COMPARATIVE STUDIES OF ESTROUS SYNCHRONIZATION, OVULATION INDUCTION, LUTEAL FUNCTION AND EMBRYO CRYOPRESERVATION IN DOMESTIC SHEEP AND APPLICATION TO RELATED NONDOMESTIC UNGULATE SPECIES

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ABSTRACT

Title of Dissertation: Comparative Studies of Estrous Synchronization, Ovulation Induction, Luteal Function and Embryo Cryopreservation in Domestic Sheep and Application to Related Nondomestic Ungulate Species

Mitchel Craig Schiewe, Doctor of Philosophy, 1989

Dissertation Directed by: Dr. David E. Wildt, Ph.D.
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The general hypothesis of this investigation is that domestic sheep can serve as valuable research models for developing, applying and comparatively examining conventional and new embryo biotechniques which can be integrated toward the preservation of related nondomestic species. A series of projects focused on: 1) analyzing the effects of various hormonal ovulation induction procedures on ovarian function and the production of transferable quality embryos; 2) determining whether atraumatic embryo collection/transfer procedures could be developed and efficiently applied without compromising subsequent fertility; and 3) evaluating novel cryoprotective solutions and cooling procedures for cryopreserving embryos. In Project I, it was determined that the number of corpora lutea (CL) and transferable quality embryos was greater ($P<0.05$) in follicle stimulating hormone (FSH-P)- and human menopausal gonadotropin (hMG)-treated sheep than in pregnant mares' serum gonadotropin (PMSG)-treated ewes. However, regardless of the type of gonadotropin treatment, a high proportion (40%) of ewes experienced premature luteal regression which was associated with embryo collection failure. Project II confirmed that the incidence of premature luteal regression could be reduced ($P<0.01$) in gonadotropin-treated ewes by synchronizing estrus with progestogen-pessaries as opposed to prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), with CL becoming dysfunctional by Day 3 after ovulation. Although the exact mechanism of luteal dysfunction was not determined, preovulatory endocrine events were not different while the number of small luteal cells and low affinity $PGF_{2\alpha}$ binding sites was increased ($P<0.05$) in ewes with normal CL on Day 3. The use of a novel laparoscopic embryo transfer procedure was proven effective for sheep in Projects I and III. In Project III, it was shown that both glycerol- and propylene glycol-treated sheep embryos could be effectively cryopreserved in ampules or straws. Survival was higher ($P<0.05$) for straw-preserved embryos, while the undesirable incidence of post-thaw zona pellucida damage was increased ($P<0.01$) in

glycerol/ampule-preserved embryos. In addition, the successful vitrification of sheep embryos was validated, although further procedural refinements are needed. In Project IV, multiple ovulation induction, nonsurgical embryo recovery and embryo cryopreservation were performed in 4 nondomestic ungulate species. The results demonstrate that FSH-P produced more CL than PMSG in the bongo and eland ($P<0.05$) and the scimitar-horned oryx ($P<0.10$). As the genetic diversity of populations of captive and wild species declines, the need for multiple embryo collection, cryopreservation and transfer will become increasingly important. However, the efficiency of implementing advanced biotechniques will depend on learning more about the basic processes of specific species.

COMPARATIVE STUDIES OF ESTROUS SYNCHRONIZATION, OVULATION INDUCTION, LUTEAL FUNCTION AND EMBRYO CRYOPRESERVATION IN DOMESTIC SHEEP AND APPLICATION TO RELATED INVESTIGATIONS OF NONDOMESTIC UNGULATE SPECIES

by

Mitchel Craig Schiewe

Dissertation submitted to the Faculty of the Department of Physiology
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This dissertation and equally important, the years invested in graduate training at the Louisiana State University, National Zoological Park and Uniformed Services University of the Health Sciences, is dedicated to Carol, for her continuous love and patience, and most importantly for becoming my wife on August 16, 1987. Furthermore, this accomplishment is dedicated to the memory of Elsa and Bill whose love and encouragement will always be cherished and honored.

