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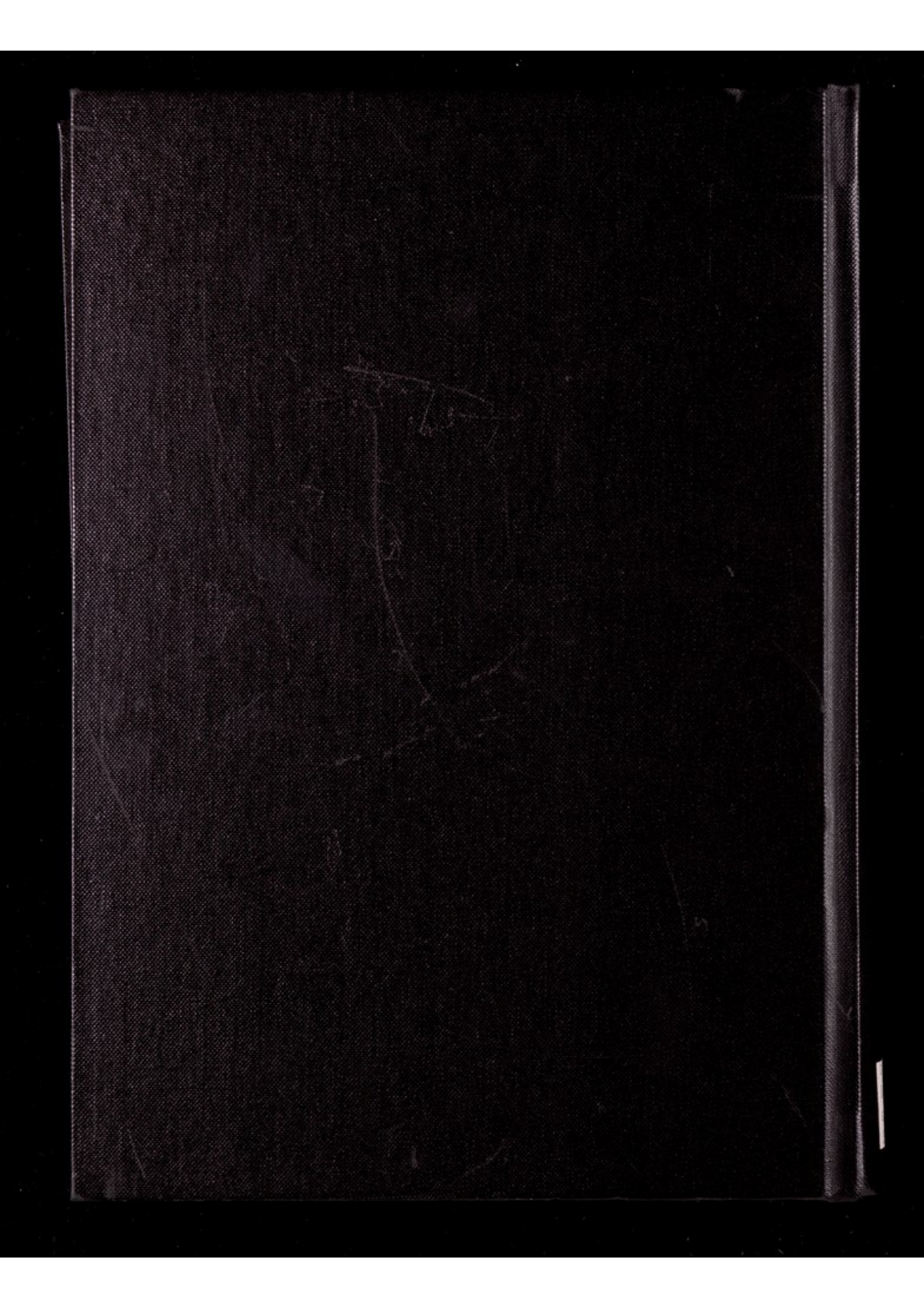
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Cytogenetic Abnormalities in Early Embryos of the Sheep and Pig

by

Erica J. Hamlet

B.V.M. & S. M.R.C.V.S.

A Thesis submitted for the Degree of

Doctor of Philosophy

in the

Faculty of Veterinary Medicine

University of Glasgow

December 1983

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Summary

This work was undertaken in normal sheep and pigs to elucidate the proportion of early embryos with cytogenetic abnormalities in order to determine the importance of chromosome abnormalities in early embryonic death in these species.

The chromosome complement of the animals used in this study was assessed using leucocyte culture. Ewe synchronisation and surgical flushing proved to be very successful in obtaining 4 and 5 day embryos with recovery rates of 61%, 73% and 84% in the 1st, 2nd and 3rd periods of study respectively. Fertilisation rates from natural service and artificial insemination in and out of the breeding season were compared with an average fertilisation rate of 54% after artificial insemination and 80% after natural mating. Estimates of embryonic death were obtained and were found to be higher in August at 66% than in peak breeding season at 25%.

A surgical technique was developed to repeatedly recover 4 to 7 day old embryos from gilts. A total of 218 embryos were surgically recovered from 9 gilts. Morphologically abnormal pig embryos were examined under a scanning electron microscope.

Embryos were cultured for chromosome analysis. The techniques used proved to be successful with pig embryos (58% analysed) but less so with sheep embryos (28% analysed). Cytogenetic abnormalities found included monosomy and trisomy, haploidy, triploidy, diploid/triploid mosaicism, tetraploidy and mixoploidy. Mixoploidy was found at the 6 cell stage in pig embryos and in leucocyte culture of normally developing 78 day

foetuses.

It was concluded that 12% of pig embryos and 9% of sheep embryos had chromosome complements likely to lead to early embryonic death.

INTRODUCTION

INTRODUCTION

Section 1

Major reviews of embryonic mortality in domestic animals have been published by Hanley (1961), Boyd (1965) and Edey (1969). Much work has been done on the causes of embryonic death. Experiments where parameters such as nutritional deprivation and heat shock have been investigated, often have conditions well beyond the range expected under commercial situations, so results from these studies, while valuable in elucidating cause, do not help in revealing the normal embryonic loss to be expected.

The method of collecting data, too, influences ~~embryonic~~ estimates obtained of embryonic death rates. Figures based on late return to oestrus ignore all the very early embryonic death which occurs prior to maternal recognition of pregnancy. In studies where numbers of corpora lutea are compared with numbers of developing foetuses, late foetal death and fertilsation failure have to be allowed for.

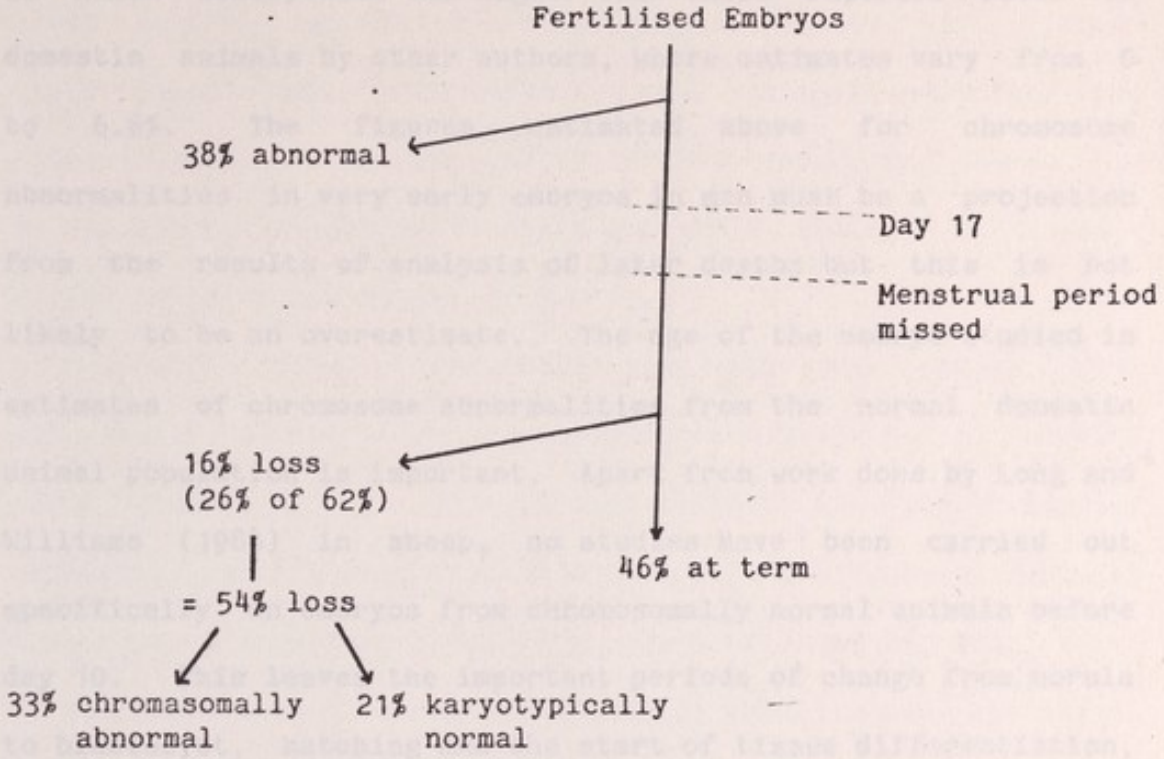
Prenatal mortality estimations in cattle with no history of infertility have ranged from 16-21% with around 20% being the accepted figure, in pigs the range is 25-50%, with 30-40% the most common figure (Hanley, 1961) and in sheep the range for the most direct estimates is 20-32% (Edey, 1969). In cattle the majority of losses occur in the early stages of pregnancy. Boyd, Bacish, Young and McCraken (1969) found embryonic death up to 26 days to account for 7.5% of ovulations and embryonic loss after 26 days to account for a further 8% of ovulations. Ayalon (1978)

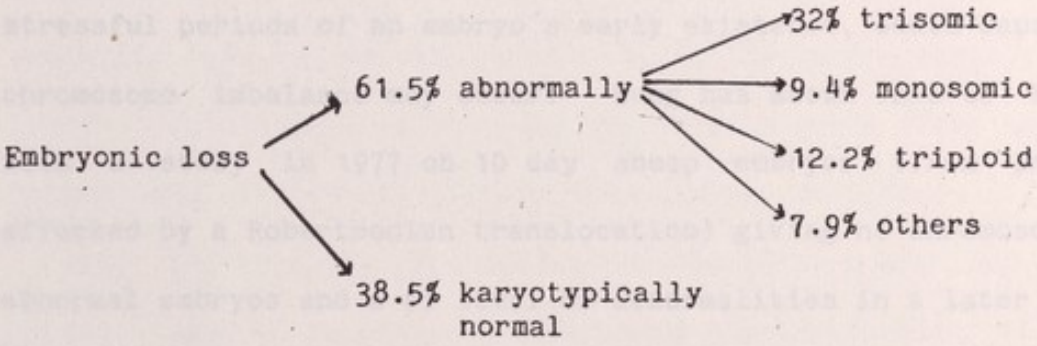
found day 7 to be critical in the development of the bovine conceptus with a 16% loss between days 4 and 10. In a large study involving slaughter of sheep at intervals after service, Quinlivan, Martin, Taylor and Cairney (1966) showed a 15.5% loss of fertilised embryos by day 18 and prenatal loss after day 30 appeared to be negligable. Embryonic mortality in pigs seems to be similarly concentrated into the period of development rather than growth. Perry and Rowlands (1962) found 22% of fertilised pig embryos apparently to be degenerating between day 6 and 9 and a loss of 28% of potential conceptuses by day 18. By 40 days after ovulation the total loss had increased to 35%, approximately 5% of which is assumed to be due to fertilisation failure. Scofield (1972) also reviews embryonic death in pigs and reports the majority of embryo loss to occur around day 9-13 with a further small peak of prenatal loss at around 21 days and another peak at day 60-70 when rapid foetal growth commences. Thus in the domestic species considered above, the majority of prenatal mortality occurs during the period of the embryo, in the first month after mating.

Estimates of embryonic death in man are difficult to obtain. However, French and Bierman (1962) found that 23.7% of presumed conceptuses, and Hertig (1975) found that 27.6% of presumed conceptuses i.e where 1 menstrual period was missed, did not result in a live birth. These figures show remarkable agreement despite the different populations studied. Only 1% of these conceptuses were still born after week 28. Hertig (1975) found 38% of embryos aged 1-17 days, from known fertile patients, to be morphologically abnormal. If it is assumed that none of these

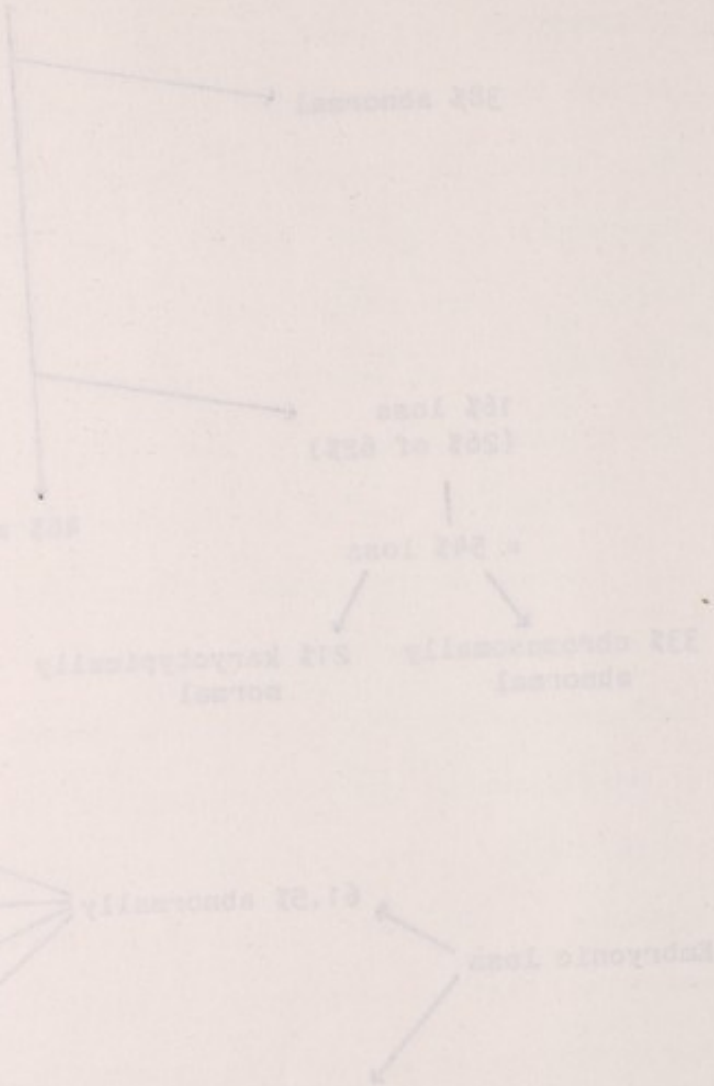
abnormal embryos cause retention of the corpora lutea and a missed menstrual period, it can be estimated that around 54% of conceptuses are lost by week 28. These figures indicated that around 45% of conceptuses are represented by a full term infant; a figure in excess of fecundability estimates of 20-30% discussed by Short (1979). The difference may be due to ovulatory or fertilisation failure lowering fecundability estimates or an underestimate of early embryonic death lowering the estimate for prenatal death discussed above.

Estimates can be made of the proportion of human embryonic and foetal death caused by gross chromosome errors from work examining the chromosome complement of early abortions. Boué, Boué and Lazar (1975), studying abortuses of less than 12 weeks of developmental age, accounting for 80% of spontaneous abortions which occur, found 61.5% to have an abnormal karyotype. Combining this datum with the figures discussed above, and assuming that chromosomal abnormalities also cause death in very early embryos as is found in laboratory animals, an estimated 33% of all conceptuses are chromosomally abnormal. The range of estimates given however vary widely. Roberts and Lowe (1975) from statistical studies, estimate 78% of human conceptuses are lost, a very large proportion of which are caused by chromosomal abnormalities. However, Polani (1969) estimates only 3.5-4% of human conceptuses to carry chromosome abnormalities. No evidence is presented.





Large scale evidence for the role of chromosome abnormalities in foetal death and abortion have not been obtained in the domestic species. However more preimplantation embryos have been studied chromosomally than is possible in man. Although not the earliest working in this field, the study by McFeely (1967) is important in setting an estimate of 10% of pig embryos to be chromosomally abnormal. This figure is



considerably lower than estimates for chromosomal abnormalities in human conceptuses but higher than those reported later in domestic animals by other authors, where estimates vary from 0 to 6.6%. The figures estimated above for chromosome abnormalities in very early embryos in man must be a projection from the results of analysis of later deaths but this is not likely to be an overestimate. The age of the embryo studied in estimates of chromosome abnormalities from the normal domestic animal population is important. Apart from work done by Long and Williams (1980) in sheep, no studies have been carried out specifically on embryos from chromosomally normal animals before day 10. This leaves the important periods of change from morula to blastocyst, hatching and the start of tissue differentiation, to occur before embryos have been analysed. During these stressful periods of an embryo's early existance, death caused by chromosome imbalance may occur. Long has shown this to happen with a study in 1977 on 10 day sheep embryos (from parents affected by a Robertsonian translocation) giving no chromosomally abnormal embryos and a 6% level of abnormalities in a later study (Long and Williams, 1980) of 2 and 3 day old embryos from chromosomally normal parents. Thus the studies in other species only sampled at or around day 10 may also be hiding a difference in the abnormality level in very early embryos and that found in well established blastocysts.

Large scale surveys have been carried out on the effects of specific chromosome rearrangements on fertility. Gustavsson (1969) and Refsdal (1976) showed that daughters of bulls heterozygous for the 1/29 Robertsonian translocation had a

significantly reduced fertility, with lower non return rates than large control population particularly during the early period after insemination. This indicates that a population with a very high incidence of heterozygosity for this chromosome change had an increased incidence in early embryonic death. This would appear to be in the region of 5-10%.

Bishop (1964) postulated that embryonic death from cytogenetic causes was a natural and unavoidable way of removing unfit genotypes. It is important to know the proportion of embryonic death due to removal of abnormal genotypes, as even in the most optimum conditions this is a portion of the basal level of embryonic loss. Though from studies on laboratory species it is known that it is possible to increase the proportion of unfit genetypes by various methods, it is important to assess the basal embryonic loss due to unfit genotypes in the farm species treated as far as possible in the same way as commercial farm animals, before the effects of different management conditions, feeding regimes and breeding programmes, on embryonic death and hence productivity can be properly understood and assessed.

Section 2

Development of the Early Embryo of the Sheep and Pig

In this study the ovum is taken to mean a fertilised or unfertilised one cell egg. The zygote is an early embryo from the 2 cell to approximately the 8 or 16 cell stage. The zygote becomes a morula when accurate counting of the cell number under a light microscope becomes difficult and the morula is termed a blastocyst at around the 50 cell stage. Once the body systems have developed sufficiently for species and sex to be determined and the period of growth rather than development has commenced the conceptus is termed a foetus.

Fertilisation

Before fertilisation the egg of the sheep and pig consists of a large ovum surrounded by a vitelline membrane and a perivitelline space. The ovum, which at this stage has not completed the second meotic division, and the first polar body are enclosed by a transparent spherical membrane - the zona pellucida. Surrounding the zona pellucida are the cells of the cumulus oophorus.

In the ewe sperm penetration takes place in the ampulla of the oviduct 2-3 hours after ovulation (Willasden, 1979). In the pig the ova is in the ampullary isthmic junction, where fertilisation takes place, within 30-45 minutes of ovulation (Hunter, 1974). Capacitation of porcine sperm is completed during the ascent of the oviduct. This ascent is a combination of active migration of the sperm and contractile waves of the oviduct and cillary currents (Hunter, 1977).

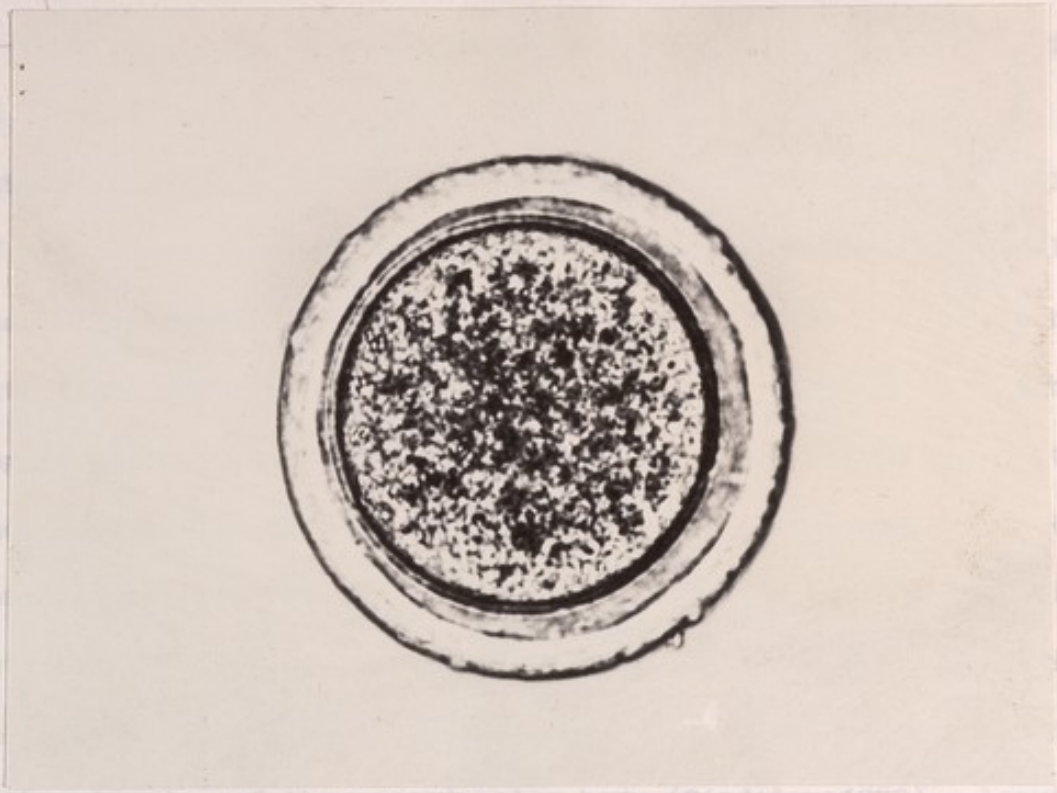


Plate 1 Unfertilised sheep ova

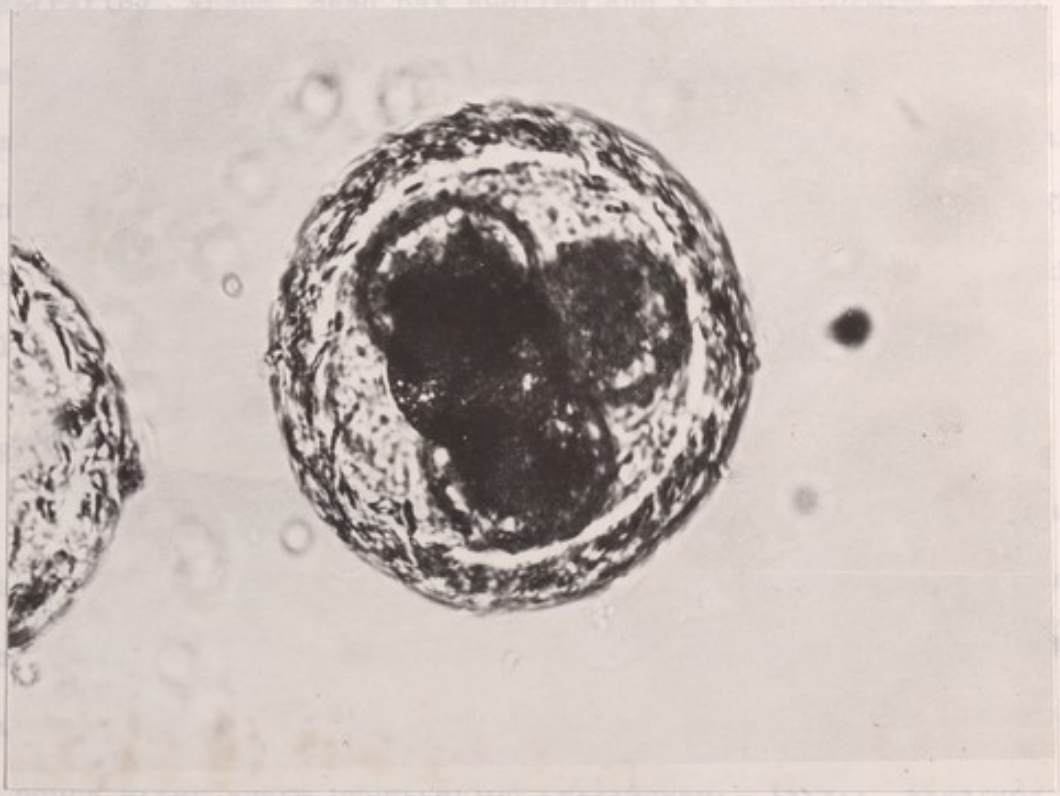


Plate 2 4 cell pig zygote, day 4

In the pig, the cumulus oophorus around the zona pelludica is lost around 3 hours after mating and ovulation, earlier if mating occurred before ovulation (Hunter, 1974) and penetration of the zona pellucida by one or more spermatozoa has also occurred (Hunter, 1972). Once a spermatozoa has penetrated the vitelline membrane a block to polyspermy develops in the inner region of the zona pellucida so that though many sperm may enter the zona pellucida none reach the perivitelline space. On the penetration of the spermatozoa the ovum is activated, with metabolic activity being increased and cortical granules released. Telaphase of the second meiotic division is completed with extrusion of the second polar body (Siedal, 1982), leaving the ovum with a haploid complement of chromosomes. By this time, in the pig 5 to 6 hours after mating (Hunter, 1972), the penetrating sperm head has swollen and is transforming into a distinct pronucleus. The two pronuclei then oppose, lose their membranes and syngamy or combination of the male and female genetic material to form a diploid complement occurs. The mitotic spindle forms from the centrosome of the sperm and the male and female derived chromosomes form on the spindle.

Zygote

The first cleavage division of the fertilised ovum occurs immediately after meiotic division of the new diploid chromosome complement, 14-16 hours after sperm penetration in the pig (Hunter, 1974). In the sheep this is reported to take place 15-18 hours after sperm penetration (Willasden, 1979). Cleavage of blastomeres is like typical somatic mitoses but without the

growth phase between divisions (Balinsky, 1960). The early cleavage stages are thought to be synchronised closely. The second cleavage division occurs only 6-8 hours after the first in the pig (Hunter, 1974) and around 12 hours after the first in the sheep (Willasden, 1979). The four cell stage lasts around 24 hours in the pig and it is at this stage of development, 46-48 hours after fertilisation, that the passage down the oviduct is complete and the zygote enters the tip of the uterine horn. The zygotes remain in the upper portion of the uterine horn for 2-3 days. The sheep zygote after the second cleavage division, divides every 16-24 hours and enters the uterus on day 3 or 4 at the 4-12 cell stage. Although the early cleavage divisions are thought to be synchronous, by the 8 cell stage asynchrony is commonly developing (Hunter, 1974) though some authors (Hancock, 1961) report asynchrony as early as 3 cells.

Morulae

Early embryos are described as morulae from after the 8 cell to around the 32 cell stages. During this period the blastomeres become difficult to count, losing their spherical shape and becoming polygonal, compacting into the centre of the area enclosed by the zona pellucida. This stage occurs around 60-90 hours after ovulation. Where day 0 is taken to be the first day of standing oestrus this is on day 4 and 5 in the pig and the sheep. The pig embryo advances more rapidly than the sheep embryo with cleavage division approximately every 16-20 hours compared with 18-24 hours for the sheep.

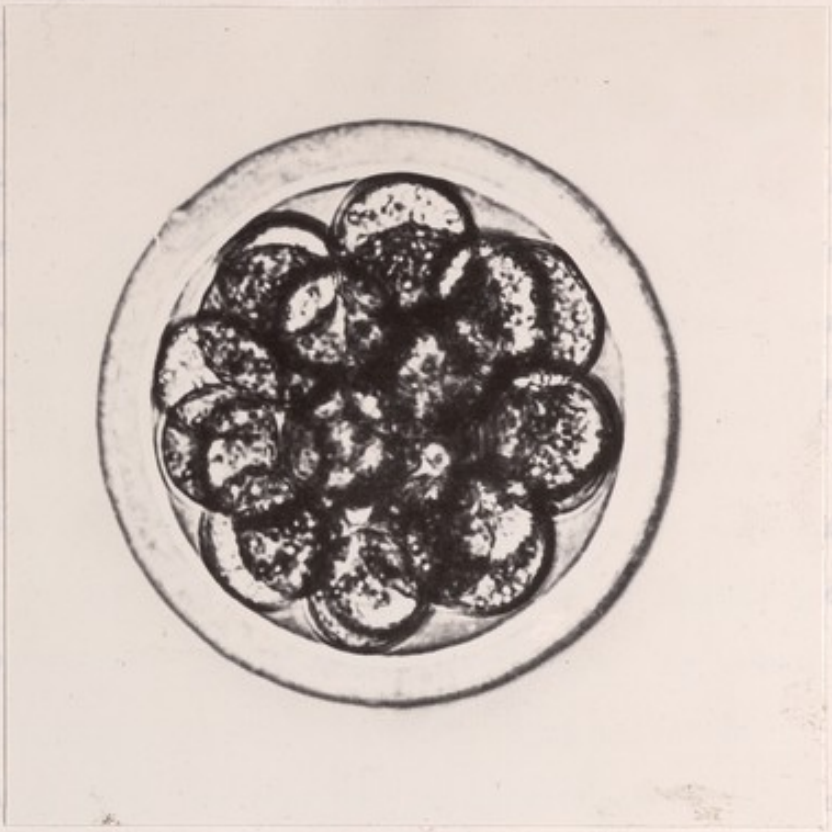


Plate 3 Sheep morula, day 5

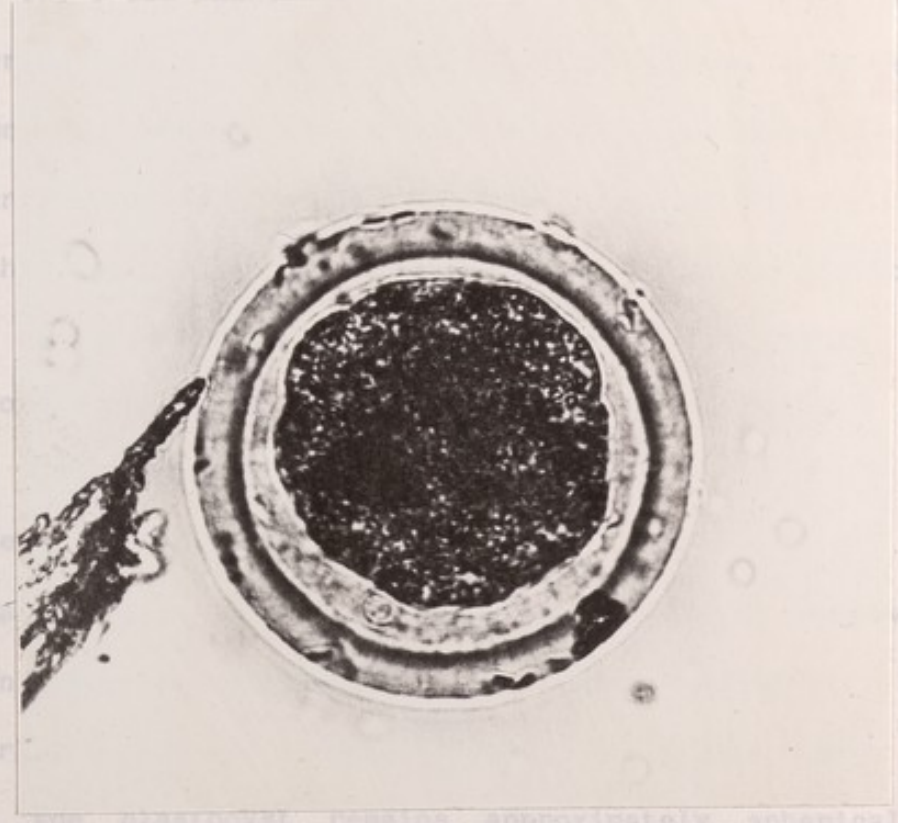


Plate 4 Compact sheep morula, day 5

Blastocyst

During the period of the blastocyst the blastomeres organise to form the inner cell mass, the blastocoel cavity and the trophoblast. This occurs around 92 hours after fertilisation or day 5-6 in the pig, and day 6 in the sheep. As the blastocoel cavity grows the trophoblast cells are pushed against the zona pellucida. At this stage the embryo can be confused with a degenerating embryo or degenerating unfertilised ovum where the cytoplasm breaks down into small particles which fill the zona. As the blastocoel cavity increases in size the whole embryo increases in size with thinning of the zona pellucida.

Hatching from, or loss of, the zona pellucida occurs on day 7 (i.e. the 6th day after ovulation) in the pig and day 8 in the sheep (Rowson and Moor, 1966). Newly hatched blastocysts contain 150-200 cells (Hunter, 1974; Willasden, 1979).

On day 6 pig embryos are still in the upper 1/3 or 1/2 of the uterine horn. On days 7 and 8 the embryos begin to spread out down the horn and around days 10 and 11 are migrating into the contralateral uterine horn (Dhindsa, Dziuk and Norton, 1967) though this migration is thought to be passive on the part of the embryo. Sheep embryos too may migrate and this is normal where double ovulation occurs in the one ovary and none in the other (Casida, Woody and Pope, 1966).

After hatching the embryo appears spherical and the trophoblast cells have a flattened surface with microvillae projecting from the apical cells (Hall, Horne and Perry, 1965; Winlenberger-Torres and Flechen, 1974). For around 2 days after hatching the blastocyst remains approximately spherical but

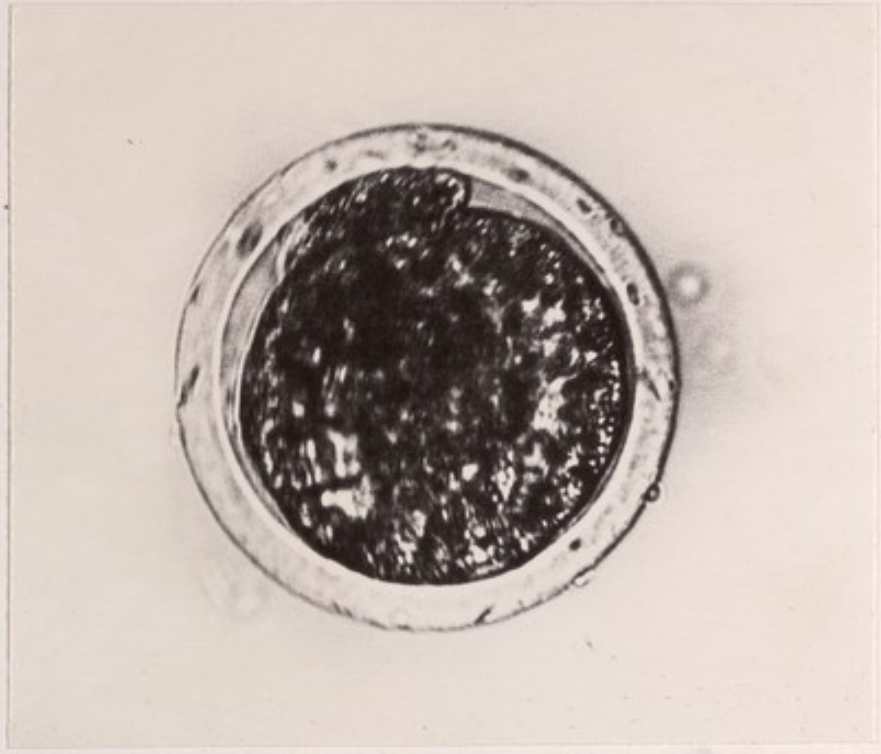


Plate 5 Early sheep blastocyst, day 5

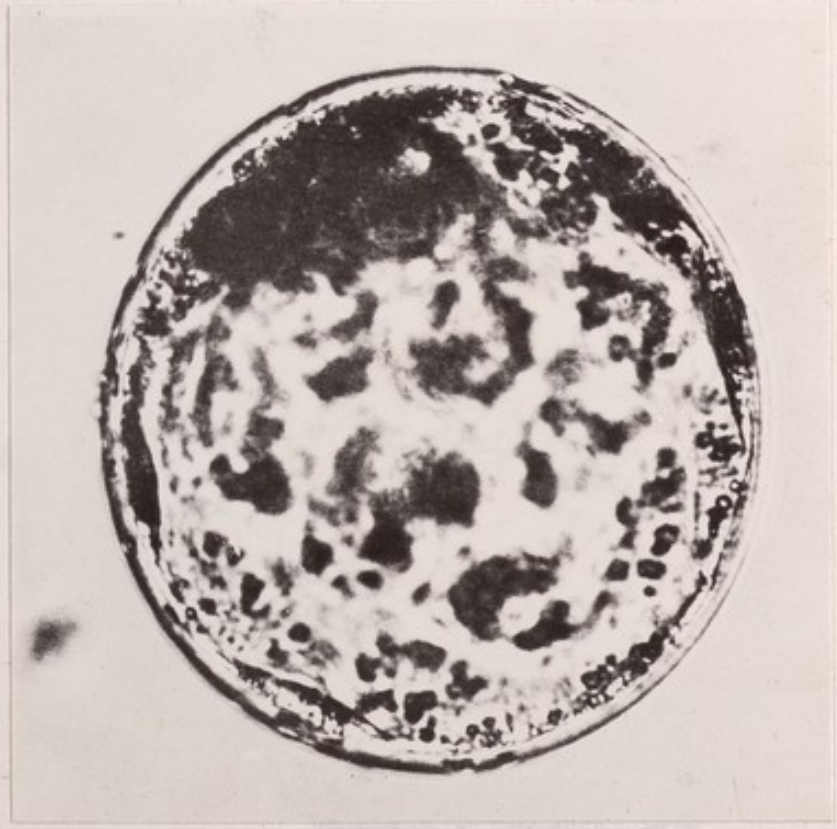


Plate 6 Expanded pig blastocyst, day 6

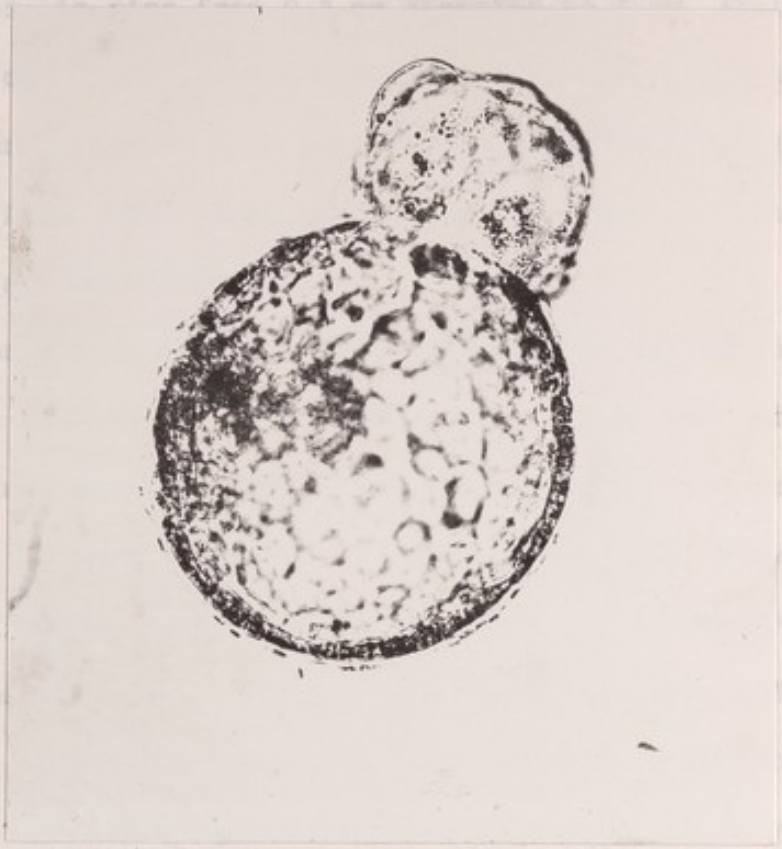


Plate 7 Hatching pig blastocyst, day 7

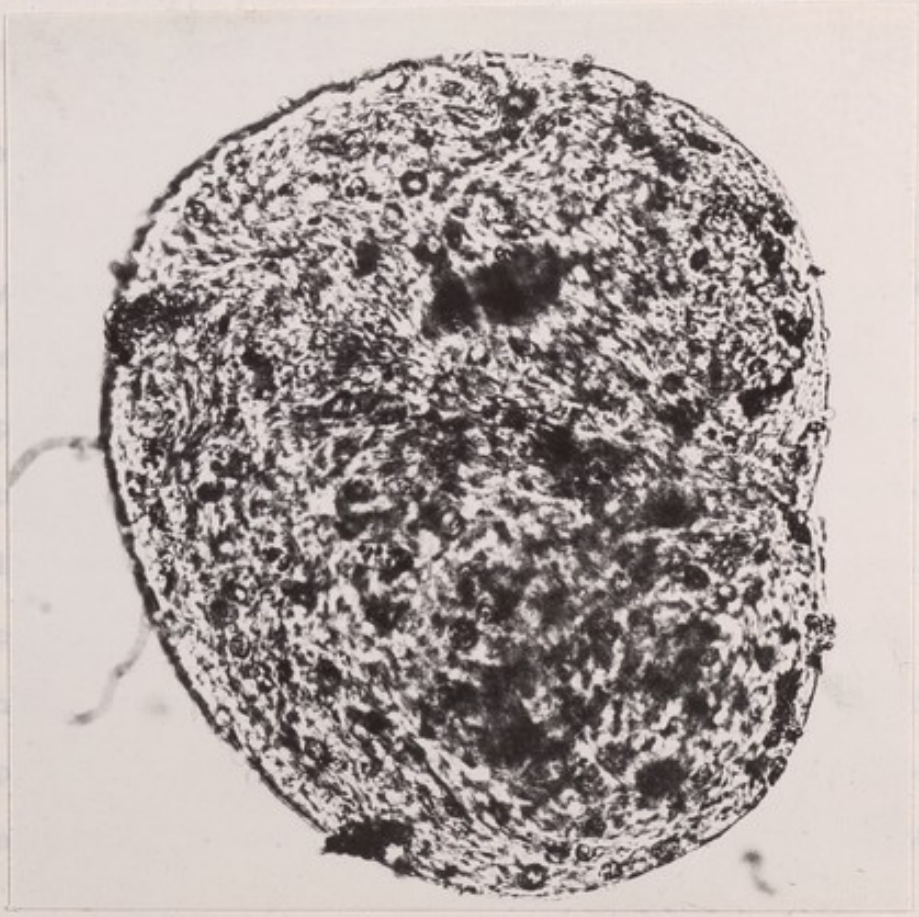


Plate 8 Hatched pig blastocyst, day 8

increases in size from 0.4 mm diameter to 1 mm (Dhindsa, Dziuk and Norton, 1967). The surface appears wrinkled and the inner cell mass becomes more prominent. Endodermal cells are being pushed from the inner cell mass to form a lining to the trophoblast ectodermal cells.

Elongation of blastocysts occurs from day 12 in the sheep (Rowson and Moor, 1966) and from day 10 in the pig (Dhindsa, Dzuik and Norton, 1967) and the primative streak begins to develop in the inner cell mass. The pig blastocyst will eventually develop to more than a metre in length (Patten, 1948; Perry and Rowlands, 1962). The appearance of mesodermal tissue follows. The development of the embryo is very rapid at this stage: the neural groove appears on day 13, by day 14 the gut, yolk sac and coelomic cavities are forming as are the mesodermal somites which eventually develop into skeletial muscles, the dermis and the vertebrae (Patten, 1948).

Implantation

The start of implantation is seen on day 13 in the pig embryo with a tenuous attachment between the microvillae of the endometrium and trophoblast, and is well advanced by day 18. As the placenta of the pig is epitheliochorial the attachment of the foetus to the endometrium is diffuse and the trophoblast is easily peeled from the endometrium (Hunter, 1974), unlike the sheep where smaller areas of more intimate attachment at the caruncles are found. Implantation is first seen on day 15 in the sheep with areas of close attachment between trophoblast and caruncular epithelium (Boshier, 1969). Plaques of multinucleate

syncytia are formed in the maternal epithelium from day 16 until the 4th week of pregnancy when placentome formation with interdigitation of foetal and maternal tissue begins.

