, J.F., LUSZCZ, L.J., DANTZLER, J.R. & HILL, J.R. (1978). Culture of ovine and bovine ova. J. Anim. Sci. 47, 908-913.
- BRADEN, A.W.H., LAMOND, D.R. & RADFORD, H.M. (1960). The control of the time of ovulation in sheep. Aust. J. Agric. Res. 11, 389-401.
- BRADFORD, G.E., QUIRKE, J.F. & HART, R. (1971). Natural and induced ovulation rate in Finnish Landrace and other breeds of sheep. Anim. Prod. 13, 627-635.
- BREM, G., BRENI, G., GOODMAN, H.M., SELDON, R.C., GRAF, F., KRUFF, B., SPRINGMAN, K., HONDELE, J., MEYER, J., WINNACHER, E.L. & KRAUSSLICH, H. (1985). Production of transgenic mice, rabbits and pigs by microinjection into pronuclei. Zuchthygiene 20, 251-252.
- BRINSTER, R.L. (1965a). Studies on the development of mouse embryos in vitro. 1. The effects of osmolarity and hydrogen ion concentration. J. Exp. Zool. 158, 49-58.
- BRINSTER, R.L. (1965b). Studies on the development of mouse embryos in vitro. 2. The effect of energy source. J. Exp. Zool. 158, 59-68.
- BRINSTER, R.L. (1969). Incorporation of carbon from glucose and pyruvate into preimplantation mouse embryos. Exp. Cell Res. 58, 153-158.
- BRINSTER, R.L. (1971). In vitro culture of the embryo. In: Pathways to Conception: The Role of the Cervix and the Oviduct in Reproduction, pp 245-277. Ed. A.I. Sherman. Charles C. Thomas, Springfield.

- BRINSTER, R.L., CHEN, H., TRUMBAUER, M., SENEAR, A., WARREN, R. & PALMITER, R. (1981). Somatic expression of herpes thymidine kinase in mice following injection of a fusion gene into eggs. Cell 27, 223-231.
- BRINSTER, R.L., CHEN, H.Y., TRUMBAUER, M.E., YAGLE, M.K. & PALMITER, R.D. (1985). Factors affecting the efficiency of introducing foreign DNA into mice by microinjecting eggs. Proc. Natl. Acad. Sci. 82, 4438-4442.
- BRINSTER, R.L. & PALMITER, R.D. (1982). Induction of foreign genes in animals. Trends Biochem. Sci. 7, 438-440.
- BROCK, H. & ROWSON, L.E. (1952). The production of viable bovine embryos. J. Agric. Sci., Camb. 42, 479-482.
- BRYANT, M.J. & TOMKINS, T. (1976). The flock mating of progestagen-synchronized ewes. 2. The influence of time of ram introduction upon mating behaviour and lambing performance. Anim. Prod. 22, 379-384.
- BUNCH, T.D., FOOTE, W.C., CALL, J.W. & CLARK, W.F. (1984). In vitro culture and maintenance of ovine embryos transported in Dulbecco's enriched phosphate-buffered saline. Theriogenology 26, 981-987.
- BUTTLE, H.R.L. & HANCOCK, J.L. (1964). Birth of lambs after storage of sheep eggs in vitro. J. Reprod. Fert. 7, 417-418.
- CAHILL, L.P. & DUFOUR, J. (1979). Follicular populations in the ewe under different gonadotrophin levels. Ann. Biol. Anim. Bioch. Biophys. 19, 1475-1481.
- CAHILL, L.P. (1984). Folliculogenesis and ovulation rate in sheep. In: Reproduction in Sheep, pp 92-98. Eds D.R. Lindsay and D.T. Pearce. Australian Academy of Science, Canberra.
- CAMOUS, S.Y., HEYMAN, Y., MEZIOU, W. & MENEZOU, Y. (1984). Cleavage beyond the block stage and survival after transfer of early bovine embryos cultured with trophoblastic vesicles. J. Reprod. Fert. 72, 479-585.
- CAPECCHI, M. (1980). High efficiency transformation by direct microinjection of DNA into cultured mammalian cells. Cell 22, 479-488.
- CAPEHART, J.S., BOWEN, M.J., BASSETT, J.W., SHELTON, J.M. & KRAEMER, D.C. (1984). A modified technique for the collection of uterine stage ovine embryos. Theriogenology 21, 227 (Abst.).
- CARNEVALE, E.M., SQUIRES, E.L. & MCKINNON, A.O. (1987). Comparison of Ham's F10 with CO₂ or Hepes buffer for storage of equine embryos at 5°C for 24h. J. Anim. Sci. 65, 1775-1781.
- CHEN, L.T. & HSU, Y.C. (1982). Development of mouse embryos in vitro: preimplantation to the limb bud stage. Science 218, 66-68.
- CHRISTENSEN, R.K. (1976). Effect of short-term progestogen treatment on induction of estrus and lambing in anestrus ewes. J. Anim. Sci. 43, 795-801.

- CHURCH, R.B., SCHAUFLE, F.J. & MECKLING, K. (1985). Embryo manipulation and gene transfer in livestock. Can. J. Anim. Sci. 65, 527-537.
- CLARKE, I.J. (1973). Repeated gonadotrophic stimulation of ewes. Proc. N. Z. Soc. Anim. Prod. 33, 204 (Abst.)
- CLARKSON, J.L. & NANCARROW, C.D. (1988). Use of trophoblastic vesicles as models for ovine blastocysts cultured in vitro. Proc. 11th Int. Cong. Anim. Reprod. A.I. 2, 148.
- COGNIE, Y., CHUPIN, D. & SAUMANDE, J. (1985). Comparison of two FSH schedules to induce superovulation in the ewe. Theriogenology 23, 185 (Abst.).
- COGNIE, Y., CHUPIN, D. & SAUMANDE, J. (1986). The effect of modifying the FSH/LH ratio during the superovulatory treatment in ewes. Theriogenology 25, 148 (Abst.).
- CONSTANTINI, F. & LACY, E. (1981). Introduction of a rabbit B-globin gene into the mouse germ line. Nature 294, 91-93.
- CONSTANTINI, F., LACY, E., ROBERTS, S., EVANS, E. & BURTENSHAW, M. (1983). A foreign b-globin gene in transgenic mice: Integration at abnormal chromosomal positions and expression in inappropriate tissue. Cell 34, 343-358.
- COONROD, S.A., COREN, B.R., McBRIDE, B.L., BOWEN, M.J. & KRAEMER, D.C. (1986). Successful non-surgical collection of ovine embryos. Theriogenology 25, 149 (Abst.)
- CROSBY, T.F., BOLAND, M.P. & GORDON, I. (1983). Effect of progesterone/progestagen treatments on the induction of pregnancy in ewes during late anestrus. J. Anim. Sci. 57, (Suppl. 1), 471 (Abst.).
- CROSBY, T.F., BOLAND, M.P., EL-KAMALI, A.A. & GORDON, I. (1980). Superovulation in the ewe using HAP. Theriogenology 13, 92 (Abst.)
- CUMMING, I.A., BUCKMASTER, J.M., BLOCKEY, M.A. deB., GODING, J.R., WINFIELD, C.G. & BAXTER, R.W. (1973). Constancy of interval between luteinizing hormone release and ovulation in the ewe. Biol. Reprod. 9, 24-29.
- CUMMINS, L.J. (1983). Ovarian function in the Booroola Merino. The role of ovarian inhibiting factors on the regulation of ovulation rate. PhD Thesis, University of New England, Armidale, N.S.W.
- CUTTEN, I.N. (1980). Influence of season of joining on the oestrous, ovulatory and lambing performance of Merino ewes with seasonal live weight fluctuations controlled. Proc. Aust. Soc. Anim. Prod. 13, 313-316.
- DAVIS, I.F., KERTON, D.J., McPHEE, S.R., GRANT, I. & CAHILL, L.P. (1984). Effect of sperm dose on conception rate following uterine AI in ewes. Proc. Aust. Soc. Reprod. Biol. 16, 101 (Abst.).
- DIELEMAN, S.J. & BEVERS, M.M. (1987). Effects of monoclonal antibody against PMSG administered shortly after the preovulatory LH surge on time and number of ovulations in PMSG/PG-treated cows. J. Reprod. Fert. 81, 533-542.

- DONEY, J.M., SMITH, W.F. & GUNN, R.G. (1976). Effects of post mating environmental stress on administration of ACTH on early embryo loss in sheep. J. Agric. Sci., Camb. 87, 133-136.
- DRIANCOURT, M.A. (1987). Follicular dynamics and intraovarian control of follicular development in the ewe. In: Follicular Growth and Ovulation Rate in Farm Animals, pp 87-105. Eds J.F. Roche and D. O'Callaghan. Martinus Nijhoff Publishers, The Netherlands.
- DUCIBELLA, T. & ANDERSON, E. (1975). Cell shape and membrane changes in the 8-cell mouse embryo: Prerequisites for morphogenesis of the blastocyst. Dev. Biol. 47, 45-48.
- DZIUK, P.J. (1965). Timing of maturation and fertilization of the sheep egg. Anat. Rec. 153, 211-224.
- DZIUK, P.J., HINDS, F.C., MANSFIELD, M.E. & BAKER, R.D. (1964). Follicle growth and control of ovulation in the ewe following treatment with 6-methyl-17-acetoxypregesterone. J. Anim. Sci. 23, 787-790.
- DZIUK, P.J., COOK, B., KALTENBACH, G. & NISWENDER, G. (1966). Control of heat in ewes by an implanted progestagen. J. Anim. Sci. 25, 922 (Abst.).
- EASTWOOD, K.C. & McDONALD, M.F. (1975). Reproductive differences in two-year-old Romney and Border Leicester x Romney ewes after gonadotrophin treatment. N. Z. J. Agric. Res. 18, 109-117.
- EBERT, K., LOW, M., OVERSTOM, E., BUONOMO, F., BAILE, C.F., ROBERTS, T.M., LEE, A., MANDEL, G. & GOODMAN, R. (1988). Molec. Endocrinol. (in press). Cited by Rexroad and Pursel (1988).
- EDEY, T.N. (1966). Nutritional stress and preimplantation embryonic mortality in Merino sheep. J. Agric. Sci., Camb. 67, 287-302.
- EDWARDS, R.G. & FOWLER, R.E. (1960). Superovulation treatment of adult mice; their subsequent natural fertility and response to further treatment. J. Endocr. 21, 147-154.
- EDWARDS, R.G., BAVISTER, B.D. & STEPTOE, P.C. (1969). Early stages of fertilization in vitro of human oocytes matured in vitro. Nature 221, 632-635.
- EDWARDS, R.G., STEPTOE, P.C. & PURDY, J.M. (1980). Establishing full-term pregnancies using cleaving embryos grown in vitro. Brit. J. Obstet. Gynaecol. 87, 737-756.
- ELSDEN, R.P. (1977). Embryo collection by surgical means. In: Embryo Transfer in Farm Animals, pp 10-12. Ed. K.J. Betteridge. Agriculture Canada, Ottawa.
- EPPLESTON, J., BILTON, R.J. & MOORE, N.W. (1984). Effect of FSH dose and treatment regime on ovulatory response in sheep. Proc. Aust. Soc. Reprod. Biol. 16, 68 (Abst.).
- EPPLESTON, J. & ROBERTS, E.M. (1986). The effect of progestagen, PMSG and time of insemination on fertility of ewes following intra-uterine insemination with frozen semen. Aust. Vet. J. 63, 124-125.