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Finally, a never-ending love and thanks goes to my family in Southern California including Donna, Benjamin and Shirley, Gilbert and Dona, Steven and Joan, Lynn and Grandma Barbara for their support and faith.

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LIST OF ABBREVIATIONS

Abbreviation	Name
ACTH	Adrenocorticotropin
AI	Artificial insemination
ANOVA	Analysis of variance
b.i.d.	Bis in die (twice a day)
BSA	Bovine serum albumin
CO_2	Carbon dioxide
C	Centigrade
cm	Centimeters
CH	Corpora hemorrhagica
CL	Corpora lutea
CLX	Corpora lutea enucleation
r	Correlation coefficient
Ci	Curies
K_d	Dissociation constant
DMSO	Dimethyl sulfoxide
fmol	Femtomole
FSH	Follicle stimulating hormone
FSH-P	Follicle stimulating hormone-pituitary extract
ED	Effective dose
EG	Ethylene glycol
EC	Embryo collection
ET	Embryo transfer
FCS	Fetal calf serum
FG	French gauge
ga	Gauge
g	Grams
HBSS	Hank's balanced salt solution
h	Hours
hMG	Human menopausal gonadotropin
i.m.	Intramuscular
i.v.	Intravenous
IU	International units
kg	Kilograms
l	Liters
LH	Luteinizing hormone
LN_2	Liquid nitrogen
MAP	6α-methyl-17α-acetoxy-progesterone
m	Meters
µg	Micrograms
µl	Microliters
µm	Micrometers
mg	Milligrams
ml	Milliliters
mm	Millimeters
mM	Millimolar
mmol	Millimole
min	Minutes
M	Molar
ng	Nanograms

Abbreviation	Name
OD	Outside diameter
PBS(1)	Phosphate buffered saline
pg	Picograms
PMSG	Pregnant mares' serum gonadotropin
POF	Preovulatory follicles
PPG	Propylene glycol
PG	Prostaglandin
QG	Quality grade
sec	Seconds
SD	Standard deviation
SEM	Standard error of the mean
s.c.	Subcutaneous
TBO	Trophoblastic outgrowth
UTJ	Uterine tubal junction
VG	Viability grade
VS3a	Vitrification solution 3a
V	Volts

INTRODUCTION

The development of embryo biotechniques in domestic and laboratory species offers new dimensions for improving the reproductive potential of captive wildlife species, many of which are threatened by extinction. An understanding of the reproductive physiology of domestic ruminant species (cattle, sheep, goats) has allowed ovulation induction and embryo transfer to be used for expanding limited gene pools and enhancing the reproductive efficiency of genetically valuable livestock populations (Dziuk, 1975; Church and Shea, 1977). Progress in embryology and much of its applied importance, however, has been dependent on earlier pioneering studies using laboratory animals (mice, rats, rabbits; Chang and Pickworth, 1969). This type of "model" strategy ensures that sufficient animal numbers are available for evaluating statistical significance (Hahn, 1984). For example, years of research on embryo culturing systems, cryopreservation and *in vitro* fertilization in the mouse and rabbit ultimately led to similar success in larger mammalian species including humans (Foote, 1987).

The use of domestic and laboratory animal models for developing a basic understanding of biological processes is essential to the application of advanced reproductive techniques to endangered wildlife. The ability to conduct empirical and comparative studies on captive nondomestic species is complicated to the point that experimental models are almost mandatory to making significant progress. The lack of physiological data about wild taxa, limited animal availability and potential captive stress susceptibility are all factors which routinely compromise our ability to conduct sound scientific research involving endangered species (Wildt, 1989). Therefore, it is logical that our ability to synchronize estrus, induce ovulation and recover and transfer embryos in nondomestic ungulates is dependent, at least initially, on developing the technical expertise in a more common, domesticated species. It is possible, however, that not all strategies or knowledge learned about the model species may be extrapolated to a taxonomically-related, rare species. Even closely related wildlife species are known to markedly differ physiologically (Wildt et al., 1988). Although some scientific techniques or concepts may not have cross-species application, the modeling approach still is beneficial because it allows characterizing the fascinating array of reproductive strategies which have evolved among and within mammalian genera.