CHAPTER 1

The Chromosome Complements of the Animals used in this Study

CHAPTER 1

SECTION 1.1

Introduction Blood Culture

In the past, examination of the chromosome complement of an individual required actively dividing cells such as were found in testicular tissue. These could also be obtained from bone marrow (Ford and Jacobs, 1958) or long term tissue culture (Tjio and Puck, 1958). Using squash preparations to display metaphase spreads (Ford and Jacobs, 1958) there was generally poorer separation of individual chromosomes than in the more recent methods using air-drying. Sectioning of cell cultures was also used with results which were readily misinterpretable.

In 1959 using fibroblast culture, Lejeune, Gautier and Turpin recognised the presence of an extra automosomein Mongoloid children, and in the same year, using bone marrow Jacobs and Strong found the extra X chromosome in Kleinfelters syndrome patients and Ford, Polani, De Almeida and Briggs noted the absence of a gonosome in Turners syndrome. These findings increased interest in chromosome studies.

The routine use of leucocyte culture to assess the chromosome complement began with the accidental discovery of mitotic agents. Phytohaemagglutinin, a haemagglutinin prepared from Phaesolus vulgaris (red kidney bean) was found by Nowell (1959) to cause human white blood cells to divide in culture after a latent period of two days. The combination of leucoctye culture with mitotic agents, the use of colchicine or colcimid to inhibit spindle formation and thus arrest cells in metaphase, and

also to cause chromatid separation and contraction (Ford and Hammerton, 1956) and the use of hypotonic solutions to cause red cell lysis and aid spreading of the chromosomes in the final preparation (Hsu and Pomerat, 1953) have made analysis of the chromosome complement of many species easy and interest grew quickly. In 1960 Moorhead, Nowell, Mellman, Ballips and Hungerford first cultured the blood of man to obtain chromosome preparations. In 1963 McConnel, Fechheimer and Gilmore, and Stone karyotyped the pig using blood cultures. Biggers and McFeely (1963) used blood cultures to examine the chromosome complement of cattle and other species and McFee, Banner and Murphee (1965) cultured leucocytes from sheep.

The method used for ovine culture was that used in the laboratory for routine cattle analysis.

The method used for porcine blood culture was developed by the author adapted from some of the many methods published to provide a simple reliable technique (Srivastava and Lasley, 1968; Zartman, Fechheimer and Baker, 1969; Harvey, 1969; Hare and Singh, 1979).

All animals used in these studies were assessed for chromosome number and the presence of any other gross cytogenetic defect. Banding was not routinely performed. An assessment of the normality of each of the adults used in these studies was important in interpreting any abnormalities found in their embryos.

Section 1.2.1

Blood Culture and Harvesting Materials

Culture Media	100 ml RPMI 1640 Medium with glutamine and 20 mM Hepes buffer made up from powder (Flow Laboratories) and preferably aged 1-3 months + 20 ml Foetal Calf Serum (Flow Laboratories) with 8,800 i.u penicillin and 8,800 mg streptomycin and 3.5 mM glutamine.
Mitogen	Phytohaemagglutinin (Wellcome) 2.5 ml reconstituted or Pokeweed (Flow Laboratories) 1.2 ml reconstituted.
Colcimid	(Calbiochem) made up in Hanks balanced salt solution
Hypotonic solution	1 gram of KCl in 600 ml deionised water.
Fixative solution	1 part glacial acetic acid (BDH) + 3 parts methanol.
Stain	10 ml Gurr Giemsa Improved R66 made up to 100 ml with buffer.

Equipment Required

Lithium Heparin monovet (Starstedt 12.5-15 iu heparin/ml blood) or Vacutainer Lithium Heparin (14.3 usp units/ml blood). Laminar Flow Cabinet, sterilised universal bottles, incubator 37-38 °C, centrifuge, water bath, centrifuge tubes, vacuum pump, glass pasteur pipettes, twin frosted microscope slides, coverslips and DPX mountant.

Section 1.2.2

Blood Culture Methods - Sheep

Blood samples were obtained from the jugular vein in either heparinised vacutainers or heparinised monovets. 10 ml of blood was taken from each animal. The blood was either cultured immediately or left overnight at 4°C.

All work in setting up the blood culture was done inside an airflow cabinet and all reasonable precautions to avoid contamination of the culture were taken. Immediately before cultures were set up the culture media was made up by adding to bottle of the RPMI media, thawed Foetal Calf Serum containing added penicillin, streptomycin and glutamine. The RPMI media was stored at 4°C and the Foetal Calf Serum at -19°C. Phytohaemagglutinin, a mitogen for white blood cells, was added at 2.5 ml to the 120 ml media and the complete media was decanted in 10 ml aliquots into sterile universals.

2 ml of whole blood was added to each universal; usually 4 cultures were made from each animal. The cultures were incubated at 37°C for 46.5 hours.

Harvesting commenced by adding colcimid to arrest dividing cells at metaphase, at 8 ug per culture i.e a final concentration of 0.66 ug/ml and returning the cultures for a further 1.5 hours incubation. No attempt was made to maintain sterile conditions at and from this point. The cultures were decanted into warmed centrifuge tubes and centrifuged at 1200 rpm for 10 minutes. The supernatant was discarded using a vacuumised pipette and 10 ml hypotonic solution at 37°C was added and the cells resuspended.

The culture was then incubated in a water bath at 37°C for 15 minutes then centrifuged for 10 minutes at 1000 rpm during which time the red cells lysed. The supernatant was again discarded and the white blood cells left were resuspended in 5 ml fixative solution. After incubation for 15 minutes at 4°C the cells were again centrifuged at 1000 rpm for 10 minutes, the fixative discarded and the cells resuspended in 2 ml fresh fixative. The cells were then left at 4°C for at least 30 minutes before being centrifuged at 800 rpm for 5 minutes. The supernatant was again discarded and the cells suspended in 1 ml fixative.

Microscope slides, 2 per culture, were cleaned in 70% alcohol, dried and labelled. Holding the microscope slide at 45°, half of each suspension was dropped by a pasteur pipette, drop at a time, from a height of approximately 10 inches above the slide. The slides were dried in a warm air cabinet and half stained for 30 minutes in a 10% Giemsa solution. After drying in a warm air cabinet the slides were mounted using coverslips and DPX.

Section 1.2.3

Pig Blood Culture - Methods

Blood samples were obtained in heparinised plastic syringes from either the jugular vein or a peripheral ear vein.

When using the jugular vein the gilt was restrained using a snare, the head held up exposing the throat. With the leg on the ipsilateral side of the gilt placed a little back, a 1.5 inch 19 gauge needle was inserted mid-way between the trachea and the point of the shoulder, pointing a little up and towards mid-line. Gentle negative pressure was then exerted with a heparinised monovet.

The peripheral ear vein was used in the boar (after sedation), in anaesthetised gilts when a cannulae was already in the ear vein, and in the one gilt in which the jugular method was found unsatisfactory. The ear was swabbed with alcohol and an elastic band placed round the base of the ear. Once the veins had filled a 21 gauge needle was used to very slowly and gently withfraw blood into a heparinised monovet.

Two methods of culturing pig blood for metaphase spreads were found to be successful. One ml of whole blood in 10 ml of culture medium gave satisfactory results. In the second, more commonly used method, the sample was centrifuged for 10 minutes at 800 rpm or left to stand for 2 hours when the majority of the plasma was withdrawn for progesterone analysis. A similar volume of culture media was added and the white cells and top 1/5 approximately of the red cells were resuspended. This suspension was then used at 0.5-1 ml to 10 ml culture media, leaving the

majority of the red cells undisturbed. Although both methods produced a good mitotic index and good metaphase spreads the second method produced slides which were slightly cleaner and of better quality.

The mitogen used for culturing pig blood was pokeweed at 1-3%.

Culture media, incubation and harvesting were all as for the sheep blood technique. Where whole blood was used, if the red cells formed a clot, the universal bottle was gently agitated to suspend white cells, then the clot was removed before centrifugation of the media. Growth obtained was still perfectly satisfactory and removing the clot aided the production of a clean preparation.



Plate 9 The chromosome complement of a ram (Ovis aries)

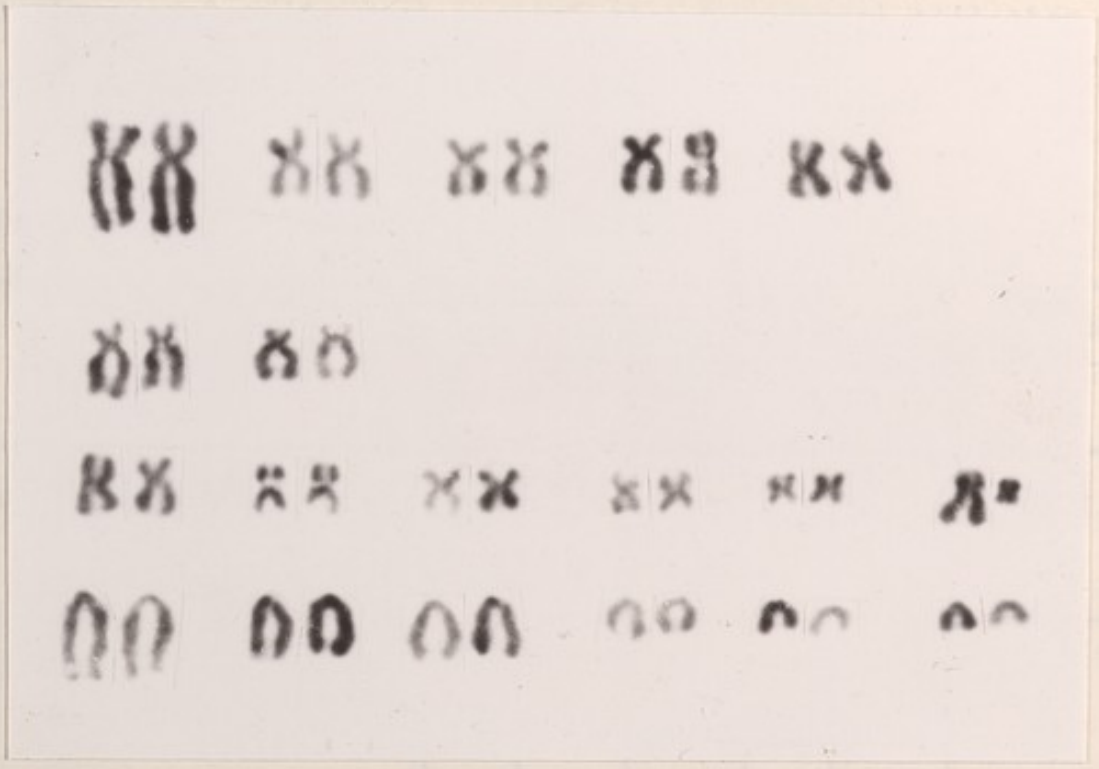


Plate 10 The chromosome complement of a boar (Sus scrota)

Section 1.3.1

Sheep - Cytogenetic Blood Results

At least 10 metaphase spreads were analysed for each of the adult animals used in this study. Metaphase spreads that appeared intact with the chromosomes well separated were chosen. Each spread was counted 3 times or until on 3 consecutive occasions the same number was obtained. Each spread was assessed for the presence of a Y chromosome. If no Y chromosome was present and the spread had the diploid number of 54 the cell was assumed to be XX. The presence of 6 large metacentric chromosomes was checked in each metaphase spread counted. Karyotyping was not carried out in each animal unless an abnormality was noted in the above criteria. If a large number of hypomodal cells were present or a hypermodal cell then 15 or more metaphase spreads were counted.

TABLE 1. Sheep cytogenetic results from blood culture

		Hypomodal		Modal	Hypermodal		Tetraploid
		<52	<53	54	55	>55	
Adult ewes	cells counted	46	41	459	7	3	2
	%	8.2%	7.3%	82.3%	1.3%	0.5%	0.4%
Ewe 148	cells counted	1		6	12	1	
	%	5%		30%	60%	5%	
9 day lambs	cells counted	1	2	25	1		
	%	3.4%	6.9%	86.2%	3.4%		

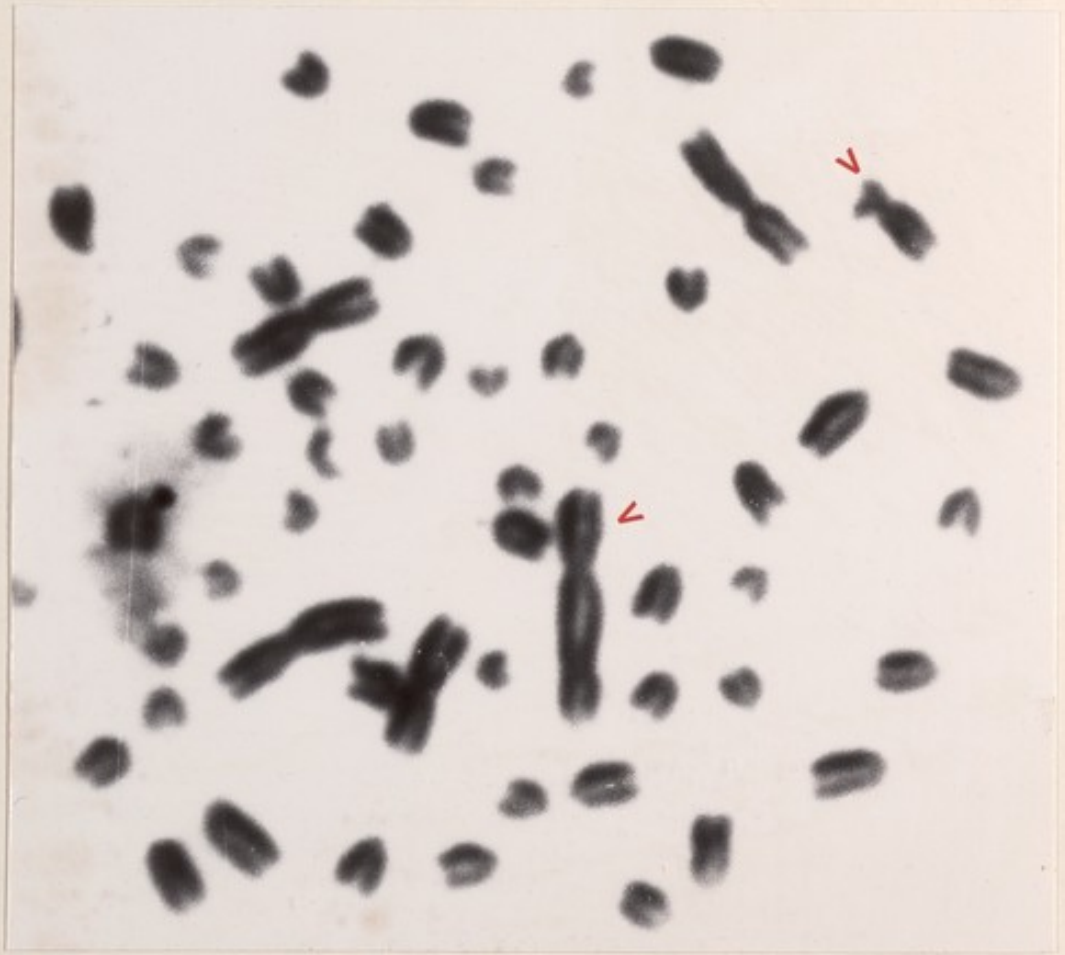
All 5 tups used and 50 of the 51 ewes examined had a modal count of 54. Four of the 5 tups and 42 of the 51 ewes had counts only of 54 or less. Hypermodal metaphase spreads were obtained from the remaining 11 sheep. Ten sheep only had at the most 2 hypermodal metaphase spreads.

One ewe was mosaic for a trisomy i.e 54XX/55XX? The results from examination of the leucocyte culture of her blood are presented seperately. In all the hypermodal cells the extra chromosome was an acrocentric. Without banding it was not possible to determine whether the extra chromosome was the same one in each cell, and if so, which chromosome it was. Unfortunately this ewe was slaughtered before further investigation could be carried out e.g. fibroblast culture and banding studies.

Two ewes showed other abnormalities in the results from blood culture.

Ewe 55 had one cell which showed a reciprocal translocation probably between the two of the largest metacentric chromosomes. Twenty metaphase spreads were counted from this ewe with a modal count of 54 and no hypermodal cells. A further 90 metaphase spreads were examined for the presence of the translocation. Only one cell was found showing the abnormality. Blood samples were cultured from 3 of the 4 lambs born to her in the spring of 1983. The fourth lamb was stillborn probably due to dystokia. The 3 live lambs were blood sampled at 9 days of age. Ten metaphase spreads were counted from each lamb and a further 40 spreads from each examined for the presence of the translocation. The results from the cells counted are presented in Table 1.





Plates 11 and 12 The chromosome spread from Ewe 55 with translocation chromosomes arrowed.

No translocation cells were found. Fibroblast cultures were attempted from the ewe but no translocation cells were found.

There was one unusual cell found in the preparations from blood culture of ewe 95. The metaphase spread had a count of 84 with each chromosome lying close to a homolog (see Fig.13). The cell with the count of 84 could either be a triploid cell (3n = 81) or a tetraploid cell with many chromsomes missing. All other metaphase spreads from this ewe were diploid.

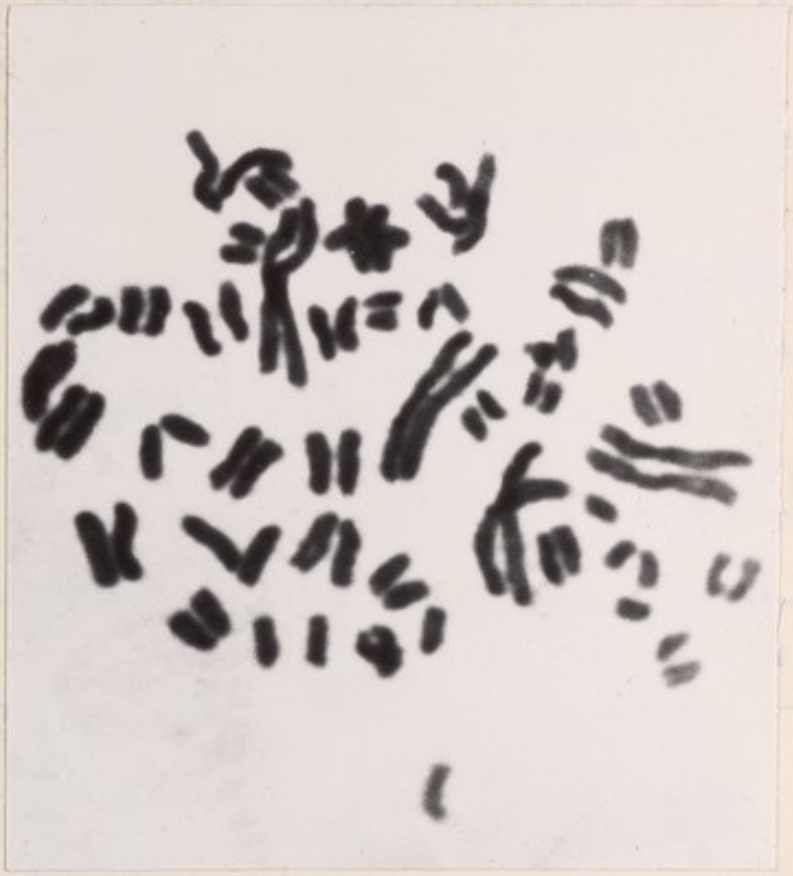


Plate 13 Metaphase spread from Ewe 95 with 84 chromosomes lying in pairs.

Section 1.3.2

Porcine Blood Culture Cytogenetic Results

Blood for culture was obtained from the 9 gilts and 1 boar used in this study. It was noted that the gilts which were particularly tame and relaxed were harder to bleed from the jugular vein than those gilts which were more nervous and thus tense.

Metaphase spreads were examined from the boar and the 9 gilts involved in surgical embryo recovery. Each metaphase spread was examined for the presence of the Y chromosome and counted.

TABLE 2 Pig Cytogenetic Results from Blood Culture

Pig No.	Sex	36	37	38	39	40	>40	Tetraploid
Boar	XY	3	2	14	1			
1	XX	4	3	13				
2	XX	1	2	15	1	1		
3	XX		5	13	2			
4	XX	1	1	17	1			
5	XX	3	5	12				
6	XX		2	16	1		1	
7	XX	4	5	10			1	
8	XX	2	2	13	2		1	
9	XX		3	16	1			
All Pigs		9%	15%	69.5%	4.5%	0.5%	1.5%	0

In every case the modal number was 38. No abnormalities in sex chromosomes were found. In 7 of the 9 gilts and in the boar, hypermodal metaphase spreads were counted. Hypomodal spreads were present in preparations from all pigs.

Section 1.4.1

Discussion - Blood Cytogenetic Results

Hypomodal cells were common in the preparations of cultured leucocytes taken from the phenotypically normal animals used in this study.

In a large survey of pigs by Harvey (1969) the modal count of 38 occurred in 85.3% of 1,758 metaphase spreads counted. Hypomodal spreads accounted for 8.7% and hypermodal but still diploid 5.6%. 0.3% of spreads in this survey were tetraploid. Other authors, Srivastava and Lasley (1968) reported a modal count of 38 in 88.5% of cells, 37 in 7.2% and 39 in 4.3% and McConnell et al (1963) report a modal count of 38 in 86.2% of cells, less than 37 in 4.6% of cells, 37 in 4%, 39 in 3.3% and greater than 39 in 2.0%. The counts recorded from the experimental pigs in this study showed fewer modal counts, slightly more hypermodal counts and more than twice as many hypomodal counts. There is no evidence however of a second cell line in one particular pig only, causing the distortion. All pigs had more hypomodal cells than the average found by Harvey.

Hypomodal cells have 3 possible causes. The most commonly accepted reason for the majority of hypomodal metaphase spreads is breakage of the cell and loss of chromosomes during the harvesting process. It is possible for a hypomodal cell line to be present in the animal sampled or to arise during culture. The mechanism for this may be anaphase lag or mitotic non-disjuntion. Mitotic non-disjunction may also be a reason for hypermodal counts. Fechheimer (1972) suggests that both non-disjunction and

anaphase lag may be genetically influenced and that it is heritable in man.

As mitotic non-disjunction should give an equal number of hypermodal and hypomodal cells other mechanisms must be present. Two possible reasons for the excess hypomodal cells are the presence of anaphase lag where one or more chromosomes fail to participate in anaphase and are excluded from the telephase nucleus, and the preparation technique causing broken cells and the loss of one or more chromosomes. The second reason may well be the more important in this study as a fairly high proportion of hypomodal cells were present in blood cultures of all species and in embryo preparations, some cells obviously broken and the chromosomes scattered. It can be concluded that the pigs used in this study were cytogenetically as well as phenotypically normal.

Bruère (1967) suggested that aneuploidy in blood cultures from sheep increases in aged animals, as aneuploidy increases in aged man (Jacobs, Court-Brown and Doll, 1961). Unfortunately, Bruère in his study used freemartin sheep as his aged group and predominately normal ewes as his low age group. Very large numbers of cells were counted. Jacobs found that aneuploidy increased more rapidly in aged abnormal subjects (Klinefelter and Turners Syndrome patients) than in normal subjects. The sheep used in this present study were predominately mature adults, certainly 3 years only and the majority probably much older as many showed signs of severe tooth wear. 18 of the ewes were less than a year old. The percentage of hypomodal and modal cells in this study compares very closely with that of Bruère's aged

sheep, hypomodal 15.5 vs. 16.45, modal 82.3 vs. 79.9. A hypermodal cell percentage of 1.8% in this study is slightly higher than that found by Bruère even in the aged freemartin sheep but not significantly so ($X^2 = 0.128$). Tetraploid cells were less common in the present study. Even in the young animals examined by Bruère, hypermodal and polyploid cells were present so it can be concluded that the presence of these cells amongst the population under study does not make that population abnormal. It is interesting to note a hypermodal cell in one of the lambs as young as 9 days.

The ewe with the high proportion of cells which were trisomies (60%) was probably a 54/55 mosaic i.e. had two cell lines. Because only leucocytes were cultured it is impossible to be sure whether this was purely a blood cell mosaicism or whether it extended to all cell types. If, due to non-disjunction at an early stage in embryonic development one daughter cell acquired 55 chromosomes or an embryo with 55 chromosomes fused in utero with one of the 54 chromosomes, it would be likely that trisomic cells would be present in many tissues. However if non-disjunction occurred in a leucocyte stem cell later in embryogenesis it is possible that the trisomy would be present only in white blood cells. Similarly the trisomy may occur only in a few cell types if originating from a chorionic vascular anastomosis between a 55XX twin and a 54XX foetus later in development. Chorionic vascular anastomosis is common in cattle twins occuring in 91.9% of twinning (Marcum, 1974) but allantoic vascular anastomosis is much rarer in lambs at 0.8-2% (Marcum, 1974; Hare and Singh, 1979).

The trisomic cells found in this ewe were unlikely to be cultural artefacts as such a high proportion of cultured leucocytes were trisomic. Trisomy is a common cause of early abortion in man. 32% of spontaneous abortions examined by Boué et al (1975) were trisomic and the authors believed that more trisomic embryos were lost before pregnancy was recognised. Although human trisomies do survive to birth and beyond, phenotypic abnormality results; trisomy 21 (Down Syndrome), trisomy 18, trisomy 13 (Patau Syndrome) and the sex chromosome trisomies i.e. Klinefelter syndrome (Gorlin 1976) are commonly found. Trisomies have been found associated with phenotypic defects in calves, puppies and kittens (Hare and Singh, 1979). Mosaicism for a trisomy does not necessarily lead to phenotypic abnormality though this has been reported in calves (Hare and Singh, 1979). A phenotypically normal bull with a 60XY/61XY leucocyte chromosome complement has been found by Harvey (1982, personal communication). Healthy adult male tortoishell cats with a 38XY/39XXY chromosome complement have been described by a number of authors (reviewed by Hare and Singh, 1979) and phenotypically normal but subfertile boars mosaic for monosomy and monosomy and trisomy, have been described by Vogt, Arakaki and Brooks (1974). It is possible that mosaicism for trisomy is present in the normal population and only exposed where phenotypcic abnormalities or infertility are investigated and population surveys are undertaken.

The cell from ewe 95 showing 84 chromosomes in pairs, though it could be a triploid cell (3n = 81) would appear to be the major

portion of a tetraploid cell. Hare and Singh (1979) suggest that the origin of a tetraploid cell where homologous chromosomes lie in pairs is endoreduplication, that is DNA replication without cell division. This has been suggested by Brown (1972) to occasionally result from prolonged culture in colcimid. However the leucocytes in this culture were only in contact with colcimid for 1.5 hours, which does not give time for 2 DNA replications. As the remaining cells from this ewe were all diploid it seems likely that this cell was a cultured artefact. Low percentages of tetraploid cells from blood cultures are reported by other authors (Harvey, 1969; Bruère, 1967; Popescu, 1968).

The reciprocal translocation found in one cell in ewe 55 appears to be found only in one cell or in a very low (< 1) percentage of leucocytes. More than 100 cells were checked for the translocation. Further studies were undertaken to assess the possible occurrence in other tissues but no further translocation cells were found. No evidence of transmission of the translocation to offspring was found.

Robertsonian translocations (where two acrocentric chromosomes become joined to form one acrocentric with or without loss of a centromere (Ferguson-Smith, 1972)) have been frequently reported in sheep (Bruère, 1969; Bruère, 1973; Bruère and Chapman, 1974; Bruère, 1974). Deletion of one arm of an acrocentric autosome was reported by Luft in 1972 in association with phenotypic abnormalities. Reciprocal translocations, where after breakage and relinking a portion of one chromosome joins another, is less commonly reported in sheep. Glahn-Luft and Wassmuth (1977) reported a 1p-24q+ reciprocal translocation. Hammerton

(1971) reviews the population studies in man and reports a 0.3% incidence of reciprocal translocations that change the somatic karyotype in the normal adult population. Reciprocal translocations have frequently been reported in the pig (King et al, 1980; Akesson and Henricson, 1972; Loeniskar, Gustavsson, Hageltern and Zech, 1976; Madan, Ford and Polge, 1978).

It is possible that the translocation arose during culture. Fechheimer (1972) discussed the possible causes of chromosome aberrations. Ionizing radiation, many chemical agents and viruses are known to cause chromatid and chromosome breaks with the possibility of rejoining to form a translocation or an insertion. Genetic factors such as Blooms syndrome also predispose to chromosome and chromatid breaks (Brown, 1972). It is impossible to be certain that none of these agents could have been present in the culture conditions where the abnormal cell was produced but there was no evidence of chromatid or chromosome breaks in other cells in this culture or in cells cultured from other animals at the same time.

The reciprocal translocation appeared to be between 2 homologous chromosomes as the other 4 large metacentric chromosomes paired very well. The ratio of the combined length of the abnormal chromosomes to chromosome 3 is 1.113 and the ratio of the other pair to chromosome 3 is 1.165. This indicates that if no chromosome material was lost during the reciprocal translocation, the reciprocal translocation pairs were chromosomes 2. However the difference in total length between the 2 largest metacentric chromosome pairs is not as great as

that found by Bruère (1966) in a study of cytogenetics in sheep at 1.046 in this study compared with 1.116 found by Bruère. Thus it is possible that the translocation chromosomes were no. 1, with a portion of a chromosome arm lost during the translocation process. This potential missing piece might make the ratio of the combined p arms to q arms (only 1.16) nearer that found by Bruère of 1.224. Bruère found the ratio of the long arms to the short of chromosomes 2 was very similar to chromosome 1 at 1.227 so the ratio of the 2 p arms to the 2 q arms of the translocation pair was low for either chromosome pair 1 or 2. This is added evidence for the possibility of loss of some chromosome material during the translocation process.

It is interesting to note that the translocation appearts to be from one homolog chromosome to another. Schneiderman and Smith (1962) and German (1964) report somatic pairing of chromosomes from culture in metaphase spreads examined and German (1964) suggests that crossing over and thus exchange of genetic material takes place in short term leucocyte culture in man. Herzog, Hahn and Ruck (1977), also show evidence of pairing in chromosomes cultured from blood from cattle suffering from hereditary paratebercul osis. Crossing over during mitosis is however not a likely explanation for the translocation as crossing over involves only one chromatid of the pair from each chromosome and normally involves breaks in the chromatids at the same distance from the centromere leaving the chromosome the same size. However the evidence that homologous chromosomes may pair during anaphase and early metaphase is interesting. This would increase the chance of a chromosome lying as a pair with its

homolog receiving a segment caused by breakage in chromosome arms.

See Figs. 1, 2 and 3 for possible mechanisms.

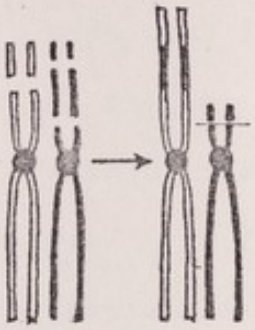


Fig.1
Insertion

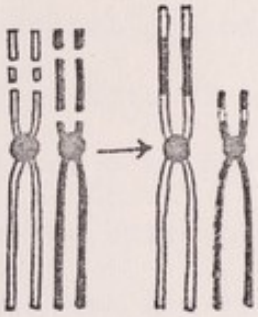


Fig.2
Reciprocal translocations

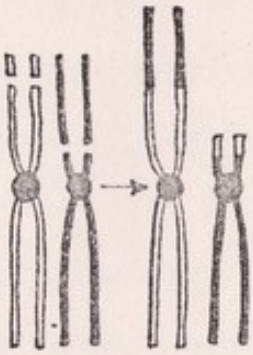


Fig.3

Banding studies would help to elucidate the origin of the translocation but these were impossible as only one cell was found.



CHAPTER 2

Surgical Embryo Recovery

from the Ewe and Gilt

and Embryo Culture

CHAPTER 2

SECTION 2.1.1

Ewe Experiments

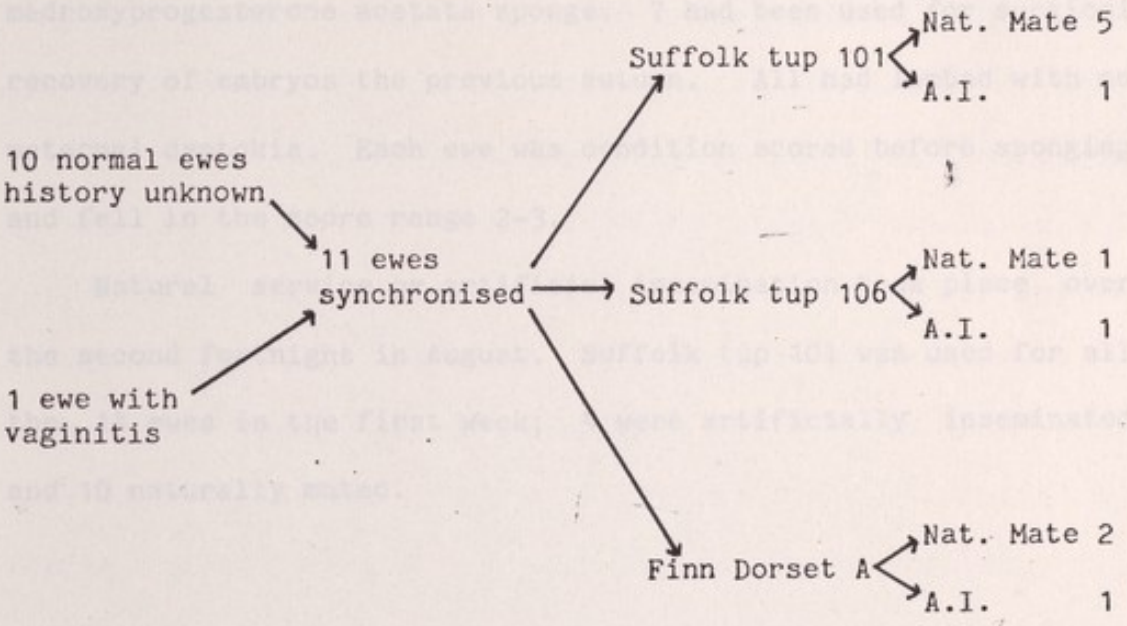
Three distinct periods of study took place; autumn/winter 1981, August 1982 and late autumn 1982 i.e. 2 periods in the peak breeding season and one pre-breeding season. A total of 45 ewes were used in the surgical groups; 6 of these were involved in all 3 studies and 17 on both of the 1982 studies. Controls both within the unit and on a nearby farm were used. The farm used was 6 miles from the unit and consisted of lowland paddocks around the steading, some low lying fields but predominantly hill pasture. The control ewes used were kept in the paddock around the farm buildings during the period of the study. All ewes in these studies were crossbred commercial adult ewes principally Scottish Greyface.

All ewes were synchronised by the same method regardless of season. A medroxy progesterone acetate impregnated intravaginal sponge (Veramix, Upjohn) were inserted for 12, 13 or 14 days with 750 i.u. pregnant mare serum gonadotrophin (PMSG) (Folligon, Intervet or Fostim, Paines and Byrne Ltd), given at the time of sponge withdrawal. Artificial insemination or natural mating took place 50-54 hours after sponge withdrawal.

Breeding Season 1. 1981

11 ewes were used in November and December 1981. 10 of these ewes were clinically normal but one ewe had a persistant vaginitis. 3 different tups were used, all were normal on

examination and produced semen of good quality on electroejaculation. The ewes were paired, the aim being for one ewe to be naturally mated and the other to be artificially inseminated with semen from the same tup on the same day. Due to technical difficulties only 3 ewes were artificially inseminated, the other 8 being naturally mated.



Embryos were surgically recovered on day 4 after oestrus from 2 ewes in a morning. The 11 ewes were flushed over a period of a fortnight. To assess the effects of surgical flushing on fertility those 6 ewes which returned to oestrus (17-19 days after the induced oestrus) were mated.

Breeding season 1 Controls

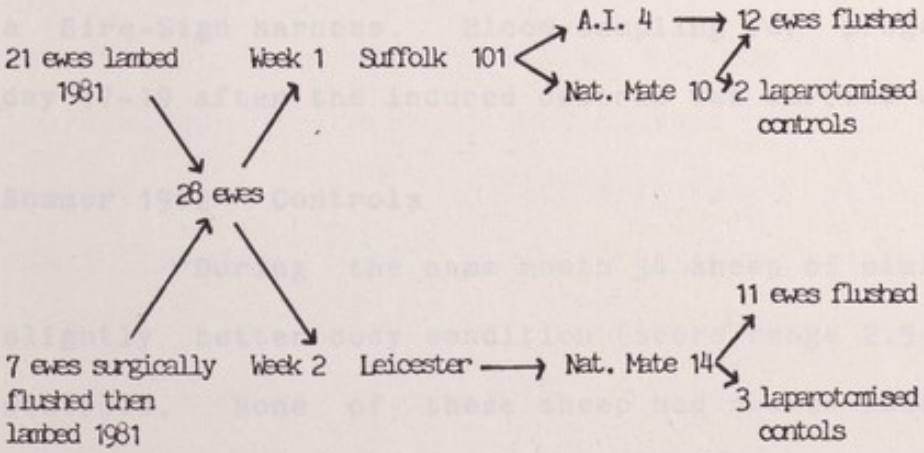
Controls were established under identical conditions in the previous month, with 12 ewes artificially inseminated and 12 naturally mated using tups 101 and 106 which were also used in the

surgical section. These ewes were monitored for a return to oestrus using a tup fitted with a Sire-Sine harness. Any ewes that returned to oestrus were served and lambing results obtained.

Summer Season 1982

28 ewes were used in the study in August. Each had been weaned from her lambs for at least one month and had basal progesterone levels (< 0.5 nm/l) before the insertion of a medroxyprogesterone acetate sponge. 7 had been used for surgical recovery of embryos the previous autumn. All had lambed with no maternal dystokia. Each ewe was condition scored before sponging and fell in the score range 2-3.

Natural service or artificial insemination took place over the second fortnight in August. Suffolk tup 101 was used for all the 14 ewes in the first week; 4 were artificially inseminated and 10 naturally mated.



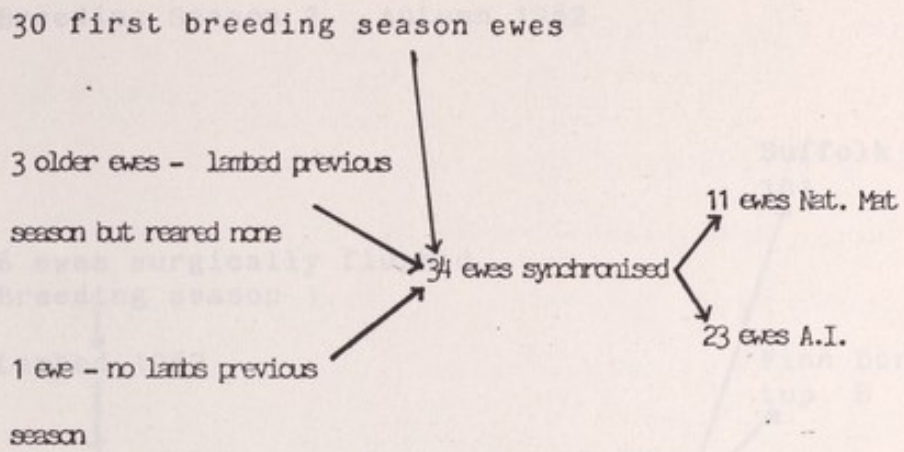
By the begining of the second week the semen Suffolk 101 produced had deteriorated in quantity to 0.5 ml and in concentration to the extent that the sample appeared watery. Because of this Border Leicester tup 1 was used for the remaining 14 ewes. This tup could not be trained to use the artificial vagina so A.I. was not carried out. 5 of the ewes, 3 mated by the Leicester and 2 by the Suffolk tup, were used for a within-experiment estimation of early embryonic death. Laporotomies were performed on these ewes in order to count the number of copora lutea present as an indication of the number of ova shed.

Surgery, on day 5 (day 0 = day of oestrus), was performed on 3 ewes daily over the period of a fortnight. Generally 2 surgical flushings and one laporotomy were performed each morning with the author remaining scrubbed and gowned between each animal. Anaesthetic preparations commenced at 9.15 am and surgery was generally completed before 12.30 pm; each flushing operation taking 30-40 minutes and each laporotomy 20 minutes. All ewes were monitored for return to service using a vasectomised tup with a Sire-Sign harness. Blood sampling for progesterone analysis day 17-19 after the induced oestrus was carried out.

Summer 1982 Controls

During the same month 34 sheep of similar breeding and slightly better body condition (score range 2.5-4) were used as controls. None of these sheep had reared lambs the previous season, 30 of them being in their first breeding season i.e. approx. 18 months old. 4 were older ewes. Intervaginal progesterone sponges were inserted in all ewes for 12 days and

750 i.u. PMSG were given at the time of sponge withdrawal. In addition 40 similar ewes were given 375 i.u. PMSG.

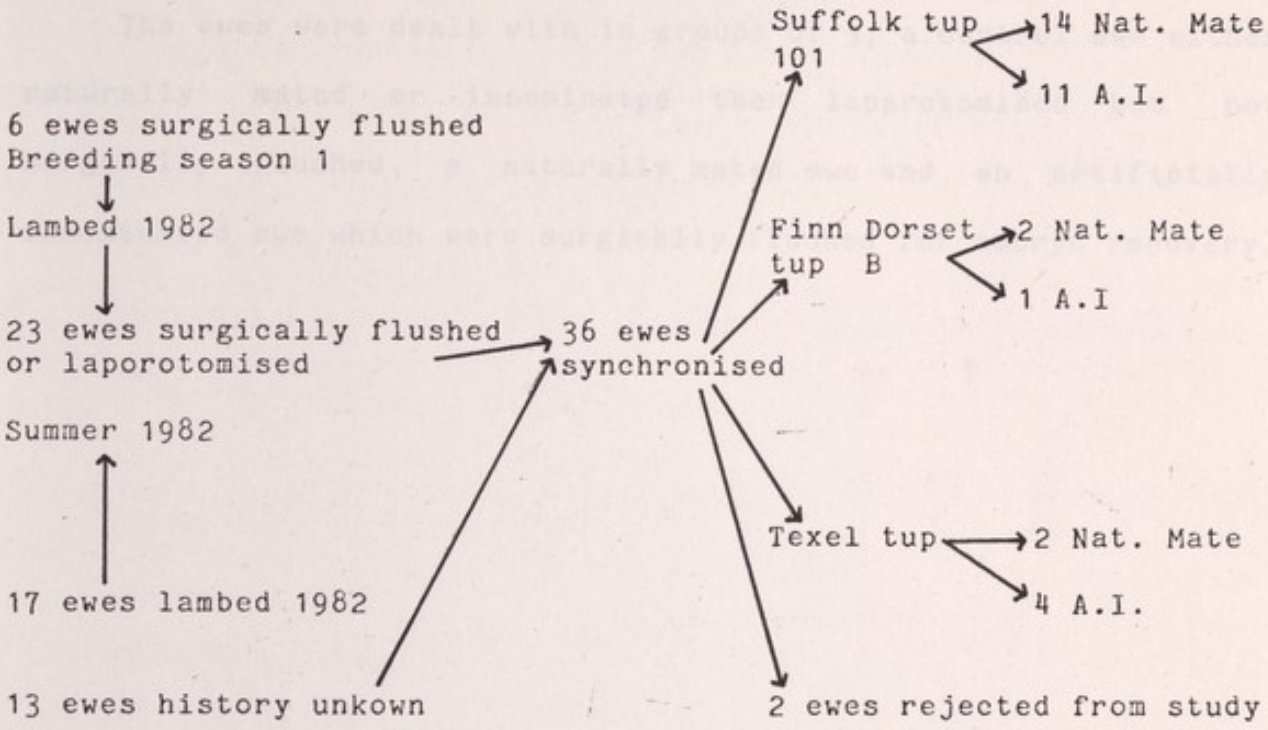


The semen used for A.I. was collected from 5 Suffolk tups selected from a group of 11 for probable fertility. Testicle size, firmness, tail size and the state of the penis was assessed as was the quality of the semen produced on electroejaculation. These tups were not trained to use an artificial vagina. Motility and concentration were assessed and a Nigrosin-Eosin smear made to examine abnormalities in sperm and any cells present. Motility was also assessed after dilution with egg yolk buffer just before insemination. Semen was not mixed so results were available for the different tups. The naturally mated ewes were confined in a small paddock with all the 5 tups used for artificial insemination. All ewes were monitored for return to oestrus with exposure to a vasectomised tup. When only 2 of the 74 ewes were marked by the tup with a crayon by 20 days after the synchronised oestrus a selection were sampled for plasma progesterone analysis to determine whether this was due to higher than expected



conception rates or ewes lapsing into anoestrus. Lambing results were obtained for these ewes.