- EPPLESTON, J., MAXWELL, W.M.C., BATTYE, K.M. & ROBERTS, E.M. (1986). Effect of thawed motility and intra-uterine dose of motile sperm on fertility of ewes. Proc. Aust. Soc. Reprod. Biol. 18, 19 (Abst.).
- EVANS, G. & ROBINSON, T.J. (1980). The control of fertility in sheep: endocrine and ovarian responses to progestagen-PMSG treatment in the breeding season and in anoestrus. J. Agric. Sci., Camb. 94, 69-88.
- EVANS, G. & ARMSTRONG, D.T. (1984). Reduction of sperm transport in ewes by superovulation treatments. J. Reprod. Fert. 70, 47-53.
- EVANS, G., HOLLAND, M.K., NOTTLE, H.B., SHARPE, P.H. & ARMSTRONG, D.T. (1984). Production of embryos in sheep using FSH preparations and laparoscopic intrauterine insemination. In: *Reproduction in Sheep*, pp 313-315. Eds D.R. Lindsay and D.T. Pearce. Australian Academy of Science, Canberra.
- EVANS, H.M. & SIMPSON, M.E. (1983). Hormones of the anterior hypophysis. Am. J. Physiol. 98, 511-519.
- EVANS, M.J., BRADLEY, A., KUEHN, M.R. & ROBERTSON, E.J. (1985). The ability of EK cells to form chimeras after selection of clones in G418 and some observations on the integration of retroviral vector proviral DNA into EK cells. Cold Spring Harbor Symp. Quant. Biol. 50, 685-689.
- EYESTONE, W.H., VIGNIERI, J. & FIRST, N.L. (1987). Co-culture of early embryos with oviductal epithelium. Theriogenology 27, 228 (Abst.).
- EYESTONE, W.H. & FIRST, N.L. (1989). Co-culture of early cattle embryos to the blastocyst stage with oviduct tissue or in conditioned medium. J. Reprod. Fert. 85, 715-720.
- FABRICANT, J.D., NUTI, L.C., MINHAS, B.S., BAKER, W.C., CAPEHART, J.S., MARROCK, P., CHALMERS, J.H., BRADBURY, M.W. & WOMACK, J.E. (1987). Gene transfer in goats. Theriogenology 27, 229 (Abst.).
- FAIRNIE, I.J., WALES, R.G. & GHERARDI, P.B. (1977). Time of ovulation, fertilization rate, and blastocyst formation in ewes following treatment with a prostaglandin analogue (ICI 88,996). Theriogenology 8, 183 (Abst.).
- FAURE, A.S., BOSHOFF, D.A. & BURGER, F.J.L. (1983). The effect of whole or halved intravaginal sponges combined with either subcutaneous or intravenous administration of PMSG on synchronization of the estrous cycle of Karakul ewes. S. Afr. J. Anim. Sci. 13, 157-160.
- FITZGERALD, J.A., RUGGLES, A.J., STELLFLUG, J.N. & HANSEL, W. (1985). A seven-day synchronization method for ewes using medroxyprogesterone acetate (MAP) and prostaglandin F-2 alpha. J. Anim. Sci. 61, 466-469.
- GANDOLFI, F. & MOOR, R.M. (1987). Stimulation of early embryonic development in the sheep by co-culture with oviduct epithelial cells. J. Reprod. Fert. 81, 23-28.

- GANDOLFI, F. & MOOR, R.M. (1988). Interactions between somatic and germinal cells during early development. Proc. 11th Int. Cong. Anim. Reprod. A.I. 5, 169-176.
- GANZ, M.B., BOYARSKY, G., STERZEL, R.B. & BORON, W.F. (1989). Arginine vasopressin enhances pHi regulation in the presence of HCO_3^- by stimulating three acid-base transport systems. Nature 337, 648-651.
- GHERARDI, P.B. & MARTIN, G.B. (1978). The effect of multiple injections of Pregnant Mares' Serum on the ovarian activity of Merino ewes. Proc. Aust. Soc. Anim. Prod. 12, 260 (Abst.)
- GHERARDI, P.B. & LINDSAY, D.R. (1980). The effect of season on the ovulatory response of Merino ewes to serum from pregnant mares. J. Reprod. Fert. 60, 425-429.
- GILBERT, D.M. & COHEN, S.N. (1987). Bovine papilloma virus plasmids replicate randomly in mouse fibroblasts throughout S phase of the cycle. Cell 50, 59-68.
- GORDON, I. (1963). The induction of pregnancy in the anoestrous ewe by hormonal therapy. Progesterone-pregnant mare's serum therapy during the breeding season. J. Agric. Sci., Camb. 60, 31-42.
- GORDON, I. (1969). Factors affecting response of anoestrous sheep to progestagen treatment. J. Ir. Dept. Agric. 66, 232.
- GORDON, I. (1971a). Induction of early breeding in sheep by standard and modified progestagen-PMS treatments. J. Agric. Sci., Camb. 76, 337-341.
- GORDON, I. (1971b). Control of reproduction in sheep: Towards programmed lamb production. J. Ir. Dept. Agric. 68, 27-75.
- GORDON, I. (1975a). The use of progestagens in sheep bred by natural and artificial insemination. Ann. Biol. Anim. Bioch. Biophys. 15, 303-316.
- GORDON, I. (1975b). Hormonal control of reproduction in sheep. Proc. Br. Soc. Anim. Prod. 4, 79-93.
- GORDON, I. (1983). Controlled Breeding in Farm Animals. Pergamon Press, Oxford.
- GORDON, J.W., SCANGOS, G.A., PLOTKIN, D.J., BARBOSA, J.A. & RUDDLE, F.H. (1980). Genetic transformation of mouse embryos by microinjection of purified DNA. Proc. Natl. Acad. Sci. 77, 7380-7384.
- GORDON, J.W. & RUDDLE, F.H. (1983). Gene transfer into mouse embryos: production of transgenic mice by pronuclear microinjection. Meth. Enzymol. 101, 411-433.
- GOSSLER, A., DOETSCHMAN, T., KORN, R., SERFLING, E. & KEMLER, R. (1986). Transgenesis by means of blastocyst-derived embryonic stem cell lines. Proc. Natl. Acad. Sci. 83, 9065-9069.
- GRAESSMAN, A., GRAESSMAN, M., TOPP, W.C. & BOTCHAM, J. (1979). Retransformation of simian virus 40 revertant cell-line, which is

- resistant to viral and DNA infections, by microinjection of viral DNA. J. Viro1. 32, 989-994.
- GREENWALD, G.S. (1963). Effect of anti-PMS serum on superovulation in the hamster. Endocrinology 73, 436-441.
- GRIFFITHS, J.G., GUNN, R.G. & DONEY, J.M. (1970). Fertility in Scottish Blackface ewes as influenced by climatic stress. J. Agric. Sci., Camb. 75, 485-488.
- HACKETT, A.J., INSKEEP, E.K., ROBERTSON, H.A., SHRESTHA, J.N.B. & WOLYNETZ, M.S. (1979). Comparison of artificial insemination and natural mating on reproductive performance of five strains of sheep during the anoestrous season in an intensive system. Can. J. Anim. Sci. 59, 675-683.
- HACKETT, A.J. & WOLYNETZ, M.S. (1981). Comparison of natural mating and artificial insemination on reproductive performance of three strains of sheep housed in total confinement. Can. J. Anim. Sci. 61, 907-912.
- HACKETT, A.J. (1982). Effect of dose of pregnant mares' serum gonadotrophin on the reproductive performance of ewes synchronized for estrus and housed in total confinement. Can. J. Anim. Sci. 62, 291-294.
- HACKETT, A.J. & WOLYNETZ, M.S. (1982). Effect of PMSG on the reproductive performance of totally confined ewes bred at a synchronized estrus. Theriogenology 17, 215-221.
- HAMMER, R.E., PURSEL, V.G., REXROAD, C.E., WALL, R.J., BOLT, D.J., EBERT, K.M., PALMITER, R.D. & BRINSTER, R.L. (1985). Production of transgenic rabbits, sheep and pigs by microinjection. Nature 315, 680-683.
- HAMMER, R.E., PURSEL, V.G., REXROAD, C.E., WALL, R.J., BOLT, D.J., PALMITER, R.D. & BRINSTER, R.L. (1986). Genetic engineering of mammalian embryos. J. Anim. Sci. 63, 269-278.
- HAMRA, A.H., MASSRI, Y.G., MARCEK, J.M. & WHEATON, J.E. (1986). Plasma progesterone levels in ewes treated with progesterone-controlled internal drug-release dispensers, implants and sponges. Anim. Reprod. Sci. 11, 187-194.
- HANCOCK, J.L. & HOVELL, G.J.R. (1961). Transfer of sheep ova. J. Reprod. Fert. 2, 295-306.
- HANCOCK, J.L. (1963). Survival in vitro of sheep eggs. Anim. Prod. 5, 237-243.
- HANRAHAN, J.P. & OWENS, J.B. (1985). Variation and repeatability of ovulation rate in Cambridge ewes. Proc. Brit. Soc. Anim. Prod., Winter Meeting, Paper no. 37.
- HARESIGN, W. (1976). Controlled breeding in sheep using the prostaglandin analogue. Anim. Prod. 22, 137 (Abst.).
- HARESIGN, W., READ, S.R., CURNOCK, R.M. & REED, H.C.B. (1986). A note on the use of laparoscopy for intrauterine insemination of frozen-thawed semen in the ewe. Anim. Prod. 43, 553-556.