The use of embryo biotechnologies (ovulation induction and embryo recovery, culture, cryopreservation and transfer) on livestock species has allowed: 1) reducing the generation interval in species with long gestation periods; 2) analyzing the contribution of

the maternal genome through multiple offspring evaluations; 3) maximizing the use of superior genetic material; 4) increasing offspring production (i.e., reproductive efficiency) of valuable individuals; 5) the widespread transportation of germ plasm; and 6) investigating many scientific questions regarding the genetics, developmental biology and physiology of preimplantation-stage embryos (Foote and Onuma, 1970; Seidel, 1981). As the genetic diversity of populations of captive and wild species declines, the need for multiple embryo collection, cryopreservation and transfer will become increasingly important. Reproductive efficiency of genetically valuable or subfertile individuals can be increased by the repeated recovery of embryos which can be transferred within (intraspecies) or between (interspecies) related species (Kraemer, 1983). Embryo cryobanking of complete genomes of known genetic background offers an important strategy for saving and then re-incorporating the genes of valuable species and individuals into later generations (Whittingham, 1974; Wildt et al., 1986). The potential of embryo cryopreservation for preserving wildlife germ plasm has been recognized for years (Polge, 1978; Bradford and Kennedy, 1980; Seidel, 1981) and would offer an efficient, inexpensive and safe approach for the transcontinental and international shipment of genetic material (Bilton, 1980; Durrant and Benirschke, 1981; Seidel, 1981). To effectively incorporate advanced reproductive techniques into wildlife propagation programs, related research on domestic species must continue and this will assist refining the biotechniques for nondomesticated species (Wildt and Bush, 1984). Although some physiological homology no doubt exists between these 2 types of animals, there is a need to perform detailed comparative studies that will help evaluate species-specific differences and sensitivities to various treatments required in embryo biotechnology.

This research project focuses on the development, application and experimental examination of conventional and new techniques to be used on domestic ruminant species. The resolution of related physiological problems will have a beneficial effect on the efficiency of integrating advanced reproductive techniques into future wildlife conservation biology programs. The general hypothesis of this investigation was that domesticated species can serve as valuable research models for developing advanced reproductive programs in related nondomestic species. The present research focused on: 1) analyzing the effects of various hormonal ovulation induction procedures on ovarian function and the production of transferable quality embryos in domestic and nondomestic ungulates; 2) determining whether atraumatic embryo collection/transfer procedures can be developed and efficiently applied to these species without compromising subsequent fertility; and 3) evaluating novel cryoprotective solutions and cooling procedures for cryopreserving embryos, including characterizing potential cryo-sensitivities and species differences that

may exist. This new knowledge will enhance the future efficacy of implementing germ plasm conservation efforts. The successful use of embryo transfer biotechniques in domestic and laboratory animals offers hope for the potential preservation of rare and endangered species.

LITERATURE REVIEW

PHYSIOLOGICAL ASPECTS OF OVULATION INDUCTION IN RUMINANT SPECIES

General Characteristics of the Estrous Cycle

Ruminant species in the Bovidae (cow), Capridae (goat), Cervidae (deer) and Ovidae (sheep) families have a polyestrous reproductive cycle and are spontaneous ovulators. Daylength (i.e., light-dark cycle) affects reproductive status of the goat, sheep and deer; during certain seasons these species experience either a period of reproductive quiescence (termed "anestrus") or regular cyclic activity (Yuthasastrakosol et al., 1975; Legan and Karsch, 1980; Lincoln and Short, 1980; BonDurant et al., 1981; Adam et al., 1985; Asher, 1985). The present discussion focuses on the estrus condition of seasonally polyestrous species and non-seasonal bovids. In general, the duration of the estrous cycle is 20 to 23 days for cattle and goats and 15 to 18 days for sheep with some breed variations within species (e.g., goats, Prasad and Bhattacharyya, 1979; sheep, Wheaton et al., 1988). Only limited information is available on deer, but data indicate that there is considerable interspecies variation in estrous cycle length (ranging from 17 to 28 days; Guinness et al., 1971; Plotka et al., 1980; Asher, 1985; Adam et al., 1985; Curlewis et al., 1988; Monfort et al., 1989).