Breeding Season 2 Autumn 1982



36 ewes were used for the study in late October and November 1982. Six of the ewes had been used in the previous 2 seasons of surgical embryo recovery and a further 17 in the summer season only all of which had lambed the previous season. The reproductive history of the remaining 13 was unknown. Each ewe had been in oestrus at least once before synchronisation was commenced. 2 ewes were removed from the study. One did not come into oestrus with the usual synchronisation treatment and the other developed severe respiratory difficulties on induction of anaethesia so surgery was abandoned. 14 ewes were naturally served and 11 artificially inseminated by Suffolk 101, the tup

used during the first week of the summer season. Frequent use of this tup caused his semen quality to deteriorate so 2 other tups were used.

Two different semen diluents were used for artificial insemination.

The ewes were dealt with in groups of 3; a control ewe either naturally mated or inseminated then laparotomised but not surgically flushed, a naturally mated ewe and an artificially inseminated ewe which were surgically flushed for embryo recovery.

Section 2.1.2

Artificial Insemination Equipment

Semen Collection and Processing

Ewe crate

Artificial vagina for sheep and goats (Arnolds)

Sperm glass tulip shaped to fit above (Camlabs)

Vaseline

Light microscope

Nova Slide 11 with grids - made for standardized urinalysis (ICL Scientific)

Incubator - 37 °C (Griffin)

Semen Diluent - see below.

Glass pipettes

Plastic pipettes

Egg Yolk Diluent

10 g lactose was dissolved in 100 ml water by heating to 80 °. The solution was cooled to 30 °C and 0.1 g streptomycin sulphate (Sigma) added. 40 ml of this was mixed with 10 ml egg yolk by gentle agitation.

Milk Diluent

Either UHT or fresh milk was heated to 92 °C in a conical flask resting on a bed of beads in boiling water to prevent burning and held at that temperature for 10 minutes. It was transferred to a sterile universal and allowed to cool to room temperature.

Insemination

Crate for restraining ewe with hind legs raised so tail 1-2 inches above level of head. During the first two sessions a metal pole secured on the top of straw bales - 2 high and placed in a corner was used. The ewes back legs were held over the bar to tilt her forward.

Vaginascope - two types were used. 1 MV speculum with lighter leg and pocket power source (Instruments de Media Vetenaire). Cold light vaginascope for ewe-length 8.75 width 0.9-1.3" battery handle (Keller Optical Products Ltd)

Plastic pipette - 1′ in length with terminal inch bent to angle of 30° from straight (Alfred Cox Surgical Ltd - specially made)

2 ml syringe and plastic tubing to fit luer fitting

Semen Collection, Treatment and Artificial Insemination Methods

Tups were trained to ejaculate into an artificial vagina using a ewe in oestrus as a teaser animal. The artificial vagina was filled with hot water so the interior was at 40-45°C. The pressure inside the artificial vagina was kept fairly high so a forefinger could just readily be inserted. A warmed glass tulip,

which fits in the end of the artificial vagina to collect the semen, was kept around 37 °C and protected by an insulated padded covering. The presenting surface of the artificial vagina was lubricated with vasaline immediately before use.

A ewe in oestrus was positioned in a set of stocks which were fitted around her head to hold her stationary. There were no sides to these stocks to distract the tup. The collector was stationed at the ewe's right side with the artificial vagina in her right hand before the tup was intoduced. As the tup mounted the ewe the penis, through the scrotum, was deflected into the artificial vagina. The ejaculate was immediately assessed for volume, motility and concentration. Two or more ejaculates were occasionally taken within a few minutes of each other particularly where the volume of the first ejaculate was low. Taking care not to chill the sample it was rapidly transferred to a laboratory and placed in a sealed test tube in an incubator at 37 °C. One drop was used to assess motility by placing it in a standardized urinalysis microscope slide and examining under X 40. A motility assessment of 3 or more was required i.e wave motion in the semen, before the sample was considered satisfactory. A Nigrosin-Eosin smear to assess abnormal sperm was done on the semen of each tup before being admitted to the experiment and periodically thereafter. Usually the sample was diluted 1 part + 2 parts diluent and then stored at room temperature for 2-5 hours before use. In the first two studies egg yolk diluent was used all the time. In the study in the autumn of 1982 both egg yolk diluent and heat treated milk diluent were used and the results obtained with their use

assessed. An approximate concentration of 1000 million sperm per ml was thus achieved.

A.I. Methods

Immediately before insemination took place the diluted semen was again assessed for motility by placing a drop on a warmed slide on a warm stage and examining under X 40. Only semen which produced a wave pattern was used. A minimum of two operators were required for the artificial insemination work. If many ewes were to be inseminated more people were generally used. With only 2 operators the semen was prepared before the ewe was positioned. A 2 ml syringe was attached to the plastic insemination pipette by a short piece of plastic tubing. Approximately 0.5 ml of air was drawn up into the syringe and 0.25 ml of diluted semen, measured by a previously marked line on the pipette, was drawn into the end of the pipette. Thus each ewe was inseminated with approximately 250 million sperm.

The ewe was prepared for insemination by raising her hind legs above her forelegs by placing a bar approximately 3 feet from the ground in front of and above her stifles. The ewes were generally fairly quiet in this position. A vaginascope slightly lubricated with KY jelly was gently inserted into the vagina. The cervix was readily visualised lying usually in the floor of the vagina, though occasionally was seen laterally or even dorsally.

The insemination pipette was inserted through the vaginascope into the cervix. The prominent lips of the cervix could always be clearly felt. When the end of the pippette was inserted into the

cervix, the vaginascope was withdrawn slightly in case a withdrawal later, after the semen had been inserted, caused the semen to reflux out of the cervix. The syringe on the end of the pipette was then squeezed so the 0.5 ml of air pushed all the semen out of the pipette. The vaginascope was carefully removed, then the pipette, and the ewe returned to the horizontal.

SECTION 2.1.3

Surgical Embryo Collection from Sheep

Equipment

Oviduct Cannulae - 2 glass tubes bent at an angle of 100° with the ends rounded, siliconised and sterilised (made from the drawn portion of a pasteur pipette).

Collection Tubes - 4 round bottomed 50 ml centrifuge tubes siliconised and sterilised.

Flushing Fluid - Ovum Culture Medium (Flow Laboratories)

- Dulbeccos Phosphate Buffered Saline (Oxoid) + 5% Bovine Serum Albumin (Sigma)

Anaesthetics - Pentobarbitone sodium BP 6% (Sagital, May and Baker) at 0.4 ml/kg i.e. 20-30 ml /sheep.

- Alphaxalon/aldolphalone acetate (Saffan, Glaxovet Ltd) at 0.75 ml/kg

- Halothane (May and Baker Ltd)

Incubator - 38° C

Surgical Equipment - Laparotomy instruments, laryngoscope, endotracheal tube, sandbags, table, anaesthetic machine, 20 ml syringes, 1" 20 gauge needles, nylon, 2/0 chromic cat gut, KY jelly (Johnson and Johnson)

Methods

The surgical collection method was based on that used by Robertson (1981).

Prior to surgery commencing collection tubes and oviduct cannulae were prepared by wrapping in foil before sterilising. Flushing fluid was made up by the addition, in the air flow cabinet, of 0.5 g Bovine Serum Albumin to 100 ml sterile Phosphate Buffered Saline (PBS). In the first series Ovum Collection Media was used as the flushing medium. The flushing medium was prepared in the morning before surgery. Using the air flow cabinet to maintain a sterile environment, a new 20 ml syringe was pushed just to protrude through the sterile wrapper, a needle attached and 10 ml of flushing fluid was drawn up into the syringe. Care was taken not to contaminate the head of the syringe. 4 syringes of flushing fluid were prepared per ewe and placed in the incubator to warm.

Ewes were starved for 2 days and deprived of water for 1 day before surgery, partly to reduce risk of regurgitation on induction of anaesthesia but mainly because surgery was easier for the author and involved less trauma to the animal when the rumen was of low volume.

In the first series of embryo recoveries alphaxalone/aldolpholone acetate at 0.75 mg/kg (Safran) was used as the induction agent and injected over a period of 45 seconds into the recurrent tarsal vein. In later series, pentobarbitone sodium was substituted. The ewe was then intubated and anaesthesia was maintained with halothane.

The ewe was positioned on her back and held in place with

sandbags. The ventral abdomen from the udder to the umbilicus was clipped and scrubbed for sterile surgery. The author and an assistant scrubbed and gowned for sterile surgery. The ewe was draped and the assistant positioned on the opposite side of the ewe to the surgeon. A mid line incision approximately 10 cm in length was made immediately anterior to the udder and the linea alba incised. The uterus was located, frequently coiled in the pelvis and was gently exteriorised through the abdominal incision. The ovaries were gently examined and the number and appearance of corpora lutea present on each noted.

Once the uterus was exposed the collecting tubes, oviduct cannulae and flushing syringes were taken from the incubator and deposited, sterile, on the instrument tray. One end of the oviduct cannulae was gently inserted through the fimbrae. The assistant kept this tube in place by holding it through the oviduct between finger and thumb. The other end of the cannulae was positioned to drain into a collecting tube. In case a portion of a flush was contaminated with blood a second tube was ready to separate any blood stained fluid (very rarely found) from clear flushings. This aided the search for embryos and reduced the possibility of haemoloysed blood cells contaminating and possibly proving detrimental to the embryos.

In the first series of ewes, flushed on day 4, only the top half of the uterine horn and the oviduct were flushed, with the surgeon holding the horn firmly between finger and thumb and inserting a 1" 20 gauge needle gently into the lumen of the uterine horn above this point. Gentle lateral movement aided the

differentiation of the correct position of the needle in the lumen from in the endometrium or myometrium. Approximately 8 ml of flushing fluid was gently pushed into the horn, passed up the horn into the oviduct and out through the cannula. In most cases, if care was taken to prevent kinking of the oviduct very little pressure was needed. Any fluid remaining was flushed out using air. The oviduct cannulae was gently withdrawn and placed in the collecting tube which was sealed, labelled and placed in an incubator at 37°C. This procedure was repeated for the other uterine horn.

In ewes flushed on day 5 or day 6 the whole horn was flushed by the same procedure twice using 8 ml fluid each time.

When flushing was complete, sterile lubricating jelly was gently smeared over the uterine horns, ovary and oviduct and the uterus was replaced in the abdomen. This appeared to reduce the formation of adhesions. Initially sterile saline only was used to moisten tissues handled but the lubricating jelly appeared to give better results.

The linea alba was closed using simple interrupted sutures in nylon. A simple subcutaneous suture of 2/0 chromic cat gut was used to eliminate dead space and the skin was closed with simple interupted sutures. All ewes healed.

Recovery from anaesthesia was very rapid in those animals where anaesthesia was induced with aphaxalone/aldolphalone acetate and slightly longer where pentobarbitone sodium was used.

In those ewes where laparotomy for observation and counting of corpora lutea took place the procedure was the same as surgical flushing up to the exteriorisation of the uterus. After

visualisation of the ovaries the uterus and the ovaries were returned to the abdominal cavity and the abdomen closed as for the surgically flushed ewes. In 4 of these laparotomised ewes the anaesthetic used was a combination of xylazine (Rompun, Bayer, UK Ltd) and ketamine (Vetalar, Parke Davis & Co).

SECTION 2.1.4

EMBRYO CULTURE

Materials

Flushing Fluid - Ovum Culture Medium (Flow Laboratories)
or Dulbeccos Phosphate Buffered Saline +
5% Bovine Serum albumin (Sigma)

Culture Media - Ovum Culture Medium + 10 or 20% heat treated sheep serum.

Equipment

Stereo dissecting microscope with X 10 and X 20 magnification.

Laminar Air Flow Cabinet

Glass embryo dishes

Binocular microscope with camera attached to photograph X 125

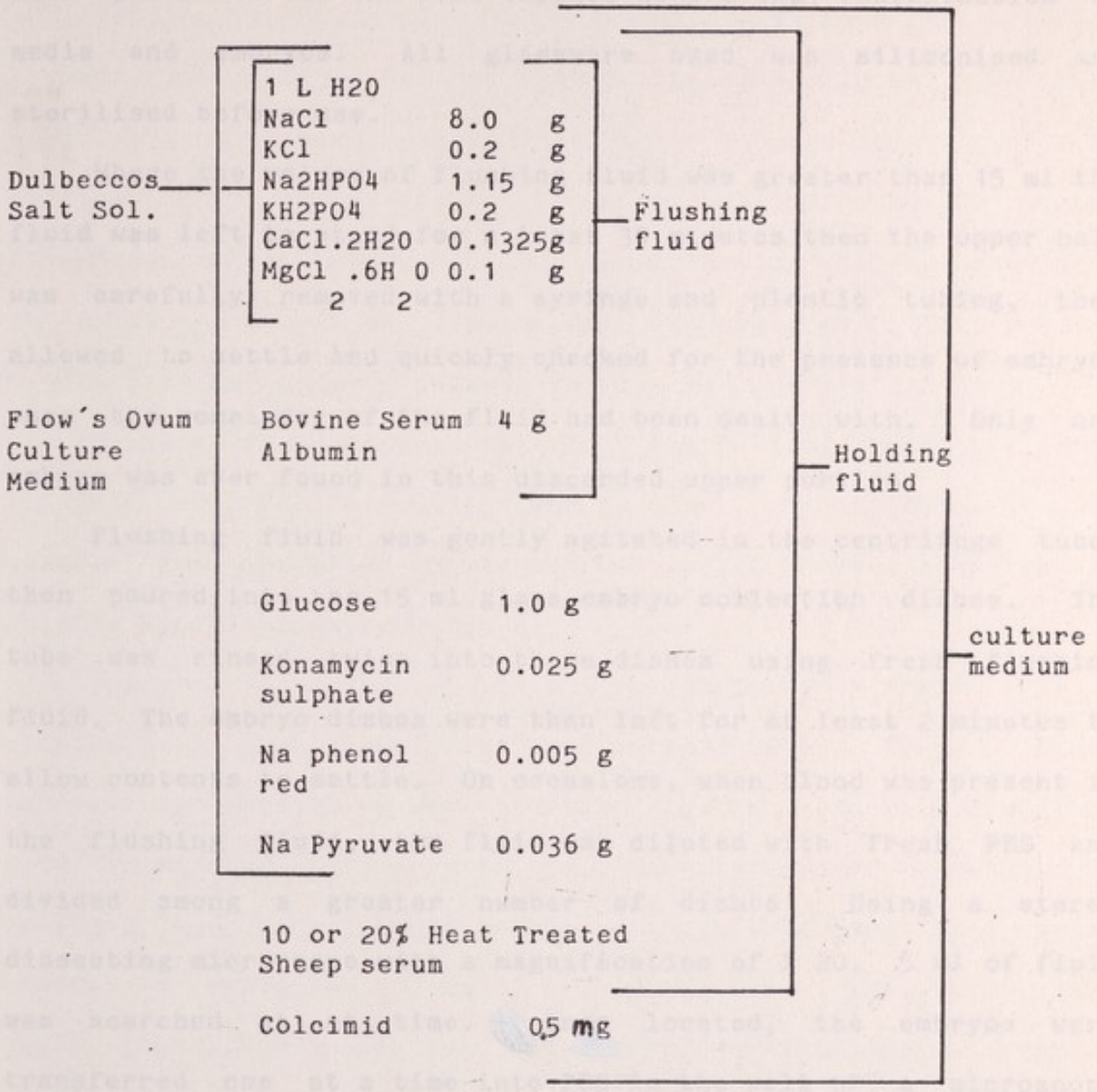
Pipette drawn at both ends - one to fit rubber tube to attach a 1 ml syringe

Microscope slides with a 10 mm fused on cell

Swinnex filters

Incubator - 38 °C

Media Composition



Method

Flushing fluid was collected into a warm sterilised 50 ml round bottomed centrifuge tube. This tube was labelled, sealed and placed in an incubator at 38°C until examined, usually within 3 hours of collection.

Wherever possible all fluid and vessels were kept at 38°C. Room temperature never fell below 20°C and thus was the minimum temperature the embryos reached. All manipulations where possible

took place in the air flow cabinet to prevent contamination of media and embryos. All glassware used was siliconised and sterilised before use.

Where the volume of flushing fluid was greater than 15 ml the fluid was left to stand for a least 30 minutes then the upper half was carefully removed with a syringe and plastic tubing, then allowed to settle and quickly checked for the presence of embryos once the remainder of the fluid had been dealt with. Only one embryo was ever found in this discarded upper portion.

Flushing fluid was gently agitated in the centrifuge tubes then poured into the 15 ml glass embryo collection dishes. The tube was rinsed twice into these dishes using fresh flushing fluid. The embryo dishes were then left for at least 2 minutes to allow contents to settle. On occasions, when blood was present in the flushing fluid, the fluid was diluted with fresh PBS and divided among a greater number of dishes. Using a stereo dissecting microscope with a magnification of X 20, 5 ml of fluid was searched at at time. Once located, the embryos were transferred one at a time into PBS in the well of a microscope slide with a 10 mm diameter fused cell. The embryo was then examined under X 125 and photographed. The embryo was exposed to bright light for as short a time as was feasable, returned to the flow cabinet, then transferred into approximately 1 ml of holding fluid in a glass embryo collection dish (4 ml). Up to a maximum of 5 embryos were placed in each dish which was then covered by another embryo dish and placed in a humidified atmosphere until all the embryos were recovered.

Embryo Culture

Holding Fluid was made up on the day of culture from freshly thawed Flow's Ovum Culture Medium plus either 10 or 20% Sheep serum which had been held at 55-60°C for 30 minutes. The heat treated sheep serum was either used freshly treated or stored frozen after heat treatment. Any serum contaminated by haemolysed red cells was discarded. The holding fluid was sterilised through a 0.22 u filter using a swinnex (Millipore) and kept at 37°C.

Culture fluid was identical to the holding fluid but with colcimid added before filtration to make a concentration of 0.5 ug/ml. The intial pH of the medium was 7.2. This rose during culture but never exceeded pH 7.7.

Embryos were kept in the holding fluid for a period from 5 minutes to 3 hours then transferred to the culture fluid. Where embryos had hatched or were hatching from the zona pellucida they were considered more susceptible to the trauma of handling so colcimid was merely added to the holding fluid containing these embryos to remove the necessity of moving them. The dishes were again closed and incubation took place in a humidified atmosphere of 16% O_2, 4% CO_2, 80% N.

In the initial experiments, following the method employed by King *et al*, embryos were incubated for approximately 4 hours. Later this was extended to 6-9 hours with an improvement in the number of embryos producing mitotic spreads. In the final stages of the work, incubation in the colcimid solution took place for up to 24 hours. Initially there was some concern that a long period in colcimid would reduce the quality of any metaphase spreads obtained as found by Ford and Hamerton (1956) but the

variation in quality of the metaphase spreads obtained was so wide from apparently identical treatments that no difference was noted with the longer culture time although the number of embryos producing mitoses rose.

SECTION 2.1.5

Harvesting of Embryos

Materials

Hypotonic solution	1 g KCl in 600 ml deionised water
1st fixative solution	1:1 methanol : glacial acetic acid
2nd fixative solution	3:1 methanol : glacial acetic acid
Stain	Gurr improved R66 Giemsa stain : 10 ml in 100 ml Giemsa Buffer

Equipment

Stereo microscope, Binocular microscope with camera, microscope slides with well, twin frosted microscope slides, drawn pipettes, coverslips, DPX mountant.

Methods

This method is based on that developed by King et al (1979).

Embryo dishes were removed as required from the incubator and the embryos manipulated, using a drawn pasteur pipette attached to a 1 ml syringe, into 0.2 ml hypotonic solution in the well of a microscope slide with a 10 mm diameter fused cell. The embryo was immediately photographed and its appearance and stage of growth noted. All procedures took place at room temperature. Hypotonic treatment lasted for 10-12 minutes for embryos of 8 cells or less, 15 minutes for morulae and early blastocysts and 20 minutes for expanded and hatched blastocysts. Embryos other than those which had hatched from the zona pellucida were transferred immediately to fresh hypotonic solution.

Twin frosted microscope slides were cleaned with 70% ethanol

and labelled. The embryo was moved from hypotonic treatment and placed in the centre of the slide in only a microdot of hypotonic fluid. Without allowing this to dry out, fixative solution was dropped from a height of 2-4 inches onto the embryo. A drawn pasteur pipette was used to give very small drops. One drop was drawn every 4-5 seconds and the slide was gently blown to spread the blastomeres. As soon as the embryo was seen to break up (1-4 drops), the cells were spread with a gentle blow and the slide left to dry. Once dry, the slides were placed in the second fixative solution for 2-12 hours, then removed, air dried and stained for 30 minutes. The stain used was Gurr's Giemsa diluted 1 in 10 with buffer, freshly made up and filtered before use. The slides were quickly assessed for staining depth, then mounted using DPX.

Once the DPX had hardened - at least 24 hours - the entire slide was scanned under X 100, the numbers of blastomeres counted, their spread and appearance noted and the position of any mitotic spreads noted.

Other fixatives and methods were tried to obtain consistently karyotypable metaphase spreads, including a drop of acetic acid only, followed by 2 drops of methanol as the first fixative, methanol only, ethanol only and formaldehyde, but the results were very inconsistant.

SECTION 2.1.6

EWE EXPERIMENTS RESULTS

Synchronisation

97 ewes in the experimental and control groups were synchronised using the method described above. 28 of these ewes were in seasonal anoestrus. Of these 97, 1 did not come into oestrus, 92 ovulated and 4 artificially inseminated controls returned to oestrus, so ovulation was not definitely established.

97 ewes synchronised →
- 12 Control, Nat. Mated Ewes - all lambed to induce oestrus
- 12 Control A.I. ewes - 8 lambed to induced oestrus, 4 returned to service
- 11 surgically flushed ewes 1st breeding season - all with corpora lutea
- 28 sugically flushed ewes or laparotomised controls, summer season. Corpora lutea present in all ewes
- 34 ewes surgically flushed or laparotomised 2nd breeding season - 33 came into oestrus and corpora lutea present

One of the 66 naturally mated ewes was not in standing oestrus until 8 hours after the introduction of the tup, otherwise all ewes were in standing oestrus at the time of tup introduction, approximately 50-54 hours after progesterone sponge withdrawal and PMSG administration. It can be concluded that the method used was very satisfactory in inducing and synchronising oestrus.

Corpora Lutea

Corpora lutea present were counted on laparotomy, the means and standard deviations calculated and presented in the table below.

TABLE 3 Variation of Corpora Lutea numbers with season

Season	Ewe Numbers	Corpora Lutea	Mean	Std. Dev.
Autumn 1981	11	36	3.27	± 1.31
Summer 1982	28	93	3.32	± 0.6
Autumn 1982	34	84	2.47	± 0.36
Total	73	215	2.95	± 0.31

Using Students t-test, the numbers of corpora lutea produced per ewe in the autumn of 1982 are significantly fewer than in the summer season 1982 ($t = 2.58$ sign. at $p = 0.05$). There is no statistically significant difference between the two breeding season corpora lutea levels, probably due to the lower sample number in the Autumn of 1981 ($t = 1.94$, $P < 0.1$).

The mean corpora lutea counts and standard deviations were calculated for the oestrus the first time ewes were synchronised and subsequent 2nd or 3rd synchronisations.

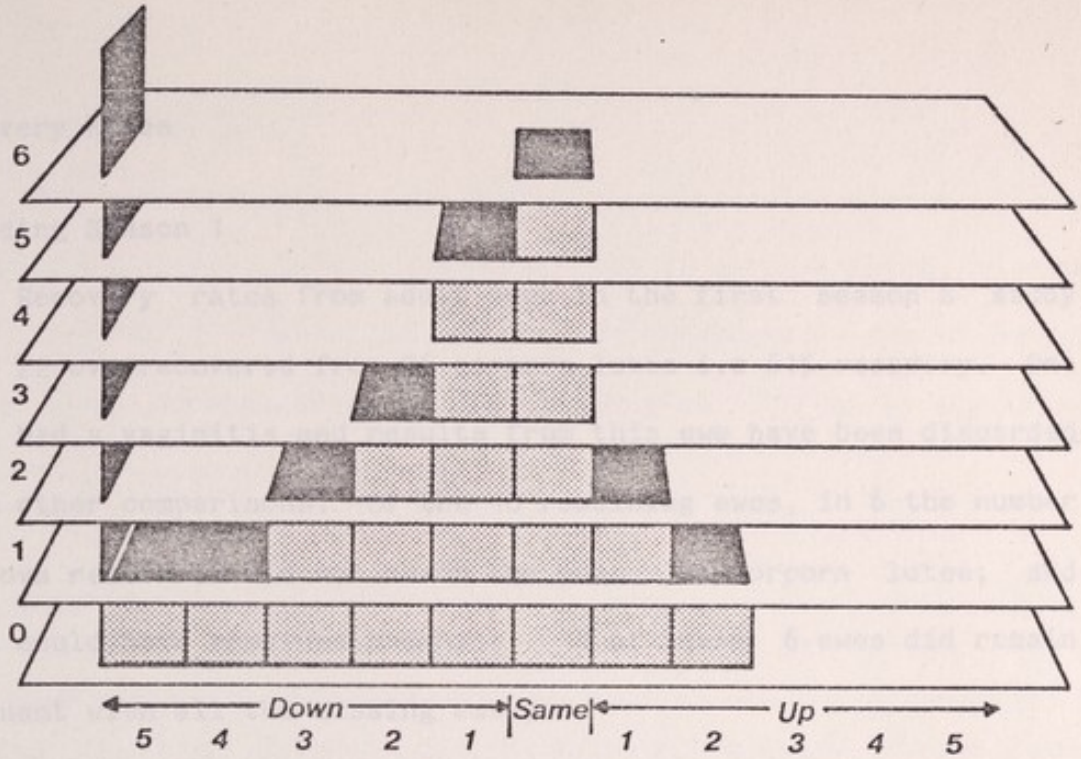
When the mean corpora lutea counts of 1st synchronised oestrus and subsequent synchronised oestrus were compared the difference is significant ($t = 2.06$, $P < 0.05$)

TABLE 4 Corpora Lutea Counts after Different Numbers of Synchronisations

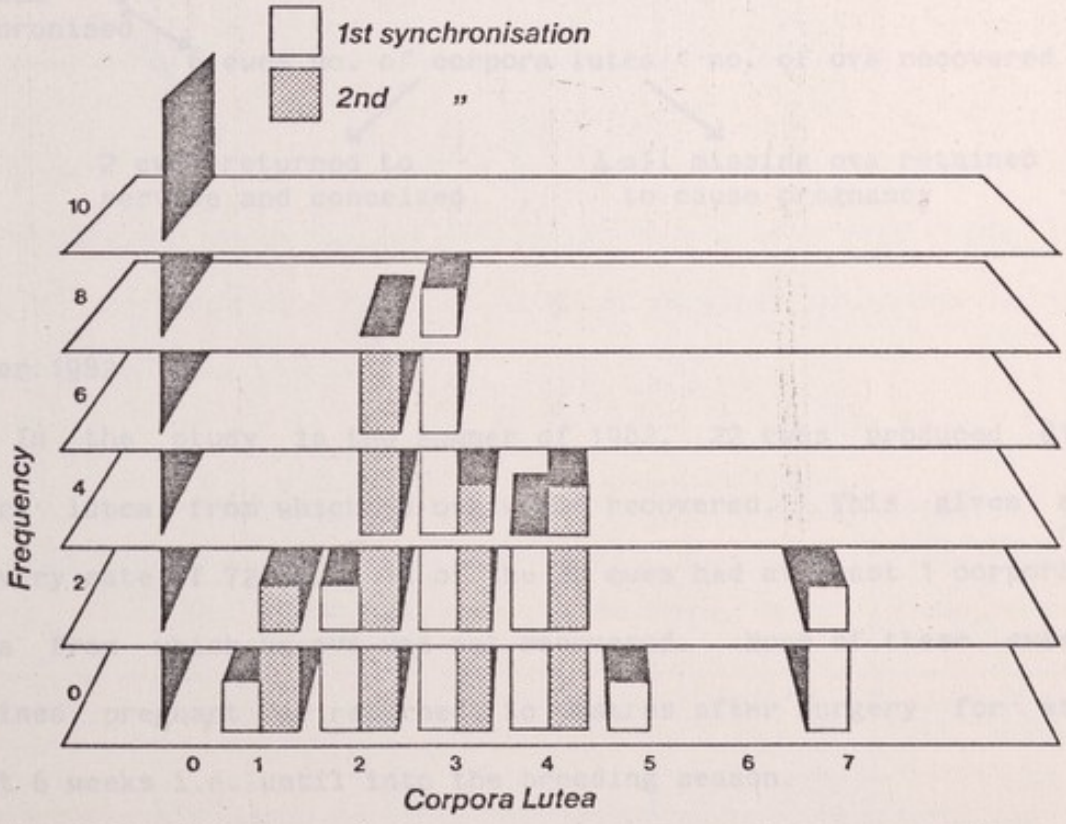
	No. of ewes	Corpora Lutea	Mean	Std. Dev.
1st synchronisation	34	117	3.44	± 0.539
2nd synchronisation	17	46	2.71	± 0.507
3rd synchronisation	6	15	2.50	± 1.1
2nd and 3rd	23	61	2.65	± 0.425

When the repeatability of the corpora lutea counts from individual ewes are assessed 13 out of 21 ewes produced the same number of copora lutea, or one more, or less, on the second synchronisation as the first. In 8/21 the difference was 2 or more (see histogram 1).

However no real conclusions can be drawn from these as the number of ewes producing 2, 3 or 4 embryos was so great (76% 1st synch., 85.7% 2nd synch.) that the majority of ewes were going to fall into these catagories on both flushes and thus show little variation of response (see histogram 2).



Histogram 1 The number of ewes with the same or changing corpora lutea counts on the first and second synchronised oestruses



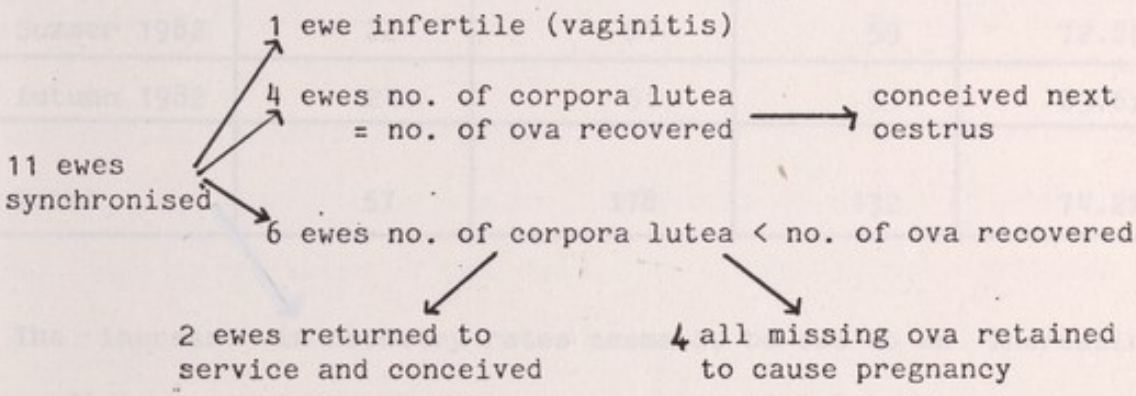
Histogram 2 Frequency of corpora lutea numbers at the first and second synchronised oestruses



Recovery Rates

Breeding Season 1

Recovery rates from adult ewes in the first season's study were 22 ova recovered from 36 corpora lutea i.e 61% recovery. One ewe had a vaginitis and results from this ewe have been discarded from other comparisons. Of the 10 remaining ewes, in 6 the number of ova recovered did not reach the number of corpora lutea, and thus could have remained pregnant. 4 of these 6 ewes did remain pregnant with all the missing embryos.



Summer 1982

In the study in the summer of 1982, 22 ewes produced 81 copora lutea from which 59 ova were recovered. This gives a recovery rate of 72.8%. 14 of the 22 ewes had at least 1 corpora lutea from which an ova was not recovered. None of these ewes remained pregnant or returned to oestrus after surgery for at least 6 weeks i.e. until into the breeding season.

Breeding Season 2

The recovery rate in the study in the breeding season of 1982 reached 83.6% i.e. 51 ova recovered from 61 corpora lutea. 9 ewes had corpora lutea where an ova was not recovered. One of these ewes died during induction of anaesthesia. Of the 8 remaining ewes only 1 lambed the missing embryo.

TABLE 5 Recovery Rates From All Adult Ewes

Season	No. of ewes	Corpora lutea	Ova Recov.	Recov. Rate
Autumn 1981	11	36	22	61%
Summer 1982	22	81	59	72.8%
Autumn 1982	24	61	51	83.6%
Total	57	178	132	74.2%

The increase in recovery rates seems to be due to an increasing proficiency in the technique as the increase in recovery rates is greater when only the first occasion of surgical interference in an individual ewe is examined.

TABLE 6 Recovery Rates From Ewes on the 1st Surgical Recovery

Season	No. of Ewes	Corpora Lutea	Ova. Recov.	Recov.Rate
Autumn 1981	11	36	22	61%
Summer 1982	16	61	43	70.5%
Autumn 1982	7	20	18	90%

An analysis was made on the recovery rates the first time a ewe was flushed and on subsequent surgery. The recovery rates were found to be not significantly different ($X^2 = 0.8$)

TABLE 7 Recovery Rates from Ewes Flushed More Than Once

Flush No.	Ewes	Corpora Lutea	Ova Recov.	Recov. rate
First	17	65	46	70.8%
Second	17	46	37	80.4%
Third	6	15	12	80.0%

The slight increase in success in the ewes flushed for the 2nd or 3rd time is likely to be due to increased proficiency in technique not to an alteration in the population studied as the difference in recovery rates from the 1st flush ewes in Autumn 1981 and 1st flush ewes in Autumn 1982 is even greater.

Fertilisation Rate

132 ova were recovered from adult ewes over the 3 study periods. Fertilisation rates were compared between ova recovered from ewes naturally mated and those artificially inseminated. 24/44 ova were fertilised in the A.I. group and 70/87 were fertilised in the natural mating group, giving a percentage fertilisation of 54.5% for A.I. against 80.5% for natural mating. This difference is highly significant at $P = 0.01$ ($X^2 = 8.45$).

The majority of the unfertilised embryos from natural service

occurred when a Leicester tup was used for a week on ewes in the summer study. Although he had sired lambs the previous breeding season and physically his testicles appeared to be normal, his fertility fell through the week. In the first 2 days, only 1 ova out of 12 recovered was unfertilised - 92% fertilisation which is similar to 96% fertilisation of other tups used. However the Leicester tup's fertilisation rate on the succeeding 3 days fell to 36%, 8/22. This fertilisation rate makes it impossible to draw any meaningful conclusions on the unflushed controls mated by the Leicester tup.

When fertilisation rates from A.I. and natural mating are compared in each season, lower statistical significance is seen probably due to reduced numbers.

TABLE 8 Fertilisation Rates

	Natural Service	Artificial Insemination	Difference
Breeding Season 1981	14/14 (100%)	4/7 (57.1%)	$X^2 = 6.52$ (P<0.05)
* Summer 1982	12/13 (92%)	6/12 (50%)	$X^2 = 3.64$ (0.1>P>0.05)
Breeding Season 1982	25/26 (96%)	14/25 (56%)	$X^2 = 9.3$ (P<0.01)
*All	51/53 (96.2%)	24/44 (54.5%)	$X^2 = 21$ (P<0.001)

* Excluding subfertile Leicester tup

Fertilisation rates in and out of breeding season were assessed. Combining figures for A.I. and natural service, the total fertilisation rate for out of breeding season was 37/59, 62.7%, and in breeding season 57/72, 79.2%. This is just significant at $P = 0.05$ ($X^2 = 3.9$). However if the figures for the Leicester tup are removed the significance disappears ($X^2 = 0.2$), 18/25 (72%) vs 57/72 (79.2%).

When the figures are broken down into natural service and A.I. there is no difference between fertilisation rates in A.I. in or out of breeding season, or in natural service in or out of breeding season if the Leicester tup's results are removed. If however they are retained, fertilisation is significantly lower in naturally mated ewes mid summer than peak breeding season ($X^2 = 11.74$, sig at $P = 0.001$).

Semen Diluent

In the Breeding Season of 1982 a different semen diluent was assessed. No statistical difference was found in fertilisation rates using egg yolk diluent in all 3 seasons and heat treated milk diluent in the autumn of 1982 ($X^2 = 0.05$).

TABLE 9 Fertilisation Rates with Different Semen Diluents

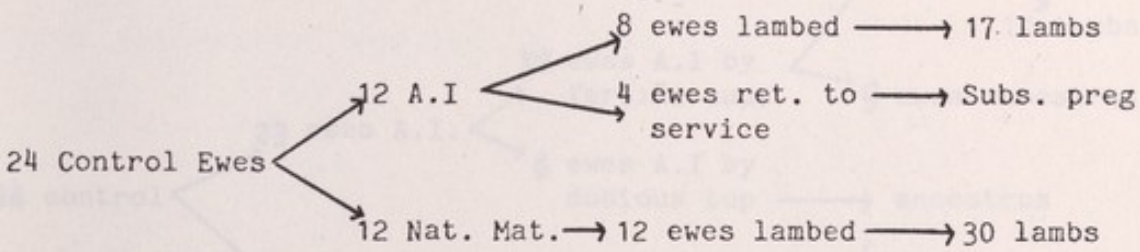
Diluent	Fertilisation Rate (%)
Egg yolk	11/24 58.3%
H.T. milk	10/20 50.0%

Control Ewes

Breeding Season 1

In the autumn 1981 study 24 controls were treated identically to the surgical group, 12 artificially inseminated and 12 naturally mated. All 12 of the naturally mated group held to first service. 8 of the 12 A.I.'d ewes held to the first service. The remaining 4 ewes were proven fertile as they held to natural service at their next oestrus. This gives a conception rate of 100% for natural service and 67% for A.I. This difference is not significant statistically at $P = 0.05$ ($X^2 = 2.7$).

30 lambs were born to the naturally mated control group and 17 to the A.I. control group giving 2.5 and 2.125 lambs per ewe lambing and 2.5 and 1.42 lambs per ewe synchronised.

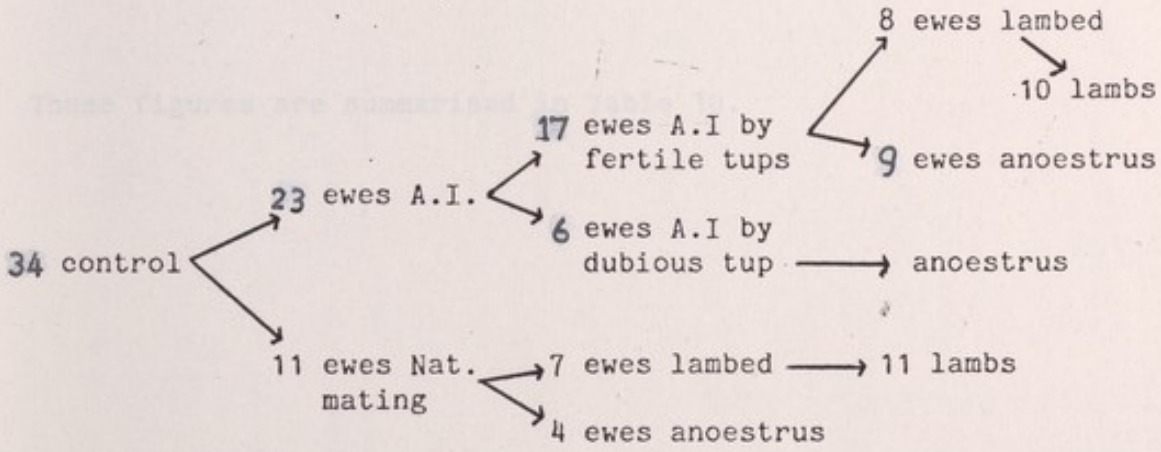


The ratio of the number of lambs born to the artificially inseminated ewes and the naturally mated ewes 17:30 i.e 57% is identical to the ratio of fertilisation rates in the surgical group 57%: 100%.

Summer 1982

In August 1982 the control group was not on the same premises as the surgical group and were in slightly better condition, but

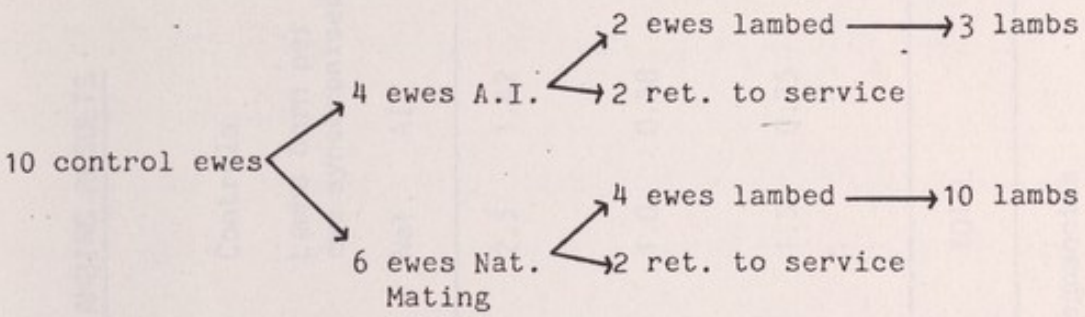
otherwise were treated the same. The conception rate to natural service was 63.6% and lambing rate per ewe served 1.0 and per ewe lambed 1.57. The conception rate to A.I. was 34.8%, lambing rate per ewe synchronised 0.46 and per ewe lambed 1.25. These artificial insemination results are low partly due to the effect of one tup which, despite having semen assessed as satisfactory, failed to get any ewes pregnant from A.I. 0/10 (4 of these ewes were in a different experiment). Removing his figures, the A.I. figures are conception rate 47%, lambs per ewe synchronised 0.58, lambs per ewe lambed 1.25. The artificial insemination conception rates may also be low because semen collected by electroejaculation was used rather than from collection by artificial vagina.



The ratio of fertilisation rates from A.I to natural mating (excluding Leicester) in the flushed sheep is 54% and the ratio of lambs per ewe synchronised in the A.I. control group to the natural mating control group of 58% is very similar.

Breeding Season 2

In the autumn of 1982 there were 10 control ewes. 6 were mated naturally and 4 A.I.'d. 4 of the 6 naturally mated ewes were pregnant and lambed 2.25 lambs per ewe lambing and 1.5 per ewe mated. 2 of the 4 A.I.'d ewes remained pregnant and 3 lambs were born giving 1.5 lambs per ewe lambing and 0.75 lambs per ewe synchronised.



These figures are summarised in Table 10.

TABLE 10 LAMBING RESULTS

Season	Controls Conception Rate Nat	AI	Controls Lambs born per ewe lambing Nat	AI	Controls Lambs born per ewe synchronised Nat	AI	Flushed Ewes Ratio of fert. rates AI	Nat	Controls Ratio of lambs born per ewe synchronised AI	Nat
Autumn 1981	$^{12}/_{12}$ (100%)	$^{8}/_{12}$ (67%)	2.5	2.125	2.5	1.42		57%		57%
Summer 1982	$^{7}/_{11}$ (63.6%)	$^{8}/_{17}$* (47%)	1.57	1.25	1.0	0.58		54% **		58%
Autumn 1982	$^{4}/_{6}$ (66%)	$^{2}/_{4}$ (50%)	2.25	1.5	1.5	0.75		58%		50%
					TOTAL			56.3%		55%

* Excluding results from tup with $^{0}/_{10}$ pregnancies

** Excluding subfertile Leicester tup

There is a remarkable agreement between the ratios of fertilisation rates of AI to natural service and the ratio of lambs born from control ewes AI'd or naturally mated. This suggests the difference in the proportion of ova fertilised by both methods is constant despite season, and fertilisation rates were similar in control and surgically flushed groups.

Early Embryonic Death Estimates

If the number of corpora lutea present in the control and experimental groups can be assumed to be approximately equal an estimate of early embryonic death can be obtained for the different seasons.