- HARVEY, T.G., JOHNSON, T.L., TERVIT, H.R. & WELCH, R.A.S. (1984). Synchronisation and artificial insemination of ewes - techniques which have possible commercial application. Proc. N. Z. Soc. Anim. Prod. 44, 7-9.
- HAWK, H.W., COOPER, B.S. & CONLEY, H.H. (1987). Inhibition of sperm transport and fertilization in superovulating ewes. Theriogenology 28, 139-153.
- HAY, M.F. & MOOR, R.M. (1975). Functional and structural relationships in Graafian follicle population of the sheep ovary. J. Reprod. Fert. 45, 583-593.
- HEDGES, D.A. & REARDON, T.F. (1973). Effects of live weight and PMS administration on reproduction rate in Merino ewes. World Conference on Animal Production 1, 5(e) 13-15.
- HENDERSON, K.M., FRANCHIMONT, P., LECONTE-YERNA, M.J., HUDSON, N. & BALL, K. (1984). Increase in ovulation rate after active immunization of sheep with inhibin partially purified from bovine follicular fluid. J. Endocr. 102, 305-309.
- HETTLE, S.J.H., HARVEY, M.J.A., CAMERON, E.R., JOHNSTON, C.S. & ONIONS, D.E. (1989). Generation of transgenic sheep by sub-zonal injection of feline leukemia virus. J. Cell. Biochem. 13B, 180 (Abst.).
- HEYMAN, Y. & MENEZO, Y. (1987). Interaction of trophoblastic vesicles with bovine embryos developing in vitro. In: The Mammalian Preimplantation Embryo, pp 175-191. Ed. B.D. Bavister. Plenum Press, New York.
- HEYMAN, Y., MENEZO, Y., CHESNE, P., CAMOUS, S. & GARNIER, V. (1987). In vitro cleavage of bovine and ovine early embryos: improved development using coculture with trophoblastic vesicles. Theriogenology 27, 59-68.
- HOLST, P.J. (1969). The ovarian response to pregnant mare serum (PMS) administered after intravaginal progestagen treatment of cyclic and anoestrous ewes. Aust. J. Agric. Res. 20, 1143-1150.
- HOLST, P.J. (1974). The time of entry of ova into the uterus of the ewe. J. Reprod. Fert. 36, 427-428.
- HOLST, P.J. & BRADEN, A.W.H. (1972). Ovum transport in the ewe. Aust. J. Biol. Sci. 25, 167-173.
- HULET, C.V. & FOOTE, W.C. (1967). Physiological factors affecting frequency rate of lambing. J. Anim. Sci. 26, 553-562.
- HULET, C.V. & FOOTE, W.C. (1969). Ovulatory response of the ewe to repeated injections of PMS. J. Anim. Sci. 29, 457-463.
- HULET, C.V. & STORMSHAK, F. (1972). Some factors affecting response of aneestrous ewes to hormone treatment. J. Anim. Sci. 34, 1011-1019.
- HUNTER, G.L., ADAMS, C.E. & ROWSON, L.E.A. (1955). Interbreed ovum transfer in sheep. J. Agric. Sci., Camb. 46, 143-149.

- HUNTER, G.L. (1968). Increasing the frequency of pregnancy in sheep. 1. Some factors affecting rebreeding during the post-partum period. Anim. Breed. Abst. 36, 347-378.
- HUNTON, J.R., FLECKER, S.E. & MAXWELL, W.M.C. (1987). Pregnancy rates following intra-uterine insemination with pellet or straw-frozen semen. J. Agric. Sci., Camb. 109, 189-191.
- JAENISCH, R. & MINTZ, B. (1974). Simian virus 40 DNA sequences in DNA of healthy adult mice derived from preimplantation blastocysts injected with viral DNA. Proc. Natl. Acad. Sci. 71, 1250-1254.
- JAENISCH, R. (1976). Germ line integration and Medelian transmission of the exogenous Maloney leukemia virus. Proc. Natl. Acad. Sci. 73, 1260-1264.
- JAHNER, D., HAASE, K., MULLIGAN, R. & JAENISCH, R. (1985). Insertion of the bacterial gpt gene into the germ line of mice by retroviral infection. Proc. Natl. Acad. Sci. 82, 6927-6931.
- JAINUDEEN, M.R. & HAFEZ, E.S.E. (1980). Reproductive failure in females. In: *Reproduction in Farm Animals*. Ed. E.S.E. Hafez, pp 449-470. Lea and Febiger, Philadelphia.
- JONMUNDSSON, J.V. & ADELSTEINSSON, S. (1985). Single genes for fecundity in Icelandic sheep. In: *Genetics of Reproduction in Sheep*, pp 159-168. Eds R.B. Land & D.W. Robinson. Butterworths, London.
- JOYCE, M.J.B. (1972). A comparison of three mating systems in ewes treated with intravaginal (SC-9880/progesterone) progestagen and PMS. Proc. 7th Int. Cong. Anim. Reprod. A.I. 2, 937-938.
- KANE, M.T. & FOOTE, R.H. (1971). Factors affecting blastocyst expansion of rabbit zygotes and young embryos in defined media. Biol. Reprod. 4, 41-47.
- KANE, M.T. (1972). Energy substrates and culture of single cell rabbit ova to blastocysts. Nature 238, 468.
- KANE, M.T. (1974). The effect of pH on culture of one cell rabbit ova to blastocysts in bicarbonate-buffered medium. J. Reprod. Fert. 38, 477-480.
- KANE, M.T. (1987). Culture media and culture of early embryos. Theriogenology 27, 49-57.
- KANE, M.T. (1988). The effect of water soluble vitamins on the expansion of rabbit blastocysts in vitro. J. Exp. Zool. 245, 220-223.
- KANE, M.T. & BAVISTER, B.D. (1988). Vitamin requirements for development of eight-cell hamster embryos to hatching blastocysts in vitro. Biol. Reprod. 39, 1137-1143.
- KEANE, M.G. (1973). Effect of body condition and level of nutrition on the response of ewes to hormone treatment in the non-breeding season. Ir. J. Agric. Res. 12, 229-237.
- KEANE, M.G. (1975). Effect of nutrition and dose level of PMS on oestrous response and ovulation rate in progestagen-treated non-cyclic Suffolk x Galway ewe lambs. J. Agric. Sci., Camb. 84, 507-511.

- KELLY, R.W., OWENS, J.L., CROSBIE, S.F., McNATTY, K.P. & HUDSON, N. (1983). Influence of Booroola Merino genotype on the responsiveness of ewes to pregnant mares' serum gonadotrophin, luteal weights and peripheral progesterone concentrations. Anim. Reprod. Sci. 6, 199-207.
- KILLEEN, I.D. & MOORE, N.W. (1970). The effect of pregnant mare serum gonadotrophin and human chorionic gonadotrophin on ovulation and fertility in the ewe. Aust. J. Agric. Res. 21, 807-814.
- KILLEEN, I.D. & MOORE, N.W. (1971). The morphological appearance and development of sheep ova fertilized by surgical insemination. J. Reprod. Fert. 24, 63-70.
- KILLEEN, I.D. (1974). The survival rate of eggs following homologous egg transfer at various stages after oestrus in the ewe. Proc. Aust. Soc. Reprod. Biol. 6, 15 (Abst.).
- KILLEEN, I.D. & CAFFREY, G.J. (1982). Uterine insemination of ewes with the aid of a laparoscope. Aust. Vet. J. 59, 95.
- KING, W.A. (1985). Intrinsic embryonic factors that may affect survival after transfer. Theriogenology 23, 161-174.
- KRAEMER, D.C. (1966). In vitro fertilization and culture of ovine ova. Diss. Abstr. 27, 285.
- KRAUBLICH, H. (1986). Criteria for useful gene transfer. Proc. 3rd World Cong. Genet. Appl. Livestock Prodn. pp 22-31, Lincoln, Nebraska.
- KUEHN, M.R., BRADLEY, A., ROBERTSON, E.J. & EVANS, M.J. (1987). A potential animal model for Lesch-Nyhan syndrome through introduction of HPRT mutations into mice. Nature 326, 295-298.
- KUMMER, V., ZRALY, Z., VEZNIK, Z., SCHLEGELOVA, J. & HRUSKA, K. (1980). Superovulation in cattle: effect of goat anti-PMSG serum. Theriogenology 14, 383-390.
- KUZAN, F.B. & WRIGHT, R.W. (1981). Attachment of porcine blastocysts to fibroblast monolayers in vitro. Theriogenology 16, 651-658.
- KUZAN, F.B. & WRIGHT, R.W. (1982a). Blastocyst expansion, hatching and attachment of porcine embryos co-cultured with bovine fibroblasts in vitro. Anim. Reprod. Sci. 5, 57-63.
- KUZAN, F.B. & WRIGHT, R.W. (1982b). Observations on the development of bovine morulae on various cellular and noncellular substrata. J. Anim. Sci. 54, 811-816.
- LAMOND, D.R. (1962). Effect of season on hormonally induced ovulation in Merino ewes. J. Reprod. Fert. 4, 111-120.
- LAMOND, D.R. & BINDON, B.M. (1962). Oestrus, ovulation and fertility following suppression of ovarian cycles in Merino ewes by progesterone. J. Reprod. Fert. 4, 57-66.
- LAND, R.B. & McLAREN, A. (1967). The response of female mice to repeated injections of Human Chorionic Gonadotrophin. J. Reprod. Fert. 13, 321-327.