The estrous cycle consists of a follicular and luteal phase and is characterized by: 1) a selective and continuous recruitment of ovarian follicles; 2) a period of sexual receptivity (estrus), a time when ovulation of one or more viable oocytes, mating and fertilization usually occurs; 3) dynamic changes in endocrine status and the oviductal and uterine environments to accommodate transport and function of spermatozoa, ova and embryos; 4) the development of ovarian luteal tissue; and 5) corpus luteum (CL) demise and reinitiation of the cycle (in the absence of fertilization or an appropriate embryonic signal). Hormones from the hypothalamus, anterior pituitary, ovaries and uterus control the physiological changes of the estrous cycle. Progesterone alone is considered to have a controlling influence over the secretion of luteinizing hormone (LH), estrus, ovulation rate and luteal regression (as examined in the ewe and reviewed by Thomas and coworkers, 1987). In the sheep and cow, the timing of the reproductive cycle appears regulated primarily by regression of the CL, a process stimulated by the local release of prostaglandin $F_2\alpha$ ($PGF_2\alpha$) from the nonpregnant uterus (Goding et al., 1972; McCracken et al., 1972; Hansel et al., 1973; Barcikowski et al., 1974; Goding, 1974; Fogwell et al., 1985; Zarco et al., 1988). The rapid decline in serum progesterone as a result of reduced luteal function

decreases the inhibition of gonadotropin-releasing hormone (GnRH) release from the hypothalamus which indirectly (through its action on the pituitary) stimulates ovarian follicular development. GnRH-induced LH concentrations progressively rise and, in conjunction with follicle stimulating hormone (FSH) released by the pituitary, stimulate the growth of preovulatory follicles. The activated growth of these secondary follicles is characterized by granulosa and thecal cell hyperplasia and an expanded antral cavity. Generally, one dominant follicle (i.e., the Graafian follicle) ovulates (Day 0) in the cow and 1 or 2 ovulate in sheep and goats, whereas non-ovulating antral follicles undergo a degenerative process termed "atresia" (Byskov, 1979). Ovulation rate is remarkably genotype-dependent, especially in sheep; certain sheep breeds routinely ovulate 3 or more oocytes (Turnbull et al., 1977, 1978; Cahill, 1981; Scaramuzzi and Radford, 1983; Bindon et al., 1986). At ovulation, the oocyte is released into the oviduct, the site of fertilization, and the remaining ovarian follicular cells (i.e., granulosa and theca cells) are transformed into luteal tissue. Luteinizing hormone stimulates the CL of ruminant species to secrete progesterone which is responsible for conditioning the uterus to form an optimal implantation environment for the developing embryo. The luteal phase is terminated by the uterine release of $PGF_{2\alpha}$ to the ovarian CL via local venoarterial (McCracken et al., 1972; Ginther, 1974; Land et al., 1976) and lymphatic (Heap et al., 1985) pathways in the absence of a perceived embryonic signal by the endometrium (Inskeep and Murdoch, 1980; McCracken et al., 1984). Intra- and interspecies variations in estrous cycle durations generally are attributed to variations in the length of the luteal phase.