In the autumn of 1981, 12 naturally mated ewes produced 30 lambs, where they may have been expected to produce 39 corpora lutea with a 100% fertilisation rate. This gives an early embryonic death estimate of $^{9}/_{39}$ = 23%.

From 12 AI ewes there were 17 lambs from an estimated 39 corpora lutea. If the fertilisation rate was 57% (see Table 8), 22 lambs might have been produced. This gives an early embryonic death estimate of $^{5}/_{22}$ = 23%.

In the summer season 11 lambs were produced from 11 ewes naturally mated where 36 corpora lutea and a fertilisation rate of 92% may have been expected to produce a possibility of 33 lambs. This gives an embryonic loss of $^{22}/_{33}$ (67%).

From 17 ewes artificially inseminated 10 lambs were born. As 56 corpora lutea and a fertilisation rate of 50% could be expected there was a possibility of 28 lambs. This gives an estimated of embryonic death of $^{18}/_{28}$ (64%).

In the autumn season of 1982 in the naturally mated laparotomised group 9 lambs were produced from 16 copora lutea with an estimated fertilisation rate of 96% giving an embryonic death rate of $^{6}/_{15}$ (33%).

7 corpora lutea were produced by the 4 artificially inseminated ewes from which 3 lambs were born. Fertilisation rate can be estimated at 56% giving an embryonic death rate of $^{1}/_{4}$ (25%).

The figures are summarised in Table 11.

TABLE 11

EMBRYONIC DEATH

		No. Ewes Mated	No. Lambs	**Est. C.L. Number rate	Est. Fert Rate	Est. E.D
Autumn 1981	Nat. Service	12	30	3.27	100%	(^{9}/39) 23%
Autumn 1981	A. I.	12	17	3.27	57%	(^{5}/22) 23%
Summer 1982	Nat. Service	11	11	3.32	92%	(^{22}/33) 67%
Summer 1982	A. I.	*17	10	3.32	50%	(^{18}/28) 64%
Autumn 1982	Nat. Service	6	9	1.78	96%	(^{5}/15) 33%
Autumn 1982	A. I.	4	3	1.75	56%	(^{1}/4) 25%

* Ewes inseminated by infertile tup removed

** Estimated in Autumn 1981 and Summer 1982 from flushed and laparotomised ewes

There was no difference between artificial insemination and natural service embryonic death rate figures for each season so the data was combined. Using the X^2 test there was no significant difference between the embryonic death rate in the 1981 and 1982 breeding seasons ($X^2 = 0.1$) but the summer season embryonic death rate was significantly higher than either breeding season ($X^2 = 20.7$ sig at $P < 0.001$ and X^2 6.38 sig at $P < 0.05$).

It was noted in the laparotomised ewes in the autumn of 1982 and the two in the summer of 1982 naturally mated by a fertile tup that pregnancy tended to occur with all the embryos produced or none at all.

TABLE 12 Lambing Results from Naturally Mated Laparotomised Control Ewes

Season	Summer		Breeding Season					
Ewe No.	49	99	55	98	58	1	9	25
Corpora Lutea	4	2	4	3	2	2	3	2
Lambs to that oestrus	0	2	4	0	2	1	0	2

Ewes 99, 55 and 98 were laparotomised at day 28 as well as day 5, to check numbers of foetuses to guard against foetal loss possibly due to overcrowding being included as embryonic loss. Ewe 98 had no evidence of embryos <u>in utero</u> but still had 3 persistant corpora lutea and did not in fact return to oestrus that breeding season.

All 5 of the ewes which remained pregnant after a flush which did not recover an ova for every corpora lutea produced retained

and lambed all the missing embryos. Thus it is possible that the "all or nothing" effect observed above is seen in these ewes also.

Anoestrus

After the induced oestrus in the summer study it was noted that only 2 ewes returned to standing oestrus with the vasectomised tups in both the surgical group and the control group. Of the 28 ewes synchronised in the surgical groups only 2 remained pregnant and produced lambs. A further 2 ewes had high levels of progesterone (>5 nmol/l) 18/19 days after induced oestrus but these had fallen by day 27 without an oestrus period being shown. Of the 74 ewes synchronised off the University premises, 34 were given 750 i.u. PMSG and 40 given 375 i.u PMSG, 2 were marked by vasectomised tups as returns to oestrus and 27 subsequently lambed. Of a sample of 26 bled for progesterone analysis on day 26, 3 of the ewes which had high progesterone levels did not lamb, 2 of these cycled in November. A further 5 of these 74 ewes which were unmarked by tups in the breeding season did not lamb.

This leaves 21 ewes in the surgical group (3 had died or been culled) and an estimated half of the farm group which lapsed into anoestrus - probably by day 19.

Sheep Embryo Culture Results

94 fertilised embryos were recovered from adult ewes, 89 of which were processed for chromosome analysis. 3 embryos were lost during handling or culture and 2 were cultured in media that became contaminated with fungus and it was found impossible to

retrieve them.

A comparison was made between different times of culture in a colcimid solution. There was found to be no statistically significant difference in the number of embryos producing metaphase spreads in different culture times (X^2 = 0.051).

TABLE 13 Effect of Culture Time on Cell Division

	4-6 hr	6-9 hr	15-24 hr
No. of Embryos	16	36	37
No. Producing Metaphase Spreads	4	8	12
% Production of Metaphase Spreads	25%	22%	32%

The ability of embryos recovered in and out of the breeding season to produce metaphase spreads on culture was compared.

TABLE 14 Effect of Breeding Season on Cell Division

	Breeding Season	Summer
No. of Embryos	54	35
No. producing metaphase spreads	15	8
% producing metaphase spreads	28%	23%

Using the X^2 test there is no significant difference between the number of embryos producing metaphase spreads in peak breeding season and summer (X^2 = 0.07).

The number of embryos producing metaphase spreads at different stages was assessed.

TABLE 15 Effect of Stage of Development on Cell Division

	4-16 cell	Morula	Blastocyst.
No. of Embryos	28	45	16
No. producing metaphase spreads	8	11	6
% producing metaphase spreads	28.6%	24.4%	37.5%

Using X^2 there was no significant difference between these groups ($X^2 = 0.46$).

Morphological abnormality

Embryos from ewe lambs were included in this comparison. Embryos were classified in the process of degeneration (IPD) if blastomeres were detached, 2 separate groups of blastomeres were developing, blastomeres were of grossly different sizes or the appearance of a morula was messy with no space between the blastomere group and zona (Linares and King, 1980).

Using these criteria, 58 embryos were considered normal, 25 IPD, 4 were considered IPD before culture but improved during culture and one was considered totally degenerated.

Culture results are shown in the table below.

TABLE 16 Effects of Morphology on Cell Division

	No. of embryos	No. with growth	% with Metaphase
Normal	58	18	31%
IPD	25	8	32%
IPD but improved	4	2	50%
Degen.	1	0	0

There was no significant difference between the numbers of embryos producing metaphase spreads in the different groups ($X^2 = 0.03$).

Tup Effect

Several different tups were used in this experiment. A comparison of the proportion of embryos producing metaphase spreads sired by different tups is shown in the table below. Embryos from ewe lambs are not included in this table.

TABLE 17 Effect of Sire on Cell Division

Tup No.	No. of fert. emb.	No. Prod. metaph. spr.	%	
Suffolk 101	54	19	19/54	35.2%
Suffolk 106	1	0	0/1	0
Leicester	19	2	2/19	10.5%
Finn Dorset B	2	1	1/2	50%
Texel	8	1	1/8	12.5%
Finn Dorset A	3	0	0/3	0
All but Suff.101	33	4	4/13	

The difference in the number of embryos sired by Suffolk 101 producing mitoses and those sired by the Leicester tup, the other tup used frequently, just fails to be statistically significant at $P = 0.05$ ($X^2 = 3.05$). However if the embryos sired by Suffolk 101 are compared with all the others combined there is a statistically significant difference in the numbers producing metaphase spreads ($X^2 = 4.47$ $P < 0.05$). Suffolk 101 was used with the majority of the ewes in both breeding seasons and with half of the ewes in the summer study. Suffolk 106 and Finn Dorset A were used in the autumn of 1981 and the Texel and Finn Dorset B in the autumn in 1982. The Leicester tup was used on half the ewes in the summer study. Thus only 18 of the 37 fertilised embryos recovered in the summer study were by the Suffolk tup. This explains perhaps the difference (not significantly great to be statistically significant) between the percentage of in season and out of season embryos producing metaphase spreads (See Table 14).

The reason for the apparent difference in the ability of embryos sired by Suffolk 101 and other sires to produce metaphase spreads cultured under these conditions is not known.

SECTION 2.1.7

DISCUSSION

Synchronisation

The induction and synchronisation regime used in these studies was found to be particularly effective. 96 out of 97 ewes, where oestrus was checked either by observed natural mating, or by laparotomy and observation of recent corpora lutea, responded successfully to induction of oestrus. Synchronisation was also very effective. This is a general finding with this type of regime. Quirke (1979) found induction and synchronisation of oestrus using this method 100% effective in July and October and only sligtly less so in April. Pregnancy rates reported by Quirke in the peak and prebreeding season were very close to those found in the control ewes in this study, though average litter sizes for both prebreeding season and peak breeding season were slightly lower in his study. Conception rates are generally found to be lower at the induced oestrus than the succeeding oestrus at 60% v 85% (Hulet and Shelton, 1980). Quinlivan and Robinson (1969) have suggested this may be due to interference with sperm transport and survival in the progestagen treated ewes as they found decreased numbers of sperm in the reproductive tract in these ewes.

It is of interest to note that the majority of non-pregnant ewes in the very large study by Quirke (1979) did not become anoestrus after the induced oestrus but returned to service. Anoestrus was common in the present study during the summer study when only 2 out of 102 ewes of the surgical group and the control group returned to service and only 29 of the remaining 100 lambed.

This difference may have been a breed effect as the ewes used by Quirke were of the Galway breed and those in this study of mixed, predominantly Scottish Greyface breeding. However, Gordon (1974) found in Ireland the Scottish Greyface and Halfbreed performed better than Galway ewes in out of breeding season programmes. Anoestrus after an induced oestrus in June, July and August was not a problem in his studies. The difference in performance between the ewes in this study and the work reported by Quirke and by Gordon may be due to climatic differences or the weather and light effect of a particular season. The variation in onset of cyclic activity in Greyface ewes in October is attributed to the effect of an individual season.

The exact timing of the lapse into anoestrus is unfortunately unknown. As progesterone levels were low with no oestrus behaviour in the surgical groups on days 16-19, anoestrus could have occurred after regression of corpora lutea with normal lifespans, or after premature regression of corpora lutea. 17 ewes were sampled on day 5 after the induced oestrus and 16 of these had raised levels of plasma progesterone indicating luteal function (> 2.3 nmol/l). The seventeenth ewe had only slightly raised levels at 1.6 nmol/l and thus could have had a regressing corpora lutea or progesterone levels may have only started to rise. Further studies involving frequent blood sampling for progesterone analysis would be of interest in elucidating this problem. Willasden (1979) reports premature luteal regression as a problem in ewes superovulated with PMSG and given prostaglandins 48-72 hours later, and also occasionally in others. Luteal

regression occurred on day 5-7. Knight, Tervit and Fairclough (1981) report premature luteal regression day 4-10 in half a sample of out-of-breeding-season-anoestrus ewes stimulated to ovulate by the presence of rams.

Ovulation Rates

A mean ovulation rate of 2.9 (range 1-7) indicates that the dose of PMSG given did not in general cause superovulation, though ovulation rates were higher than might be expected from a natural oestrus. Ovulation rates for greyface ewes are influenced by a large number of factors including age, nutrition, condition and stage in the breeding season (Gunn and Doney, 1975; Hulet and Shelton, 1980) but a mean for this breed would be expected in the range 1.75-2.3 (Gunn, Doney and Smith, 1979).

Using superovulatory doses of PMSG, 1,000 - 5,000 i.u., a very much greater variability in corpora lutea numbers is found (Willasden, 1979) with ovulation numbers varying from less than 5 to greater than 20. Within and between animal variations in response to PMSG were noted by Trounson (1983) and have caused difficulties in the reliable production of large numbers of embryos for transfer or experiment. Trounson suggests this variation in response to PMSG may be due to individual variation in the number of follicles capable of responding to PMSG. Perhaps because the dose of PMSG used in this study was low, in those animals where at least 2 synchronisations were performed 81% of the ewes fell in the range 2-4 corpora lutea. Thus within animal variation was low in this study.

The mean ovulation rate, however, was significantly lower in

the second and third synchronisation than the first. As 23 of the 36 ewes used in the second breeding season study had been synchronised within the preceeding 4 months, this may be the reason for the lower ovulation rates in the breeding season of 1982 than that of 1981. Saumande, Chupin, Mariana, Orlavant and Mauleon (1978) found in heifers, ovarian response to PMSG declined over 4 or 5 administrations then started to rise slowly. Seasonal factors were shown not to influence this and the author suggested possible mechanisms were exhaustion of follicles capable of responding to stimulation or formation of anti PMSG antibody. Saumande was unable to detect the presence of these antibodies. However Jainudeen, Hafez, Gollinck and Moustaya (1966) reported the presence of anti PMSG antibodies, formed after 2 PMSG injections 6 months apart in heifers, that appeared to block the ovarian response to exogenous PMSG and not to endogenous gonadotrophins. Boland and Gordon (1982) found no difference in the mean ovulation rate from ewes treated for the first, second or third times with horse anterior pituatary extract - an alternative source of exogenous FSH. Is is possible that this or some other source of exogenous FSH may prove to give a more repeatable superovulatory response than PMSG.

Recovery Rates

Recovery rates obtained in these studies, particularly in 1982, compared very well with those reported by other authors. Hanrahan and Quirke (1980) report recovery rates of 78 and 79% and Wright, Bondioli, Grammer, Kuzan and Menino (1981) reported recovery rates of 62 and 70%. Boland, Crosby and Gordon (1983)

found recovery rates were higher using local rather than general anaesthesia but the rate of 70% was still below those obtained in this study. Boland and Gordon (1982) report a decrease in the percentage ova recovered in sequential surgical flushes with a 65.3% success on the first occasion and 22.9% on the third. This was not noted in the present study where recovery rates were over 70% for the first and 80% for subsequent flushes.

This rise in the recovery rate for second or third flushes was not due to an intrinsic property of the sheep but due to an increasing proficienct of the surgeon. When the data for ewes flushed for the first time in the first and third studies are compared there is a difference of nearly 30% in the recovery rates (61% and 90%). Indeed there is a very slight, but not statistically significant ($X^2 = 0.03$) reduction in the recovery rates of ova from sheep flushed for the third time (80%) compared with sheep flushed for the first time (90%) during the 2nd breeding season study.

It was found, particularly in the first surgical recovery study, that embryos left in the uterus after surgical flushes, where fewer ova were recovered than corpora lutea counted, in 18% of ewes retained the ability to cause a pregnancy. This is a recognised phenomenon and Willasden (1979) in his review article on embryo transfer in sheep recommends that prostaglandin F2α should be given if a ewe is to be reused as a donor. Pregnancy from embryos left in the uterus after flushing was more common in the first season (66.7%), probably because only the upper third of the uterine horn was flushed for day 4 recovery, whereas in the 1982 studies almost the entire horn was flushed for day 5

recovery. It is possible that even on day 4, embryos had passed further down the horn than anticipated and thus were missed causing the lower recovery rate obtained on that day. There may also be a seasonal effect as none of the 14 possible ewes in the summer study remained pregnant whereas 1 of 8 in autumn 1982 did so. Thus $^{0}/_{14}$ out of breeding season and $^{5}/_{14}$ in the breeding season remained pregnant ($X^2 = 4.0$; $P < 0.05$).

In the second breeding season study, laparotomy and visual inspection of the ovaries were carried out in the control animals. $^{4}/_{6}$ of the naturally mated ewes and $^{2}/_{4}$ of the artificially inseminated ewes lambed to this service. This is not significantly lower ($X^2 = 1.0$) than the very good results obtained from the control ewes the previous breeding season ($^{12}/_{12}$ and $^{8}/_{12}$ pregnant) where no surgical interference was made. This indicates that no deleterious effect on pregnancy resulted from the surgery. The very low number of pregnancies maintained after surgery in the summer study could have been due partly to the very poor fertilisation rates found in the second week of the study and partly due to the effect of season on corpora lutea maintenance. If after the induced oestrus there was premature regression of the corpora lutea between day 4 and day 10 (Knight et al, 1981) then the potentially viable embryo would have no opportunity to inhibit luteolysis by production of trophoblastiin around day 14 (Martel, Lacroix, Loudes, Saunier and Wintenberger-Torres, 1979).

During the early period of the oestrus cycle prostaglandin F2α (PGF2α) is present in endometrial tissue though only at approximately $^{1}/_{4}$ of the levels found by day 14 in the pregnant

ewe (Wilson, Cenedella, Butcher and Inskeep, 1972). Distension of the uterine horn of the guinea-pig has been shown to cause the release of PGF2α *in vitro* (Poyser, Horton, Thompson and Los, 1971) and PGF2α has been shown, when infused slowly into the ovarian vein (an ovarian autotransplant was used) to cause luteolysis followed by a normal oestrus (McCracken, Glew and Scaramuzzi, 1971). However either distension of the uterus does not cause PGF2α release in the ewe, or the insult provided by flushing was too slight to cause this.

Stress, particularly physical stress such as is involved in 24-48 hours deprivation of food, in anaesthesia, and in handling the uterus and ovaries, does not seem to be a major cause of embryonic death at day 3-5. It may be that this period of development in the embryo is less susceptible to the influence of stress than the period around ovulation, fertilisation and the first cleavage divisions, that is day 0-2. Dutt (1963) certainly found this to be so with heat stress.

Fertilisation Rates

Fertilisation rates after natural service were generally high ranging from 92-100% if the Leicester tup is examined separately. These figures are in agreement with the literature. Quinlivan *et al* (1966) report a 93% fertilisation rate in parous ewes and Boyd (1965) reviewing embryonic death, reports fertilisation in normal animals to be 81-96%. It has been observed that natural mating does not always give a satisfactory fertilisation rate where superovulation has caused 10 or more ova to be produced (Willasden, 1979), where surgical insemination gives

more satisfactory results despite the possible hazards associated with interference with the infundibula and oviduct around ovulation and the stress caused by surgery. The failure of fertilisation from natural mating after superovulation appears to be due to a defect in transport of sperm in the cervical canal. In this study, no ovulation rate greater than 7 was found and in each case fertilisation of ova occurred.

Fertilisation rates after artificial insemination (54.5%) were significantly lower than in the naturally mated ewes. However, the conception rate for artificially inseminated ewes was higher than this with 66.7% of ewes conceiving in the 1st breeding season, as fertilisation did not occur in all the ova or none of an oestrus. In 33% of ewes, only a proportion of oocytes ovulated were fertilisied. Thus insemination techniques cannot be the only cause of the reduction in fertilisation. It is possible that though approximately 250 million sperm were delivered into the external os of the cervix, insufficient sperm reached the oviducts to fertilise all oocytes or that timing was at fault as only one insemination was made in each ewe. Higher conception rates are reported after double insemination 68.2% v 63.2% (Gordon, 1974). However double insemination becomes less practical as a commercial on farm technique. In his report, Gordon found fertilisation with 400 million sperm in one dose gave higher conception rates than 2 inseminations of 200 million sperm. The partial fertilisation found in 33% of ewes after A.I. in this study could also have been due to a combination of a spread in the ovulation of ova and the single dose insemination.

During these studies 2 tups were found with less than optimum fertility. The results from both were obtained only during the pre-breeding season study. One of these tups was examined both physically and his semen examined after electroejaculation before and after dilution with buffer. The other tup was examined physically and had a history of acceptable fertility. The Suffolk tup was used for control ewes. $^{0}/_{10}$ of the ewes inseminated with a satisfactory ejaculate became pregnant compared with 33% from other tups. On semen examination, total abnormalities of 2% were noted, a very few leucocytes present, and satisfactory motility. It is possible that the presence of leucocytes, though sparse, indicated the presence of an infection deleterious to fertilisation or embryo survival.

The Leicester tup used on the surgical group had a very satisfactory semen picture at the start of the week but this was not rechecked during the week as it was thought that electroejaculation may have affected his libido. The fertilisation results obtained from the first 6 ewes mated over 2 days were very satisfactory (92%) but this fell dramatically in the next 3 days and 9 ewes to 36%. This indicates that semen produced later in the week was of inferior quality, possibly immature due to to reduced semen production out of breeding season. It was interesting to note that the quality of semen from the Suffolk tup used the previous week had deteriorated in quantity and concentration after a week's use. As his semen was collected by artificial vagina this was noted and he was not used further in that study. The weather had been particularly hot for a fortnight 2-4 weeks before the tups were used. Braden and

Mattner (1970) report that heating the testes of rams to 39.5 °C did not seem to affect the sperm already present in the epididymis but damaged those developing in the testes. This causes low fecundity days 14-34 after the heating due to decreased numbers of sperm and an increased proportion of dead and tailess sperm. However the same effect was noticed in the Suffolk tup after heavy use in the breeding season. This emphasises the importance of sufficient tups for the number of ewes particularly where synchronisation of ewes is undertaken. The recommended rate for the commercial regime of progestogen sponge and PMSG administration is one tup to ten ewes (Data sheets). This may be an overestimation of the capacity of some tups particularly out of breeding season.

Embryonic Death Estimates

It was interesting to note that from the surgically flushed group 56.3% of the number of ova fertilised by natural mating were fertilised by artificial insemination and in the control groups, ewes artificially inseminated produced 55% of the number of lambs the naturally mated ewes did. It can be assumed that the number of corpora lutea was similar in the artificially inseminated and naturally mated controls and that fertilisation rates should be the same for the control and the surgically flushed groups. Thus it can be deduced that embryonic and foetal death rates were the same for the artificially inseminated and naturally mated ewes with the reduction in conception rates and lambing percentages in artificially inseminated ewes being due to fertilisation failure.

The surgically laparotomised naturally mated control ewes showed a tendancy to produce lambs from all the ova ovulated or none (7 out of 8 ewes). Moore and Shelton (1964) also found an all or none effect after embryo transfer experiments in sheep. Wilmut (1983, personal communication) in sheep and Edwards (Hunter Lecture, Glasgow, 1983) in man, suggest that the presence of a healthy embryo establishing a pregnancy aids the establishment of a second, possibly less viable, embryo.

The estimated embryonic death rate of 23% for the first breeding season in both artificially inseminated and naturally mated ewes, and 33% and 25% respectively for the second breeding season gives a total in-breeding-season embryonic and foetal death estimate of 25%. Wilmut and Sales (1982) suggest 15-30% of fertilised eggs die in the first three weeks of pregnancy. Edey (1969) in a review suggests 20-30% foetal embryonic death with the peak before day 18 and very few deaths after day 30. It may be considered that the figures for the breeding season of 1982 are likely to be the most accurate in the study, as the corpora lutea were counted in these control ewes rather than estimated, but as the corpora lutea estimates were always made from a similar group of ewes in the same conditions, treated identically and examined over the same period, they should be accurate.

However the embryonic death rate for the summer season was much higher at 65.6% and again consistent in artificially inseminated and naturally mated ewes. There is a possible source of error in these results because though the surgically flushed ewes used to provide the fertilisation rates and corpora lutea

estimates were of similar breeding and treated identically to the lambing ewes, they were in different locations and the lambing ewes were in slightly better bodily condition at mating than the surgical group. There was evidence of late embryonic/early foetal death both in the surgical group where 2 ewes had high progesterone levels past day 19 which fell by day 27 and in the lambing group where of 14 sampled out of 34 ewes, 1 had a high level of progesterone on day 26 but failed to lamb to that service. It is possible that the high rate of embryonic death was related to the return to anoestrus after the induced heat found in both farm controls and the in unit surgical group. If premature regression of the corpora lutea occurred before day 12 even a viable embryo would have no chance to initiate maternal recognition of pregnancy and maintenance of the corpora lutea. Another possible bias against the summer control group was that the majority of ewes in the breeding season studies were known to be fertile whereas the control group in the pre-breeding season were ewes which had not reared lambs the previous season. This was not necessarily due to infertility: most ewes were in their first breeding season and others had lambs which had died. Thus certainly a portion of the extra embryonic death found out of breeding season may not be directly attributable to the embryo but be caused by the ewe. Low levels of fertility very early and very late in the breeding season have been investigated by several authors (Dutt, 1954; Hulet, Voigtlander, Pope and Casida, 1956; Laffey and Hart, 1959). In all cases, fertilisation rates were found to be low, but embryonic death estimates particularly for the first 18 days, were raised and were reported to be caused by

abnormal ova, i.e. mishapen, broken zonas and lack of development of some blastomeres. Some of these however may be artefacts caused by recovery and examination.

It would appear that a large proportion of embryonic or foetal death in this study occurred in the early stages of embryonic development. 4 ewes not pregnant in the A.I. control group the first season returned to service 17-19 days after the induced oestrus, showing that either lack of fertilisation or early embryonic death before day 12 (Moor and Rowson, 1966) had occurred where the embryo had not developed sufficiently to prolong the life of the induced corpora lutea. In the second breeding season study 4 of the 10 control ewes did not lamb to the induced oestrus, 2 of which returned to service at 17-19 days, 1 never returned to service but was not pregnant on laparotomy at 28 days post service, and the fourth ewe returned to oestrus on day 35. The stage at which embryos were lost when they were twin or the third or fourth ova in a ewe which lambed is unknown. An attempt was made to elucidate this in 2 ewes where laparotomies were carried out at day 28 after the original laparotomy at day 5. However 1 ewe had lost all 3 possible foetuses by then and the other retained all 4 embryos ovulated to lambing.

From all ewes in the study on the premises only 3/93 returned to service after an elongated cycle length or maintained a high level of progesterone beyond a normal cycle length. Cessation of luteal function occurred by day 27 in 2 cases and day 35 in the third. Dooley, Wodzicka-Tomaszewska and Edey

(1974) found progesterone levels indicating a functioning corpora lutea for an average of 10 days after removal of a 15 day embryo and 15 days after death of a 15 day embryo was induced *in situ*, but when a 10 day embryo was killed, cycle lengths were normal. Moor and Rowson (1966) found removal of a 12 day sheep blastocyst did not prolong cycle length though removal of a day 13, 14 or 15 embryo did. Rowson and Moor (1968) report that ewes returned to oestrus in an average of 7-8 days after removal of a foetus (day 30 plus) unless the placenta was retained when the return to oestrus was prolonged. From these reports embryonic death probably occurred around day 13-17 in the 3 ewes discussed above. Though late embryonic or foetal death may have occured in ewes lambing fewer lambs than ova produced these 3 ewes are the only positive evidence for late embryonic death.

Culture

The proportion of embryos producing metaphase spreads after culture was found to be unaffected by length of culture in colcimid, season, stage of development and morphological appearance.

It was expected that more embryos would be found to produce metaphase spreads after culture in colcimid for 15-24 hours than for 9 hours or less. The increase actually found was not statistically significant. The replication rate of sheep embryos in culture is close to 24 hours (Tervit, Whittingham and Rowson, 1972) and synchronisation in division is close in these early stages. It was thought possible that long exposure to colcimid might cause overcontraction of chromosomes and thus poor

preparations. However no difference was found in the quality of the metaphase spreads obtained, which even under apparently identical treatments varied widely. Long and Williams (1978) found after culture for 24 hours in a colchicine solution that 56.3% of 2 or 3 day embryos produced metaphase spreads. Culture was more successful in the 2-4 cell stage than 8 cell.

The recovery media used in this study was a very standard one: phosphate buffered saline plus bovine serum albumin. The culture media used was the same as that used by Whittingham (1971) and Willasden (1979) supplemented with 10-20% heat treated sheep serum. This differs from the synthetic oviduct fluid (SOF) described by Tervit et al (1972) only in the inclusion of lactale and a bicarbonate buffer in the SOF. However Tervit and Gould (1977) found that after culture for 3 days SOF produced better results in development and viability after transfer than enriched PBS. This difference though pronounced in day 3 embryos was very much less so in day 5 embryos. However Wright, Anderson, Cupps, Drost and Bradford (1976) found that simple salt solutions such as Brinsters modified ovum culture medium, synthetic oviduct fluid and supplemented Whittens media supported growth of sheep embryos better than more complex culture media e.g. Modified Eagles medium, TCM 199 and HF-10, and that there was little difference between them in results obtained. The disadvantage of SOF is that it has to be made up daily from fresh stock solutions whereas enriched PBS is available commercially (Flow Laboratories).

There was no difference in the proportion of embryos classified as normal or in the process of degeneration (IPD) producing metaphase spreads on culture but the one embryo

recovered that was considered totally degenerated before culture did not divide. The criteria used for catagorising embryos as normal, IPD or degenerate were those described by Linares and King (1980) for cattle blastocysts. 4 sheep embryos which would be described as degenerating i.e. with separated blastomeres or 2 distinct cell groups within the zona or blastomeres of very different sizes, produced metaphase spreads after processing none of which showed cytogenetic abnormalities. King, Linares, Gustavsson and Bane (1979) found no difference in the proportion of normal or abnormal embryos producing metaphase spreads on culture but considered the preparations from abnormal embryos to be of poorer quality and contain fewer cells in mitosis.

Significantly more embryos sired by Suffolk 101 produced metaphase spreads after culture than embryos from the other tups combined. This could be either the cause or the effect of the lower metaphase spread rates in the summer season when he was used least frequently i.e. embryos from Suffolk 101 could be more viable in these culture conditions or the lower (but not significantly so) growth rate in the summer could have biased the other tups' figures down if season was having an effect on the viability of embryos. This is supported by the very high estimated embryonic death rate from summer matings in control animals.

If it were that embryos from Suffolk 101 were more viable in culture it may be that a survivability factor could be passed by either parent to the embryo. This may prove of significance in embryo transfer, where a maternal effect is found. Church and Shea

(1975) report some cows to be consistently poor donors because of failure of embryos after transfer and Elsden, Nelson and Seidel (1979) found on investigating embryo transfer from cows with undiagnosed infertility that not only were ova production, fertilisation and recovery rates lower than for other donors but the percentage of apparently normal embryos transferred surviving was also reduced. Hare, Singh, Betteridge, Eaglesome, Randal and Mitchell (1978) found an apparent donor influence in success in sexing day 13-15 cattle embryos. Thus there would appear to be a maternal factor involved in the ability of an embryo to survive, at least after disturbance. Bulman (1979) found a paternal influence in the proportion of late embryonic deaths in cattle and Logue (1981) found that semen assessment could not predict the lambing results from a particular tup. Perhaps a paternal contribution to embryo viability is as important as fertilising ability in semen and Suffolk 101 passed a 'survivability' factor to his offspring.

SECTION 2.2.1

Ewe Lambs - Materials and Methods

Nine Blackface ewe lambs were purchased at the beginning of November, previous reproductive history unknown. Blood samples were taken on the day of purchase and 9 days later, and analysed for progesterone levels. Only 1 ewe lamb had a plasma progesterone level above 3 nmol/l and was thus considered to be cycling. The ewe lambs were then penned with a vasectomised tup fitted with a sire-sine harness and crayon to check for the onset of cycling.

Ten Finn Dorset ewe lambs were purchased mid December. These ewe lambs had been kept with a vasectomised tup fitted with a marker crayon, and had not been observed in oestrus. These ewe lambs were not assessed for progesterone levels as the history was considered sufficient. Once purchased they were penned with a vasectomised tup as above.

First Oestrus Lambs

Five prepubertal Scottish Blackface ewe lambs, weight 35-41 kg, were brought into their first oestrus by inserting an intravaginal medroxy progesterone acetate impregnated sponge (Veramix, Upjohn Ltd) for 14 days. Either 500 or 750 i.u. of PMSG (Folligon, Upjohn Ltd) were given at the time of sponge removal. The ewe lambs were mated by natural service using either one of 2 known fertile tups 48-60 hours after sponge withdrawal. The ewe lambs were starved for 2 days, anaesthetised using alphaxone/aldolphalone (Saffan, Glaxovet), and surgically

flushed for the recovery of embryos on day 4 by the method described previously for adult ewes. It was noted that the fimbrae of the oviduct bursae of these animals were particularly short and thick.

Cycling Lambs

A further 4 ewe lambs of a Finn Dorset cross, weight 29-31 kg which had at least one oestrus cycle were prepared and flushed by the method above, using 750 i.u. PMSG for each ewe lamb. One of these ewe lambs died on induction of anaesthesia due to peracute pulmonary oedema. The uterus was removed shortly after death and flushed for embryos in the laboratory. Despite the presence of 3 corpora lutea, no embryos were recovered. As the flushing method for this ewe was not standard, her figures have been removed from the results analysed.

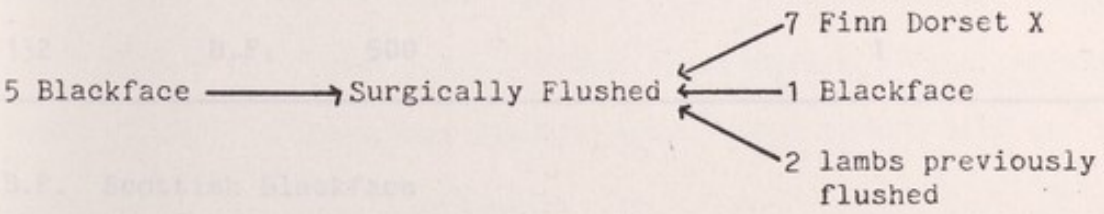
Subsequently a further 3 cycling ewe lambs were synchronised by giving 500 i.u. PMSG on day 11 of their oestrus cycle and 13.4 mg dinoprost - a synthetic prostaglandin F2α on day 13 (2ml Lutalyse, Upjohn Ltd). These ewe lambs were flushed on days 3, 4 and 6 post oestrus. Two of the Finn Dorset cross ewe lambs previously flushed were synchronised with progesterone sponges, given 500 i.u. PMSG, served naturally and flushed on days 4 and 5. One of these 2 ewe lambs showed no adhesions as a result of previous surgical interference, the other showed some adhesions around the ovaries.

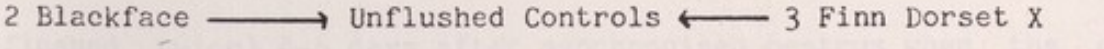
Controls

A control group of 7 ewe lambs, 5 Finn Dorset cross and 2 Blackface, were synchronised using progesterone sponges. They

were given 500 i.u. PMSG on day 14, the day of sponge removal, and confined with a fertile tup. Due to a graze on the brisket the tup was not fitted with a harness. 3 Finn Dorset cross lambs and the 2 Blackface lambs were then left as unflushed controls, put in with a tup with a harness and marker crayon. These ewes were examined for evidence of return to oestrus and blood sampled for progesterone analysis to diagnose pregnancy. The remaining 2 Finn Dorset cross lambs were surgically flushed on day 4. One of these ewes was found to be approximately 21 days pregnant, the other had corpora lutea present that were buried and many days more mature than day 4. The conclusion had to be drawn that the cyclic status of the Finn Dorset cross ewe lambs was not necessarily as thought, as some had certainly been cycling and even mated before purchase. Thus there were only 2 controls, the 2 Blackface lambs, for fertility at first oestrus.

1st Oestrus Lambs — Cycling Lambs





Thus in total 5 1st oestrus ewe lambs were flushed and 10 flushes were carried out at the second or subsequent oestrus. 5 controls were left unflushed, 2 of which were definitely 1st oestrus.

SECTION 2.2.2

Results of Ewe Lamb Experiments

Recovery Rates

No embryos were recovered from the 5 Blackface ewe lambs surgically flushed after their first oestrus. 4 the ewe lambs produced corpora lutea (3 had one corpora lutea and one ewe lamb had 2 corpora lutea), and one ewe lamb just had a follicle present on laparotomy.

TABLE 18 Corpora Lutea Produced by Ewe Lambs at their 1st Oestrus

Ewe Lamb	Breed	PMSG Dose	Corpora Lutea No.	Follicle No.	No. Recovered
128	B.F.	500	1		-
129	B.F	500	1		-
130	B.F.	750	2		-
131	B.F.	750	1		-
132	B.F.	500		1	-

B.F. Scottish Blackface

7 embryos were recovered from 7 flushes (5 ewe lambs, 2 flushed twice) 4-6 days after synchronised oestrus when the ewe lamb had had at least one oestrus period before synchronisation. 11 corpora lutea were present i.e. there was a 63.6% recovery rate.

TABLE 19 Corpora Lutea Produced by Ewe Lambs at a Second or Subsequent Oestrus

Lamb	Breed	PMSG Dose	Corpora Lutea No.	Follicles	No. Recovered
2	F.D	750	2	1	1
5	F.D	750	1	1	-
11	F.D	750	2	5	2
140	B.F	500	1	1	1
23	F.D	500	1		1
2	F.D	500	3	1	1
11	F.D	500	1	1	1

F.D - Finn Dorset X
B.F - Scottish Blackface

This difference in recovery rates between first oestrus and cycling ewe lambs i.e. 0 v 63.6% is not quite significant using the X^2 test at P = 0.05 (X^2 = 3.37). The recovery rates for embryos from adult ewes during this season was 22/36 61%. If a comparison is made between recovery rates from all sheep flushed that season which had cycled before synchronisation and recovery rates from the ewe lambs flushed after their first oestrus the difference is significant using the X^2 test at P = 0.05 (X^2 = 4.7).

The difference between the ovulation rates of adult ewes that season (mean 3.1) and lambs (mean 1.6) is statistically significant at P = 0.01 using the t-test (t = 3.23).

All embryos recovered from ewe lambs were fertilised and ranged from 12 cells (day 4) to an expanded blastocyst (day 6).

A trial was attempted to determine a pregnancy rate for mating at an induced first oestrus. Unfortunately doubt was cast over the reproductive history of the Finn Dorset X lambs because of 2 Finn Dorset X lambs flushed at what was thought to be their first oestrus, one was pregnant and the other had evidence of 'old' corpora lutea. Thus only 2 control ewe lambs were mated at what was certainly their first oestrus. Both conceived, one lambed a single, the other twins.

Culture Results

2 embryos recovered from ewe lambs looked morphologically abnormal, one had an oval zona pellucida and blastomeres of uneven size, the other had blastomeres of unequal size but improved after culture. 5 of the 7 embryos cultured produced metaphase spreads (71.1%). 4 embryos were cultured in colcimid solutions for less than 8 hours, 3 of which produced metaphase spreads and 3 were cultured for more than 15 hours, 2 of which produced metaphase spreads.

TABLE 20 Effect of Culture Time

	Embryos Cultured	Produced Metaphase Spreads
colcimid < 8 hours	4	3
colcimid > 15 hours	3	2
Total	7	5

$^{24}/_{89}$ (27%) of embryos recovered from adult ewes produced metaphase spreads which is significantly fewer ($X^2 = 4.16$) than embryos from ewe lambs, $^{5}/_{7}$ (71%).

SECTION 2.2.3

Discussion

The total failure to recover embryos from ewe lambs at their first oestrus is surprising when the recovery rate of ova from ewe lambs at their second or a subsequent oestrus was very similar to the recovery rate from adult ewes in that season. As the 2 control lambs conceived to service at their first oestrus it was shown that ova are ovulated and do reach the uterus. Quirke and Hanrahan (1977) found the recovery rate from ewe lambs after slaughter similar to that from adult ewes, though these ewe lambs may not have been synchronised for their first oestrus. Wright et al (1976) collected embryos from prepuberal lambs but recovery rates and methods are not given. Trounsen, Willasden and Moor (1977) surgically recovered embryos from prepuberal Welsh Mountain ewe lambs. Recovery rates are not given but ovulation rates ranging from means of 0.5 to 7.8 were found depending on the length of progesterone priming 3-18 days before PMSG injection. The lambs in Trounsen's study were given twice the dose of PMSG that the lambs in the present study received.

Ovulation results were significantly lower in ewe lambs than adult ewes despite identical treatment. Indeed ewe lambs were receiving a higher dose of PMSG per kilogram bodyweight than the adult ewes in most cases. The lower ovulation rates were possibly due to a lower number of follicles sufficiently developed to be able to respond to PMSG stimulation in ewe lambs.

The proportion of embryos producing metaphase spreads on culture was significantly higher in embryos recovered from ewe

lambs than those from adult ewes. This finding is surprising in view of reports by Wright et al (1976) where, though cleavage indices in those embryos which grew in culture were similar in embryos from ewe lambs and adult ewes, 16% fewer embryos from ewe lambs cleaved than embryos from adults. This showed that fewer ewe lambs embryos were viable in culture than embryos from adult ewes, but those that did divide did so at the same rate. Quirke (1979) found fertilisation rates in ewe lambs similar to those found in adult ewes under similar conditions but found that approximately 60% of cleaved ova from ewe lambs were lost by day 28, most of these between day 12 and 28. Quirke and Hanrahan (1977) and Quirke (1979) found that embryos from ewe lambs had a lower survival rate after transfer than embryos from adult ewes, whether the recipient was an adult ewe or a ewe lamb, showing that low pregnancy results of ewe lambs (of the Galway breed) is due principally to a failure of the embryo itself rather than a fault in the uterus or endocrinology of the ewe lamb. However, Onuma and Foote (1969) found fertilised ova from prepubertal superovulated calves grew better in culture if recovered at the 1 cell stage than if cleavage had taken place before recovery and Seidal, Larsen, Spilman, Hahn and Foote (1971) found that ova from pre and post pubertal heifers developed better if recovered at the 2 cell stage rather than 4 cells or more. These authors suggested this may be due not to intrinsically less viable ova but a potentially harmful property of the reproductive tract of the immature bovine. Unlike the ova from immature heifers the reduction in viability of the embryos from ewe lambs on transfer

found by Quirke and others seemed to be due to the embryo itself and could be due to cytogenetic defects. However in this study the embryos proved to significantly more viable in culture than those from adult ewes, despite apparently identical culture conditions.

Materials - Pigs

In the major part of this study gilts were purchased and kept in standard conditions on the hospital premises. In a preliminary study a sow due to be culled was mated on the farm of origin and slaughtered 4 days after oestrus before flushing of the uterus for embryo recovery.

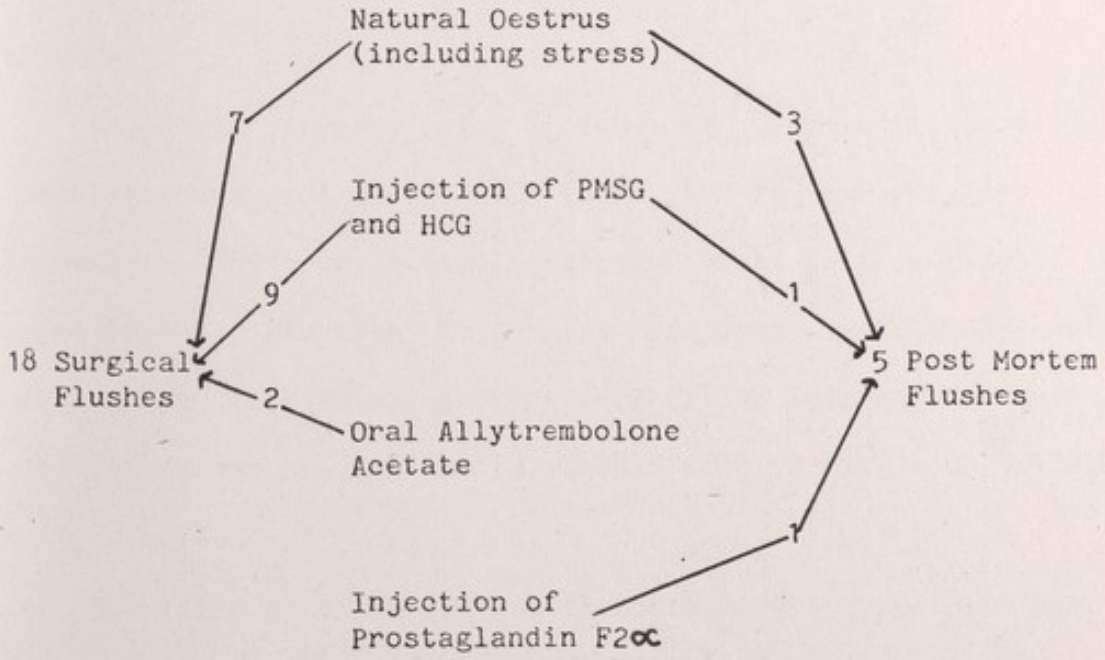
A total of 9 gilts were obtained in groups of 3 from bacon pig farms at around 80 kg and 5 months of age. These gilts were from Large White boars and commercial hybrid sows. No gilt was kept beyond 11 months of age. No gilt had cycled at the beginning of the study, progesterone assays being used with the teasing regime to monitor cyclic status. A young boar was purchased and was used for the duration of the study.