- LAND, R.B. & WILMUT, I. (1977). The survival of embryos transferred in large groups to sheep of breeds with different ovulation rates. Anim. Prod. 24, 183-187.
- LAND, R.B. & WILMUT, I. (1987). Gene transfer and animal breeding. Theriogenology 27, 169-179.
- LANGFORD, G.A., MARCUS, G.J., HACKETT, A.J., AINSWORTH, L., WOLYNETZ, M.S. & PETERS, H.F. (1979). A comparison of fresh and frozen semen in the insemination of confined sheep. Can. J. Anim. Sci. 59, 685-691.
- LANGFORD, G.A. (1982). Influence of PMSG and time of artificial insemination on fertility of progestogen-treated sheep in confinement. J. Anim. Sci. 54, 1205-1211.
- LANGFORD, G.A., MARCUS, G.J. & BATRA, T.R. (1983). Seasonal effects of PMSG and number of inseminations on fertility of progestogen-treated sheep. J. Anim. Sci. 57, 307-312.
- LASTER, D.B. & GLIMP, H.A. (1974). Influence of breed on response to exogenous hormones in estrous and anestrus ewes. J. Anim. Sci. 39, 1129-1135.
- LATHE, R., CLARK, A.J., ARCHIBALD, A.L., BISHOP, J.O., SIMONS, P. & WILMUT, I. (1986). Novel products from livestock. In: *Exploiting New Technologies in Animal Breeding: Genetic Developments*, pp 91-102. Eds C. Smith, J.W.B. King and J.C. McKay. Oxford University Press, Oxford.
- LAWSON, R.A.S. & CAHILL, L.P. (1983). Modification of the embryo-maternal relationship in ewes by progesterone treatment early in the oestrous cycle. J. Reprod. Fert. 67, 473-475.
- LAWSON, R.A.S., PARR, R.A. & CAHILL, L. (1983). Evidence for maternal control of blastocyst growth after asynchronous transfer of embryos to the uterus of the ewe. J. Reprod. Fert. 67, 477-483.
- LIGHTFOOT, R.J. & SALAMON, S. (1970). Fertility of ram spermatozoa frozen by the pellet method. 1. Transport and viability of spermatozoa within the genital tract of the ewe. J. Reprod. Fert. 22, 385-398.
- LINDNER, G.M., DICKEY, J.F., HILL, J.R. & KNICKERBOCKER, J.J. (1979). Effect of bovine serum albumin concentration on the development of ovine embryos cultured in Brinster's and Whitten's Medium. J. Anim. Sci. 49 (Suppl. 1), 314 (Abst.).
- LINDSAY, D.R., COGNIE, Y., PELLETIER, J. & SIGNORET, J.P. (1975). Influence of the presence of rams on the timing of ovulation and discharge of LH in ewes. Physiol. Behav. 15, 423-426.
- LINDSELL, C.E., RAJKUMAR, K., MANNING, A.W., EMERY, S.K., MAPLETOFT, R.J. & MURPHY, B.D. (1986). Variability in FSH:LH ratios among batches of commercially available gonadotrophins. Theriogenology 25, 167 (Abst.).
- LOHSE, J.K., ROBL, J.M. & FIRST, N.L. (1985). Progress towards transgenic cattle. Theriogenology 23, 205 (Abst.).

- LUNSTRA, D.D. & CHRISTENSEN, R.K. (1981). Synchronization of ewes during anoestrus: influence of time of year and interval to onset of estrus on conception rate. J. Anim. Sci. 53, 448-457.
- MACHLIN, L.J. (1972). Effect of porcine growth hormone on growth and carcass composition of the pig. J. Anim. Sci. 35, 794-800.
- MAHADEVAN, M.M., FLEETMAN, J., CHURCH, R.B. & TAYLOR, P.J. (1986). Growth of mouse embryos in bicarbonate media buffered by carbon dioxide, Hepes or phosphate. J. In Vitro Fert. Embryo Transfer 3, 304-307.
- MARTIN, E., COGNIE, Y., ESPINOSA, E. & JUARISTI, J.L. (1976). Determination of ovulation time in Rasa Aragonesa ewes after treatment by ear-implant with SC 21099. Proc. 8th Int. Cong. Anim. Reprod. A.I. 1, 168.
- MATTNER, P.E., ENTWISTLE, K.W. & MARTIN, I.C.A. (1969). Passage, survival and fertility of deep-frozen ram semen in the genital tract of the ewe. Aust. J. Biol. Sci. 22, 181-187.
- MAURER, R.R., WHITENER, R.H. & FOOTE, R.H. (1969). Relationship of in vivo gamete aging and exogenous hormones to early embryo development in rabbits. Proc. Soc. Exp. Biol. Med. 131, 882-885.
- MAXWELL, W.M.C., CURNOCK, R.M., LOGUE, D.N. & REED, H.C.B. (1980). Fertility of ewes following artificial insemination with semen frozen in pellets or straws, a preliminary report. Theriogenology 14, 83-89.
- MAXWELL, W.M.C., BUTLER, L.G. & WILSON, H.R. (1984). Intra-uterine insemination of ewes with frozen semen. J. Agric. Sci., Camb. 102, 233-235.
- MAXWELL, W.M.C. (1986a). Artificial insemination of ewes with frozen-thawed semen at a synchronized oestrus. 1. Effect of time of onset of oestrus, ovulation and insemination on fertility. Anim. Reprod. Sci. 10, 301-308.
- MAXWELL, W.M.C. (1986b). Artificial insemination of ewes with frozen-thawed semen at a synchronized oestrus. 2. Effect of dose of spermatozoa and site of intra-uterine insemination on fertility. Anim. Reprod. Sci. 10, 309-316.
- MAXWELL, W.M.C. & BARNES, D.R. (1986). Induction of oestrus in ewes using a controlled internal drug release device and PMSG. J. Agric. Sci., Camb. 106, 201-203.
- MAXWELL, W.M.C. & HEWITT, L.J. (1986). A comparison of vaginal, cervical and intrauterine insemination of sheep. J. Agric. Sci., Camb. 106, 191-193.
- MCCONNELL, D.J. (1986). Methods and achievements of genetic engineering: prospects in agriculture. A review. Livestock Prod. Sci. 15, 109-131.
- MCDONALD, M.F. (1969). Egg transplantation studies in Romney ewes. Proc. N. Z. Soc. Anim. Prod. 29, 95-101.

- McGOVERN, P.T., WILLIAMS, H.L. & HANCOCK, J.L. (1969). The time of ovulation in the ewe following treatment with human chorionic gonadotrophin. J. Reprod. Fert. 20, 537-540.
- McGOVERN, P.T. & LAING, J.A. (1976). Ovulation rate in ewes treated with pregnant mares' serum gonadotrophin and luteinizing hormone releasing factor. J. Agric. Sci., Camb. 86, 127-129.
- McINTOSH, J.E.A., MOOR, R.M. & ALLEN, W.R. (1975). Pregnant mare serum gonadotrophin: rate of clearance from the circulation of sheep. J. Reprod. Fert. 44, 95-100.
- McKELVEY, W.A.C. & ROBINSON, J.J. (1984a). Collection of embryos from ewes by laparoscopy. Vet. Rec. 115, 158 (Abst.).
- McKELVEY, W.A.C. & ROBINSON, J.J. (1984b). Normal lambs born following transfer of embryos by laparoscopy. Vet. Rec. 115, 230.
- McKELVEY, W.A.C., ROBINSON, J.J. & AITKEN, R.P. (1985). A simplified technique for the transfer of ovine embryos by laparoscopy. Vet. Rec. 117, 492-494.
- McKELVEY, W.A.C., ROBINSON, J.J., AITKEN, R.P. & ROBERTSON, I.S. (1986). Repeated recoveries of embryos from ewes by laparoscopy. Theriogenology 25, 855-865.
- McKNIGHT, G.S., HAMMER, R.E., KUENZEL, E.A. & BRINSTER, R.L. (1983). Expression of the chicken transferrin gene in transgenic mice. Cell 34, 335-341.
- McLAREN, A. (1980). Fertilization, cleavage and implantation. In: Reproduction in Farm Animals, pp 226-246. Ed. E.S.E. Hafez. Lea & Febiger, Philadelphia.
- McMILLAN, W.H. (1987). Oestrous cycle control in ewes - timing of CIDR withdrawal and ram introduction. Proc. 4th AAAP Animal Science Congress, p. 20, Hamilton, New Zealand.
- MEINECKE-TILLMAN, S., EVERS, P. & MEINCKE, B. (1987). Relationships between PMSG-plasma concentrations and ovarian response after superovulatory treatment in Merino ewes. Theriogenology 27, 259 (Abst.).
- MICHALSKA, A.E. (1988). Production and characterization of transgenic mice and pigs carrying the porcine growth hormone gene. PhD Thesis, University of Adelaide.
- MINHAS, B.S., CAPEHART, J.S., BOWEN, M.J., WOMACK, J.E., McCRADY, J.D., HARMS, P.G., WAGNER, T.E. & KRAEMER, D.C. (1984). Visualization of pronuclei in living bovine zygotes. Biol. Reprod. 30, 687-691.
- MINTZ, B. (1962). Formation of a genotypically mosaic embryo. Am. J. Zool. 2, 432 (Abst.).
- MOOR, R.M. & CRAGLE, R.G. (1971). The sheep egg: Enzymatic removal of the zona pellucida and culture of eggs in vitro. J. Reprod. Fert. 27, 401-409.

- MOOR, R.M., OSBORN, J.C. & CROSBY, I.M. (1985). Gonadotrophin-induced abnormalities in sheep oocytes after superovulation. J. Reprod. Fert. 74, 167-172.
- MOORE, N.W., ROWSON, L.E.A. & SHORT, R.V. (1960). Egg transfer in sheep: factors affecting the survival and development of transferred eggs. J. Reprod. Fert. 1, 332-349.
- MOORE, N.W. & SHELTON, J.N. (1962). The application of the technique of egg transfer to sheep breeding. Aust. J. Agric. Res. 13, 718-724.
- MOORE, N.W. & SHELTON, J.N. (1964a). Response of the ewe to a horse anterior pituitary extract. J. Reprod. Fert. 7, 79-87.
- MOORE, N.W. & SHELTON, J.N. (1964b). Egg transfer in sheep. Effect of degree of synchronization between donor and recipient, age of egg, and site of transfer on the survival of transferred eggs. J. Reprod. Fert. 7, 145-152.
- MOORE, N.W. & HOLST, P.J. (1967). The evaluation of progesterone and SC9880 impregnated intravaginal sponges used with PMS for the induction of breeding in the anoestrous crossbred ewe. In: Control of the Ovarian Cycle in the Sheep, pp 133-145. Ed. T.J. Robinson. Sydney University Press, Sydney.
- MOORE, N.W. (1970). Preliminary studies on in vitro culture of fertilized sheep ova. Aust. J. Biol. Sci. 23, 721-724.
- MOORE, N.W. & SPRY, G.A. (1972). The culture of fertilized sheep ova. J. Reprod. Fert. 28, 139 (Abst.).
- MOORE, N.W. & BILTON, R.J. (1973). The storage of fertilized sheep ova at 5°C. Aust. J. Biol. Sci. 26, 1421-1427.
- MORTIMER, S.I. (1987). Australian estimates of genetic parameters for wool production and quality traits. In: Merino Improvement Programs in Australia, pp 159-173. Ed. B.J. McGuirk. Australian Wool Corporation, Melbourne.
- MULLIS, K.B. & FALOONA, F.A. (1987). Specific synthesis of DNA in vitro via a polymerase catalysed chain reaction technique. Meth. Enzymol. 155, 335-350.
- MURRAY, J.D., NANCARROW, C.D. & WARD, K.A. (1988). Techniques for the transfer of foreign genes into animals. Proc. 11th Int. Cong. Anim. Reprod. A.I. 5, 20-27.
- MUTIGA, E.R. & BAKER, A.A. (1982). Superovulatory responses in Merino ewes to three PMSG dose levels. Theriogenology 17, 100 (Abst.).
- MUTIGA, E.R. & BAKER, A.A. (1984). Transfer of sheep embryos through a laparoscope. Vet. Rec. 114, 401-402.
- MUTIGA, E.R., BAKER, A.A. & JILLELLA, D. (1983). Limitations of intrauterine balloon catheters for ova collection in sheep. Theriogenology 20, 213-220.
- MUWALLA, M., FOOTE, W.C., BUNCH, T.D. & CALL, J.W. (1988). The influence of culture and transport on pretransfer development and

- posttransfer survival of sheep embryos. Theriogenology 29, 369-379.
- NAGLEE, D.L., MAURER, R.A. & FOOTE, R.H. (1969). Effect of osmolarity on in vitro development of rabbit embryos in a chemically defined medium. Exp. Cell Res. 58, 331-333.
- NANCARROW, C.D., MURRAY, J.D., BOLAND, M.P., SUTTON, R. & HAZELTON, I.G. (1984a). Effect of gonadotrophin releasing hormone in the production of single-cell embryos for pronuclear microinjection of foreign genes. In: Reproduction in Sheep, pp 286-288. Eds D.R. Lindsay and D.T. Pearce. Australian Academy of Science, Canberra.
- NANCARROW, C.D., MURRAY, J.D., BOLAND, M.P., HAZELTON, I.G. & SUTTON, R. (1984b). Towards gene transfer in ruminant embryos: effect of centrifugation. Theriogenology 21, 248 (Abst.).
- NANCARROW, C.D., MARSHALL, J.T., WILMUT, I., HAZELTON, I.G. & MURRAY, J.D. (1987a). Comparative efficiency of FSH and PMSG for sheep zygote production. Proc. Aust. Soc. Reprod. Biol. 19, 66 (Abst.).
- NANCARROW, C., MARSHALL, J., MURRAY, J., HAZELTON, I. & WARD, K. (1987b). Production of sheep transgenic with the ovine growth hormone gene. Theriogenology 27, 263 (Abst.).
- NANCARROW, C.D., WARD, K.A. & MURRAY, J.D. (1988). The future for transgenic livestock in Australian agriculture. Aust. J. Biotech. 2, 39-44.
- NICHOLS, J. & GARDNER, R.L. (1989). Effect of damage to the zona pellucida on development of preimplantation embryos in the mouse. Human Reprod. 4, 180-187.
- NICOLAU, C., LE PAPE, A., SORIANO, P., FARGETTE, F. & JUHEL, M.-F. (1983). In vivo expression of rat insulin after intravenous administration of the liposome-entrapped gene for rat insulin 1. Proc. Natl. Acad. Sci. 80, 1068-1072.
- NIE, N.H., HULL, C.H., JENKINS, J.G., STEINBRENNER, K. & BENT, D.H. (1975). Statistical Package for Social Sciences, 2nd edn. McGraw-Hill Book Co., New York.
- NOTTLE, M.B., ARMSTRONG, D.T., HOLLAND, M.K. & SHARPE, P.H. (1984). Superovulation and embryo transfer in the Merino. Proc. Aust. Soc. Reprod. Biol. 16, 70 (Abst.).
- OGAWA, S., SATOH, K. & HASHIMOTO, H. (1971). In vitro culture of rabbit ova from the single cell to blastocyst stage. Nature 233, 422-424.
- ORTAVANT, R., THIBAUT, C. & WINTENBERGER, S. (1949). Contribution a l'etude de la superovulation experimentale chez la brebis. Annls Endocr. 10, 170-173.
- O'SHEA, T., CUMMINS, L.J., BINDON, B.M. & FINDLAY, J.K. (1982). Increased ovulation rate in ewes vaccinated with an inhibin-enriched fraction from bovine follicular fluid. Proc. Aust. Soc. Reprod. Biol. 14, 85 (Abst.).
- O'SHEA, T., AL-OBODI, S.A.R., HILLARD, M.A., BINDON, B.M., CUMMINS, L.J. & FINDLAY, J.K. (1984). Increased ovulation rate in Merino ewes

- and advancement of puberty in Merino lambs immunized with a preparation enriched in inhibin. In: *Reproduction in Sheep*, pp 335-337. Eds D.R. Lindsay and D.T. Pearce. Australian Academy of Science, Canberra.
- PALMITER, R.D., BRINSTER, R.L., HAMMER, R.E., TRUMBAUER, M.E., ROSENFELD, M.G., BIRNBERG, N.C. & EVANS, R.M. (1982). Dramatic growth of mice that develop from eggs microinjected with metallothionein-growth hormone fusion genes. Nature **300**, 611-615.
- PALMITER, R.D., NORSTEDT, G., GELINAS, R.E., HAMMER, R.E. & BRINSTER, R.L. (1983). Metallothionein-human GH fusion genes stimulate growth of mice. Science **222**, 809-814.
- PALMITER, R.D. & BRINSTER, R.L. (1986). Germ-line transformation of mice. Ann. Rev. Genet. **20**, 465-500.
- PAPAIIOANNOU, V.E., MCBURNEY, M.E., GARDNER, R.L. & EVANS, M.J. (1975). Fate of teratocarcinoma cells injected into early mouse embryos. Nature **258**, 70-73.
- PAPKOFF, H. (1978). Relationship of PMSG to the pituitary gonadotrophins. In: *Current Topics in Veterinary Medicine*. 1. pp 73-86. Ed. J.M. Sreenan. M. Nijhoff, The Hague.
- PAPKOFF, H. (1981). Variations in the properties of equine chorionic gonadotrophin. Theriogenology **15**, 1-11.
- PEEL, C.J., BAUMAN, D.E., GOREWIT, R.C. & SNIFFEN, C.J. (1981). Effect of exogenous growth hormone on lactational performance in high yielding dairy cows. J. Nutr. **11**, 1662-1671.
- PEEL, C.J., FRONK, T.J., BAUMAN, D.E. & GOREWIT, R.C. (1982). Lactational response to exogenous growth hormone and abomasal infusion of a glucose-sodium caseinate mixture in high-yielding dairy cows. J. Nutr. **112**, 1770-1778.
- PERKINS, J.L. & GOODE, L. (1966). Effects of stage of estrous cycle and endogenous hormones upon the volume and composition of oviduct fluid in ewes. J. Anim. Sci. **25**, 465-471.
- PETERS, D.F., ANDERSON, G.B. & CUPPS, P.T. (1977). Culture and transfer of sheep embryos. J. Anim. Sci. **45**, 350-354.
- PETTERS, R.M. (1986). Recombinant DNA, gene transfer and the future of animal agriculture. J. Anim. Sci. **62**, 1759-1768.
- PETTERS, R.M., JOHNSON, B.H. & SHUMAN, R.M. (1989). Gene transfer to swine embryos using an avian retrovirus. J. Cell. Biochem. **13B**, 182 (Abst.).
- PIGON, H.M., CLEGG, T. & COLE, H. (1960). The formation of antigonadotrophin in sheep and its effect on the endocrine and reproductive system. Acta Endocrinol. **35**, 253-260.
- PINKERT, C.A., PURSEL, V.G., MILLER, K.F., PALMITER, R.D. & BRINSTER, R.L. (1987). Production of transgenic pigs harboring growth hormone (MTbGH) or growth hormone releasing factor (MThGRF) genes. J. Anim. Sci. **65** (Suppl.1), 260 (Abst.).