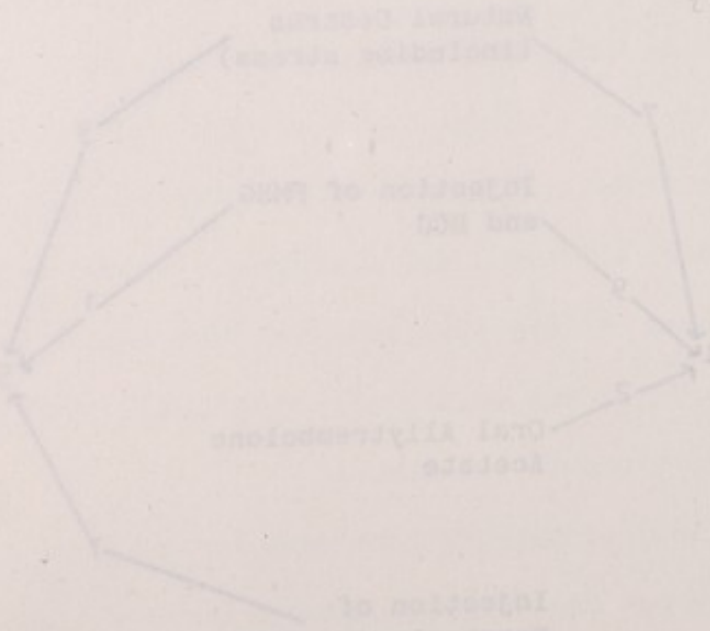
Three gilts and the boar were kept at one time; the 3 gilts being loose housed together with a small area of direct contact with the boar. All pigs were kept on straw with ad lib water through nipple drinkers. Commercial sow nuts were fed. The gilts were teased for a minimum of 5 minutes daily until they either stood for service or objected to the boar mounting. Natural light was provided through large skylights. After surgery the gilts were returned to the same area but were kept singly in a deeply bedded insulated pen with a heat lamp until fully recovered when they were returned to the others.

A variety of methods were used for oestrus induction. All methods used bar one, allyltrenbolone acetate, are available and are used by the commercial pig farmer. In the anoestrus gilt a

mixture of 400 i.u. Pregnant Mare Serum Gonadotrophin (PMSG) and 200 i.u. Human Chorionic Gonadotrophin (HCG) (Folligon and Chorulon, Intervet Lab Ltd, to simulate PG600, Intervet Lab Ltd.) was very successful and consistent in inducing oestrus 4 days after injection. Allyltrenbolone acetate (Regumate, Hoechst) was also used in anoestrus gilts but with less success than in cycling gilts. Mild stress, such as transport, jugular bleeding and introduction of new pigs also stimulated cycling gilts to come into oestrus. These were classified as natural oestruses, which also occurred in the cycling gilts. Prostaglandin F2∝ (Lutalyse, Upjohn) injection on day 13 of her cycle was used to bring 1 gilt into oestrus.

Surgical flushing was carried out after a total of 7 natural oestruses, 9 oestruses induced using injection of PMSG and HCG and 2 oestruses following oral administration of 20 mg allyl trenbolone acetate for 18 days





Flushing post mortem followed 3 natural oestruses, one induced by injection of PMSG and HCG and one by injection of prostaglandin. 3 pigs were slaughtered in anoestrus and one pregnant.

All gilts were served naturally by the same boar. In all but 2 cases where the gilt only stood for the boar on one day, each gilt was mated at least once on each of the 2 days of standing oestrus. No more than 2 gilts were in oestrus and served in one day.

Surgical flushing took place on days 4-7 (the first day of standing oestrus = day 0).

SECTION 2.3.2

Surgical Embryo Collection from Pigs

Materials

Flushing Fluid - Sterile Dulbeccos Phosphate Buffered Saline + 5% Bovine Serum Albumin

Anaesthetics - Azaperone (Suicalm, Janssen) 8-12 ml per gilt

- Metomidate (Hypnodil, Janssen) 7-10 ml per gilt

- Halothane

Equipment

Oviduct Catheter - dogs urinary catheter, sterilised

Collection Catheter - Foley catheter with whistle tip, sterilised

Collection tubes - round bottomed glass centrifuge tubes 50 ml, siliconised and sterilised

Butterfly canulae - 21 gauge

Surgical laparotomy instruments, V shaped wooden crate, incubator.

Methods

Prior to surgery all instruments were sterilised, all catheters wrapped in film and sterilised in an ethylene oxide chamber. The collection vessels were foil wrapped and sterilised. Flushing fluid was prepared immediately before surgery and four 20 ml syringes were filled keeping the syringe sterile. These and collection vessels were warmed in an incubator to 37 C before use.

The gilts were starved for 20 hours before surgery to reduce the volume of abdominal contents. 8-12 ml suicalm, depending on

approximate weight, was given intramuscularly. The gilts were left in darkness and quiet for 20-30 minutes until the sedative effect reached maximum. The gilts were usually recumbent but were able to walk. Disturbing them as little as possible, an ear was swabbed and the veins raised by an elastic band round the base of the ear. A butterfly cannulae was inserted into a vein, the elastic band cut and 7-10 ml hypnodil slowly injected. Frequently the gilts shook their heads when the hypnodil was administered and care had to be taken that the cannulae did not come out of the vein. Initially the cannulae was taped in place and incremental doses of hypnodil were given to maintain anaesthesia throughout surgery - each dose lasting approximately 25 minutes. However this was discovered to cause ischaemia in portions of the ear and in later cases, after induction, anaesthesia was maintained with halothane by mask.

The gilts were positioned on their backs in a wooden cradle. The ventral abdomen was clipped and scrubbed. The author prepared for sterile surgery. The surgical procedure did not require a scrubbed assistant. The gilt was draped and a mid line incision approximately 12-15 cm in length was made down and through the linea alba. When previous laparotomies had occurred a paramedian incision was made to avoid the fibrous tissue around the sight of previous surgery. The uterus was easily located and exteriorised and both ovaries gently examined. The number and state of the corpora lutea and follicles (if any) on each ovary were assessed. It proved impossible to accurately predict how many ovulations had occurred as on dissection on post-mortem many

corpora lutea proved to be double, some were almost entirely buried and some follicular structures had a thin layer of lutinised tissue. Estimation of ovulation numbers was particularly difficult after 2 or 3 surgical flushings when some fibrous tissue tended to obscure portions of the ovary.

One small incision was made in the body of the uterus and a whistle tipped Foley catheter inserted through this and directed up one horn. The free end of the catheter was directed into a collection vessel. Approximately 15 ml air was used to inflate the cuff which sealed the base of the uterine horn. A catheter was inserted through the ipsilateral infundibulum and 2-5 cm down the oviduct. The oviduct was gently but firmly held round this between the thumb and index finger of the left hand and 20 ml of flushing fluid were syringed down the catheter using the right hand. This was followed by around 10 ml of air. The oviduct catheter was removed and the fluid gently stripped down the uterine horn and out of the Foley catheter at the body of the uterus. To avoid unnecessary damage to the delicate oviduct in the second flush 10 ml flushing fluid was injected into the top of the uterine horn, followed by 10 ml of air and this too was gently stripped down the horn. After the second flush the collection vessel was sealed and place in an incubator. The Foley catheter was deflated and without removing the catheter through the incision in the body of the uterus the catheter was moved from the flushed horn to the other. The cuff was reinflated and the flushing procedure repeated for this side

After removal of the Foley catheter from the uterus the incision was closed using inversion sutures with 2/0 chromic

catgut. This healed and was never a problem in future flushes. The ovaries, oviducts and uterus were covered with a layer of KY jelly before being returned to the abdomen. Simple interupted sutures of nylon were used to close the abdomen. A simple continuous subcutaneous suture in chromic catgut closed dead space before simple interupted skin sutures. Procaine penicillin was given I/M. In 2 pigs a stitch abcess formed - these did not prevent healing of the wound.

Collection Post Slaughter Method

After 1-3 surgical flushes, gilts were brought into oestrus or came into oestrus naturally and slaughtered in a commercial abattior 4 to 7 days after the first day of standing oestrus. The gilts were stunned using a captive bolt pistol and bled. Immediately after bleeding the uterus, ovaries and cervix were removed, placed in an insulated container and transported to the lab. The uterus and oviducts were flushed by the same method as the surgical flush as soon as was possible. All uteri were flushed within 2 hours of slaughter and most within 1 hour.

Embryos recovered were recovered and treated as the surgically flushed embryos. Care was taken to avoid any sudden drop in temperature, which never fell below 25 C.

SECTION 2.3.3

Results

Recovery and Growth

A total of 261 pig embryos were recovered, varying in stage from 4 cells to the newly hatched blastocyst. All ova recovered were fertilised. Fertilisation was assumed when sperm were visible in the zona pellucida when the embryo was examined under X 100 magnification in a light microscope. Motile sperm tails could be seen protruding around the zona pellucida in several day 4 embryos recovered by surgical flushing. It was very common to find lightly stained sperm in the final preparation. In a few preparations some sperm heads were swollen and stained very darkly.

It was found impossible to calculate a recovery rate. Once the method was established none of the pigs remained pregnant after surgery although one of two gilts flushed by a trial method, with fluid going up the horn and oviduct instead of down, did remain pregnant. In this gilt there was a corpora lutea count of 18, 5 embryos were recovered and she was found to have 10 foetus at day 78 despite the fact that this trial method of surgical flushing caused considerably more traumatic damage to the uterine horns than the method described and subsequently used. Corpora lutea counts at surgery were found to be a very poor estimate of the number of ova shed as numbers recovered frequently exceeded the estimated ovulation rate. Once the gilts had been surgically flushed 2 or 3 times adhesions tended to build up around the ovary and although corpora lutea counts were difficult,

ovulation, fertilisation and transport of the ova down the fimbrae still occurred. However numbers of embryos recovered tended to be reduced.

247 of the 261 embryos recovered were cultured and processed for metaphase spreads. 4 were lost during handling or culture and 10 were used for investigations into abnormal embryos with electron microscopy.

128 of these 247 cultured embryos produced metaphase spreads, that is 51.8%. Significantly fewer ($P < 0.001$) embryos recovered post-mortem produced metaphase spreads than those recovered after surgery 6/31 v 122/211 i.e. 16.7% v 57.8% ($X^2 = 187.8$). Thus in all other comparisons of different treatments only embryos recovered after surgical flushing were considered, to avoid bias induced by the poor growth rate of post-mortem recovered embryos.

The percentage of embryos producing metaphase spreads from each gilt varied widely (17-80%) but as in 4 gilts only one surgical flush was made prior to slaughter and these were on different days post oestrus, and the embryos from pigs later in the same series were cultured for longer than those nearer the beginning of the study no valid conclusions can be drawn from these differences.

The length of time in culture with colcimid made a difference in the number of embryos producing mitosis. When the culture period was less than 10 hours 48.4%, 61/126, produced metaphase spreads and when over 15 hours 71.8%, 41/85, produced metaphase spreads. The difference is significant at $P = 0.001$ ($X^2 = 10.4$).

In the following tables the effect of high litter numbers

i.e. 18 or more embryos recovered or corpora lutea counted whichever was the higher, is compared with more normal ovulation numbers in the gilt of 13 or less.

TABLE 21 Effect of Ovulation Number

Ovulation Number	Embryos Cultured	No. Producing Metaphase Spread	% Producing Metaphase Spread
> 18 (18–35)	130	75	57.7%
< 13 (5–13)	81	48	59.3%

There is no difference in the proportion of embryos producing metaphase spreads from high or low ovulation rates.

The method of oestrus induction was examined in relation to the ability of the embryos to produce metaphase spreads in culture.

TABLE 22 Effect of Method of Oestrus Induction

Method of oestrus Induction	No. of Gilts Induced	No. Recovered	Av. Recov. per Flush	No. (%) producing Metaphase Spreads
Natural Oestrus	4	33	8.25	16 (48.5%)
Regumate	2	15	7.5	8 (53.3%)
Inj. PMSG + HCG	8	163	20.4	99 (60.7%)

There is no significant effect of method of oestrus induction on the proportion of embryos producing metaphase spreads in culture. Individually comparing the 3 methods, the greatest X^2 was 1.1.

The ovulation rate was significantly higher ($t = 4.34$, $P < 0.001$) in oestruses induced by injection of PMSG and HCG than by other methods.

TABLE 23 Effects of Oestrus Number on Cell Division

Oestrus No.	Embryos Cultured	No. Producing Metaphase Spreads	% Producing Metaphase Spreads
Ist Oestrus	144	87	60.4%
Subsequent Oestrus	67	36	53.7%

There is no significant difference in the production of metaphase spreads of embryos recovered at the 1st oestrus or at a subsequent oestrus ($X^2 = 0.59$).

Embryos at the compact monula and early blastocyst stage appeared to respond better in culture than at other stages. Hatched blastocysts were very difficult to identify in the flushing fluid though unmistakable at X 100 magnification. The trophoblast showed a very regular flattened pattern of cells. A similar sized piece of endometrial debris presents the appearance of many small adherent spheres.

TABLE 24 Effect of Stage of Development on Cell Division

Stage of Embryo	No. Cultured	No. Producing Metaphase spreads	% Producing metaphase spread
4-16 cells	49	27	55.1%
Monula/early blastocyst	60	42	70%
Expanded blastocyst	79	42	53.2%
Hatched blastocyst	23	11	47.8%

Using the X^2 test the proportion of embryos producing metaphase spreads at the monula/early blastocyst stage of development is significantly higher ($P = < 0.05$) than the other stages combined ($X^2 = 4.4$).

When the data is rearranged so that embryos recovered on different days after oestrus were compared the results become clearer.

TABLE 25 Effect of Day of Recovery on Cell Division

Days of Embryo Recovery	No. Cultured	No. Producing Metaphase spreads	% Producing Metaphase spread
Day 4	41	28	68.3%
Day 5	58	41	70.7%
Day 6	84	43	51.2%
Day 7	28	10	35.7%

Whilst day 5 embryos which are expected to be at the monula/blastocyst stage of development were found to produce significantly more metaphase spreads in culture than day 6 embryos

(X^2 = 4.62) and day 7 embryos (X^2 = 8.15), the proportion of day 4 embryos producing metaphase spreads was also significantly higher than day 7 embryos (X^2 = 5.88) and higher than day 6 embryos but this increase is not statistically significant at $P < 0.05$ (X^2 = 2.6).

This is a more accurate way of assessing the best time to attempt this technique as retarded embryos, and thus less likely to produce metaphase spreads, may have originated from day 6 and 7 flushings and been included in the 4 cell to early blastocyst groups reducing the success rate and the differential success between the earlier and later stages.

SECTION 2.3.4

Pig Foetuses - Materials and Methods

Gilt 3 remained pregnant after surgical flushing by a trial method. Only 5 embryos were recovered despite an estimate of 18 corpora lutea present in the ovary. Pregnancy was not affected by an injection of 10 mg of dinoprost - a synthetic prostaglandin F2α (2 ml lutalyse, Upjohn Ltd) 15 days after oestrus. The gilt showed a pruritic reaction with patchy cuticular vasodilation, 15 minutes after the injection.

The gilt was slaughtered 78 days post oestrus, the uterus recovered immediately post mortem and brought back to the lab. An hour and a half after slaughter foetal processing started.

Cultures were attempted from tail, liver and spleen of each foetus, the blood of each foetus and from the amnion.

Organ Cultures

The tail tip, liver and spleen of each foetus were recovered and placed in 0.25% trypsin in culture media (RPMI 1640 Medium, Flow) for 1 hour. The organs were then removed from the media and placed in a universal bottle containing 10 ml culture media and 20% foetal calf serum and incubated at 37 C for 44 hours. Colcimid was added for the final 2 hours of culture.

Harvesting would have taken place in a manner similar to blood cultures but it was found that each culture was badly contaminated some with bacteria, others with both bacteria and fungi.

Amnion

5 cm^2 of amnion was removed from round the foetus with sterile scissors and forceps and placed in a petri dish with 5 ml culture medium and colcimid at 0.08 ug/ml for 1-1.5 hours at room temperature. The culture media was then removed and a hypotonic solution added for 12 minutes. The hypotonic solution was replaced with 1:3 glacial acetic acid: methanol fixative for approximately 2 hours. The fixative was removed and the amnion gently blotted before the addition of 2 ml of 60% acetic acid. The amnion was gently agitated to loosen cells. After 4 minutes, the acetic acid suspension was dropped onto warmed slides. After drying the slides were stained for 30 minutes in a 10% Giemsa solution.

Blood

1-4 ml of foetal blood was obtained from the umbilical cord vein of each foetus. A 21 gauge needle on a heparinised plastic syringe (Monovet, Starsted) was used. This blood was processed exactly as the adult pig blood samples, with 0.5-2 ml of whole blood per culture. The cultures were harvested by the standard method except that a stronger hypotonic solution than normal i.e. 1 g KCl in 175 ml H_2O instead of 1 g in 600 ml was used for 2 out of the 4 slides made from the cell suspension. On one of the slides produced with the stronger hypotonic method G banding was attempted according to Chen's modification of Sun, Chu and Chong's method (Chen, 1981).

All slides were stained in a 10% filtered Giemsa solution and when dry protected by the addition of DPX and a coverslip.

Results

Very few cells were available in some of the preparations from the foetal amnion. One of these slides - of Foetus 2 - showed a metaphase spread.

Some of the blood cultures from the foetuses produced many metaphase spreads. Poor results were obtained from the cultures from foetuses 3 and 4.

Sexing of the embryo from the metaphase spreads was possible in 8 out of the 10 foetuses. The quality of the metaphase spreads obtained from these blood cultures w as very much higher than in general had been obtained from early embryos; much nearer that obtained in blood culture from adult animals.

The results of the banding was a little disappointing probably due to the limited number of metaphase spreads obtained. Some spreads were partially banded and a metaphase spread from each of Foetus 5 and Foetus 7 showed a banding pattern in all the chromosomes.

SECTION 2.3.5

Discussion - Pig Results

It has been noted that gilts may not maintain cyclic activity after their first oestrus and fail to ovulate and show a second oestrus (Segal and Baker, 1973). Paterson (1982) discussed the maintenance of cycic activity in gilts treated with 400 i.u. PMSG and 200 i.u. HCG and found a variable response, but only around half the gilts showed a second oestrus after the induced oestrus. He found the presence of a mature boar improved results. Despite the presence of a mature boar maintenance of cyclic activity proved to be a problem in the gilts in this study, not only in those induced using gonadotrophins but also where allyl trembolene was fed and in those gilts which came into season naturally at the same time as those which were induced. That this was a true anoestrus not a behavioural anoestrus was shown by basal (< 1 ng/ml) progesterone levels. The reasons for this anoestrus in this study are unknown as the gilts were kept under conditions which should have allowed for high reproductive performance i.e. penned as a small group, direct boar contact but not overexposed, natural daylight, temperate condition, and were kept in good bodily condition but not overfat. It is possible that confinement and the very small group number (3) affected the cyclic activity.

The fertilisation rate for porcine ova in this study was 100%. Polge (1978) states the fertilisation rate in most sows is 100% but there is complete failure of fertilisation in 5% of sows and Perry and Rowlands (1962) found 95.5% of all ova recovered in their experiments were fertilised after natural mating. However

Hunter (1966) found in gilts ovulating 20 or more, a fertilisation rate of only 90% after artificial insemination and a further 10% of gilts with no fertilised ova.

Mating or aritificial insemination appears to be most advantageous around 12-16 hours before ovulation. The time of ovulation cannot be reliably determined by the onset of oestrus though it is estimated at 34 hours ± 6 hours after the onset of standing oestrus (Polge, 1978) and it occurs 36-40 hours after the LH surge. However the relationship between the onset of behavioural oestrus and the LH surge is variable (Foxcroft and Van de Wiel, 1982). Add to this an uncertainty over the time of onset of oestrus when gilts are teased only once or twice a day then mating at least twice during standing oestrus seems desirable to prevent fertilisation of aged ova or possibly fertilisation with aged sperm. Hunter (1977) discusses the role of the uterus and isthmus in regulating the numbers of sperm present in the ampullary-isthmic junction, the site of fertilisation, and thus reducing the incidence of polyspermy. Sperm transport efficiency is reduced and polyspermy and sygyny occur more frequently when semen is introduced more than 6 hours after ovulation (Polge, 1978). To minimise any abnormalities caused by late fertilisation, whenever possible gilts in this study were mated twice, once on each day of standing oestrus.

Generally in stained preparations of cultured embryos a large number of sperm were present. Hunter (1977) discusses the block to polyspermy. As the fertilised ova passes down the oviduct more sperm may enter the zona pellucida, though not pass through it.

Some embryos may acquire more than 200. This is probably the origin of the motile sperm tails seen around the zona in some day 4 and day 5 recovered embryos. Swollen sperm heads were present in a few of the preparations. These were approximately twice the diameter of normal sperm heads and stained darkly with Giemsa. The nuclear material appeared to have been activated by some means. Hunter (1972) describes swollen sperm heads in the cytoplasm of ova with a more advanced male pronuclei present. It is possible these sperm penetrated through the zona pellucida before or despite the block to polyspermy and on entering the ovum the nucleoprotein started to swell and decondense but these sperm heads did not develop as far as pronuclei.

Recovery rates were not established in this study as it was not found possible to accurately count corpora lutea - particularly in the higher ovulation rates - without damage to the ovary and risk of adhesions forming. Occasionally more embryos were recovered than corpora lutea were counted. Recovery rates are generally found to be higher in early embryo recovery, where ova are flushed from the fallopian tubes within 2 days of ovulation, than later when the uterine horn is flushed also. Recovery rates are easier to determine after slaughter when the number of corpora lutea can be more accurately determined by dissection of the ovary. Perry and Rowlands (1962) achieved a 100% recovery from the fallopian tubes but only 89% success where recovery was from the uterine horn. Hunter (1964) reports a 75% recovery of day 4 ova after slaughter. Pope, Christenson, Zimmerman-Pope and Day (1972) achieved a 87% recovery rate after surgical flushing of the fallopian tube and uterotubal junction

day 2. King, Gustavsson, Popescu and Linares (1980) had a recovery rate after slaughter of 87% of preimplantation embryos ranging from day 2 to 16.

Very poor results were obtained in culture of embryos obtained post mortem. Only 16.7% of these embryos produced metaphase spreads after culture whereas 57.8% of embryos cultured after surgical recovery produced metaphase spreads. Pope and Day (1977), Davis and Day (1978) and Robl and Davis (1981) used embryos from surgical recovery but other authors McFeely (1967), Axelson (1968), Smith and Marlowe (1971), Bouters, Bonte and Vanderplassche (1974), Moon, Rashid and Mi (1975), King et al (1980) and Long and Williams (1982) successfully cultured pig embryos recovered after slaughter. Dolch and Chrissman (1981) found pig blastocysts recovered after slaughter unsatisfactory and blamed lack of mitoses on acidic maternal environment post mortem.

It is possible that the delay of 1 to 2 hours after slaughter before recovery is too long and autolysis of maternal tissue had begun and was affecting the viability of the embryo. The delay was unavoidable because of the distance across the city from the slaughter house to the laboratory. It is interesting to note that in a small study, 7 sheep embryos were recovered after slaughter and none of these produced metaphase spreads after culture. The morphology of embryos recovered post mortem was no different from those recovered after surgery.

An increased proportion of pig embryos produced metaphase spreads after culture in a colcimid solution for 15 to 24 hours than cultures in colcimid for less than 10 hours. 16-20 hours are

required for the number of cells in a pig embryo to double. Division of cells in the early stages of development tends to be synchronised (Hunter, 1974). Those embryos which did not produce metaphase spreads may have just divided before recovery and with synchronisation of division would not replicate in culture before processing. However these embryos may have ceased dividing or were less viable than those which produced metaphase spreads in culture. It is interesting to note that no statistically significant advantage was found in culturing sheep embryos for 15-24 hours rather than 4 -9 hours, though there was a small increase in the proportion of embryos producing metaphase spreads. King et al (1979) also found no difference in the number of day 6 and 7 cattle embryos producing metaphase spreads after 2-4 or 4-9 hours of culture in medium with colchicine.

No difference was found in the proportion of embryos producing metaphase spreads from flushes where there was a high ovulation rate (> 18) or a lower rate (< 13). Ovulation rates for gilts on their first oestrus are expected to be 10-12, (Baker and Coggins 1968; Thompson and Savage 1978), and rise by an average of 1.1 corpora lutea at each subsequent oestrus until the fourth post pubertal oestrus (Anderson and Melampy, 1971). Litter sizes increase in gilts mated at second and third oestruses; 1st oestrus 7.8, 2nd oestrus 9.8, 3rd oestrus 10.4 respectively (MacPherson, Hovell and Jones, 1977).

All of the 35 embryos produced at a first oestrus by one gilt were fertilised. 2 embryos appeared to have only one cell and the others varied from 4-30 cells predominately 6-12 cell. Standing oestrus lasted for 3 days in this gilt - longer than any other in

this study, which if ovulation was extended over a longer period than normal, explains the difference in embryo stages found. Despite this difference in embryo cell numbers, 28 of these 35 embryos were shown to be viable at day 4-5 by producing metaphase spreads on culture. Moon et al (1975) had a lower success rate in culture with 41 day-10 blastocysts recovered from a mature sow with 47 corpora lutea. The 6 embryos not recovered could have been unfertilised, failed to develop, degenerated, or simply not been recovered. Under a variety of culture conditions, Moon et al found only 15/35 (36.6%) produced successful preparations. This could be due to technique or because by day 10 a large proportion of these superovulated embryos were no longer viable. Boyd (1965) suggests that there is no effect of ovulation rate on embryonic death up to 18 ova. However Blichfeldt and Torbjorn (1982) found that in gilts mated at their second oestrus the survival rate of embryos declines above an ovulation rate of 13. Hunter (1966) found in superovulated gilts (mean ovulation 36.5) only 48% of corpora lutea or corpora albicans were represented by a normal embryo on day 25, whereas 77.8% of recovered ova were normal 2-4 days after the start of standing oestrus showing a 30% decrease in viable embryos over this period of early development. The viability of embryos from the superovulated gilts found by Hunter was lower than that of the embryos discussed above where on day 4, 33 out of 35, i.e. 94.3%, were morphologically normal and 80% divided in culture.

The gilts producing large ovulation numbers in this study were all induced to ovulate by injection of 400 i.u PMSG and 200

i.u. HCG. There was no significant difference in the proportion of embryos producing metaphase spreads from different methods of oestrus induction or first or subsequent oestruses.

In this study it was found that significantly more embryos recovered 4 and 5 days after the first day of standing oestrus produced metaphase spreads after culture than day 6 and 7 embryos (69.7% v 47.3%). Other authors have worked principally with pig embryos either younger (King et al, 1980) or older (McFeely, 1967; Long and Williams, 1982, day 10; Moon et al, day 11; Akesson and Hennesson, 1972, days 10, 11 and later; Smith and Marlowe, 1971,day 25; Moon, 1977, 9-27 days; King et al, 1980, 9-40 days). Bouters et al (1974) examined some day 6 embryos from boars with reduced fertility and his success varied with the sire investigated. It may be possible that expanding and hatching blastocysts are more sensitive to changes in environment than earlier and later stages. King et al found the technique for the preparation of metaphase spreads from early embryos unsatisfactory for embryos days 6-9. The reduction in success in embryos recovered later than day 5 may have been because of a reduction in viability i.e. the beginnings of early embryonic death.

Success in obtaining mitotic spreads varied between authors. McFeely (1967) obtained metaphase spreads from 97% of the day 10 pig blastocysts he examined whereas Moon et al (1975) obtained successful preparations from 36.5% of day 10 blastocysts. Most of the data presented by King is pooled collectively as preimplantation where 57.5% of embryos produced mitotic spreads. In that figure a 68.21% success was achieved with day 2 and 3 embryos. It appears therefore that the proportion of embryos

producing metaphase spreads after culture seems to vary widely between authors and the stage of embryo examined.

SECTION 2.4.1

INVESTIGATION INTO MORPHOLOGICALLY ABNORMAL PIG EMBRYOS

Introduction

One of the gilts (No. 7) consistently produced morphologically abnormal embryos. The first time this gilt was flushed, 11 morphologically normal expanded blastocysts were surgically recovered 6 days after her first oestrus. The gilt came into oestrus naturally. At each subsequent flush (2 surgical and 1 post mortem) the embryos produced were classified as in the process of degeneration. Oestrus was induced in the gilt, using injection of PMSG and HCG, as she had apparently lapsed into anoestrus, and she was surgically flushed on day 5. A small dense area was noticed in the peritoneum on the previous suture line. This felt calcified. 10 embryos were obtained, all looked degenerate on recovery but improved on culture and many could be identified as morula or early blastocysts. 6 produced metaphase spreads on processing. 9 of the embryos had brown conical/pyrimidal semi-translucent accumulations very firmly attached to the zona. In some embryos several of these accumulations were seen on a single zona. These structures were very dense. Very many tiny hard crystal-like structures were found in the flushing fluid retrieved from this pig. It was deemed interesting to discover what the crystals and the accumulations on the zona were.

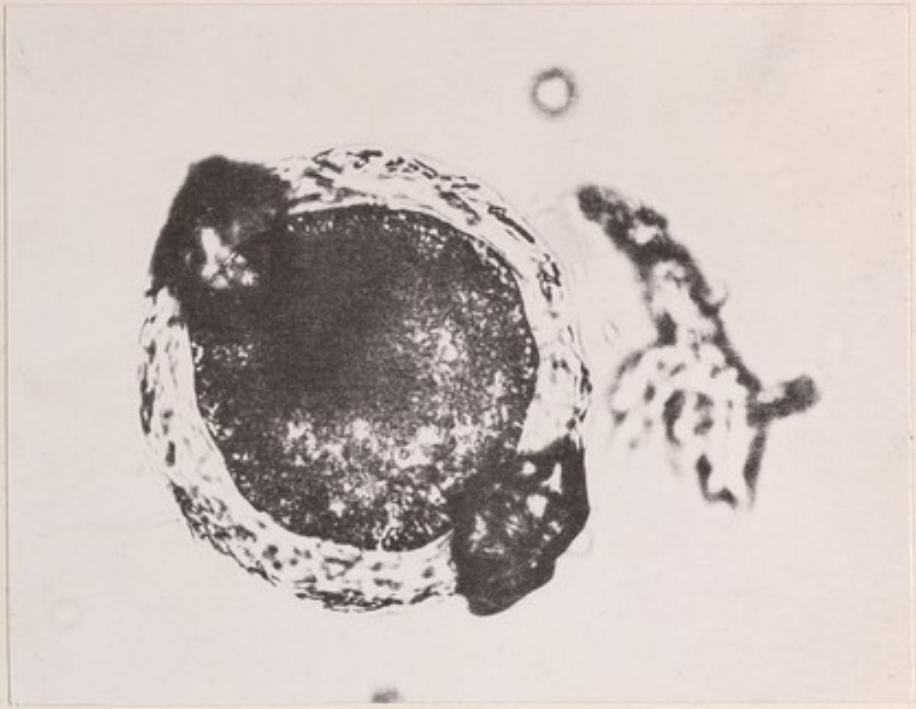


Plate 14 Blastocyst from Pig 7, with accumulations on the zona pellucida

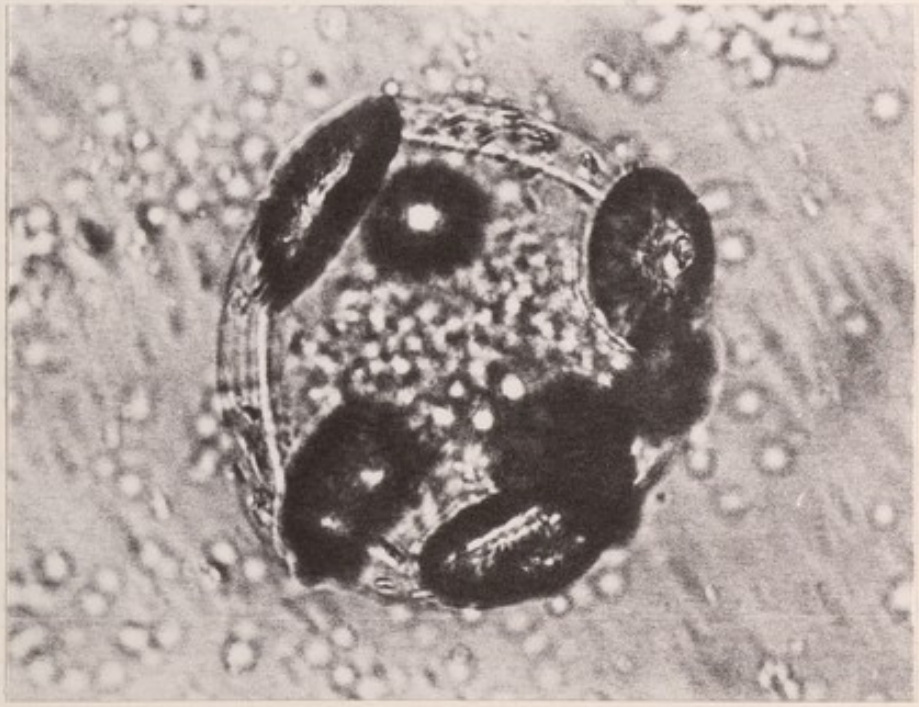


Plate 15 Empty zona pellucida with adherent accumulations

SECTION 2.4.2

Materials and Methods

Slides were prepared from the crystal-rich fluid by drying approximately 0.2 ml of fluid with as much of the granular material as possible on cleaned microscope slides. The slides were fixed with methanol and one was stained with a 10% solution of Gurrs Giemsa stain for 30 minutes. On examination, the material appeared to by crystalline, predominantly lenticular in shape and did not take up the stain. The morphology was not sufficient to provide a clue to identity. The material was not soluble in either water or methanol. Samples of the fluid were sent for mycological and bacteriological culture with no significant positive results. Further crystalline material and 3 affected embryos were obtained from a further surgical flush from a natural oestrus. Attempts were made, after photographing the embryo under a light microscope, to obtain scanning electron mitographs. Unfortunately the material was lost in processing.

Using an EDAX microanalysis detector and a Phillips 50113 Scanning Electron Microscope, a partial identification of the crystalline material retrieved from the flushing fluid was obtained. A slide of the crystals was prepared as above, sputter coated with carbon to make the material conductive, then the slide was reviewed in the scanning electron microscope. Pictures of the crystalline material were obtained. The microscope was focussed on an area particularly rich in crystals, so they filled almost the entire screen. The EDAX microanalysis detector was used to collect the spectra present. The process was repeated for an

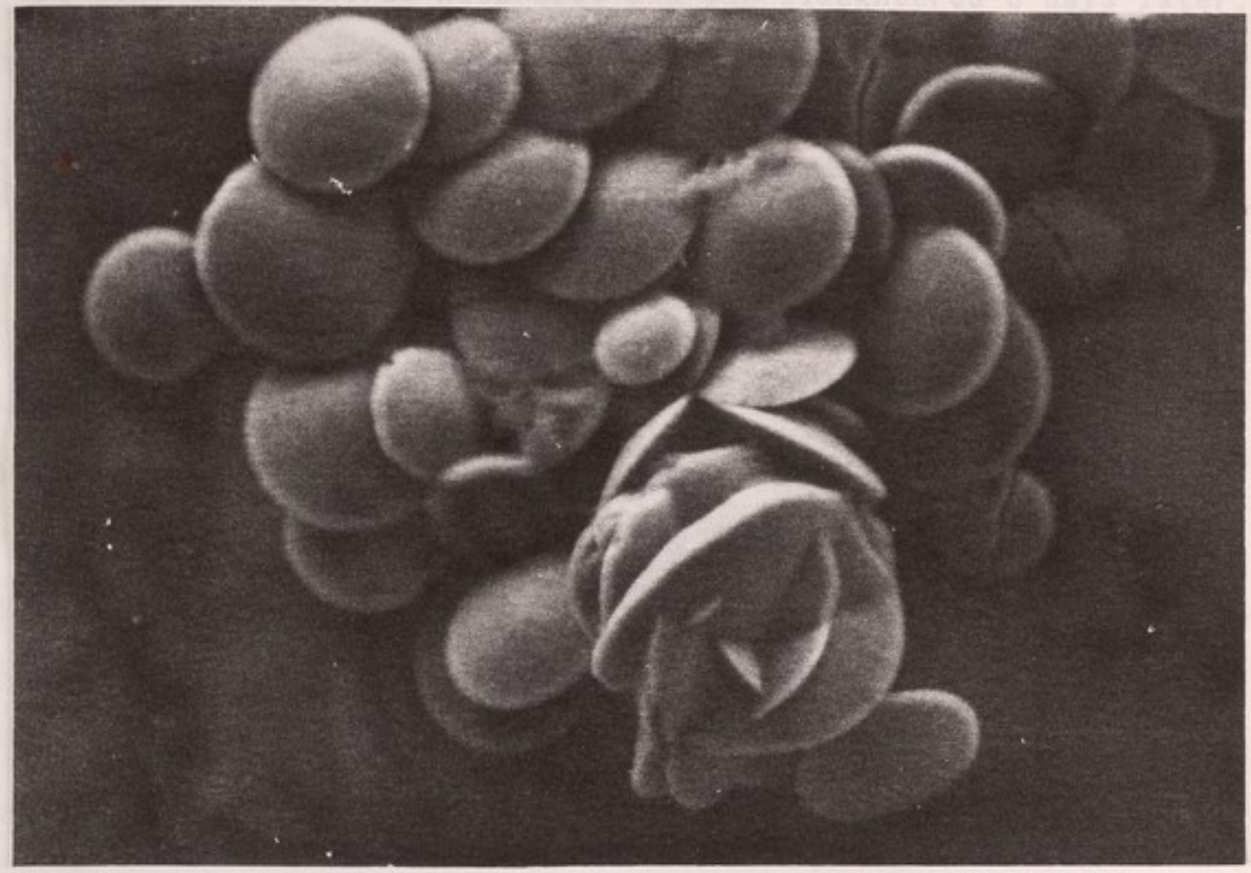


Plate 16 Crystals from flushing fluid from Pig 7 viewed under the electron microscope (x 250 approx)

empty area of microscope slide and the spectra obtained was compared with the spectra obtained for the crystals.

Further embryos were obtained from pig 7 post mortem. The gilt came into oestrus naturally and was slaughtered 6 days later. Unfortunately under the terms of the Home Office license obtained by the author the gilts were to be surgically flushed a maximum of 3 times. The uterus was obtained withing minutes of death and flushed using the normal method within 1 hour. 3 embryos and 2 empty zona pellucida were recovered, all with the dense accumulation attached to the zona. Very little of the crystalline material was found in the flushing fluid. One of the embryos and 1 of the empty zona pellucida were fixed in 4% gluteraldehyde in buffer, placed in the centre of an aluminium stubb and allowed to dry. The stubbs were then coated with carbon and examined under the scanning electron microscope. The embryo was lost in processing but pictures of the dense pyramidal material attached to the empty zona were obtained and again the EDAX microanalysis detector was used to collect the spectra. These accumulations of material were in the same positions relative to each other on the pictures obtained as the dense material was on the zona. The remaining 2 embryos and empty zona pellucida were prepared for scanning electron microscopy according to the method of Kelly (1981) a modification of the method described by Reeve and Ziomek (1981). Photographs were obtained of one of these embryos.

SECTION 2.4.3

Results

Examination of the spectra produced by the microanalysis

detector proved useful in partially identifying the crystalline material recovered from the flushing fluid. The only peaks of any significance which appeared in the first spectra collection of the crystals, and not the second, of the background slide, were Calcium K peaks. An X-ray map was made which showed great accumulations of calcium in the areas covered by the crystals. The microanalysis detector cannot read the presence of elements such as carbon, oxygen and hydrogen, and as no other element in any significant quantity but calcium was shown to be present it was postulated that the crystals could be $CaCO_3$ or some similar material.

The microanalysis detector was used on the material obtained from the empty zona with the same results. An X-ray map of the calcium content of the material from the empty zona was obtained.

The flushing fluid used to flush the uterus of pig 7 post mortem was analysed for calcium levels. A sample of the flushing fluid prior to use was used as a control and a sample of the fluid syphoned from the top of the collecting tubes after flushing were analysed by atomic absorbtion (using a 1L 257 Atomic Absorber). The level of calcium in the fluid before flushing was 0.17 mM/l mainly as $CaCl_2.2H_2O$ and post flush calcium level was 0.49 mM/l.

Plasma samples obtained on each day of the surgical flushing and on the day of slaughter, were analysed for calcium levels by atomic absorbtion. Plasma obtained from a control gilt treated identically to pig 7 was analysed at the same time. All samples were within the normal range.

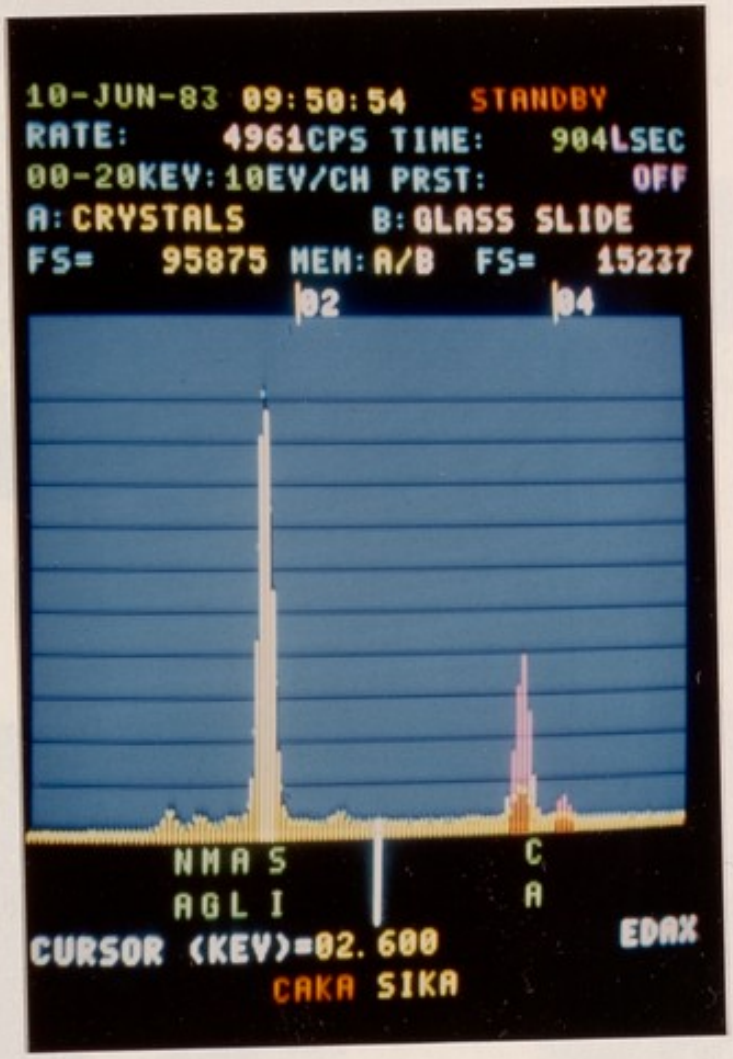


Plate 17 Spectra collected by EDAX microanalysis detector from a crystal rich area of slide (red) with spectra from crystal free area of slide superimposed (yellow)

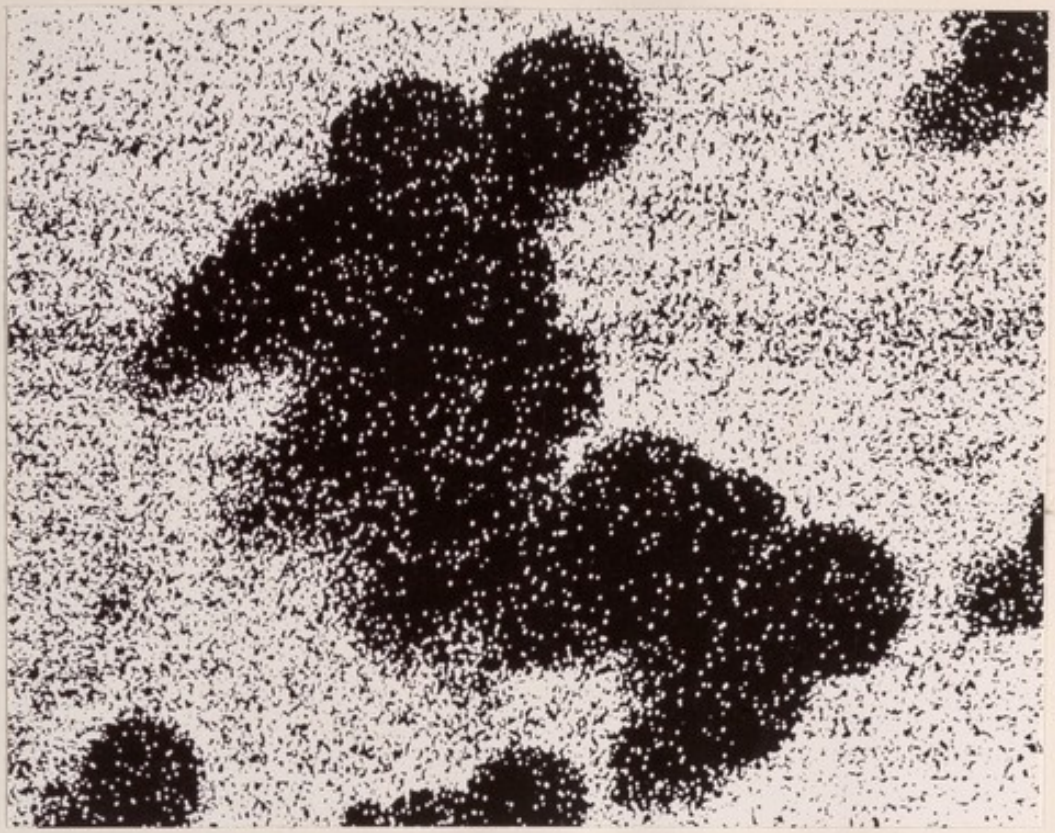


Plate 18 X ray map of crystals from uterine flushings with a calcium window. Light areas - no calcium present.