- PIPER, L.R., BINDON, B.M., CURTIS, Y.M., CHEERS, M.A. & NETHERY, R.D. (1982). Response to PMSG in Merino and Booroola Merino crosses. Proc. Aust. Soc. Reprod. Biol. 14, 82 (Abst.).
- PIPER, L.R. & BINDON, B.M. (1985). The single gene inheritance of the high litter size of the Booroola Merino. In: Genetics of Reproduction in Sheep, pp 115-125. Eds R.B. Land and D.W. Robinson. Butterworths, London.
- POLGE, C. (1982). Embryo transplantation and preservation. In: Control of Pig Reproduction, pp 277-291. Eds D.J.A. Cole and G.R. Foxcroft. Butterworths, London.
- POPE, W.F. (1988). Uterine asynchrony: a cause of embryonic loss. Biol. Reprod. 39, 999-1003.
- PURSEL, V.G., REXROAD, C.E., BOLT, D.J., MILLER, K.F., WALL, R.J., HAMMER, R.E., PINKERT, C.A., PALMITER, R.D. & BRINSTER, R.L. (1987). Progress on gene transfer in farm animals. Vet. Immunol. Immunopath. 17, 303-312.
- PURSEL, V.G., HAMMER, R.E., BOLT, D.J., WALL, R.J., PALMITER, R.D. & BRINSTER, R.L. (1988). Transgenic swine transmit foreign gene to progeny. J. Anim. Sci. 63 (Suppl. 1), 203 (Abst.).
- PURVIS, I.W., ATKINS, K.D. & PIPER, L.R. (1987). Genetic parameters for reproductive traits. In: Merino Improvement Programs in Australia, pp 229-242. Ed. B.J. McGuirk. Australian Wool Corporation, Melbourne.
- QUIRKE, J.F. & HANRAHAN, J.P. (1975). Effect of gonadotrophin-releasing hormone and human chorionic gonadotrophin on the response of the ewe to pregnant mare serum gonadotrophin. J. Reprod. Fert. 43, 167-170.
- QUIRKE, J.F. (1979). Oestrus, ovulation, fertilization and early embryo mortality in progestagen-PMSG treated Galway ewe lambs. Ir. J. Agric. Res. 18, 1-11.
- QUIRKE, J.F., JENNINGS, J.J., HANRAHAN, J.P. & GOSLING, J.P. (1979). Oestrus, time of ovulation, ovulation rate and conception rate in progestagen-treated ewes given Gn-RH, Gn-RH analogues and gonadotrophins. J. Reprod. Fert. 56, 479-488.
- QUIRKE, J.F. & GOSLING, J.P. (1981). Reproductive performance and plasma progesterone concentrations in ewe lambs and mature ewes following treatment for synchronization of oestrus during the breeding season. Ir. J. Agric. Res. 20, 9-20.
- QUIRKE, J.F., HANRAHAN, J.P. & GOSLING, J.P. (1981). Duration of oestrus, ovulation rate, time of ovulation and plasma LH, total oestrogen and progesterone in Galway adult ewes and ewe lambs. J. Reprod. Fert. 61, 265-272.
- RADFORD, H.M., AVENELL, J.A. & SZELL, A. (1984). Human chorionic gonadotrophin induces multiple ovulations in sheep. In: Reproduction in Sheep, pp 342-344. Eds D.R. Lindsay and D.T. Pearce. Australian Academy of Science, Canberra.

- RALPH, M.M., SEAMARK, R.F. & GEYTENBECK, P.E. (1984). Ovulation rate of ewes subsequent to FSH administration early in the oestrous cycle. Proc. Aust. Soc. Reprod. Biol. 16, 66 (Abst.).
- RASSOULZADEGAN, M., LEOPOLD, P., VAILLY, J. & CUZIN, F. (1986). Germ line transmission of autonomous genetic elements in transgenic mouse strains. Cell 46, 513-519.
- RENARD, J.-P. & BABINET, C. (1987). Genetic engineering in farm animals: the lessons from the genetic mouse model. Theriogenology 27, 181-200.
- RESTALL, B.J. & WALES, R.G. (1966). The fallopian tube of the sheep. 3. The chemical composition of the fluid from the fallopian tube. Aust. J. Biol. Sci. 19, 687-698.
- REXROAD, C.E. & WALL, R.J. (1987). Development of one-cell fertilized sheep ova following microinjection into pronuclei. Theriogenology 27, 611-619.
- REXROAD, C.E. & POWELL, A.M. (1988a). Co-culture of ovine eggs with oviductal cells and trophoblastic vesicles. Theriogenology 29, 387-397.
- REXROAD, C.E. & POWELL, A.M. (1988b). Co-culture of ovine ova with oviductal cells in medium 199. J. Anim. Sci. 66, 947-953.
- REXROAD, C.E. & PURSEL, V.G. (1988). Status of gene transfer in domestic animals. Proc. 11th Int. Cong. Anim. Reprod. A.I. 5, 28-35.
- RHODES, L. & NATHANIELSZ, P.W. (1988). Comparison of a controlled internal drug release device containing progesterone with intravaginal medroxyprogesterone sponges for estrus synchronization in ewes. Theriogenology 30, 831-836.
- ROBERTS, E.M. (1966). The use of intravaginal sponges impregnated with 6-methyl-17 acetoxy progesterone (MAP) to synchronize ovarian activity in cyclic Merino ewes. Proc. Aust. Soc. Anim. Prod. 6, 32-37.
- ROBERTS, E.M. & EDGAR, D.G. (1966). The stimulation of fertile oestrus in anoestrous Romney ewes. J. Reprod. Fert. 12, 561-564.
- ROBERTS, E.M., JACKSON, N. & PHILLIPS, J.M. (1975). A revised list of family groups of Australian Merino stud flocks. Wool Tech. Sheep Breed. 22, 6-9.
- ROBERTSON, E., BRADLEY, A., KUEHN, M. & EVANS, M. (1986). Germ-line transmission of genes introduced into cultured pluripotential cells by retroviral vector. Nature 323, 445-448.
- ROBINSON, J.J., FRASER, C. & MCHATTIE, I. (1975). The use of progestagens and photoperiodism in improving the reproductive rate of the ewe. Ann. Biol. Anim. Biochim. Biophys. 15, 345-352.
- ROBINSON, T.J. (1951). The control of fertility in sheep. 2. The augmentation of fertility by gonadotrophin treatment of the ewe in the normal breeding season. J. Agric. Sci., Camb. 41, 6-63.

- ROBINSON, T.J. (1956). The artificial insemination of the Merino sheep following the synchronization of oestrus and ovulation by progesterone injected alone and with pregnant mare serum gonadotrophin (PMS). Aust. J. Agric. Res. 7, 194-210.
- ROBINSON, T.J. (1961). The time of ovulation and efficiency of fertilization following progesterone and pregnant mare serum treatment in the cyclic ewe. J. Agric. Sci., Camb. 57, 129-135.
- ROBINSON, T.J. (1962). Comparative studies of several gonadotrophins, progestin and oestrogen treatments in the anoestrous ewe. J. Endocr. 24, 33-35.
- ROBINSON, T.J. (1965). Use of progestagen-impregnated sponges inserted intravaginally or subcutaneously for the control of the oestrous cycle in the sheep. Nature 206, 39-41.
- ROBINSON, T.J. & SMITH, C.A. (1982). Potential fertility of ewes at a controlled oestrus. Proc. Aust. Soc. Reprod. Biol. 14, 87 (Abst.).
- ROCHE, J.F. & CROWLEY, J.P. (1971). The effect of controlling the interval from mating to ovulation on pregnancy rate in ewes treated with progesterone. J. Reprod. Fert. 24, 307-309.
- ROGERS, G.E., SIVAPRASAD, A.V., D'ANDREA, R.J., BAWDEN, C.S. & KUCZEK, E.S. (1989). Towards a new genotype with increased wool growth by transgenesis with microbial genes for cysteine synthesis. J. Cell. Biochem. 13B, 183 (Abst.).
- ROWSON, L.E.A. & MOOR, R.M. (1966). Embryo transfer in the sheep: the significance of synchronizing oestrus in donor and recipient animal. J. Reprod. Fert. 11, 207-212.
- RYAN, J.P., BILTON, R.J., HUNTON, J.R. & MAXWELL, W.M.C. (1984). Superovulation of ewes with a combination of PMSG and FSH-P. In: *Reproduction in Sheep*, pp 338-341. Eds D.R. Lindsay and D.T. Pearce. Australian Academy of Science, Canberra.
- SAKKAS, D., BATT, P., CAMERON, A. & TROUNSON, A. (1988). In vitro development of goat preimplantation embryos in coculture with oviductal epithelial cells. Proc. Aust. Soc. Reprod. Biol. 20, 48 (Abst.).
- SCARAMUZZI, R.J., DAVIDSON, W.G. & VAN LOOK, P.F.A. (1977). The effect of active immunization against androstenedione on oestrus and ovulation in sheep. Nature 269, 817-818.
- SCARAMUZZI, R.J. & HOSKINSON, R.M. (1984). Immunoneutralization of steroid hormones for increasing fecundity. In: *Immunological Aspects of Reproduction in Mammals*, pp 501-523. Ed. D.B. Crighton. Butterworths, London.
- SCHAEFFER-RIDDER, M., WANG, Y. & HOFSCHEIDER, P.H. (1982). Liposomes as gene carriers: Efficient transformation of mouse L cells by thymidine kinase gene. Science 215, 166-167.
- SCHIEWE, M.C., BUSH, M., STUART, L.S. & WILDT, D.E. (1984). Laparoscopic embryo transfer in domestic sheep: a preliminary study. Theriogenology 22, 675-682.