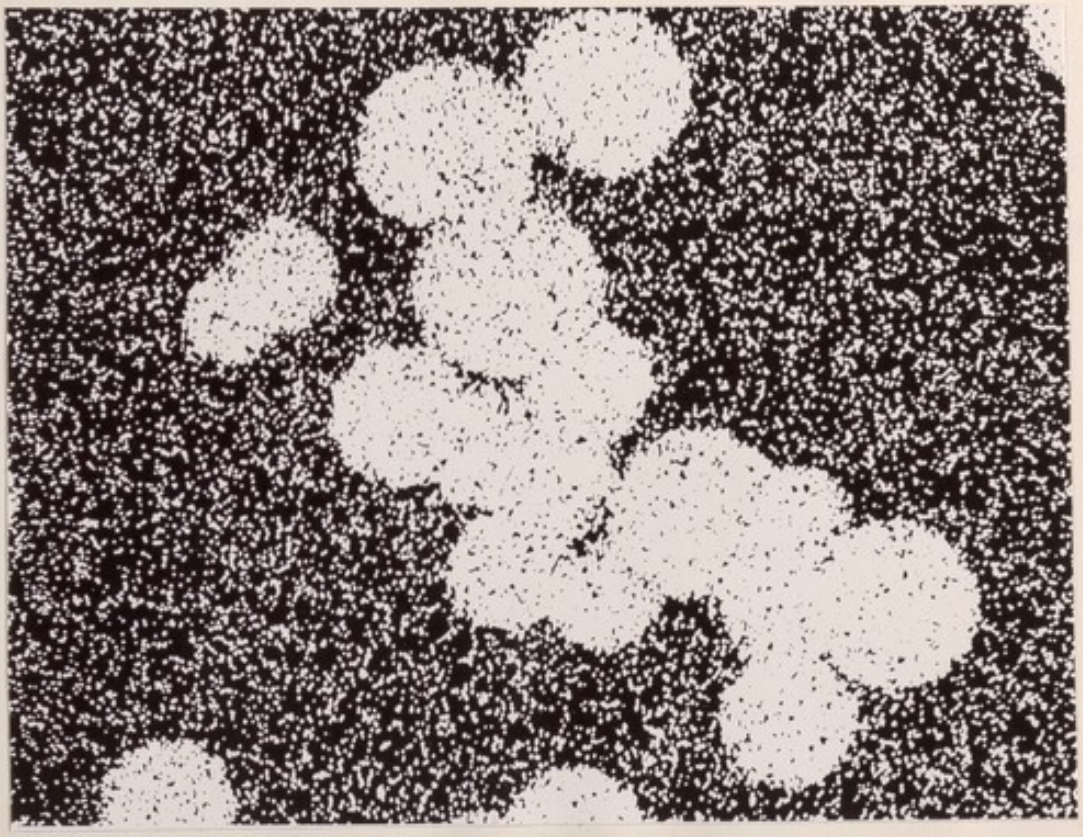


Plate 19 X ray map of crystals from uterine flushings with a silicon window. Light areas - no silicon present.

TABLE 26 Plasma Calcium Levels

Pig No.	Date	Ca Level in plasma
7	25.10.82	2.6 mM/l
7	14.1.83	2.75 mM/l
7	28.2.83	2.72 mM/l
7	22.3.83	2.82 mM/l
8	22.3.83	2.93 mM/l

Scanning electron micrograph pictures were obtained of an embryo with calcium crystals adherent to the zona. X-ray confirmation of the identity of these crystals was not possible as gold/palladium was used to coat the embryo prior to photography. This interferes with the spectra gathered. However under X 640 the characteristic lenticular shape of the calcium crystals could be seen on the surface of the zona pellucida. Under the light microscope, the accumulations on the zona of this embryo appeared identical to those on the empty zona which was processed using the carbon coating method so that spectra could be gathered and were shown to contain calcium.

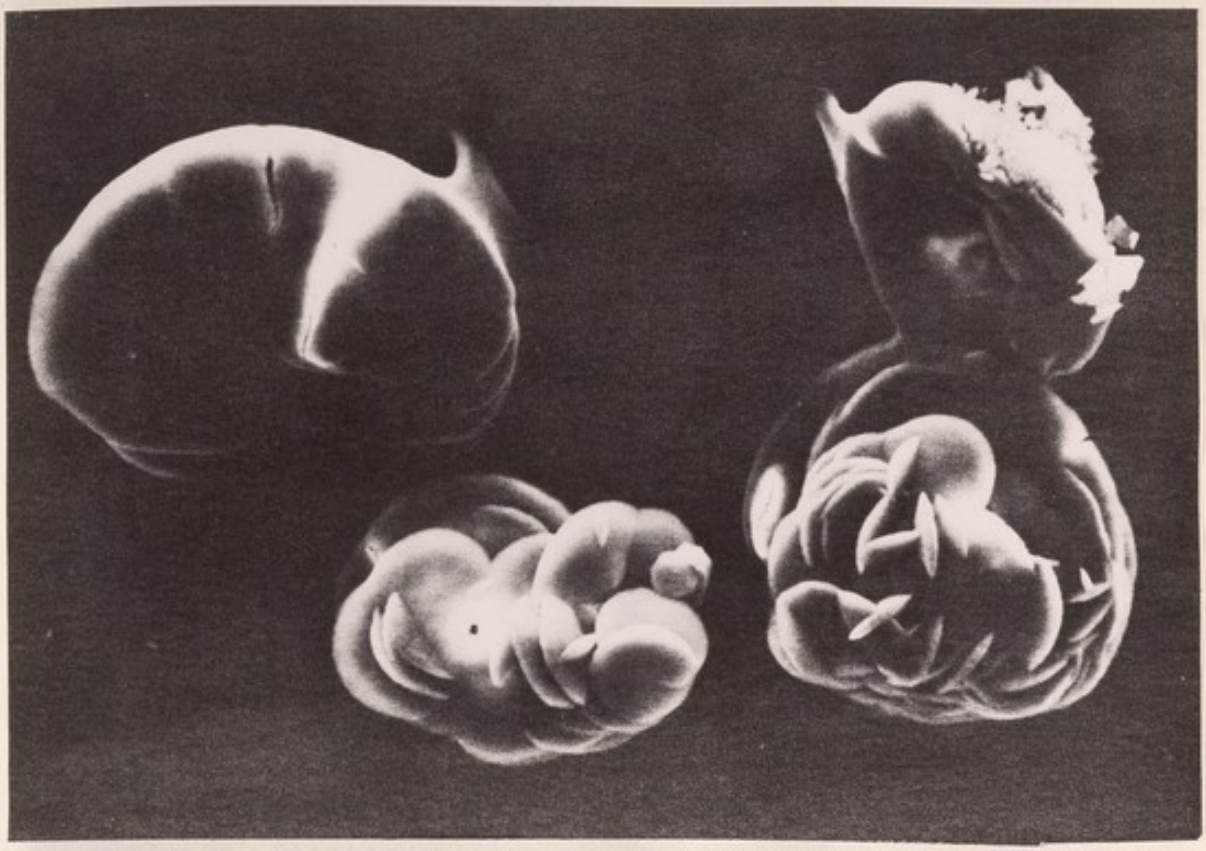


Plate 20 Electron micrograph of crystals from an empty zona pellucida (x 300 approx)

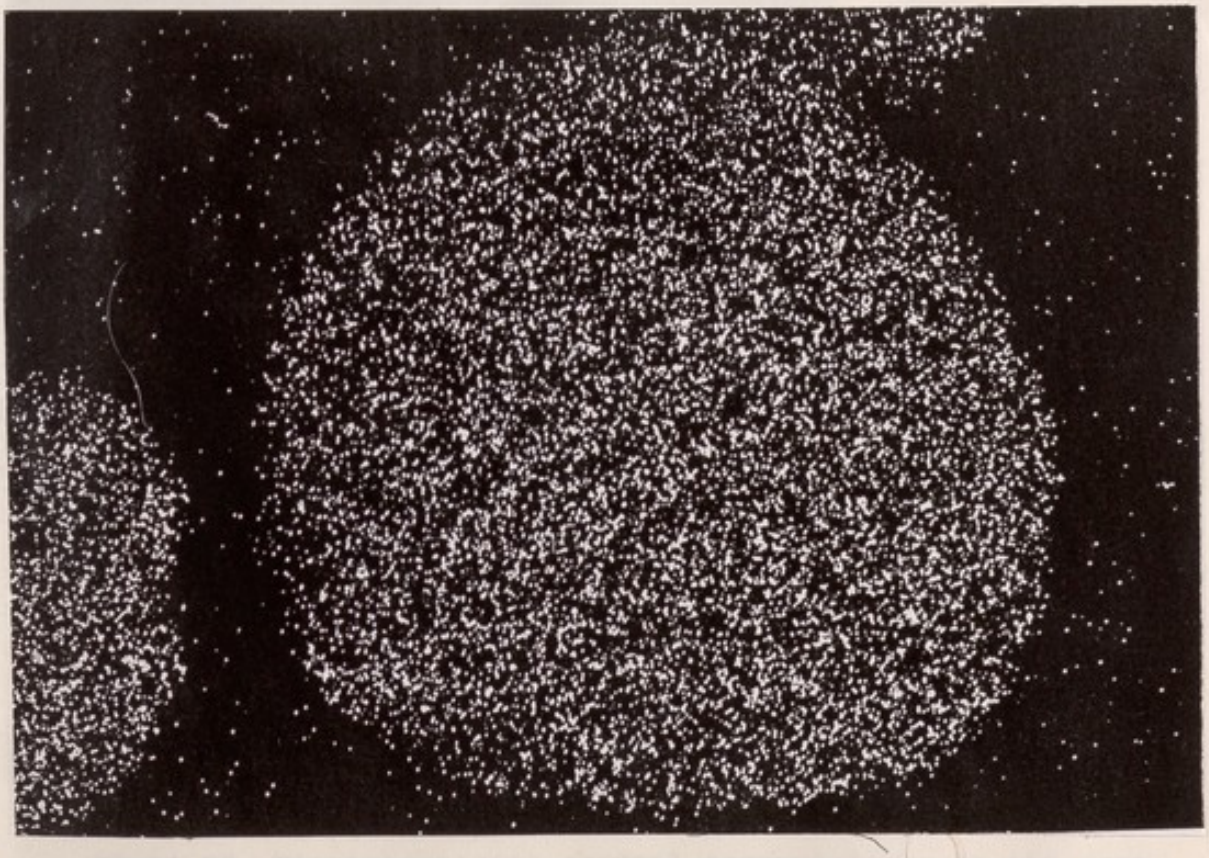


Plate 21 X ray map of crystals from an empty zona pellucida

Light areas = calcium (x 500 approx)

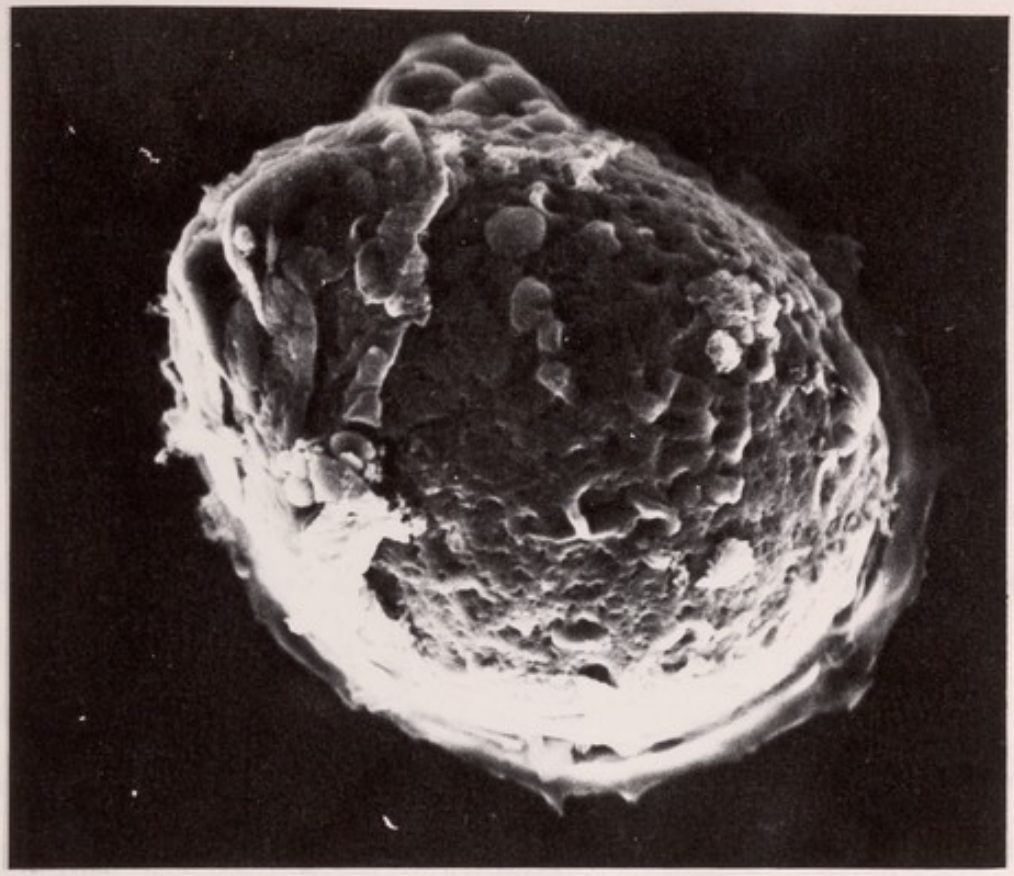


Plate 22 Blastocyst with crystal accumulations on the zona pellucida (x 500 approx)

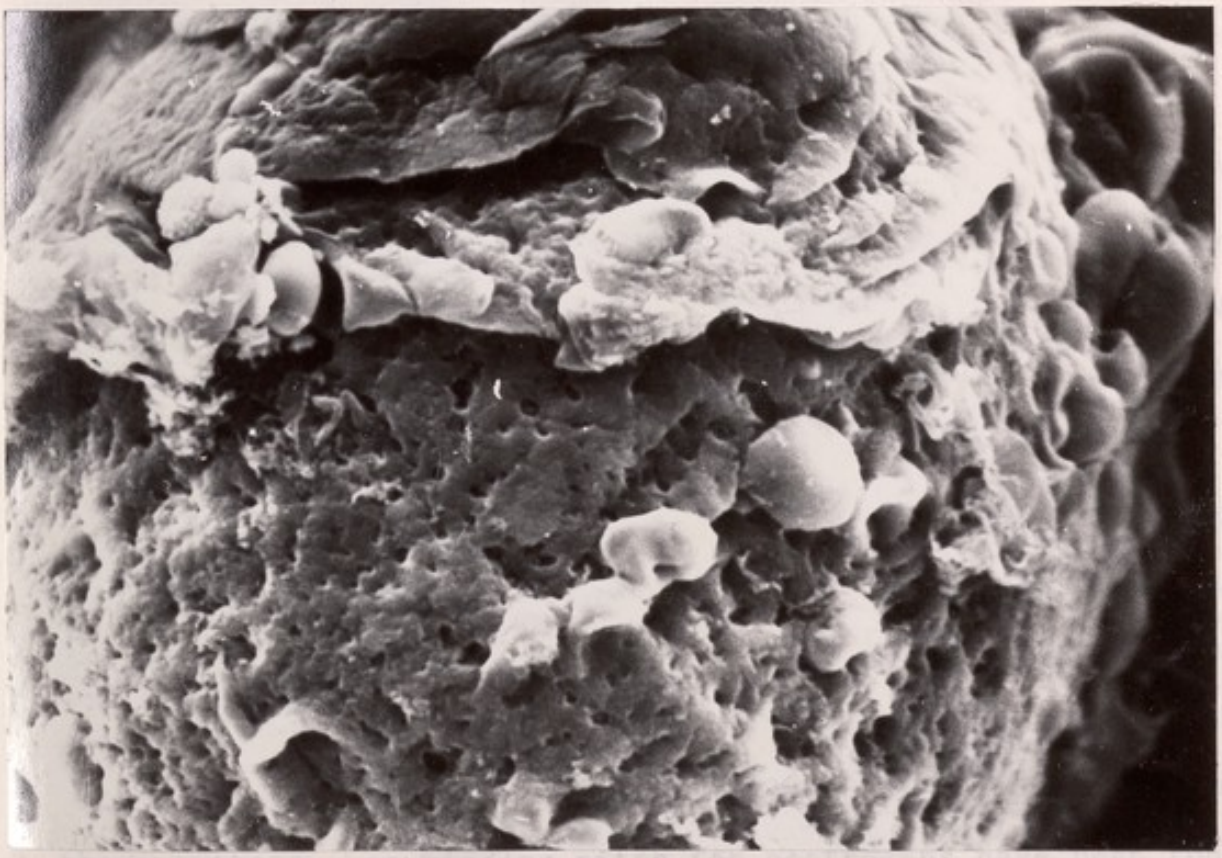


Plate 23 Blastocyst with crystal accumulations on the zona pellucida (x 950 approx)

Discussion

Some investigations were made into the abnormal morphology of embryos produced by gilt 7 at the second and subsequent oestruses. Crystalline material recovered from these flushes was identified as calcium salts. The exact nature of these salts was not elucidated but was probably of an organic nature such as calcium carbonate. The heavy deposits on the zona pellucida of these embryos were morphologically identical to the crystals above and were also formed from calcium. Fluid recovered after flushing this gilt had a greatly increased calcium content. As the gilt was in good health throughout the experiment and plasma calcium levels were within the normal range it seems likely there was an abnormally high level of active calcium secretion into the lumen. Macroscopically and histologically the uterus appeared to be normal.

Calcium is required for the development of early embryos and reduction in the concentration of calcium in the culture media reduces development (Brinster, 1968). However concentrations of calcium below that found in serum are used routinely in embryo culture.

The concentration of calcium in uterine flushing in pregnant gilts rises during the period of maternal recognition of pregancy on days 11-12 (Geisert, Renegar, Thatcher, Roberts and Bazar, 1982). This could be caused by the simultaneous rise in oestrogens, produced by the early filamentous blastocysts, found by these authors, as binding sites for oestrogens have been found to

be present on the cell membrane of rat uterine epithelium (Pietras and Szego, 1977). Although appearing morphologically normal it is possible that there was some disturbance in the secretion of calcium in the uterus of this gilt causing an abnormally high amount of calcium in the uterine lumen. If the saturation level was reached then calcium would crystallise out and form the crystalline material found on flushing. The slightly viscous adhesive nature of the zona pellucida (Flechon and Renard, 1978), with its rough porous seeming surface would be a good place for crystals to form. If oestrogens in the lumen of the uterus cause calcium secretion it is possible that the abnormality was not primarily that of the uterine epithelium but in a pathology causing an increase in uterine oestrogens. This could not however have been caused by the embryos as they were still in the zona pellucida and oestrogen secretion from the pig blastocyst does not commence until day 11 when the blastocysts were changing from spherical to the tubular shape.

It can be seen from the photographs that the calcium crystals built up on the surface of the zona pellucida. The fate of these embryos if they had been left <u>in utero</u> is unknown but is unlikely that, even if hatching proceeded, the unprotected embryo could tolerate the very high concentrations of calcium that would be present. Although at the time of collection the morphology of the embryos presented the appearance of the beginnings of degeneration, the morphology improved after culture in standard media and 6 of the 10 embryos produced metaphase spreads on processing. Thus at day 5 these embryos were viable. In a later recovery, however, 2 of the 4 products of ovulation recovered were

empty zona pellucida each with several large crystal accumulations adhering. Although empty zona pellucida had been recovered from other pigs flushed, this was a rarity. It is possible that these empty zona from gilt 7 were only recovered because the large dense bodies adherent to the zona pellucida made them more visible. Normally empty zona are difficult to visualise. However it is possible that the dense crystals caused early splitting of the zona pellucida and loss of the embryo from within.

CHAPTER 3

Cytogenetic Analysis of Pig and Sheep Embryos

CHAPTER 3

SECTION 3.1

Introduction

Cytogenetic abnormalities in the embryos of farm animals have been reported by several authors. Embryos from pigs were examined very early in the history of modern cytogenetics. 16 and 17 day old pig embryos from delayed matings were examined by Bomsel Helmreich (1961) who found 2.7% triploid and a total abnormality rate of 4.1%.

McFeely (1967) found 10% of 88 10 day old pig blastocysts had grossly detectable cytogenetic abnormalities, mainly triploids though tetraploids and a diploid/triploid mosaic were also present. This work was repeated by Day (1981) who found a lower proportion of abnormalities (6.6%), particularly polyploids (1.2%) in his control group but a similar proportion of abnormalities to McFeely in a delayed mating group. As well as polyploids Day found haploidies, monosomies, a trisomy and a monosomic/diploid mosaic. Dolch et al (1981) found only 1.8% tetraploid cells and 0.07% octoploid cells in 10 day blastocysts from prepuberal gilts treated with gonadotrophins. However Moon et al (1975) found 4 out of 15 day 12 blastocysts to be polyploid, three of them mixoploids. Moon (1977) found only a 1.48% true abnormality in 340 pig blastocysts 9-27 days old. These were triploids, a haploid and a translocation mosaic. There were also monosomic/diploid mosaics. 50% of his direct preparations were mixoploids and this increased to 95% after culture. Long and Williams (1982) investigated the origin of

TABLE 27

Cytogenetic examinations of pig embryos

Day after first day standing oestrus	Reference	Diploid	Polyploid e.g. 3n	Mosaics e.g.2n/3n	Mixoploid	Other abnormalities
10	McFeely (1967)	77 (89.5%)	7 (8.1%)	1 (1.2%)		1 (1.2%)
12	Moon, Rashid & Mi (1975)	22 (84.6%)	1 (4.2%)		3 (12.5%)	
11-16	Moon (1977)	165 (50%)	3 (0.9%)			2 (0.6%)
16-19	Moon (1977)	185 (96.4%)	3 (1.4%)			4 (2%)
11	Day (1981)	156 (93.4%)	2 (1.2%)			9 (5.4%)
11	Day (1981) delayed mating	52 (86.7%)	4 (6.7%)			4 (6.7%)
10	Dolch & Chrisman (1981)	169 (100%)			1.9% of cells	
10	Long and Williams (1982)	11 (36.5%)			20 (64.5%)	
10	" " " (1)	35 (89.7%)	2 (5.1%)		2 (5.1%)	
10	" " " (2)	20 (52.6%)	1 (2.6%)		12 (44.7%)	

(1) only inner cell mass of embryo cultures

(2) trophoblast only cultured

these polyploid cells in day 10 pig blastocysts. They found that the majority of polyploid cells were located in the trophoblast rather than the inner cell mass. The incidence of triploidy in this study was 2.9%. Smith and Marlowe (1971) examined 76 day 25 embryos and found only one embryo to be abnormal - a monosomy.

Pig embryos from translocation heterozygotes have been examined. King, Gustavsson, Popescu and Linares (1980) studied embryos derived from matings where one or both parents were heterozygous for a reciprocal (13Q-14Q+) translocation and found 20/69 (29%) of preimplantation embryos were unbalanced. No unbalanced embryos were found post implantation but estimates of embryonic death had risen. Akessen and Henricson (1972) working with embryos sired by a boar with a different reciprocal translocation found only 12.5% of analysed embryos unbalanced and the unbalanced embryos were found as late as 80 days of gestation.

Bouters, Bonte and Vandeplassche (1974) examined the chromosome compliment of embryos of various ages from 7 boars with reduced fertility. One boar produced embryos where cell division was not complete as the chromosomes of daughter cells remained linked and another boar produced embryos with an autosomal translocation.

Thus the level and type of chromosome abnormalities reported from pig embryos varies from author to author. These authors investigating translocation imbalance did not record incidences of other abnormalities. It is not known whether other abnormalities did not occur or they were not reported. Similarly the incidence of mixoploidy varies from 50% of embryos by Moon to 1.9% of cells

by Dolch. Cytogenetic abnormalities appear to be more common in studies with preimplantation blastocysts. Very little work, however, has been done on pig embryos still in the zona pellicida i.e. prior to day 7. King et al (1980) studied a few day 1 to day 6 embryos but did not present the results for these separately from older preimplantation blastocysts, while Bouters et al (1974) studied some day 6 embryos from the subfertile boars under investigation but gave no detailed results.

Cattle

Cytogenetic analysis of cattle embryos has been carried out not only to determine abnormality rates but also to sex normal blastocysts prior to transfer (Hare, Singh, Betteridge, Eaglesome, Randall, Mitchell, Bilton and Trounson, 1980; Wintenberger-Torres and Popescu, 1980; Popescu, Cribbu, Boscher, De Dapper, Da Costa, Henry, Wintenberger-Torres and Rebours, 1981). McFeely (1968) examined cattle blastocysts at day 12 to 16 and found 1 of 8 to contain tetraploid cells. Hare and Singh (1980) examined day 12 to 18 blastocysts or fragments of blastocysts prior to transfer and found around 40% of blastocysts were mixoploid, the proportion of polyploid cells increasing from day 13. Excluding mixoploids of 2n, one triploid, one diploid/triploid and one diploid/hexaploid embryo were found i.e. a 1.9% abnormality rate.

Eldridge, Larson, James and Cowan (1978) examined embryos from cows heterozygous for 1/29 Robertsonion Translocations and both Popescu (1980) and King et al (1980) examined preimplantation embryos sired by bulls heterozygous for 1/29

Robertsonion translocation. Eldridge et al found no abnormalities in the 13 day 8-12 blastocysts analysed. King et al examined embryos aged 1-15 days and found 2 of 38 embryos from the heterozygous bull to be trisomic and 4 to be possible monosomics. Popescu working with day 13 embryos found 2/52 monosomic for chromosome 1. No cytogenetic abnormalities were found by either author in embryos from normal bulls.

King et al noted a 2n/n 2 cell embryo and one that was polyspermic and found up to 25% of cells in day 15 embryos were polyploid.

In a survey of the chromosome content of bovine foetuses collected from an abbatoir Fechheimer and Harper (1980) found 7 out of 224 foetuses (3.125%) abnormal; 5 were sex chromosome chimeras, one a translocation mosaic and the other had a malformed gonosome.

Sheep

Chromosome complements of early sheep embryos have been studied at 2-3 and 15-20 days. The present study was intended to help fill the gap. Long and Williams (1978 and 1980) found 6% of 2-3 day sheep embryos had chromosomal abnormalities, these being one 2n/1n mosaic and 4 trisomics. An unfertilised egg was also trisomic, one embryo had a chromatid break and one unfertilised ova and 4 embryos were monosomic, which if real and not cultural or preparation artefacts gave 14.6% abnormal. However Williams and Long (1980) found 23.1% of superovulated day 2 and 3 sheep embryos were abnormal. The mean ovulation rate was 6.5. The abnormalities were extra sperm pronuclei, non-extruded polar

bodies, triploidy (3.3%), trisomy (3.3%), one haploid, one 53XY/54XY mosaic and one mixoploid 2n/4n. No abnormalities were found in day 16-20 sheep blastocysts (Long, 1977) and only one out of 86 embryos from superovulated sheep was found to be abnormal at day 20 (Williams and Long, 1980).

These findings indicate that chromosome abnormalities from embryos of sheep and cattle with normal chromosome complements may be lower than found in pig embryos. These abnormalities may be increased by superovulation and the majority of gross cytogenetic defects have proved lethal by day 20-25.

SECTION 3.2

EWE EMBRYO CYTOGENETIC RESULTS

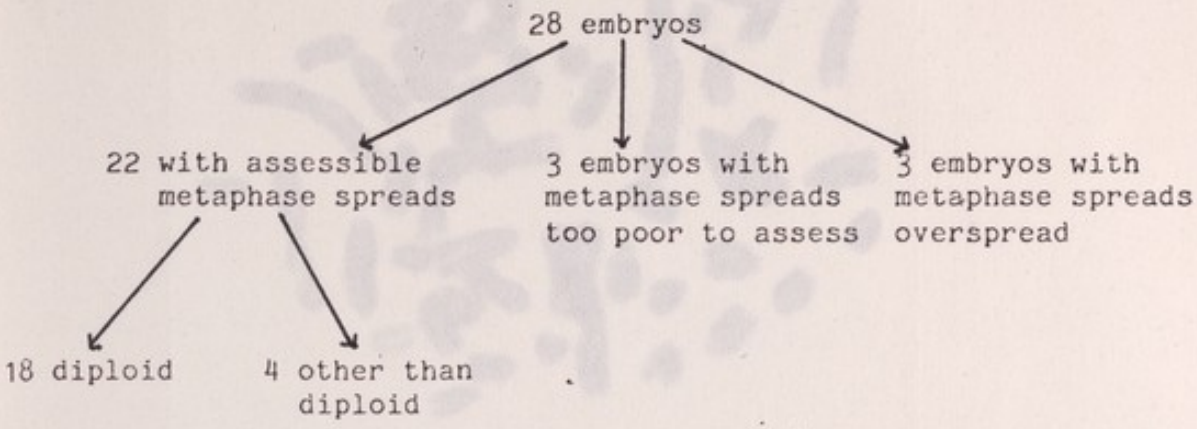
28 embryos produced metaphase spreads after culture and processing. A chromosome count of 26 or 27 was considered to be a haploid spread only where there was no evidence of loose chromosomes or other small chromosome groups in the vicinity. Frequently an accurate count of individual chromosomes could not be made due to inadequate spreading of part of the metaphase spread causing overlay of chromosomes. Provided only a small ambiguity was present e.g. a count of 48 plus a small group of overlayed chromosomes, the spread was considered to be diploid. Spreads were assumed to be tetraploid even though 108 chromosomes were not counted if there were between 100 and 110 chromosomes in a well defined group all at the same stage of contraction. Where there was doubt over the number of chromosomes in a mitotic spread that metaphase spread was photographed and re-examined.

Sexing

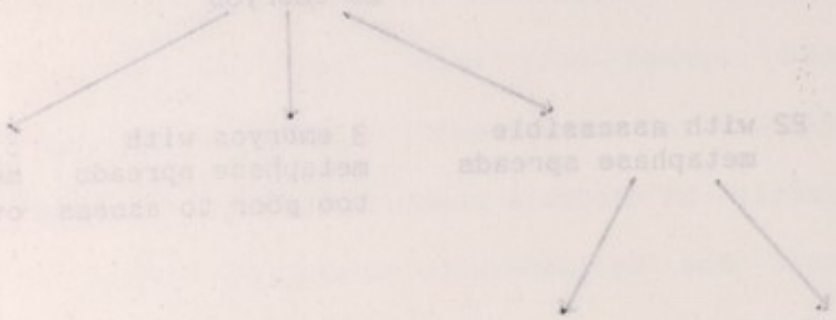
The Y chromosomes could be positively identified in metaphase spreads from 4 embryos. A count of 54 was made in 4 metaphase spreads where no Y chromosome could be identified. Due to excessive chromosome contraction and lack of chromotid separation it was found impossible to be sure that no Y chromosome was present or to positively identify two X chromosomes, which are acrocentrics only very slightly larger than the autosomes (Ford, Pollock and Gustavsson, 1980).

Of the 28 embryos that grew 22 had metaphase spreads where at least the ploidy was assessible. Three embryos had only metaphase

spreads that were too poor to assess the ploidy with certainty and in 3 embryos the mitoses were overspread and counting impossible.



Eighteen of the 22 embryos had purely diploid metaphase spreads. In 5 of these 22 embryos a definite count of 54 was made. In another embryo one metaphase spread of 53 chromosomes and another of 55 chromosomes were found. A Y chromosome was present in both spreads. When karyotyped the missing chromosome in the first spread was a medium or small acrocentric. The extra chromosome in the second spread was also an acrocentric though due to the early stage of contraction of these chromosomes it was impossible to tell which chromosome it was. Unless banded the chromosomes of the sheep from nos. 4 to 26 can be distinguished only by a gradual decrease in size (Ford et al, 1980). The X chromosome is the largest acrocentric. It would seem likely that the missing chromosome and the extra chromosome were autosomes and not the X chromosome.



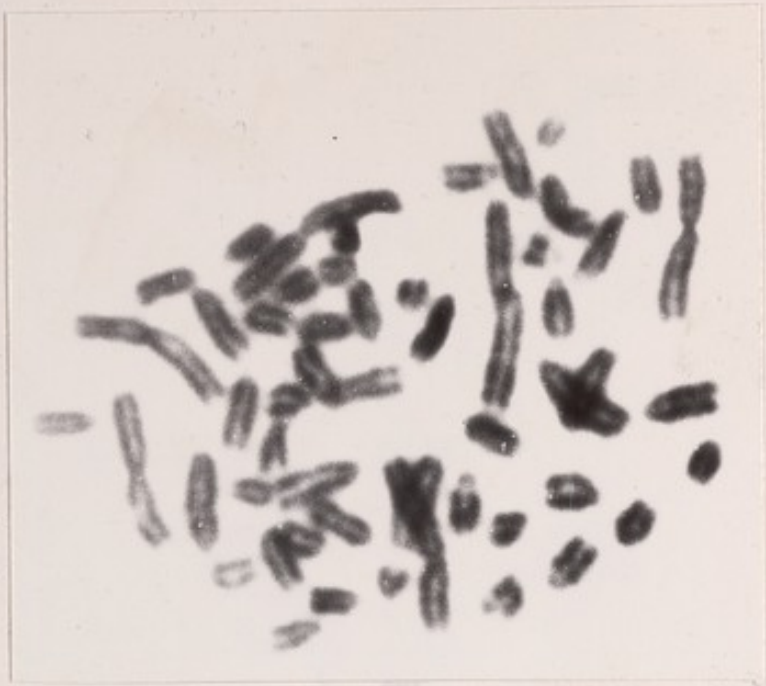


Plate 24 Sheep embryo E5 1/12/81 - monosomic spread

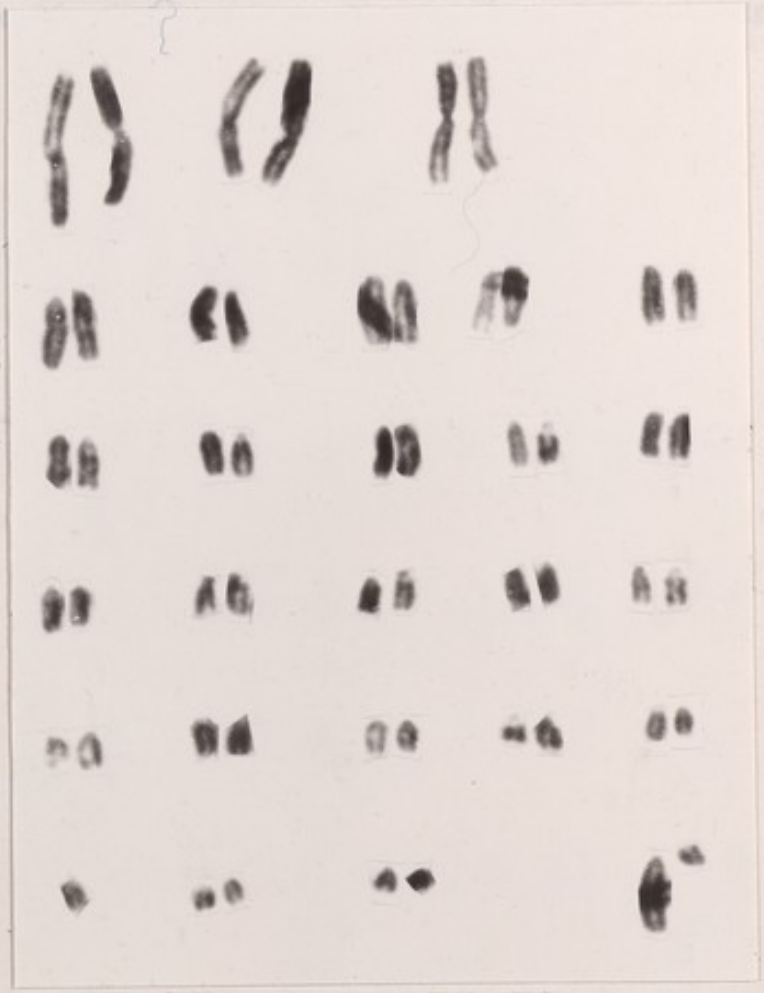


Plate 25 Karyotype from monosomic spread of sheep embryo E5 1/12/81

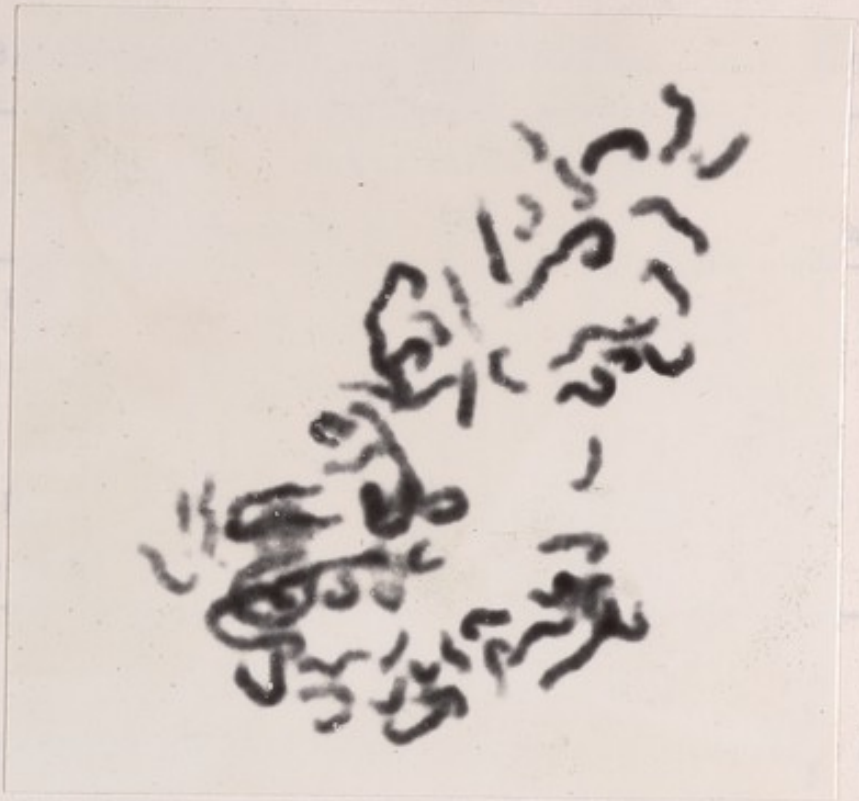


Plate 26 Sheep embryo E5 1/12/81 - trisomic spread

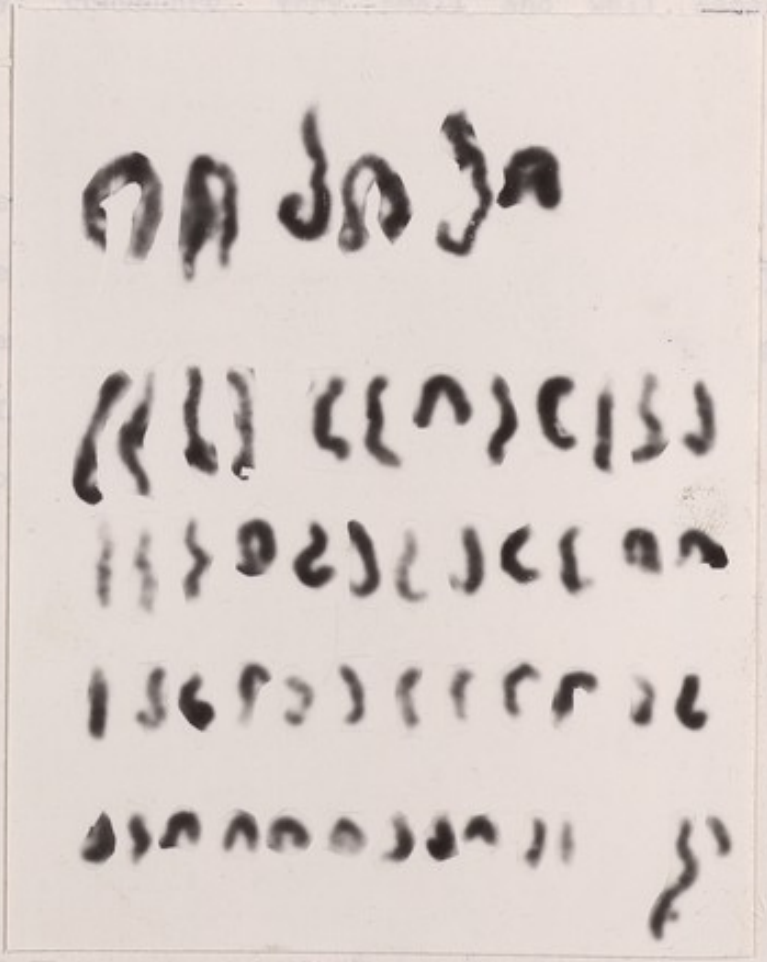


Plate 27 Karyotype from trisomic spread of sheep embryo E5 1/12/81

TABLE 28. Deviations from the chromosome complement of 54

Embryo	No. of cells counted					
	n	monosomic	trisomic	2n	3n	4n	>4n
1/12/81 E5		1	1	1			
30/11/81 E3	1						
30/12/81 E1	1						
20/1.82 E1				2		3	
27/30/82 E2						3	

Two embryos had one metaphase spread each with only a haploid count. The chromosomes in these haploid spreads were smaller in morphology; condensed, very small and well separated though contained in an area only 1/4 the diameter of the neighbouring nuclei.

Two embryos produced metaphase spreads with greater than 2n chromosomes. One embryo was a 2n/4n mixoploid with 3 tetraploid spreads and 2 diploid spreads, one of the diploid spreads being 54XY. The other embryo produced 3 tetraploid spreads in preparation.

TABLE 29

Data on embryos with analysable metaphase spreads

Deviation		Stage of Embryo	Morphology	Ewe No.	Tup No.	Time in Colc.	No. of C.L. in oestrus	Ewe Age	Season
Monosomy/ Trisomy		16 cell	good	110	101	4½	7	Adult	Breeding 1
Haploid		8 cell	good	108	101	4	3	Adult	Breeding 1
Haploid		8 cell	good	711	101	6	2	Ewe lamb	Breeding 1
2n/4n		exp. blast	good	723	106	21	1	Ewe lamb	Breeding 1
4n		exp. blast	good	110	101	18½	4	Adult	Breeding 2
Diploids	17	3x 4-16 cell	12x good		14x 101	9 8	3.8		6x summer
		12x morula	5x poor		2x 106	8 16			11x Breeding season
		2x early blast			1x Leicester				

Though all the embryos with chromosome deviations found were found from the breeding season studies this was not statistically significant (X^2 = 0.97).