- SCHWARTZ, F.L. & ULBERG, L.C. (1972). Viability of sheep embryos after culture. J. Anim. Sci. 35, 254 (Abst.).
- SEIDEL, G.E. & SEIDEL, S.M. (1981). The embryo transfer industry. In: *New Technologies in Animal Breeding*, pp 41-80. Eds B.G. Brackett, G.E. Seidel and S.M. Seidel. Academic Press, New York.
- SHELTON, J.N. & MOORE, N.W. (1966). Survival of fertilized eggs transferred to ewes after progesterone treatment. J. Reprod. Fert. 11, 149-151.
- SHELTON, J.N. & MOORE, N.W. (1967). The response of the ewe to pregnant mare serum and to horse anterior pituitary extract. J. Reprod. Fert. 14, 175-177.
- SHRESTHA, J.N.B, AINSWORTH, L. & HEANEY, D.P. (1983). Influence of breed on the reproductive performance of ewes treated with fluorogestone-acetate-impregnated intravaginal sponge and pregnant mare's serum gonadotrophin during the breeding season. Can. J. Anim. Sci. 63, 1-6.
- SIEGAL, S. (1956). *Non-parametric Statistics for the Behavioural Sciences*, pp 116-193. McGraw-Hill Kogakusha Ltd, Tokyo.
- SIGNORET, J.P. & COGNIE, Y. (1975). Determination of the moment of ovulation in ewe and sow. Influence of environment and hormonal treatment. Ann. Biol. Anim. Biochim. Biophys. 15, 205-214.
- SIMONS, J.P. & LAND, R.B. (1987). Transgenic livestock. J. Reprod. Fert. 34, 237-250.
- SIMONS, J.P., McCLENAGHAN, M. & CLARK, A.J. (1987). Alteration of the quality of milk by expression of the sheep beta-lactoglobulin in transgenic mice. Nature 328, 530-532.
- SIMONS, J.P., WILMUT, I., CLARK, A.J., ARCHIBALD, A.L., BISHOP, J.O. & LATHE, R. (1988). Gene transfer in sheep. Biotechnology 6, 179-183.
- SIRARD, M.A. & LAMBERT, R.D. (1985). *In vitro* fertilization of bovine follicular oocytes obtained by laparoscopy. Biol. Reprod. 33, 487-494.
- SMITH, C. (1986). Use of embryo transfer in genetic improvement of sheep. Anim. Prod. 42, 81-88.
- SMITH, C., MEUWISSEN, T.H.E. & GIBSON, J.P. (1987). On the use of transgenes in livestock improvement. Anim. Breed. Abstr. 55, 1-10.
- SMITH, C.L. (1984). Dose effect of follicle stimulating hormone for superovulation of crossbred Targhee ewes. Theriogenology 21, 262 (Abst.).
- SMITH, J.F. (1976). Selection for fertility and response to PMSG in Romney ewes. Proc. N. Z. Soc. Anim. Prod. 36, 247-251.
- SMITH, J.F., CRUICKSHANK, G.F., MCGOWAN, L.T., PARR, J. & MORTIMER, B.J. (1988). Seasonal changes in oestrus, ovulation and conception of Coopworth ewes treated with CIDRs and PMSG. Proc. N. Z. Soc. Anim. Prod. 48, 99-102.

- SMITH, P.A., BOLAND, M.P. & GORDON, I. (1978). Conception rate in ewes: effect of method of breeding and number of inseminations. J. Agric. Sci., Camb. 91, 511-512.
- SORIANO, P., DIJKSTRA, J., LEGRAND, A., SPANJER, H., LONDOS-GAGLIARDI, D., ROERDINK, F., SCHERPHOF, G. & NICOLAU, C. (1983). Targetted and nontargetted liposomes for in vivo transfer to rat liver cells of a plasmid containing the preproinsulin 1 gene. Proc. Natl. Acad. Sci. 80, 7128-7131.
- SOUTHERN, E.M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Molec. Biol. 98, 503-517.
- SPITZER, J.C. & CARPENTER, R.H. (1981). Estrus and pregnancy rates following synchronization with cronolone intravaginal sponge or norgestomet ear implant in cycling ewes. Theriogenology 16, 287-294.
- STEFFAN, J., POISSONNET, P. & THIBIER, M. (1982). Control of oestrus in ewe lambs and yearlings with medroxyprogesterone acetate and fluorogestone acetate. Anim. Reprod. Sci. 5, 191-198.
- STEWART, C.L., STUHLMANN, H., JAHNER, D. & JAENISCH, R. (1982). De novo methylation, expression, and infectivity of retroviral genomes introduced into embryonal carcinoma cells. Proc. Natl. Acad. Sci. 79, 4098-4102.
- STEWART, C.L., VANEK, M. & WAGNER, E.F. (1985). Expression of foreign genes from retroviral vectors in mouse teratocarcinoma chimaeras. The EMBO Journal 4, 3701-3709.
- STONE, B.A., QUINN, P. & SEAMARK, R.F. (1984). Energy and protein sources for development of pig embryos cultured beyond hatching in vitro. Anim. Reprod. Sci. 7, 405-412.
- SUTTON, R., NANCARROW, C.D., WALLACE, A.L.C. & RIGBY, N.W. (1984). Identification of an oestrus-associated glycoprotein in oviductal fluid of the sheep. J. Reprod. Fert. 72, 415-422.
- TAIT, R.M. (1971). Influence of nutritional status on hormone induced superovulation in normally cycling ewes. Can. J. Anim. Sci. 51, 771-775.
- TARKOWSKI, A.K. (1961). Mouse chimeras developed from fused eggs. Nature 190, 857-860.
- TERVIT, H.R. & McDONALD, M.F. (1969). Abnormalities and dimensions of ova from New Zealand Romney ewes. N. Z. J. Agric. Res. 12, 21-30.
- TERVIT, H.R., WHITTINGHAM, D.G. & ROWSON, L.E.A. (1972). Successful culture in vitro of sheep and cattle ova. J. Reprod. Fert. 30, 493-497.
- TERVIT, H.R. & ROWSON, L.E.A. (1974). Birth of lambs after culture of sheep ova in vitro for up to 6 days. J. Reprod. Fert. 38, 177-179.
- TERVIT, H.R. & HAVIK, P.G. (1976). A modified technique for flushing ova from the sheep uterus. N. Z. Vet. J. 24, 138-140.

- TERVIT, H.R. & GOOLD, P.G. (1978). The culture of sheep embryos in either a bicarbonate-buffered medium or a phosphate-buffered medium enriched with serum. Theriogenology 9, 251-255.
- THIBAULT, C. (1966). La culture in vitro de l'oeuf de vache. Ann. Biol. Anim. Biochim. Biophys. 6, 159-164.
- THOMPSON, J.G.E. & WALES, R.G. (1987). Observations on the loss of the cellular vestment surrounding superovulated sheep oocytes. Anim. Reprod. Sci. 15, 169-175.
- TORRES, S. & COGNIE, Y. (1984). Superovulation and egg transfer in the ewe. Reprod. Nutr. Develop. 24, 623-631.
- TORRES, S., COGNIE, Y. & COLAS, G. (1987). Transfer of superovulated sheep embryos obtained with different FSH-P. Theriogenology 27, 407-419.
- TROUNSON, A.O. & MOORE, N.W. (1972). Ovulation rate and survival of fertilized ova in Merino ewes selected for and against multiple births. Aust. J. Agric. Res. 23, 851-858.
- TROUNSON, A.O. & MOORE, N.W. (1974a). Fertilization in the ewe following multiple ovulations and uterine insemination. Aust. J. Biol. Sci. 27, 301-304.
- TROUNSON, A.O. & MOORE, N.W. (1974b). Attempts to produce identical offspring in the sheep by mechanical division of the ovum. Aust. J. Biol. Sci. 27, 505-510.
- TROUNSON, A.O. & MOORE, N.W. (1974c). The survival and development of sheep eggs following complete or partial removal of the zona pellucida. J. Reprod. Fert. 41, 97-105.
- TROUNSON, A.O., WILLADSEN, S.M. & MOOR, R.M. (1976). Effect of prostaglandin analogue Cloprostenol on oestrus, ovulation and embryonic viability in sheep. J. Agric. Sci., Camb. 86, 609-611.
- TURNER, H.N. & YOUNG, S.S.Y. (1969). Quantitative Genetics in Sheep Breeding. The Macmillan Company of Australia Pty Ltd, Melbourne.
- VAN DER HORST, C.J.G. & BRAND, A. (1969). Occurrence of hypotaurine and inositol in the reproductive tract of the ewe and its regulation by pregnenolone and progesterone. Nature 223, 67-68.
- VAN DER PUTTEN, H., BOTTERI, F.M., MILLER, A.D., ROSENFELD, M.G., FAN, H., EVANS, R.M. & VERMA, I.M. (1985). Efficient insertion of genes into the mouse germ line via retroviral vectors. Proc. Natl. Acad. Sci. 82, 6148-6152.
- VAN DER WESTHUYSEN, J.M., VAN NIEKERK, C.H. & HUNTER, G.L. (1970). Duration of oestrus and time of ovulation in sheep: effect of synchronization, season and ram. Agroanimalia 2, 131-138.
- VAN NIEKERK, C.H. & BELONJE, P.C. (1970). The time of ovulation after the synchronization of oestrus in Merino ewes with medroxyprogesterone acetate. Proc. S. Afr. Soc. Anim. Prod. 9, 177-178.