The average number of ovulations was 4.7, in the oestrus cycle when flushing took place, in adult ewes from which the embryos producing deviating metaphase spreads were recovered. This is not significantly higher (t = 0.78) than embryos from adult ewes producing diploid (normal) spreads (3.8). In ewe lambs there was no difference, 1.5 and 1.3 being the respective means. Adult ewes in these studies had been shown to have significantly higher ovulation rates than ewe lambs so the comparisons were made separately.

Although 4 of the 5 embryos with chromosome complements deviating from normal were sired by tup 101 this is not significant (X^2 = 0.07) as 19 out of the 22 analysable embryos were sired by 101.

The embryos with polyploid metaphase spreads were expanding blastocysts. Thus 2 of the 4 expanding blastocysts producing analysable metaphase spreads had counts greater than 2n.

Of the 7 embryos recovered from ewe lambs, 5 produced metaphase spreads on processing. All were analysable. Two of these 5 were abnormal, one haploid, the other mixoploid, giving a total deviation rate of 40%. The total abnormality rate for embryos from adult ewes was 17.6%. There is no statistical difference (X^2 = 0.54).

There is no relationship between cytogenetic deviations and morphological abnormality in embryos of this age (X^2 = 0.97).

(see Table 30).

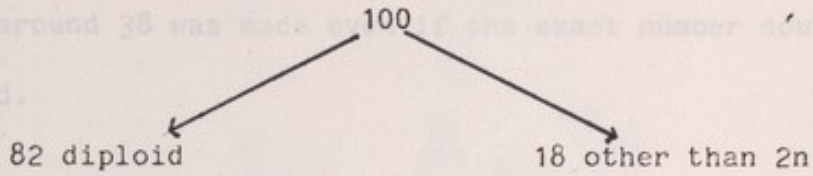
TABLE 30. Relationship between morphology and cytogenetic deviations

	Embryos assessed	
	cytogenetic normal	cytogenetic deviators
morphologically normal	11	5
in process of degeneration	6	0

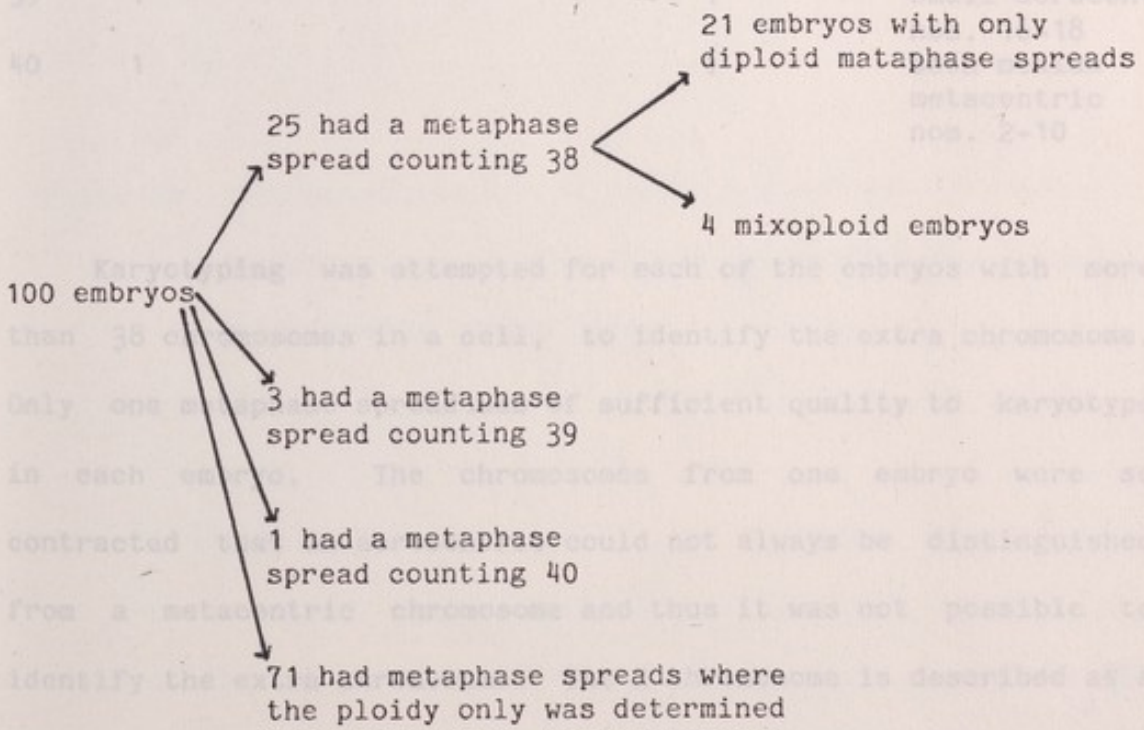
SECTION 3.3

RESULTS OF PIG EMBRYO MITOSES

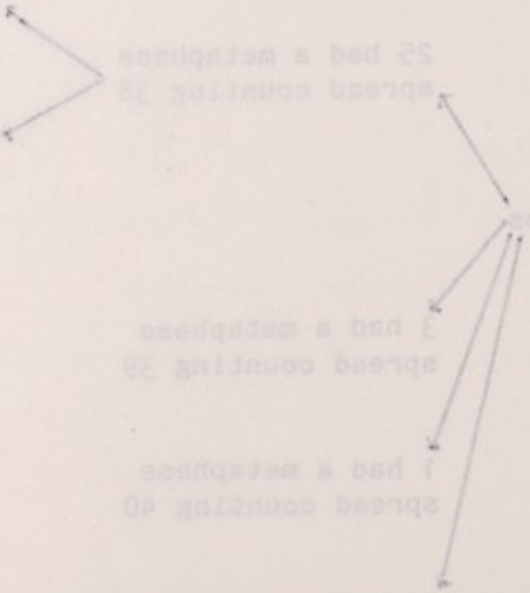
125 embryos with metaphase spreads were examined. 12 embryos had spreads which were uncountable due to the lack of spreading and 13 had metaphases too overspread or mixed for counting.



27 embryos were sexed, 9 being female end 18 male. A male cell was identified by the presence of a very small metacentric chromosome, the Y chromosome (Ford et al, 1980). A female cell was diagnosed by the presence of 38 chromosomes with the absence of a Y chromosome.







In many embryos the metaphase spreads were not of sufficient quality for a definite number to be counted or for them to be sexed. A count of around 38, i.e 36-39 was very common where chromosomes overlay one another or chromatids were widely separated or large secondary constrictions were present. A metaphase spread was considered to be diploid where an estimated count of around 38 was made even if the exact number could not be determined.

Trisomies

TABLE 31. Trisomies

No. diploid spreads		Y chromosome	No. cells counted	Extra chromosome
39	1	Y	1	? No. 1
39	1	Y	1	?
39	1		1	small acrocentric nos. 16-18
40	1		1	both medium metacentric nos. 2-10

Karyotyping was attempted for each of the embryos with more than 38 chromosomes in a cell, to identify the extra chromosome. Only one metaphase spread was of sufficient quality to karyotype in each embryo. The chromosomes from one embryo were so contracted that an acrocentric could not always be distinguished from a metacentric chromosome and thus it was not possible to identify the extra chromosome. The X chromosome is described as a metacentric similar in size to no. 9 (Ford et al, 1980) thus the two extra medium sized metacentrics found in the embryo with 40

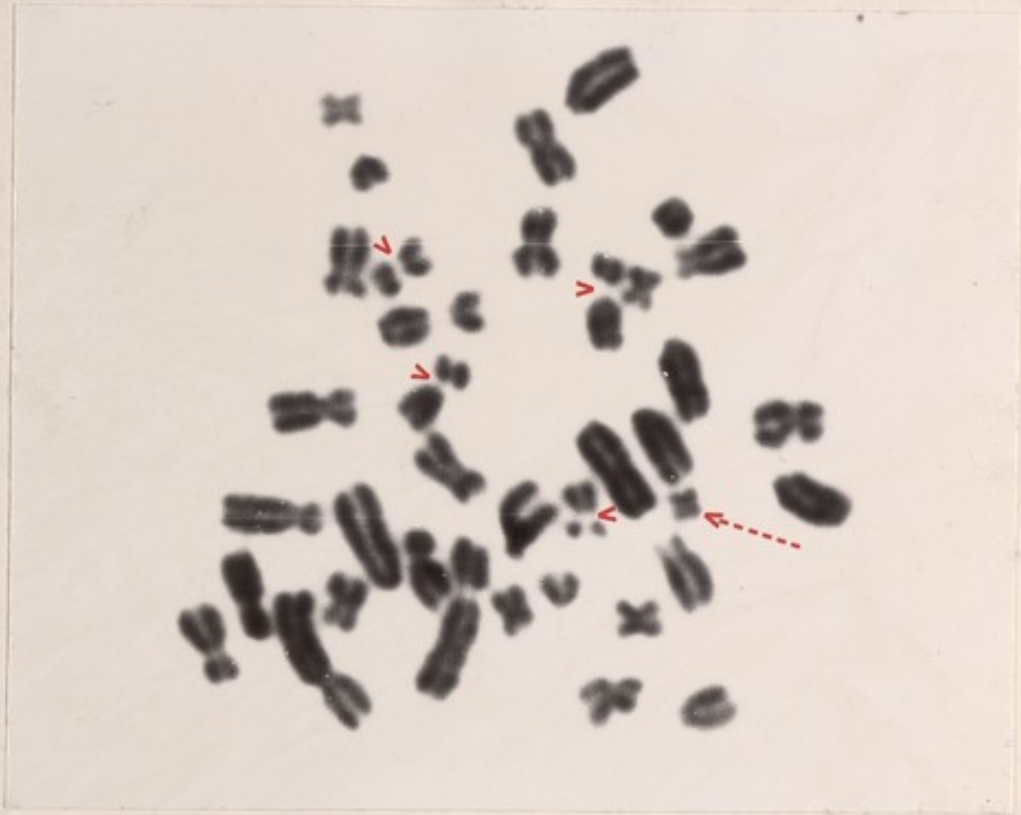


Plate 28 38XY metaphase spread from a pig embryo. Secondary constrictions in metacentric chromosomes are arrowed Y chromosome broken arrow

chromosomes may have been either X chromosomes or autosomes thus the embryo may have been 40XX ?2A+, 40XXX ?A+ or 40XXXX.

The karyotype of the embryo with an apparent extra chromosome 1 showed another abnormality. One chromosome no 13, the largest acrocentric appeared to be approximately half its normal length. The chromatid arms could have folded back on themselves to make the appearance merely a preparation artefact, or a fragment of this chromosome could have been lost or the missing portion could have formed a reciprocal translocation with a medium sized metacentric forming a large metacentric chromosome similar in appearance to chromosome 1. Any interpretation of the metaphase spread however leads to an interpretation of trisomy (see Plate 29).

4 embryos had haploid metaphase spreads. (Table 32).

TABLE 32. Embryos with haploid metaphase spreads

Embryo	No. haploid spread	Sex Chr.	Other spreads	Cell No.	Stage	Gilt
10/5/82 E2	1		None	35	early blast	1
1/7/82 P5 E6	1		None	67	exp. blast	5
38/1/83 E10	1	19Y (x1)	38XY (x1)	39	exp. blast	8
25/10/82 E3	1		2n(x2) 4n(x1)	69	exp, blast	7

In both of the metaphase spreads where the haploid spread was the only one present the chromosomes were of a very distinctive spidery appearance not seen in any of the other metaphase spreads, and staining very lightly. Identification of the Y chromosomes was impossible.

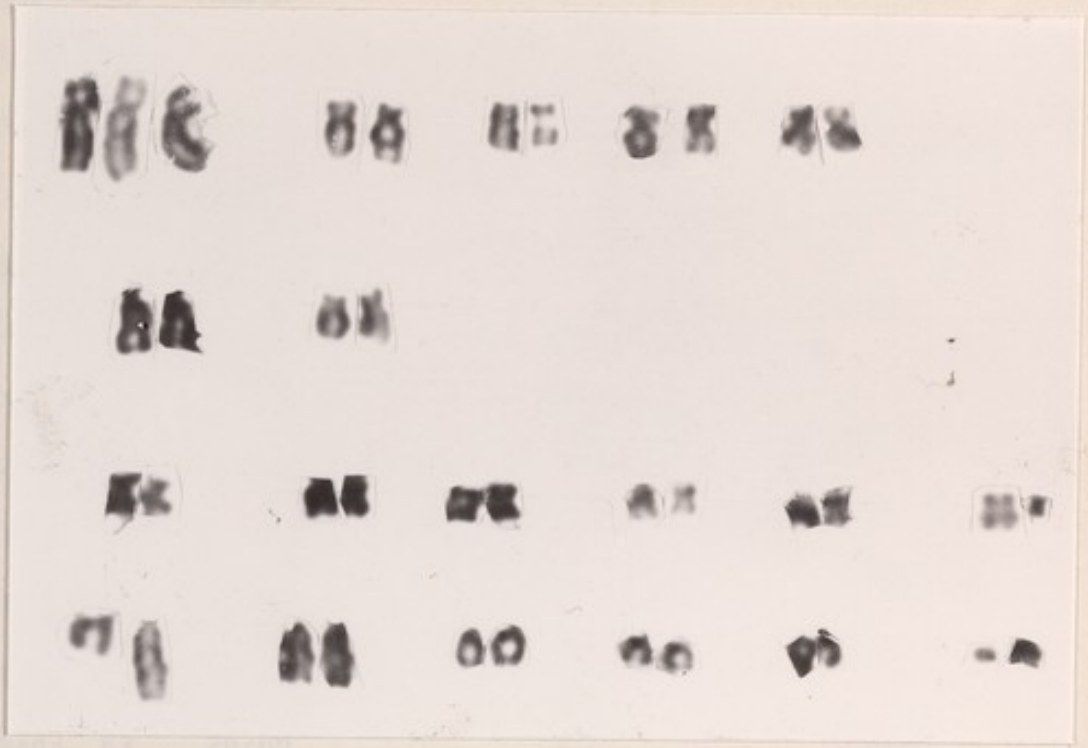


Plate 29 Trisomic pig embryo metaphase spread with apparent extra chromosome 1.

14 embryos were mixoploids (Table 33)

TABLE 33. Mixoploid Embryos

Embryo	ploidy	n	2n	3n	4n	>4n
19/10/82 E3	2n/3n		2	2		
19/10/82 E22	2n/3n		1	1		
18/1/83 E7	2n/3n		4	3		
19/10/82 E34	3n/6n			1		1 (6n)
10/8/83 E3	2n/4n		2		1	
1/7/82 E11	2n/4n		2		2	
1/7/82 E16	2n/4n		3		1	
1/7/82 E3	2n/4n		1		1	
19/10/82 E27	2n/4n		1		1	
18/1/83 E6	2n/4n		5		1	
18/1/83 E8	2n/4n		1		1	
18/1/83 E3	2n/6n		1			1 (6n)
18/1/83 E10	n/2n	1	2			
25/10/82 E3	n/2n/4n	1	2		1	

Also found was one embryo with a tetraploid metaphase spread only and one embryo with a triploid spread only.



Plate 30 Tetraploid metaphase spread 4n = 78 from a mixoploid pig embryo E8, 18/1/83

TABLE 34. Pig embryos with chromosome complements deviating from 38

	Nos. of Embryos
39	3
40	1
haploid	2
n/2n	1
n/2n/4n	1
2n/4n	7
2n/6n	1
2n/3n	3
3n/6n	1
3n	1
4n	1
Total	22

7 of the 9 mixoploids based on 2n and multiples were expanded or hatched blastocysts. Only 2 were embryos that had not developed an inner cell mass and blastocoel cavity.

More embryos with chromosome deviations were found from flushes where the ovulation rate was high, 25.4% vs. 16.2%, and also where PMSG and HCG was used to induce oestrus before collection 26.8% vs. 9.7%. These differences do not reach statistical significance.

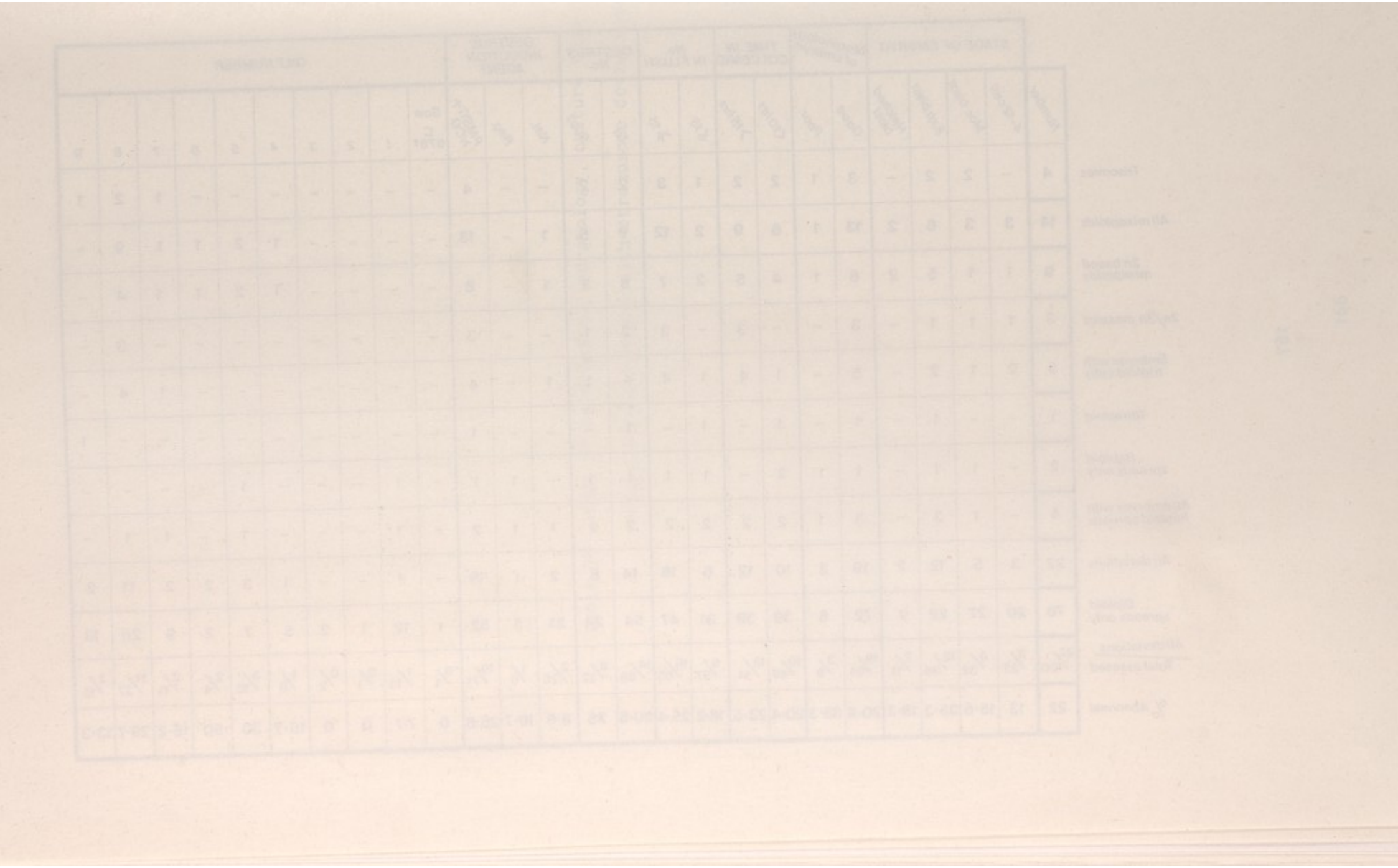
33% of morphologically abnormal embryos had a chromosome

complement deviating from diploid. This was higher but not statistically significantly different from morphologically normal embryos.

More deviations of chromosome number from 2n = 38 were found in embryos which had developed at least to the expanded blastocyst than in the younger, less advanced embryos 31% vs. 14.5%. This difference was principally due to the greater number of embryos containing tetraploid metaphase spreads among the more advanced blastocysts. However these were present even in the 4-12 cell stage. The differences in frequency of 2n based mixoploids in different stages of embryo development just fails to be significant ($X^2 = 2.96$, $P<0.01$).

Table 35 Stage of development, morphology, culture time and origin of pig embryos with chromosome deviations

		STAGE OF EMBRYO				Morphology of embryo		TIME IN COLCEMID		No. IN FLUSH		OESTRUS No.		OESTRUS INDUCTION AGENT			GILT NUMBER									
	Number	4-12 cell	Mor. blast	Exp.blast	Hatched blast	Good	Poor	<10 hrs	>15 hrs	≤13	≥15	1st	Subs	Nat.	Reg.	PMSG+ HCG	Sow C 6751	1	2	3	4	5	6	7	8	9
Trisomies	4	–	2	2	–	3	1	2	2	1	3	2	2	–	–	4	–	–	–	–	–	–	–	1	2	1
All mixoploids	14	3	3	6	2	13	1	6	9	2	12	9	5	1	–	13	–	–	–	–	1	2	1	1	9	–
2n based mixaploids	9	1	1	5	2	8	1	4	5	2	7	6	3	1	–	8	–	–	–	–	1	2	1	1	4	–
2n/3n mosaics	3	1	1	1	–	3	–	–	3	–	3	2	1	–	–	3	–	–	–	–	–	–	–	–	3	–
Embryos with triploid cells	5	2	1	2	–	5	–	1	4	1	4	4	1	1	–	4	–	–	–	–	–	–	–	1	4	–
Tetraploid	1	–	–	1	–	1	–	1	–	1	–	1	–	–	–	1	–	–	–	–	–	–	–	–	–	1
Haploid spreads only	2	–	1	1	–	1	1	2	–	1	1	1	1	–	1	1	–	1	–	–	–	1	–	–	–	–
All embryos with haploid spreads	4	–	1	3	–	3	1	2	2	2	2	2	2	1	1	2	–	1	–	–	–	1	–	1	1	–
All deviations	22	3	5	12	2	19	3	10	12	6	16	14	8	2	1	19	–	1	–	–	1	3	2	2	11	2
Diploid spreads only	78	20	27	22	9	72	6	39	39	31	47	54	24	21	5	52	1	12	1	2	5	7	2	9	26	13
All deviations / Total assesed	22/100	3/23	5/32	12/34	2/11	19/91	3/9	10/49	12/51	6/37	16/63	14/68	8/32	2/25	1/6	19/71	0/1	1/13	0/1	0/2	1/6	3/10	2/4	2/11	11/37	2/15
% Abnormal	22	13	15·6	35·3	18·2	20·9	33·3	20·4	23·5	16·2	25·4	20·6	25	8·6	16·7	26·8	0	7·7	0	0	16·7	30	50	18·2	29·7	13·3



SECTION 3.3.2

Pig foetuses - Cytogenetic results

Results from chromosome analysis of blood cultures of 10 pig foetuses were obtained. Only 8 of the 10 foetuses examined could be sexed by examining the metaphase spreads. 4 embryos were XX - and 4 XY.

All of the 10 foetuses had 2n metaphase spreads present and 9 of these had metaphase spreads of 38 chromosomes. It was impossible to determine the exact number in the diploid spread of foetus 4.

Five of the 10 foetuses had only diploid spreads, 2 had diploid and tetraploid spreads, 2 had diploid and tetraploid spreads plus 1 spread greater than 4n which might have been either a 5n or a 6n with chromosomes missing. A further foetus had 2n, 4n and a 6n spread present. In all foetuses except No. 3 and 4 there were mitoses present which were too poor to be assessed and occasionally overspread.

The results are summarised in Table 36.

Where the exact number of chromosomes could not be counted but were assessed at around 38, or the count was close to 38, the metaphase spread was assessed as diploid. There were no diploid counts of over 38 i.e. trisomes found.

TABLE 36. Chromosome results from pig foetuses

Foetus Number	Diploid Number	No. of spreads	Sex	2n	3n	4n	>4n	Result
1	38	7	XX	14				38XX
2	38	5	XX	14		3	1	Mixoploid 2n/4n/6n
3	38	1		2				38?
4				1				Diploid
5	38	5	XX	11		5		Mixoploid 2n/4n
6	38	14	XY	32		3		Mixoploid 2n/4n
7	38	7	XY	21		3	1	Mixoploid 2n/4n/?
8	38	3	XY	27		6	1	Mixoploid 2n/4n/?
9	38	6	XY	14				38XY
10	38	1	XX	13				38XX

Thus at least half of these foetuses, apparently developing normally, were mixoploids. It is possible that foetuses 3 and 4 may have proved to be mixoploid if more metaphase spreads had been present in preparations from these embryos.

The percentage of metaphase spreads greater than 2n for foetuses 2,5,6,7 and 8 respectively was 22.3, 31.2, 8.5, 16 and 20.6%. The average percentage of metaphase spreads greater than 2n in those embryos whhich were mixoploid was 18.6%. The percentage of metaphase spreads greater than 2n in all those sampled was 13.9%.

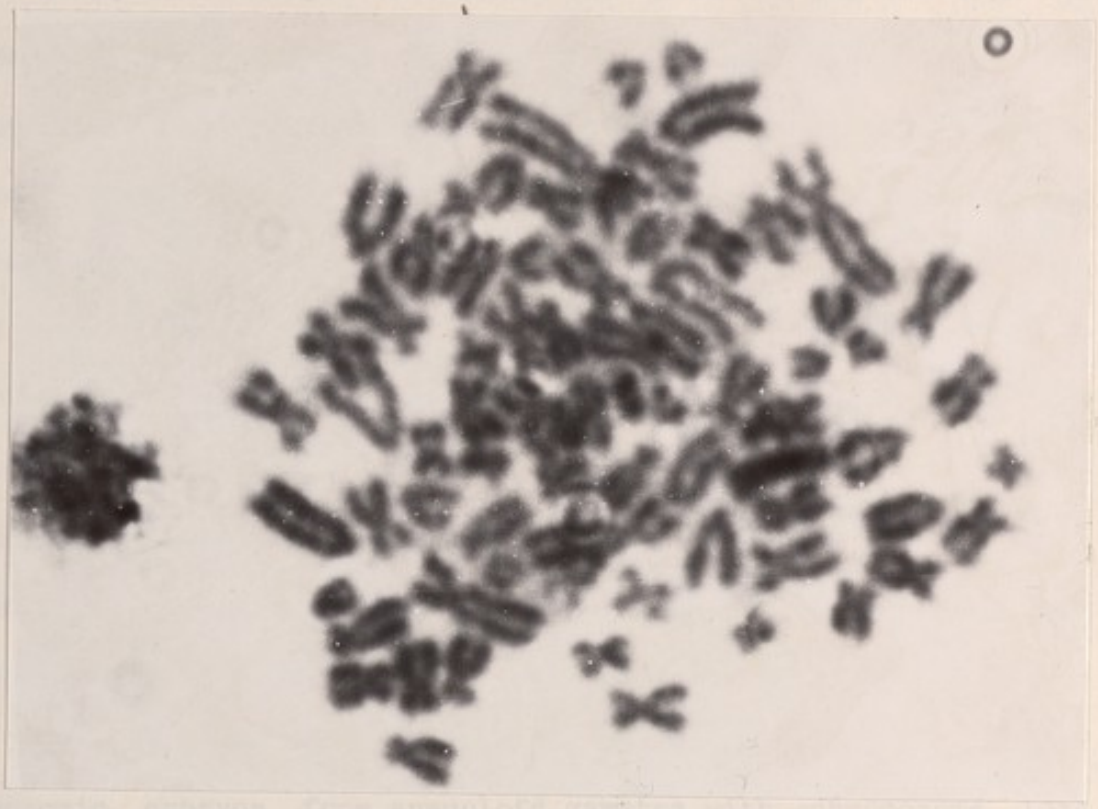


Plate 31 Tetraploid metaphase spread from leucocyte culture of pig foetus 5

SECTION 3.4

DISCUSSION

Section 3.4.1

Monosomy and Trisomy

Aneuploid embryos accounted for 4% of the pig and 4.5% of sheep embryos examined. This may be an underestimate of anuploidy as metaphase spreads with less than the diploid number were interpreted with caution as loss of a chromosome or chromosomes could be artefacts. Ford and Evans (1973), Cattenach and Mosely (1973) and Logue and Harvey (1978) suggested that to avoid errors caused by technical artefacts non disjunction rates are best calculated by doubling the incidence of trisomies and expressing this as a percentage of 2n $\pm$ 1 counts. This would give an estimate of aneuploidy in the pig embryos caused by non disjunction, of 9.8%. However even this may be an underestimate of the level of aneuploidy as it ignores the possibility of monosomic embryos from aneuploid gametes with chromosome loss caused by anaphase lag where no equivalent trisomic gamete is formed. The fact that monosomic embryos were not found may have been because monosomy is lethal early in embyrogenesis and any monosomic embryos were amongst those with a mitotic index of 0. In the mouse, monosomy has been found to be lethal earlier in gestation than trisomy (Ford and Evans, 1973; Epstein and Travis, 1979) with monsomy and trisomy found in equal numbers on day 3 but the frequency for monosomics 1 and 19 had fallen by day 4 while the incidence of trisomics was unaffected.

Trisomy is common in the newborn population in man; trisomy

21 occurs in 0.145% of births, trisomy 18 in 0.0125%, trisomy 13 0.01-0.025% of births, trisomy XXX at 0.08% of female births, XXY at 0.118% of male births and XYY at 0.11% of male births. Trisomics 8, 9, 14 and 22 have been rarely found at or after birth. The most common monosomy found is of the X chromosome at 0.08% of births (de Grouchy and Turleau, 1977). Using figures for embryonic death in man discussed earlier trisomy and monosomy are thought to account for 22% of all conceptions.

Monosomy and trisomy are also reported in embryos from mice (Vickers, 1969; Maudlin and Fraser, 1977), rabbits (Shaver and Carr, 1967; Fechheimer and Beatty, 1974; Fujimoto, Pohlavan and Duckelow, 1974), rats (Butcher and Fugo, 1967), pigs (Day, 1981; monosomy only Smith and Marlow, 1971), sheep (Long and Williams, 1978; Williams and Long, 1980) and effective trisomy and monosomy in unbalanced pig and cattle embryos from sires heterozygous for translocations (Akessen and Henricson, 1972; King, Linares and Gustavsson, 1980; King, Gustavsson, Popescu and Linares, 1980; Popescu, 1980).

Mechanisms of origin

There are three possible mechanisms for the production of monosomic embryos.

1. Monosomy may be a technical artefact with loss of a chromosome from cells during processing.

2. Anaphase lag occurs when one or more chromosomes fail to participate in anaphase and is excluded from both daughter nuclei. Harvey (1969) postulated that this may be more common in some chromosomes, i.e. small acrocentric in the pig, than in

others. Cattanach and Mosely (1973), working with mice heterozygous for Robertsonian translocations, also found non disjunction to involve some chromosomes more commonly than others.

3. The third cause, non disjunction, where one chromosome moves to the 'wrong' side of the spindle in anaphase and telaphase would produce a monosomic and a trisomic daughter cell. If non disjunction occurred in the second meiotic division gametes lacking a chromosome or with an extra chromosome would be formed. These gametes after fertilisation and syngamy with a normal haploid gamete from the opposite sex would produce either a monosomic or trisomic embryo. Non disjunction could however take place at the first or a subsequent mitotic division of the embryo. This is the likely mechanism for the monosomic/trisomic sheep embryo found. As only two metaphse spreads were countable it is possible that the other cells contained either trisomic or monosomic numbers of chromosomes from non disjunction at the first mitotic division or the diploid number of 54 if non disjunction had occurred in a later mitotic division.

Three pig embryos found were trisomic, one apparently for chromosome 1, one for a small acrocentric and the extra chromosome could not be identified in the third embryo. A further embryo had 40 chromosomes, both the additional ones being medium sized metacentrics. Aneuploidy in these four pig embryos could have been caused by non disjunction in the first or an early mitosis. As in each case only one metaphase spread was counted it is possible there was a monosomic cell line also present. However the trisomy could have arisen by fertilisation of a normal ova by a trisomic sperm or of an ova with a

chromosome complement of n + 1 by a normal sperm.

Fujimoto et al.(1974) found the distribution of the extra chromosome in trisomic cell lines to be uneven; in rabbits more trisomics were found with an extra acrocentric chromosome than other types. Boué et al (1975) and Hammerton (1971) also reported this in examining the karyotype of human abortuses, with an extra chromosome in the D, E and G groups being much more common than A, B and F groups. This could either be due to some trisomics proving lethal very early in the development of the embryo or some chromosomes being more subject to non disjunction than others.

In studies using tobacco mouse crossbreds Cattenach and Mosely (1973) found that trisomy for different chromosomes proved lethal at different stages of gestation with neither size nor position of the centromere determining the duration of development before death. Akesson and Henricson (1972) found unbalanced pig embryos from parents with a reciprocal translocation very much later than did King et al (1980) working with stock with a different reciprocal translocation. This indicates that in pigs also, effective monosomy and trisomy for different chromosomes is lethal at different stages of embryonic development. In the present study the extra chromosome or chromosomes in the aneuploid pig embryos were from different chromosome groups so selective non-disjunction did not seem to have occurred.

Causes

Fujimoto et al (1973) found that the genotype of the dam affected the incidence of trisomy. Genetic influences appear to play a part in mosaic monosomies and trisomies in man (reviewed

by Fechheimer, 1972). Two of the four aneuploid pig embryos came from the same gilt at the same oestrus. However these 2 trisomics from the 13 analysable embryos from this oestrus are not significantly more frequent than the 2 trisomies found in the other 87 analysable embryos ($X^2 = 2.2$).

Logue and Harvey (1978) found the level of aneuploidy in gametes higher in bulls heterozygous for the 1/29 Robertsonian translocation than in normal bulls. These aneuploid gametes have been shown to cause monosomic and trisomic embryos (Popescu, 1980; King et al, 1980). Bruère, Scott and Henderson (1981) also found an increased level of aneuploidy in gametes from rams heterozygous for Robertsonian translocation but Long (1977) found no corresponding aneuploid embryos at day 10. Akesson and Henricson (1972) and King et al (1980) have found pig embryos with monosomy and trisomy associated with reciprocal translocations. The effect of translocations on the chromosome complement of embryos seems to depend on the species of animal as Bruère (1974) found no reduction of fertility associated with Robertsonian translocations while Gustavsson (1969) and Refsdal (1976) did find a reduction in fertility in cattle and Akesson and Henricson (1972) also found a reduction in fertility in pigs.

This difference in the effect on fertility of aneuploid gametes may be due to a prezygotic selection of spermatozoa. King et al (1980) found the majority of unbalanced embryos were caused by adjacent-2 segregation leading to non-disjunction in primary spermatocytes. Translocation heterozyosity, though it appears to

increase the incidence of monosomy and trisomy in pigs is not likely to be the cause of 3 of the 4 trisomic pig foetuses found in this study as both sire and dams of these embryos were cytogenetically normal. However the embryo which apparently had an extra chromosome number 1 may have had a reciprocal translocation between a portion of the largest acrocentric chromosome, which may have had a portion missing, and one of the medium sized metacentric chromosomes. This would produce a chromosome of approximately the same size as chromosome 1. If this reciprocal translocation arose spontaneously in the gonads a quadrivalent instead of the normal bivalent would have formed leading to an increased chance of non-disjunction and the formation of an aneuploid gamete. However as only one karyotype is available for this embryo it is impossible to be sure if the reduction in the length of the arms of chromosome is a preparation artefact or if the chromosome had broken to form a fragment not seen in this preparation or was due to a reciprocal translocation.

Delay in fertilisation does not increase the incidence of trisomy in mice and rabbits (Shaver and Carr, 1967; Vickers, 1969) and the increase in monosomy and trisomy in pig embryos found after delayed mating by Day (1981) is very slight, 5/167 (3%) vs 3/60 (5%) and not statistically significant ($X^2 = 0.09$). However Butcher and Fugo (1967) showed that experimentally induced delayed ovulation increased the incidence of monosomy, trisomy and monosomic mosaics in rat embryos. There is no reason to believe that delayed ovulation took place in these studies as the pig embryos were at the expected developmental stage.

Butcher and Fugo's data are, however, interesting in that not only was the incidence of monosomy and trisomy increased, presumeably by errors in non-disjunction at oogenesis, but also monosomic mosaics were increased, which must be formed by a non-disjunction error of the zygote at the second or subsequent mitotic division. Thus an increased 'tendancy' to error rather than an immediate error must be caused by the delay in ovulation.

Maternal age is known to play a significant part in the incidence of non-disjunction during oogenesis in man increasing the incidence of triploidy with maternal age (Penrose, 1933; Sigler, Lilienfield, Cohen and Westlake, 1965; Wahrman and Fried, 1970; Boué et al, 1975) but does not increase the incidence of monosomy (Boué et al, 1975). The ewe which produced the monosomic/trisomic embryo was aged, but the dams of the trisomic pig embryos were only gilts at their first or second oestrus.

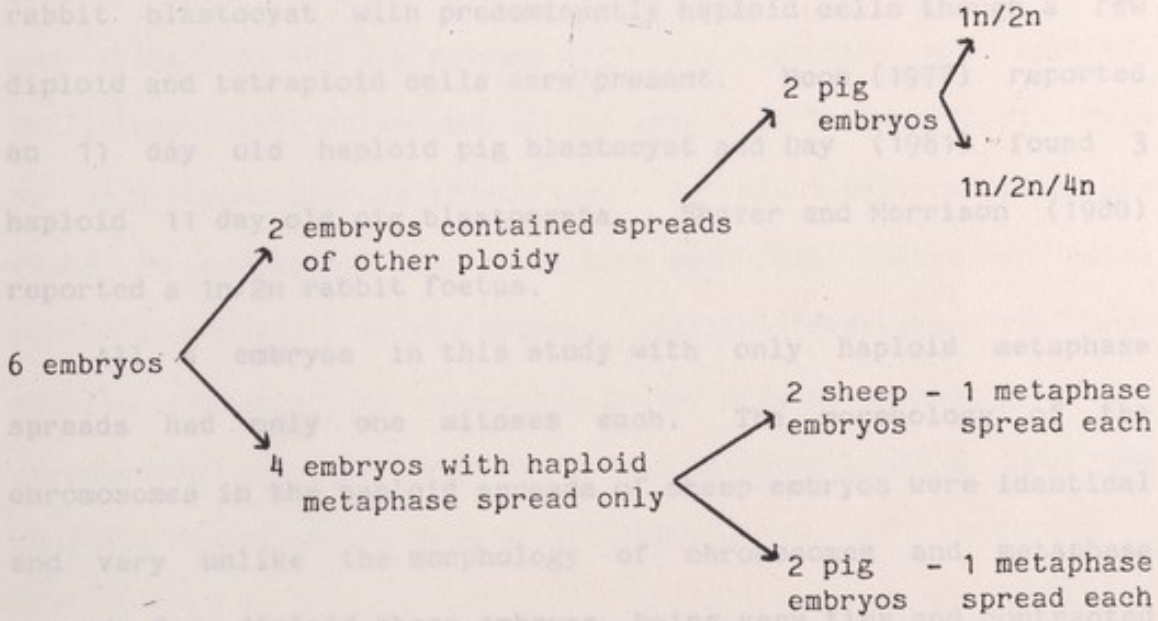
The monosomic/trisomic sheep embryo is not likely, however, to have been produced because of a fault in meiosis but as a result of an error in the first or an early mitotic division of the embryo. Aged ewes were found by Bruère (1967) to have a greater incidence of aneuploidy in cultured white blood cells than young ewes, presumably caused by mitotic non-disjunction or anaphase lag. It is interesting to speculate whether this tendancy to misdivision in mitoses would extend to the gametic products of aged ewes. Working with neonatal pigs derived from sperm irradiated by X rays, or where the gonads of the sire or grandsire were irradiated, Zartman, Fechheimer and Baker (1969) found a greater proportion of structural abnormalities from these

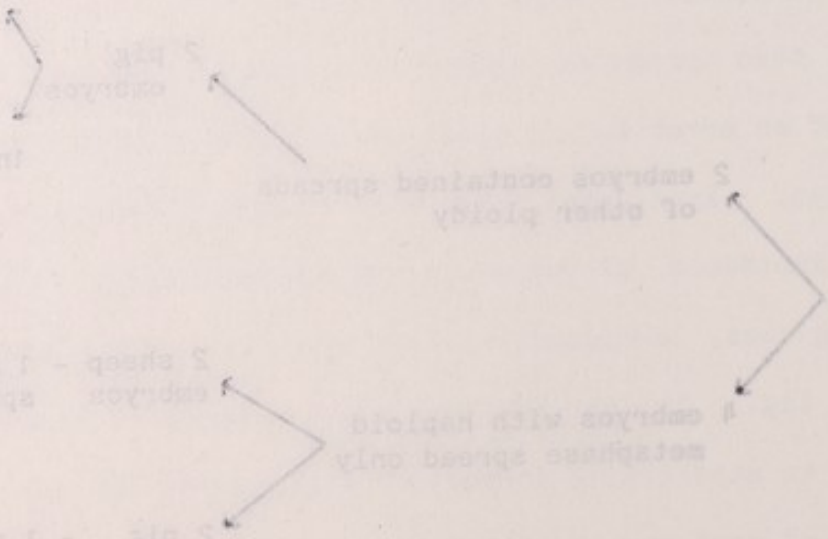
pigs than from control pigs. Mosaic conditions were common and the results were interpreted to mean that a tendancy to chromosome breakage was present, passed from an irradiated gamete or through a generation after irradiation to the embryo, and chromosome breakage occurred at varying stages in the development of the embryo. If a tendancy to chromosome breakage can be passed from an irradiated gamete to an embryo then perhaps a tendancy to misdivision could also be passed on to the embryo from gametes derived from aged parents.

Section 3.4.2

Haploids

6 embryos contained haploid spreads





Haploid metaphase spreads only

The 4 embryos with only a haploid spread varied in developmental stage. The 2 sheep embryos had 8 interphase nuclei and the pig embryos had 35 and 67 interphase nuclei being an early blastocyst and an expanded blastocyst respectively.

These 4 embryos may have been haploid in all their cells or the non-dividing interphase nuclei may have been diploid and the haploid spreads relics of a second polar body or a sperm pronucleus. True haploidy in an embryo may be due to parthenogenetic division, fertilisation of an ovum by a sperm with inactive genetic material, expulsion of the male pronucleus after activation of the female pronucleus prior to combination, or expulsion of the female pronucleus after penetration of the sperm. True haploid embryos have been reported by several authors. Hanson-Melander and Melander (1970) reported a smaller than normal rabbit blastocyst with predominantly haploid cells though a few diploid and tetraploid cells were present. Moon (1977) reported an 11 day old haploid pig blastocyst and Day (1981) found 3 haploid 11 day old pig blastocysts. Shaver and Morrison (1980) reported a 1n/2n rabbit foetus.

All 4 embryos in this study with only haploid metaphase spreads had only one mitoses each. The morphology of the chromosomes in the haploid spreads of sheep embryos were identical and very unlike the morphology of chromosomes and metaphase spreads from diploid sheep embryos, being very tiny and contracted but well spread. The chromosomes of the two pig haploid spreads were spidery rather than constricted and again unlike diploid pig embryo metaphase spreads.

Long and Williams (1980) found a 2n/1n mosaic sheep embryo and suggested the origin of the 1n metaphase spread was from an extra male nuclei caused by dispermy. King et al (1980) found a one cell ova from a superovulated cow with 4 pronuclei in metaphase, assumed to be polyspermic male pronuclei. King et al also found a 2n/1n mosaic 2 cell cow embryo thought to be due to activity of a retained polar body. Long and Williams (1980) suggested that chromosomes from a retained polar body in sheep tend to be long and spidery, whereas the haploid chromosome spread from the 2 cell cow embryo described by King et al (1980) as a retained polar body, had very small contracted chromosomes. Williams and Long (1980) found both embryos with non-extruded polar bodies and embryos with extra sperm pronuclei in 2 day old superovulated sheep embryos. In the present study the haploid spreads from the 2 sheep embryos were very small and contracted and those from the pig embryos were very elongated and spidery. It is impossible to be sure of the origin of these haploid cells as no Y chromosome was identified. As only 1 haploid spread was found in each embryo they could have been polar bodies or extra sperm pronuclei. It is unlikely that they expressed the true genetic content of the embryo due to the unusual morphology of the chromosomes and the very small area covered compared with the anaphase nuclei of the rest of the embryo.