- VAN RENSBERG, S.J. (1964). Ovum production: action of various gonadotrophins in sheep and goats. Onderstepoort J. Vet. Res. 31, 97-106.
- VELLE, W. & HELLE, O. (1979). Experiences with estrus synchronization in sheep over a twelve-year period using oral MAP treatment for ten days. J. Anim. Sci. 48, 1015-1019.
- VILLALON, M., ORTIZ, M.E., AGUAYO, C., MUNOZ, J. & CROXATTO, H.B. (1982). Differential transport of fertilized and unfertilized ova in the rat. Biol. Reprod. 26, 337-341.
- VIPOND, J.E. & KING, M.E. (1979). Synchronization of oestrus as an aid to management in small flocks. Anim. Prod. 28, 447.
- VISSER, D. & SALAMON, S. (1973). Fertility of ram spermatozoa frozen in a Tris-based diluent. Aust. J. Biol. Sci. 26, 513-516.
- VIZE, P.D., MICHALSKA, A.E., ASHMAN, R., LLOYD, B., STONE, B.A., QUINN, P., WELLS, J.R.E. & SEAMARK, R.F. (1988). Introduction of a porcine growth hormone fusion gene into transgenic pigs promotes growth. J. Cell Sci. 90, 295-300.
- VOEKLE, S.A., AMBORSK, G.F., HILL, K.G. & GODKE, R.A. (1985). Use of uterine-cell monolayer culture system for micromanipulated bovine embryos. Theriogenology 24, 271-281.
- WAGNER, E.F., STEWART, T.A. & MINTZ, B. (1981). The human beta-globin gene and a functional viral thymidine kinase gene in developing mice. Proc. Natl. Acad. Sci. 78, 5016-5020.
- WAGNER, E.F. & MINTZ, B. (1982). Transfer of nonselectable genes into mouse teratocarcinoma cells and transcription of the transferred human beta-globin gene. Mol. Cell Biol. 2, 190-198.
- WAGNER, T.E., HOPPE, P.C., JOLLIICK, J.D., SCHOLL, D.R., HODINKA, R.L. & GAULT, J.B. (1981). Microinjection of rabbit beta-globin gene into zygotes and its subsequent expression in adult mice and their offspring. Proc. Natl. Acad. Sci. 78, 6376-6380.
- WAGNER, T.E., MURRAY, F.A., MINHAS, B. & KRAEMER, D.C. (1984). The possibility of transgenic livestock. Theriogenology 21, 29-44.
- WAGNER, T.E. (1985). The role of gene transfer in animal agriculture and biotechnology. Can. J. Anim. Sci. 65, 539-552.
- WAGNER, T.E. (1986). Biotechnology and livestock improvement. Proc. Brit. Soc. Anim. Prod., Winter meeting, pp 22.
- WAGNER, T.E. & JOCHLE, W. (1986). Recombinant gene transfer in animals: the potential for improving growth in livestock. In: Control and Manipulation of Animal Growth, pp 293-313. Eds P.J. Buttery, N.B. Haynes & D.B. Lindsay. Butterworths, London.
- WAGNER, T.E., CHEN, X.Z. & HAYES, W.B. (1986). Mammalian gene transfer and gene expression. In: Molecular and Cellular Aspects of Reproduction. Ed. D.S. Dhindsa. Plenum Press, New York.

- WALES, R.G. (1970). Effects of ions on the development of the preimplantation mouse embryo in vitro. Aust. J. Biol. Sci. 23, 421-429.
- WALKER, S.K., SMITH, D.H., LITTLE, D.L., WARNES, G.M., QUINN, P. & SEAMARK, R.F. (1984). Artificial insemination and transfer of embryos by laparoscopy. In: *Reproduction in Sheep*, pp 306-309. Eds D.R. Lindsay & D.T. Pearce. Australian Academy of Science, Canberra.
- WALKER, S.K., WARNES, G.M., QUINN, P., SEAMARK, R.F. & SMITH, D.H. (1985). Laparoscopic technique for the transfer of embryos in sheep. Aust. Vet. J. 62, 105-106.
- WALL, R.J., PURSEL, V.G., HAMMER, R.E. & BRINSTER, R.L. (1985). Development of porcine ova that were centrifuged to permit visualization of pronuclei and nuclei. Biol. Reprod. 32, 645-651.
- WALL, R.J. & HAWK, H.W. (1988). Development of centrifuged cow zygotes cultured in rabbit oviducts. J. Reprod. Fert. 82, 673-680.
- WALTON, J.R., MURRAY, J.D., MARSHALL, J.T. & NANCARROW, C.D. (1987). Zygote viability in gene transfer experiments. Biol. Reprod. 37, 957-967.
- WARD, K.A., MURRAY, J.D., NANCARROW, C.D., SUTTON, R. & BOLAND, M.P. (1982). The practical aims and recent progress in the transfer of recombinant DNA to ruminants. Rev. Rural Sci. 6, 39-47.
- WARD, K.A. (1982). Possible contributions of molecular genetics to animal improvement. In: *Future Developments in the Genetic Improvement of Animals*, pp 17-39. Eds J.S.F. Barker, K. Hammond & A.E. McClintock. Academic Press, Sydney.
- WARD, K.A., MURRAY, J.D., NANCARROW, C.D., BOLAND, M.P. & SUTTON, R. (1984). The role of embryo gene transfer in sheep breeding programs. In: *Reproduction in Sheep*, pp 279-285. Eds D.R. Lindsay & D.T. Pearce. Australian Academy of Science, Canberra.
- WARD, K.A., FRANKLIN, I.R., MURRAY, J.D., NANCARROW, C.D., RAPHAEL, K.A., RIGBY, N.W., BYRNE, C.R., WILSON, B.W. & HUNT, C.L. (1986). The direct transfer of DNA by embryo microinjection. Proc. 3rd World Cong. Genetics Appl. Livestock Prod., pp 6-21, Nebraska.
- WARE, C.B., NORTHEY, D.L., BOLAND, M.P. & FIRST, N.L. (1988). Early cycle FSH-p priming as a prelude to superovulating gonadotropin administration in ewes and heifers. Anim. Reprod. Sci. 16, 97-105.
- WARNES, G.M. (1976). Biochemical aspects of the ovine oviduct. PhD Thesis, University of Adelaide.
- WATERMAN, R.A. & WALL, R.J. (1988). Lipid interactions with in vivo development of mammalian zygotes. Gam. Res. 21, 243-254.
- WELCH, R.A.S., ANDREWES, W.D., BARNES, D.R., BREMNER, K. & HARVEY, T.G. (1984). CIDR dispensers for oestrus and ovulation control in sheep. Proc. 10th Int. Cong. Anim. Reprod. A.I. 1, 354.

- WHITE, D.H., RIZZOLI, D.F. & CUMMING, I.A. (1981). Embryo survival in relation to number and site of ovulations in the ewe. Aust. J. Exp. Agric. Anim. Husb. 21, 32-38.
- WHITTEN, W.K. (1957). Culture of tubal ova. Nature 179, 1081-1082.
- WHITTEN, W.K. (1971). Nutritional requirements for the culture of preimplantation embryos in vitro. In: Advances in Biosciences, Vol. 6. Pergamon Press, New York.
- WHITTINGHAM, D.G. & BIGGERS, J.D. (1967). Fallopian tube and early cleavage in the mouse. Nature 213, 942-943.
- WHITTINGHAM, D.G. (1971). Survival of mouse embryos after freezing and thawing. Nature 233, 125.
- WHITTINGHAM, D.G. & BAVISTER, B.D. (1974). Development of hamster eggs fertilized in vitro and in vivo. J. Reprod. Fert. 38, 489-492.
- WHITTINGHAM, D.G. (1975). Fertilization, early development and storage of mammalian ova. In: The Early Development of Mammals, pp 1-24. Eds M. Balls & A.E. Wild. Cambridge University Press, Cambridge.
- WILLEMSE, A.H. (1968). Relationship between the day of the oestral cycle at the time of intravaginal application of a MAP impregnated sponge and synchronisation rate and conception rate in Texel sheep. Proc. 6th Int. Cong. Anim. Reprod. A.I. 2, 1539-1541.
- WILMUT, I. & SALES, D.I. (1981). Effect of an asynchronous environment on embryonic development in sheep. J. Reprod. Fert. 61, 179-184.
- WILMUT, I., SALES, D.I. & ASHWORTH, C.J. (1985a). The influence of variation in embryo stage and maternal hormone profiles on embryo survival in farm animals. Theriogenology 23, 107-119.
- WILMUT, I., SALES, D.I. & ASHWORTH, C.J. (1985b). Physiological criteria for embryo mortality - is asynchrony between embryo and ewe a significant factor. In: The Genetics of Reproduction in Sheep, pp 275-289. Eds R.B. Land & D.W. Robinson. Butterworths, London.
- WILMUT, I., SALES, D.I. & ASHWORTH, C.J. (1986). Maternal and embryonic factors associated with prenatal loss in mammals. J. Reprod. Fert. 76, 851-864.
- WILMUT, I., ASHWORTH, C.J., SPRINGBETT, A.J. & SALES, D.I. (1988). Effect of variation in embryo stage on the establishment of pregnancy, and embryo survival and growth in ewes with two embryos. J. Reprod. Fert. 83, 233-237.
- WOODY, C.O., BEAUCHENE, S.L., FECCIA, R.C., SEPE, P.A., HIGGINS, F.M., COWAN, W.A. & RIESEN, J.W. (1983). Regulation of the oestrous cycle in ewes with progestin containing implants: influence of dose and day of cycle at treatment. Theriogenology 19, 677-684.
- WOYCHIK, R.P., CAMPER, S.A., LYONS, R.H., HOROWITZ, S., GOODWIN, E.C. & ROTTMAN, F.M. (1982). Cloning and nucleotide sequencing of the bovine growth hormone gene. Nucleic Acid Res. 10, 7197-7199.

- WRIGHT, R.W., ANDERSON, G.B., CUPPS, P.T. & DROST, M. (1976a). Blastocyst expansion and hatching of bovine ova cultured in vitro. J. Anim. Sci. 43, 170-174.
- WRIGHT, R.W., ANDERSON, G.B., CUPPS, P.T., DROST, M. & BRADFORD, G.E. (1976b). In vitro culture of embryos from adult and prepubertal ewes. J. Anim. Sci. 42, 912-917.
- WRIGHT, R.W. & BONDIOLI, K.R. (1981). Aspects of in vitro fertilization and embryo culture in domestic animals. J. Anim. Sci. 53, 702-729.
- WRIGHT, R.W., BONDIOLI, K., GRAMMER, J., KUZAN, F. & MENINO, A. (1981). FSH or FSH plus LH superovulation in ewes following estrus synchronization with medroxyprogesterone acetate pessaries. J. Anim. Sci. 52, 115-118.
- WRIGHT, R.W. & O'FALLON, J.V. (1987). Growth of domesticated animal embryos in vitro. In: The Mammalian Preimplantation Embryo, pp 251-271. Ed. B.D. Bavister. Plenum Press, New York.
- ZIGLER, J.S., LEPE-ZUNIGA, J.L., VISTICA, B. & GERY, I. (1985). Analysis of the cytotoxic effects of light-exposed HEPES-containing culture medium. In Vitro Cell Dev. Biol. 21, 282-287.