Causes

Polyspermy is normally prevented in the ewe and sow by a change in the properties of the inner region of the zona pellucida (Hancock, 1963; Hunter, 1977). This is less effective in the aged

ova and so polyspermy occurs more frequently when insemination or mating takes place after ovulation than before. Fertilisation of aged ova, however, is not likely to be the cause of abnormalities in the gilts used in this study. One of the two gilts producing these haploid embryos was mated on both days of standing oestrus and the other on the only day she would stand, both by a highly fertile boar. Both ewes were naturally mated and as ovulation occurs near or after the end of standing oestrus (Cumming, 1979; Whyman, Johnson, Knight and Moor, 1979) the ova from these ewes were not likely to be aged before fertilisation.

Polyspermy also occures in pigs where a larger than normal number of sperm (10^5 - 10^6) are present in the oviduct around ovulation (Polge, 1978). Normally, however, the isthmus acts to regulate the number of sperm passing into the oviduct (Polge, 1978; Hunter, 1981). Frequently very large numbers of sperm were seen in and around the zona of recovered pig embryos. This was much less pronounced in sheep embryos. In pigs polyspermy also occurs commonly when ovulation is induced during the luteal phase. This appears to be due to progesterone dominated uterine environment rather than an intrinsic property of the embryo (Hunter, 1967).

In pigs, primary oocytes caused to ovulate by HCG injection appear to have a poor block to polyspermy (Polge and Dziuk, 1965). However the sperm in these immature ova showed no signs of developing into pronuclei, or the ova developing further (Hunter 1966). Superovulation using gonadotrophins increases the proportion of immature ova shed (Hunter, 1964 and 1966). The

embryos with haploid spreads in this study were not likely to be from immature ova as they had developed normally, one even becoming an expanded blastocyst. From the data of Long and Williams (1980) and Williams and Long (1980) extra sperm pronuclei and non-extruded polar bodies are more common in ova from superovulated than normal oestruses in sheep. Although oestrus had been synchronised using low doses of gonadotrophins, the affected sheep embryos came from sheep with 2 and 3 corpora lutea and thus could not be considered to have superovulated. The two porcine embryos came from gilts with ovulation rates of 9 and 19.

Retention of the second polar body occurs in fertilised aged ova (Shaver and Carr, 1967; Vickers, 1969). Heat shock can also cause retention of the second polar body (Fechheimer, 1972).

Though these mechanisms and polyspermy may cause triploid embryos, if the extra pronucleus was merely retained and not included with the two normal pronuclei it would be possible to obtain a normal diploid embryo carrying the remains of the extra pronucleus. The 4 embryos in this study may have had a normal genotype and thus continued their development to karyotypically normal young animals.

Two further pig embryos had a haploid metaphase spread, 1 also had 2 diploid metaphase spreads and the other 2 diploid and 1 tetraploid metaphase spreads. The origin of these haploid spreads may have been an extra sperm pronuclei. As both of these haploid spreads had a Y chromosome they could not have been caused by a retained polar body. However the morphology of the chromosomes in these haploid spreads was very unlike those discussed above, being very similar in size and staining density to the other chromosomes

present in the embryos. It is possible that these haploid spreads were caused by random loss of chromosomes either during mitoses or during preparation of the slide. However, though the quality of the chromosomes was too poor to karyotype with confidence, approximately the correct proportion of large to small and metacentric to acrocentric chromosomes were present in one of these metaphase spreads.

Brown (1972) suggested that haploid spreads may arise through abnormal spindle formation in a tetraploid cell leading to a triploid and a haploid daughter cell. The triploid cell line may have been lost or none been in division during the culture period.

Braden (1957) found immediate cleavage of an ova after fertilisation to occur in 0.9% of ova in one strain of mice. At the 2nd polar body formation, the cell split into two to give one cell with a haploid chromosome complement from a female pronucleus, the other with genetic material from both sire and dam. This is a different mechanism from mere retention of a polar body which would either cause the formation of a triploid nucleus or fail to be included in syngamy and remain as an inactive inclusion. This is not a possible mechanism for the n/2n embryos as a Y chromosome was present and found. It would appear to be under genetic control from the strain differences noted by Braden and the incidence even in the high strain group is low. There is no information on the possible morphology of the haploid chromosome groups.

SECTION 3.4.3

TRIPLOIDY

Two triploid embryos were found in this study. One of these was also mixoploid, having a 6n spread. Triploidy has been reported in early pig embryos by other authors (Bomsel-Helmreich, 1961; McFeely, 1967; Moon, 1977; Long and Williams, 1982) and in other species; the mouse (Beatty and Fishberg, 1952; Vickers, 1969; Maudlin and Fraser, 1977), the rat (Piko and Bomsel-Helmreich, 1960), the rabbit (Venge, 1956; Shaver and Carr, 1967 and 1969; Fujimoto, Puhlavan and Dukelow, 1974; Fechheimer and Beatty, 1974), cattle (Hare and Singh, 1980) and sheep (Williams and Long, 1980). It would appear that triploidy may almost always be lethal as authors working with older embryos and foetuses from pigs and cattle did not find them (Smith and Marlow, 1971; Akessen and Henricson, 1972; Fechheimer and Harper, 1980). However Walker, Andrews, Gregson and Gault (1973) reported cases of triploidy in man surviving till birth but never long thereafter.

Mechanisms of origin

Triploid embryos could arise through a variety of mechanisms.

1. Fertilisation of a normal ovum by 2 sperm and their incorporation into one nucleus.
2. Fertilisation of a normal ovum by a diploid sperm.
3. Fertilisation of a diploid ovum by a normal sperm.
4. Incorporation or suppression of polar body expulsion.
5. Initial mitotic failure i.e. duplication but non-division of the male or female pronucleus and then incorporation into a

triploid nucleus.

Causes

The presence or absence of one or two Y chromosomes aid in determinining the origin of the triploidy. However the metaphase spreads from the triploid embryos in this study were of insufficient quality to be sure of the sex.

Kajii and Niikawa (1977) Q and R banded chromosome preparations from human abortuses and their parents and used chromosome markers to help determine the origin of the triploidy. One triploid foetus was found to be due to maternal 1st meiotic division failure, 5 were due to dispermy, 2 were due to an error in paternal 1st meiotic division or the 1st mitotic division and 2 others were paternal but origin unknown. Lauritson (1982) also using chromosome markers, estimated that triploidy in man arises from an extra paternal set of chromosomes in 83% of cases and in 17% of cases from the mother. Jacobs, Angell, Buchanan, Hassold, Matsuyama and Manuel (1978) found 66% of triploid abortuses to be caused by dispermy, 24% by diploid sperm and 10% by a diploid ovum caused by failure of the 1st meiotic division. Thus in man the extra haploid set of chromosomes is of paternal origin in 83-90% of cases, though the major cause of triploidy (50-66%) would appear to be failure of the ova's block to polyspermy.

Polyspermy and suppression of expulsion of the second polar body have both been observed in domestic animals (Hancock 1968; Hunter, 1977; Polge, 1978; Williams and Long, 1980).

Salisbury and Baker (1966) found the proportion of diploid spermatozoa present in bull semen depended on the sire line of the

bull investigated, varying between 0. and 0.7%. Fechheimer (1972) reports that the highest incidence of diploid sperm from rabbits was found from very young males. However the triploid embryos in this study were found from matings where the boar was mature i.e. 17 and 20 months old. Maudlin and Fraser (1977) found 1% of mouse ova were fertilised *in vitro* by diploid spermatozoa and 2 of the triploid human abortuses examined by Kajii and Niickawa (1977) were caused by diploid sperm. Thus, in some species at least, diploid sperm are capable of fertilisation, though Fechheimer and Beatty (1974) found no evidence that diploid sperm used in their study with rabbits were a cause of triploid embryos found.

Superovulation, caused by injection of PMSG, has been found to increase the incidence of triploidy in various species. In the mouse, using *in vitro* fertilisation Maudlin and Fraser (1977) found the incidence of triploidy increased with the dose of PMSG used. Fujimoto *et al* (1974) found no abnormalities in embryos from non-superovulated rabbits but 4% of embryos produced after superovulation were triploid. In 2 and 3 day old embryos from superovulated sheep Williams and Long (1980) found 3.3% to be triploid, a further 8.8% to have extra sperm pronuclei and 2.2% non-extruded polar bodies. In a similar study using donor sheep which were not superovulated Long and Williams (1980) found abnormal embryos but no triploids. In this present study, however, one of the triploid embryos came from an oestrus where the ovulation rate was high and the other from a low ovulation number. Triploidy may be higher in superovulated embryos because high levels of gonadotrophins may increase the proportion of

immature ova shed (Hunter, 1964 and 1966) which have a poor block to polyspermy (Polge and Dzuick, 1974). It is thought however these immature ova may not develop (Hunter, 1966).

Delayed fertilisation has been shown by Vickers (1969) to increase the incidence of triploidy in mice; a 7-13 hour delay producing a nine-fold increase in incidence. It is not known what the mechanism was. In the rabbit, Shaver and Carr (1967) showed a delay of 8-14 hours in insemination after injection of HCG increased the incidence of triploidy from 0 to 8.5%. The same authors postulated in a later study (1969) that the principle mechanism for triploidy in these circumstances is digyny. Day (1981) found triploidy in a delayed mating group of pigs to be 5% and the incidence in a normal mating group to be only 1.2%. However Bomsel-Helmreich (1961) found only 2.7% of 16-17 day embryos from delayed mating to be triploid. It is possible that it is around this stage of development that triploidy becomes lethal in pig embryos as those found by these authors were severely retarded and at around day 12 of development. This would explain the apparently low percentage of triploid embryos found by this author after delayed mating. Controls were not established in this study.

The 2 triploid embryos found in this present study (2%) are close to the normal incidence found by Day. As the gilts used in this study were mated on each day of standing oestrus delayed fertilisation is not likely to be a cause of triploidy. Triploidy caused by delay in fertilisation is attributed both to loss of the block to polyspermy and non-expulsion of the second polar body (Fechheimer, 1972).

Genetic factors may increase the incidence of triploidy. The silver strain of mouse was found by Beatty and Fischberg (1952) to have 3.9% of triploid embryos whereas control mice had only an incidence of 0.6%. Braden (1957) found this increased incidence in silver strain mice was due primarily to non-expulsion of the second polar body. Williams and Long (1980) found all three of the triploid embryos in their study were from the same ewe. Thus genetic factors are possible. The same sire was used for all the embryos in this study and the two gilts may have been related as they came from the same farm. However the level of triploidy in embryos from these 2 gilts (2/48) is not significantly higher than from other gilts (0/52) ($X^2 = 0.59$).

Heat or cold stress may be a cause of suppression of polar body expulsion (Fechheimer, 1972). This is not likely to be a cause of triploidy in these studies as the gilts were in good health throughout the study and the environment was temperate.

Polyspermy has been found to result from a larger than normal number of sperm present in the oviduct around ovulation (Polge, 1978). This could be a cause of the triploidies found in this study if the normal regulating mechanisms in the isthmus were not operating effectively. Many sperm were present in the preparations from these two embryos but many of the diploid embryos also had a lot of sperm present in their preparations.

Thus the cause of the triploidies in this study cannot be elucidated. As the incidence of triploidy is very similar to that found by Day (1981) in his study of normal animals, it is possible that this is a normal level of 'errors' which can be increased by

any of the factors discussed above.

SECTION 3.4.4

Diploid/Triploid

Three pig embryos were found in this study with a 2n/3n mosaic chromosome complement. 2n/3n mosaics have been reported in embryos from cattle (Hare and Singh, 1980), pigs (McFeely, 1967; Long and Williams, 1982), rats (Piko and Bomsel-Helmreich, 1960; Bomsel-Helmreich, 1965) and rabbits (Hansen-Melander and Melander, 1970; Fechheimer and Beatty, 1974; Robinson and Shaver, 1979).

Mosaicism for triploidy is not necessarily lethal as viable individuals have been reported from a number of species, namely the cat (Chu, Thurline and Norby, 1966; Gregson and Ishmael, 1971; Centerwall and Benirschke, 1973), the mink (Nes, 1966), the pig (Melander, 1963), man (Lejeune, Sulman, Berger, Rothere, Rossier and Job, 1967) and cattle (Dunn, McEntee and Hansel, 1970; Hare and Singh, 1980). Although the calves reported were associated with phenotypic defects such as hermaphroditism and skeletal defects and phenotypic abnormalities particularly mental retardation have been reported in man (Mange and Mange, 1980) the mink and cats were only investigated due to infertility in the mink and tortoishell coat colour in the apparently male cat. As the cats showed no phenotypic abnormality apart from the incompatibility of the tortoishell coat colour (requiring 2 X chromosomes) and maleness (requiring a Y chromosome) it is possible that many more diploid/triploid mosaics may be present in the animal population than have been discovered at present. XX/XXX diploid/triploid mosaics may be difficult to identify as there may be no phenotypic abnormality such as evidence of

intersexuality present. As Polani (1969) found predominantly diploid cells, Lejeune et al (1967) found 635/665 of cells to be diploid in blood cultures from diploid/triploid mosaics in man, Dunn et al (1970) found only 1 triploid cell in 700 counted from blood cultures in a calf with a diploid/triploid mosaicism in other tissues, Centerwall and Benirschke (1973) found only diploid cells on blood cultures of a cat when the skin had 19/46 metaphase spreads triploid, and Gregson and Ishmael (1971) found no triploid cells in the 12 metaphase spreads obtained in blood cultures from a cat with diploid and triploid cells found in fibroblast culture, it would be easy to miss a diploid/triploid mosaic on blood culture for routine analysis.

Large scale surveys of the chromosome complement of domestic animals use blood culture routinely and normally 10-30 metaphase spreads are counted (Gustavsson, 1969; Amrud, 1969; Harvey, 1971; Pollock, 1974; Blazak and Eldridge, 1977). Thus with a frequency of 0-4.5% of triploid cells in leucocyte culture diploid/triploid mosaics may remain undetected, even in the small percentage of the domestic animal population which is chromosomally analysed.

Mechanisms

Various mechanisms are postulated for the origin of a diploid/triploid mosaic (Nes, 1960; Melander, 1963; Chu et al, 1964; Lejeune et al, 1967; Dunn et al, 1970) and are examined below.

1. Fusion of a diploid and a triploid zygote in utero
2. Vascular anastomosis between a diploid and a triploid twin
3. Incorporation of a haploid polar body nucleus into one of the

daughter nuclei at the first zygotic division

4. Incorporation of an extra sperm pronucleus into one of the daughter nuclei at the first zygotic division

5. Fertilisation of a second polar body and polyspermic fertilisation or fertilisation with a diploid sperm of the ovum and division of both cell lines.

6. Polyspermic or diploid fertilisation of the 2nd polar body and fertilisation of the ovum.

7. Fertilisation of the 1st polar body (2n) with one sperm and fertilisation of the ovum followed by normal expulsion of the second polar body.

8. Fertilisation of the ovum with 2 sperm or a diploid sperm and parthenogenetic development of the first polar body.

9. A triploid embryo arising by any of the mechanisms discussed previously and in an early mitotic division losing a haploid set of chromosomes either due to multipolar spindle formation or irregularily due to anaphase lag or non-disjunction.

Mechanisms 3 to 8 whilst theoretically possible, seem unlikely due to their complexity. Mechanism 1 may occur but cannot be the cause of the diploid/triploid pig embryos found in this study as these embryos were still in the zona pellucida. Mechanism 2 also cannot be the cause of the mosaicism found in these embryos but it would appear to be the likely cause of a diploid/triploid infant found by Lejeune et al (1967) where a mascerated co-twin was present at birth. It is possible that this mosaicism may have arisen through one of the other mechanisms mentioned, with early cleavage of the embryo into monozygotic

twins. Perhaps the mascerated twin had a higher proportion of triploid cells and thus failed to develop normally.

Mechanism 9 was thought by Dunn et al (1970) to be the cause of a diploid/triploid calf. Triploid cells were very scarce in blood and bone marrow culture but were present in greater concentration in other tissues. A diploid XXY cell was found in a preparation of bone marrow. Dunn et al (1970) and Lejeune et al (1967) suggest that diploid/triploid mosaics may have originated as triploids, a diploid cell line been produced, probably due to multipolar spindle formation and irregular segregation of chromosomes at anaphase, and that diploid cell line once formed was favoured, particularly in rapidly multiplying tissues. Melander (1963) suggested that the diploid cell line once formed was favoured, particularly due to the sex chromosomes. If this is the cause of the diploid/triploid mosaics found in this study then the divisions which gave rise to the diploid cell lines must have occurred at very early mitoses as one of the diploid/triploid embryos in this study was only at the 8 cell stage. The other 2 embryos were more advanced, one having 12 cells and the other being an expanding blastocyst.

It is interesting to note, though it fails to be statistically significant at $P = 0.05$ ($\chi^2 = 2.85$), that all 3 diploid/triploid mosaics were produced by the same gilt, 2 at the same oestrus. It is possible that some genetic influence such as in suppression of polar body extrusion in silver strain mice (Braden, 1957), is occurring with this gilt. One of 2 apparently triploid embryos came from this gilt also.

All 3 diploid/triploid embryos were from oestruses induced by PMSG and HCG with an ovulation rate of 20 or over. This however is not statistically significant ($X^2 = 0.54$). Thus as in triploidy, diploid/triploid mosaics in this study appear to be more common, though the numbers are not sufficiently high to give statistical significance, from those ovulations which could be said to be superovulated. This would be expected if mechanism 9 is the cause of the origin of diploid/triploid mosaics. Indeed all the mechanisms for the origin of these mosaics necessitate arising of a triploid cell line plus another 'mistake'. The incidence of diploid/triploid mosaics at 3% however seems unreasonably high compared to the incidence of triploidy, 2%, if the very complex mechanisms 3-8 are the cause of the mosaics. However if mechanism 9 is the cause, the true incidence of triploidy after fertilisation may be 5% with more than half the triploids becoming mosaics where eventually the diploid line may predominate and the embryo form an apparently normal young animal, if no sex mosaicism is involved. However Hare et al (1980) transplanted a 12-15 day bovine blastocyst found by chromosome analysis of a trophoblast fragment to be a diploid/triploid mosaic. Though the embryo was diagnosed as a diploid/triploid mosaic from analysis of the trophoblast this should be a true reflection of the chromosome complement of the embryo, as though giant cells are known to form in the trophoblast these have been found to be multiples of the ploidy of the inner cell mass. This diploid/triploid embryo was among the 69% of biopsied embryos which did not result in a pregnancy after transfer. As only 17/63 embryos from which a portion of trophoblast had been biopsied

resulted in a pregnancy, when transferred, there was only a 1 in 3.7 chance of it causing a pregnancy, even if the mosaic embryo could be considered to be as viable as diploid embryos . Thus because no pregnancy resulted from its transfer it cannot be taken to mean the embryo was not viable. Piko and Bomsel-Helmreich (1965) found diploid/triploid mosaics later in gestation in rats than triploid embryos. Beatty (1957) suggested that as only a few cells of the inner cell mass form the embryo proper, rather than the embryonic and foetal membranes, there is a natural anti-mosaic mechanism in the mammalian egg as these very few cells may all be of the same chromosome complement in a mosaic embryo.

Though the low numbers of diploid/triploid domestic animals reported may mean the diploid/triploid embryos found in this study were not viable, the data discussed above indicates that diploid/triploid mosaics, whether originally triploid embryos or derived by other mechanisms, may not necessarily fail to develop but the diploid cell line may predominate and allow the maturation of a viable neonate. If no phenotypic abnormality was present, for example intersexuality, if a male cell line were involved, the individual may go undetected even if examined for chromosome complement by blood culture.

SECTION 3.4.5

TETRAPLOIDY

Tetraploidy was found in one pig and one sheep expanded blastocyst. Though these embryos may have been mixoploids as discussed below, the sheep embryo had 3 tetraploid metaphase spreads and no diploid metaphase spreads and therefore was likely to be tetraploid. As the tetraploid pig embryo had only one metaphase spread present after processing, it is possible that diploid cells were present but were not represented by metaphase spreads.

Tetraploid early embryos have been reported in the pig (McFeely, 1967; Moon et al, 1975) and the mouse (Beatty and Fischberg, 1952; Edwards, 1958; Vickers, 1969)

Mechanisms of Origin of Tetraploidy

1. Fertilisation of an ovum and suppression of cell cleavage at 1st mitosis.
2. Trispermic fertilisation of an ovum.
3. Fertilisation of both an ovum and a 2nd polar body and combination at syngamy.
4. Fertilisation of the 1st polar body (diploid) and inclusion of the ovum, after expulsion of the second polar body.
5. Diploid fertilisation of the ovum and inclusion of the 2nd polar body.
6. Parthenogenetic development and suppression of extrusion of both polar bodies.

Tetraploid blastocysts, where the sex is reported, appear to be principally XXYY or XXXX. Mechanism 1 would always give this result and mechanisms 2, 3, 5 and 6 may produce XXXX or XXYY embryos. Kajii and Niikawa (1977) found, on investigating the origin of a tetraploid human abortus, that there was duplication of the maternal and paternal sets of chromosomes. Mechanism 1 would always given this result and mechanisms 3 and 5 may also. Mechanism 1 seems therefore to not only be the simplest mechanism but also the one that fits best with the very limited experimental evidence. The other mechanisms mentioned, though possible seem unrealistically complicated. In this study, fertilisation was assumed to have taken place by the presence of large numbers of sperm in the zona pellucida of both embryos, and though parthenogenetic development initiated by fertilisation by a sperm with inactive genetic material is possible it is not likely.

Causes

Experimentally tetraploidy has been induced in mice by prevention of 1st cleavage division through culturing ova in vivo in a dilute solution of colcimid shortly after fertilisation (Edwards, 1958). This has also produced diploid/tetraploid mosaics presumably by suppression of a cleavage division after the first. Both the pig and sheep tetraploid embryos in this study were expanded blastocysts on recovery and though the sheep embryo was cultured in the presence of colcimid for 18.5 hours the pig embryo was only in culture for 9.5 hours. Thus with the interval between cell division in pig embryos 16-20 hours and in sheep embryos 18-24 hours there was not sufficient time for colcimid to

disrupt spindle formation and prevent cell cleavage, and allow another replication cycle so 4n chromosomes were present at the metaphase analysed.

Heat shock and cold shock have been reported by Beatty (1957) to cause suppression of cleavage division in mammalian embryos. To produce a true tetraploid embryo this shock would have to occur around 15 hours after fertilisation i.e: late on day 1 after oestrus in the sheep and on day 2 in the pig where the first day of standing oestrus is day 0. During the entire period of the experiment the ewe and sow were kept under temperate conditions and were clinically healthy. Thus temperature shock is not likely to be a cause of tetraploidy in this study.

Polyspermy or a combination of polyspermy and activation or failure of expulsion of polar bodies would be required for mechanisms 2-5. Aging of ova before fertilisation can cause suppression of polar body release in the rabbit (Shaver and Carr, 1969) and reduce the block to polyspermy in the pig and sheep ova (Hancock, 1963; Hunter, 1977). However the pig ova in this study were not likely to be aged before fertilisation. Ovulation normally takes place on the second day of standing oestrus and as mating took place on both days of standing oestrus even if ovulation took place abnormally early, fertilisation would take place before any aging of the ova. Oestrus was induced in the dam of the sheep embryo by prior use of a medroxy progesterone acetate sponge and PMSG injection. Standing oestrus normally occurs on the second day after sponge withdrawal and ovulation around the end of standing oestrus. As the tup was not

allowed free access to the ewe it is theoretically possible that if the ewe was mated at the end of standing oestrus and if ovulation occurred abnormally early the ova may have been aged but as the progesterone source was removed only 50-54 hours before mating it seems unlikely there was sufficient time for ovulation to take place before mating.

MIXOPLOID DISCUSSION

Nine mixoploid pig embryos and 1 mixoploid sheep embryo were found with metaphase spreads of 2n and multiples of 2n. Eight of the pig embryos and the sheep embryo had diploid and tetraploid metaphase spreads and the ninth pig embryo had a diploid and a 6n spread. Five 78 day pig foetuses were also found to be mixoploid on blood culture. The polyploid cells were principally tetraploid but some cells with chromosome counts greater than 4n were found.

2n based mixoploids or diploid preimplantation embryos with a proportion of polyploid cells have been described in the pig (Moon et al, 1975; Moon, 1977; Dolch and Chrisman, 1980; Long and Williams, 1982), sheep (Williams and Long, 1980), cattle (Hare et al, 1980; King et al, 1980), rabbit (Shaver and Carr, 1967; Fechheimer and Beatty, 1973; Fujimoto et al, 1974) and in a human foetus (Kohn and Robinson, 1970).

The polyploid cells found in day 4 to 6 embryos in this study were principally 4n though 2n and 6n metaphase spreads were found in preparations from 1 pig blastocyst. The origin of the mixoploidy is uncertain. These mixoploid embryos may have originally been tetraploid and by division without chromosome replication, or divisions involving multipolar spindle formation, formed a diploid cell line. The most probable method for formation of a tetraploid embryo is failure of cell division after chromosome replication at 1st mitosis. Other mechanisms involve trispermic fertilisation, fertilisation of a diploid ovum by two sperm or a diploid sperm and fertilisation of an ovum and a polar body and fusion to form a tetraploid cell. As 9% of early

pig embryos, 50% of pig foetuses and 4.5% of sheep embryos assessed were 2n based mixoploids it seems an unacceptably high percentage for the mechanism of origin to be basically those of tetraploid embryos with another 'mistake' added. It could also be postulated that two independent cell lines were present initially, for example, a diploid sperm or two sperm fertilising a first polar body and fusing with the diploid product of normal fertilisation. However the mechanism to provide this possibility also seems unrealistically complicated.

It would seem more likely that the embryos originated as normal diploids and the tetraploid cell lines arose early in development. Brown (1972) and Hare and Singh (1980) suggested that polyploid cells can be formed by chromatid replication and separation but without effective anaphase disjunction and cell division i.e. by endoreduplication. Polyploidy could also be caused by fusion of 2 cells and their nuclei or simultaneous division of their nuclei on 1 spindle to form 1 tetraploid cell.

Thus either a very small proportion, or up to half of cells in a mixoploid individual would be likely to be polyploid where the original cell population was diploid, whereas more than half the cells examined in an early embryo might be expected to be tetraploid if an error at 1st mitotic division or anomolies of fertilisation were the cause of the tetraploid cell line. In the present study either an approximately equal number of diploid and polyploid metaphase spreads were found or the diploid cell line appeared to predominate. This finding is in agreement with other authors though working with more advanced preimplantation blastocysts, and thus different material. Dolch and Chrisman

(1980) found only 1.8% of cells examined from day 10 pig embryos to be polyploid. King et al (1980) found up to 20% of cells in 15 day old cow embryos to be polyploid. Hare et al (1980) found 7.6% of day 13 cattle embryo cells to be polyploid increasing to 25.9% in day 18 embryos. However the polyploid sheep embryo found by Williams and Long (1980) was only at a 2 cell stage and had one 2n metaphase spread and one 4n.

It has been observed by Moon (1977) that culture of a 10 day pig embryo for 24 hours has increased the proportion of mixoploid embryos from 50% of blastocysts in direct preparations to 95% of embryos after culture. Hare et al (1980) noted a similar increase in 12-18 day cow embryos where 38.7% of embryos processed directly were polyploid mosaics and 66.6% of those embryos cultured for 18-22 hours were polyploid mosaics. However the average percentage of polyploid cells in each mixoploid blastocysts did not increase being 17.6% and 23.7% respectively. Walker, Lee and Gregson (1970) found that increased culture time increased the proportion of polyploid cells in culture from human amniotic fluid and Nikagoma, Linuma and Matsumaga (1972) found altering the technique reduced the incidence of polyploid cells in these cultures. It is possible that the increase in the percentage of embryos with polyploid cells after culture is a statistical artefact and more mixoploid embryos were present pre-culture than were found due to the low incidence of polyploid cells. However Hare et al (1980) counted an average of 17.9 cells per embryo analysed directly and an average of 4.2 polyploid cells in each mixoploid embryo. Thus though it is possible polyploid

cells were missed in embryos classified as purely diploid, this is not likely to account for the missing 28% of mixoploid embryos between the non-cultured and cultured groups.

In the present study no direct preparations were made due to the relatively low number of cells in the preimplantation embryos used, but length of culture made no difference to the proportion of mixoploid pig embryos; culture for less than 10 hours 8.2% mixoploid, greater than 15 hours 9.8% mixoploid. The 2 cell sheep embryo with 2n/4n chromosome complement reported by Williams and Long (1980) was cultured for 24 hours in a media containing colcimid. The differences in polyploid cell incidence found by Walker et al with human amniotic fluid cannot be directly related to this study or studies of very early embryos as the cultures were long term varying from 5 to 40 days.

Fechheimer (1972) discusses some of the causes of polyploidy. These include irradiation of stem cells, many chemical agents, viruses, rickettsia, hormones such as growth hormone, thyroid hormone, androgens and oestrogens and genetic influences leading to endoreduplication. Experimentally polyploidy has been induced in embryos by the use of colchicine and heat and cold treatment. Colchicine or colcimid is used in all embryo preparations for chromosome analysis but for varying lengths of time. Hare et al (1978) in direct preparation cultured the embryo for only 1 hour in the presence of colcimid whereas Williams and Long (1980) cultured embryos for around 24 hours in the presence of colcimid. Colcimid is unlikely to be the cause of tetraploid cells when embryos are exposed for only 1 hour, but when the embryos are exposed for 24 hours i.e. over

more than one normal replication cycle, it is possible that a cell in mitoses prevented from segragation at anaphase by the spindle disrupting action of colcimid, may aquire a tetraploid cell content, revert to the synthetic phase and replicate chromosomes again to present as a tetraploid cell in mitoses on embryo processing at the end of 24 hours culture.

Heat shock (45.5 °C) and cold shock (room temperature) have been reported by Beatty (1957) to cause suppression of cleavage division in mammalian embryos around the time of fertilisation and the early cleavage stages. It is possible that during the recovery and manipulation process in this study cold shock may have occurred and certainly the embryos did reach room temperature (20-25 °C). The temperature of the culture media never rose above 38 °C and as the ewe and sow were in good health during the entire experiment the embryo could not receive heat shock. If a slow reduction of temperature of holding media to room temperature were sufficient an insult to cause suppression of a cleavage division it is likely that tetraploidy or mixoploidy would be more commonly reported in early embryos in the literature. It is, however, possible that the susceptibility to heat and cold shock is greater in some stages of embryonic development, for example around fertilisation and 1st cleavage division, than others. Irradiation and hormone influences are not likely to be the cause of the increased incidence of mixoploidy embryos after culture in this study. The number of chemical agents causing chromosome deviations is very large. Any of these agents contaminating culture media or used in the cleaning of implements used in

recovery, handling and culture may be the cause of the increased incidence of mixoploid embryos after culture. However laboratories take care to prevent contamination and if contamination had occurred it is likely the the effect would be noticed on routine blood cultures as well as the embryos unless instruments used in embryo recovery were the cause of the problem. As several authors report this increase with cultures contamination is not likely to be the explanation.

Viruses and rickettsia cannot be ruled out as possible causes of the increase in polyploidy found after culture.

The data presented by Hare et al (1980) showed the increase in polyploidy after culture is an increase in the proportion of embryos with polyploid cells, not an increase in the proportion of polyploid cells in the affected embryos. This indicates the cause of this increase would have to affect apparently normal diploid embryos rather than increasing the incidence of polyploid cells in embryos already mixed.

Genetic influences do seem to significantly affect the incidence of mixoploidy in embryos. Hare et al (1980) found a donor factor influenced the proportion of mixoploid day 12-18 cattle embryos. Miller, Fechheimer and Jaap (1971) reported 14/23 eggs from one hen which were diploid/triploid mosaics. A genetic role in the high incidence of polyploid cells from blood cultures of cattle with the 'double muscle' condition seem likely (Popescu, 1968) but no embryos have been examined. Popescu found 16-25% of cells polyploid on blood culture of cattle with muscular hypertrophy, 11-14% of polyploid cells from crossbred calves and under the same culture conditions control cattle were found to

have 4-11% of cells polyploid. This study must be regarded with caution as there was an abnormally high proportion of polyploid cells in the control animals. Genetic influences are possible in the origin of the mixoploid embryos in this study as the same boar was the sire of all embryos and foetuses examined. Though the 9 mixoploid pig embryos came from 5 different gilts, these gilts could have been related as they came from the same commercial pig farm in 2 batches 4 months apart. It was not possible to establish parentage of the gilts due to management conditions on the farm involved. All the 78 day foetuses came from one litter, the dam of which was unrelated to the other 5 gilts.

Binucleate or multinucleate cells are found in the trophoblast on or after day 16 in the sheep (Boshier, 1969) and day 15 in the cow embryo (Staples, McEntee and Hansel, 1969). The stage at which these cells arise in the pig is uncertain. Cytogenetic and histological studies have revealed the presence of tetraploid metaphase spreads in the trophoblast of pig embryos at day 10 (Long and Williams, 1982). Very little work has been done on cytogenetic examination of pig embryos prior to day 10 and the authors who have worked in this area, King et al (1980) and Bouters et al (1974), do not report polyploid cells or mixoploid embryos. The earliest point of origin of the trophoblast polyploid cells is uncertain, Hare et al (1980) found tetraploid cells on day 13 but not in the few bovine embryos examined on day 12, and these polyploid cells increased in incidence until day 18 (the latest day in their study). Barlow and Sherman (1972) found that in rat embryos maternal stimulation was not required for the formation of trophoblast binucleate cells

as they would form in culture and in embryos cultured for 4-6 days but which had failed to hatch from the zona pellucida. The time of origin of these cells is largely unknown. The majority (7/9) of the 2n based mixoploid pig embryos in this study and the mixoploid sheep embryo, were expanded or hatched blastocysts, that is the blastocoel cavity had started to form, as had some form of differentiation between inner cell mass and trophoblast tissues. If, as Barlowe and Sherman (1972) postulate, it is the formation of the blastocoel cavity that is the stimulation to the formation of these polyploid cells, then the tetraploid cells found in 8 of the 10 2n based mixoploids in this study could be very early trophoblast polyploid cells. The remaining 2 2n based mixoploid pig embryos, however, were a 6 cell zygote and a very early blastocyst prior to the formation of a visible blastocoel cavity. Thus the tetraploid cells in these embryos require a different explanation. Reeve and Ziomek (1981) on examining a 16 cell mouse embryo with a scanning electron microscope, reported a morphological difference between the cells on the surface and the inside population with the outside population cells developing a microvillus pole. The authors postulated that these cells would become trophectoderm. It is possible that as there is a morphological difference between the possible potential trophoblast cells and cells of the inner cell mass at this early stage in development, that a potential for polyploidy, or even polyploidy itself had been achieved.

There are 3 possible explanations for the appearance of the 2n based mixoploid preimplantation embryos in this study.

1. The mixoploidy occurred at or around fertilisation due to an error of cleavage or abnormality of fertilisation.
2. Conditions of recovery or culture induced the appearance of polyploid cells.
3. The potential for the development of trophoblast polyploid cells arises very early in development and either polyploid cells arise earlier than previously supposed or cells with the potential for endoreduplication or cell fusion are caused to become polyploid by culture conditions.

Older embryos in this study were also found to be mixoploid. Blood cells from 78 day foetuses were studied and 50% (5/10) of foetuses were found to be mixoploid, mainly tetraploid, with the incidence of polyploid cells varying from 0 to 31%. It is possible that a greater proportion of these pig foetuses were mixoploid as only 8 of the 10 produced more than 10 metaphase spreads. With an average incidence of polyploid cells in the mixoploid embryos of 18%, it is possible that the 2 embryos with only 1 and 2 metaphase spreads and even those with 13 or 14, had polyploid cells which were not in division at the time of preparation.

Long and Williams (1982) found that the majority of polyploid cells were in preparations of the trophoblast of 10 day old pig embryos rather than the equivalent inner cell mass where polyploid cells were found in only 2/35 embryos. However the results from the present study of blood cultures of the pig foetuses show that polyploid cells in normally developing foetuses are not confined to the trophoblast. It is possible

that these polyploid cells were cultural artefacts induced by 48 hour culture. However the incidence of polyploidy in blood cultures from adult pigs under identical culture conditions was less than 1% - indeed a polyploid cell was very rarely found. Thus the leucocyte population of these mid to late term pig foetuses must either by mixoploid or have an inbuilt potential for polyploidy not found in leucocytes of adult pigs. The immune system develops relatively early in the pig foetus, with cells staining for immunoglobulins present in Peyer's patches in the intestines of 55 day foetuses and immunoglobulin producing cells present in the thymus day 65-70 (Porter, 1975).

The thymus itself, from which T lymphocytes originate, starts to develop at 28 to 36 days of gestation (Patten, 1948; Marrable, 1971). In man, non-granular leucocytes are in circulation at 2 months gestational age and leucocytes at 3 months and these cells, while immature, can multiply (Arey, 1954). While poke-weed mitogen was used in this study in the culture of blood from the 78 day foetuses, it is possible that it was not necessary if the white cells in circulation in this age of pig retained the ability to divide.

12-18 day old cattle embryos with 2n based mixoploid trophoblasts were shown by Hare et al (1980) to be viable on transfer. The 78 day pig foetuses in this present study with mixoploid leucocyte populations were developing normally and were indistinguishable from diploid litter mates. Kohn and Robinson (1970) reported a 5 month human foetus, aborted due to a high percentage of tetraploid cells in two successive cultures of amniotic fluid, which had 3/100 tetraploid cells in culture of the

skin and 2/668 tetraploid cells in cord blood. This foetus was normal anatomically and there was no evidence to indicate it would not have developed normally to birth. Karyotypically and phenotypically normal infants have been found when up to 100% of cells cultured after amniocentesis were found to be tetraploid (Walker, Lee and Gregson, 1970).

Thus it can be concluded that 2n based mixoploid embryos, particularly pig embryos, can develop normally. Though it is possible that late foetal death may have occurred there is no reason to believe these pig foetuses would not have become normal neonates. However authors who have surveyed newborn or young pigs (Akesson and Henricson, 1972; King et al, 1980) do not report finding polyploid cells in either leucocyte or skin cultures. This would appear to indicate that the polyploid cells found on day 78 either disappear or lose their potential for polyploidy in culture by birth.

SECTION 3.5

CONCLUSIONS

22 pig embryos were found where the chromosome complement deviated from the normal diploid count of 38. This gave a deviation rate of 22%.

5 sheep embryos were found where the chromasome complement deviated from the normal diploid count of 54. This gave a deviation rate of 22.7%.

The various abnormalities found, monosomy and trisomy, trisomy, haploidy, triploidy, tetraploidy, diploid/triploid mosaicism and 2n based mixoploidy have been discussed earlier in this chapter and the future viability of the embryo assessed. It was concluded that monosomy and trisomy, triploidy and tetraploidy were very likely to cause embryonic death, haploidy (other than where chromosomes assumed to be remnants of sperm pronuclei and polar bodies were the only spreads present) and diploid/triploid mosaicism were possibly a cause of embryonic death. The presence of polyploid cells in a basically diploid embryo and the presence of pronuclei in a normally dividing embryo were thought not to be a likely cause of embryonic death.

The percentage of embryos with chromosomal deviations thought to cause embryonic death is reduced to 7-12% in pigs and 9.1% in sheep.

This figure of 12% is higher than those obtained by other authors. McFeely (1967) found a 10% abnormality rate but other authors give lower estimates. This difference may be due to an increased accuracy in this study because the embryos studied were

very much younger than those in other studies, in most cases still inside the zona pellucida. As the age at which an embryo is studied increases it is reasonable to assume that the level of chromosomal abnormalities in that population will decrease as the lethal effect of the chromosome imbalances takes effect. Thus the earlier a population is studied the nearer the estimate will be to the true abnormality rate immediately after fertilisation. 12% is still well within the range of known embryonic death in the pig of 30-40%.

An estimate of 9% of sheep embryos chromosomally abnormal is higher than that observed by Long and Williams (1980) for younger embryos (6%) but not significantly so ($X^2 = 0.006$). Very much smaller numbers were analysed in the sheep study (22) than the pig embryo study (100) and statistical variations in these samples are inevitable.

The figures found in this study of 12% in pigs and 9% in sheep confirm the widespread belief that approximately 1/3 of embryonic death in these domestic species at least is due to chromosome imbalances.

APPENDIX 1

APPENDIX 1

PROGESTERONE ASSAY

Materials

Progesterone 10 ng and 1.0 ng/ml in alcohol for Standard Curve

Progesterone 1, 2, 6, 7 - ^{3}H (n) - tracer. 250 uCi in 100ml alcohol

Anti-Progesterone Serum - raised in sheep.

The above were stored at -20 ^{o}C

Phosphate Buffered Saline + 0.1% Gelatin

Petroleum ether

Charcoal

Scintillation fluid

Soda glass assay tubes

Scintillation vials

Dispensers and pipettes.

Method

1. Seven standard solutions were made up using double dilution techniques from the standard progesterone solution. These solutions were stored at -20 ^{o}C for several assays.
2. Extraction: 0.5 ml (sheep plasma) or 0.1 ml (pig plasma) of sample was pipetted into a test tube and 5 ml or 2.5 ml of ether was added. The sample and ether were throroughly mixed using a vortex for 3 minutes. After centrifugation to separate ether from plasma the samples were frozen, the ether containing the progesterone extracted from the sample

was decanted and dried down. Contol samples containing no progesterone and high, medium and low levels of progesterone were treated similarly.

3. Standards were prepared from the stock solutions made up in (1) and dried.

4. 100 ul tracer and 500 ul antibody (diluted 1:40,000) were added to each tube which was then thoroughly mixed and left to incubate for at least 2 hours at 4°C.

5. Charcoal was added to each tube to remove unbound progesterone. The contents of the tube were then thoroughly mixed then left for 10 minutes incubation at 4°C. The tubes were centrifuged and the supernatant decanted into a scintillation tube with 10 ml scintillation fluid.

6. A scintillation counter was used to count the radioactive emmision of each sample and standard over 1 minute. The standards were used to produce a standard curve from which the sample values were read. The samples with known progesterone content were used to provide an extraction value. Accuracy was checked from duplicated standards and known samples. The results obtained were then corrected for the sample volume and extraction value.

Control tubes were included to check the function of each stage of the assay.

APPENDIX II

APPENDIX II

PREPARATION OF EMBRYOS FOR SCANNING ELECTRON MICROSCOPY

Materials

Buffer 0.1 M Sodium cacodylate pH 7.2

Fixative 4% Gluteraldehyde in buffer

Poly-1-lysine solution A 0.1% solution of poly-1-lysine (Type 1- hydrombromide P1886, Sigma) in distilled water. To be used immediately.

Equipment

6 mm diameter glass cover slips (McFarlane Robson)

Ovum collecting dish (Camlab)

Aluminium stubs (Agar Aids)

Sputter coater (Emscope)

Scanning Electron Microscope (Phillips SEM 500)

Microanalysis Detector (EDAX)

Method

1. Transfer embryos from flushing fluid to fixative and leave for 1-2 hours. Store as necessary in the fixative until processing.
2. Attach eggs to coverslips using poly-1-lysine solution. The coverslips were scored on the working surface, washed in ethanol and dried. A drop of poly-1-lysine solution was placed in the centre of the coverslip and left for 15

minutes at room temperature. Excess poly-1-lysine was removed and the the coverslip washed immediately 3-4 times with buffer solution. A drop of buffer was left on the coverslip and an embryo transferred using a drawn pipette into the drop. After a few minutes, adhesion of the embryo to the coverslip was checked by gently moving the coverslip. The coverslip was then flooded with buffer.

3. Most of the buffer was removed prior to dehydration in graded concentrations of acetone (30-50-70-90%) for 5-10 minutes each, and 3 changes of 100% acetone leaving the coverslip in the acetone for 10 minutes each change. Still covered with 100% acetone the coverslips were transferred to the critical point dryer for approximately 1 hour. The coverslips were stuck onto aluminium stubs using conductor silver paint and coated with a thin layer of gold or carbon in a sputter coater.

4. The embryo was examined under the scanning electron microscope at an acceleration voltage of 15 KV. The slide was examined using a television monitor and photographs taken.

5. When carbon coated crystals of embryos were examined, spectra were collected from the area pictured using the Microanalysis Detector. A spectra was collected for the background and subtracted from that obtained for the area under investigation. Photographs were taken.

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