Folliculogenesis

Mammalian ovaries contain a complete population of primordial follicles at birth. Although some follicular growth and atresia occurs before puberty, ovulation with the release of a follicular oocyte only coincides with the pubertal onset of estrus (Ryan and Foster, 1980). At sexual maturity, follicular growth becomes a continuous cyclic process with two potential outcomes, atresia or ovulation. Waves of follicular development occur throughout the estrous cycle, every 6 to 7 days in cattle (Rajakoski , 1960; Matton et al., 1981; Spicer and Echternkamp, 1986) and 4 to 5 days in sheep (Smeaton and Robertson, 1971; Turnbull et al., 1977). A cohort of follicles progress simultaneously into the antral stage, but only 1 or 2 ovulate and the remainder become atretic.

The mechanisms of follicular development are not completely understood but are known to involve complex interactions between hormonal changes and cellular events (as previously reviewed by Mauleon and Marianna, 1977; Peters, 1979; Cahill and Mauleon, 1980; Armstrong et al., 1981; Baird and McNeilly, 1981; Cahill, 1981; Staigmiller and

England, 1982). In the ewe, granulosa cells of small (i.e., secondary) follicles (1 to 3 mm in diameter) contain only FSH receptors and are responsive to the consistent secretion of FSH throughout the estrous cycle. The presence of LH receptors on theca and granulosa cells is not evident until follicles are medium-sized (4 to 6 mm in diameter) (Armstrong et al., 1981; Webb and England, 1982). During the luteal phase, circulating FSH stimulates follicular growth (Richards et al., 1978), but, due to progesterone-suppressed LH activity, the large antral follicles degenerate (Moor et al., 1984). As progesterone levels decrease during luteal regression, the frequency of pituitary LH pulsatility increases (Pant et al., 1977; Rahe et al., 1980; Baird et al., 1981). The elevated tonic LH release during the follicular phase activates medium- to large-sized follicular development (Moor et al., 1984). There are parallel increases in the production of theca cell androgen (Baird, 1978) which is converted to estrogens by an aromatase enzyme system in the follicle's granulosa cell layers (Armstrong et al., 1981). In the ewe, the primary follicular estrogen is estradiol-17β (Moore et al., 1969). Increasing estradiol-17β concentrations in the blood downregulate further FSH release from the anterior pituitary (Baird, 1983) which inhibits further development of less advanced follicles (Moor et al., 1984). The "estrogenic" follicles contain more granulosa cells and measurable antral estrogen than non-activated follicles (McNatty, 1982), and the estradiol-17β and pituitary FSH combined promote further acquisition of LH receptors by the granulosa cells (Richards and Midgley, 1976). Within 36 h of CL luteolysis, 1 or 2 large antral follicles with high concentrations of intrafollicular steroids and inhibin-like factors become the dominant Graafian follicle(s). These follicular components effectively inhibit pituitary FSH secretion, and as a result, other antral follicles in the growing population become atretic (McNatty et al., 1982).

Secretion of estradiol-17β by the Graafian follicle(s) stimulates the onset of behavioral estrus and appears to influence intra-oocyte maturation (Mauleon and Marianna, 1977; Moor and Trounson, 1977). High circulating estrogen levels eventually trigger a massive release of pituitary gonadotropin (i.e., the "LH surge") which is responsible for the final oocyte maturation and ovulation (Gay et al., 1970; Hay and Moor, 1975; Moor et al., 1975; Mauleon and Marianna, 1977; Baird and McNeilly, 1981). The preovulatory LH surge apparently inhibits further follicular androgen and estrogen production (Baird and McNeilly, 1981) and causes a distinct shift in steroid biosynthesis by the dominant follicle; luteinization of the granulosa cells converts hormone production from estrogen to progesterone dominance (Murdoch and Dunn, 1982). At the time of ovulation, the dominant Graafian follicle releases a mature ovum which is potentially capable of fertilization. The cells of the ruptured follicle are transformed into luteal tissue responsible

for the formation of the CL and production of luteal phase progesterone secretion (Hansel et al., 1973).

Luteal Function

Progesterone secretion of CL origin increases shortly after ovulation and continues as the primary steroid of the luteal phase. If an embryo is present, progesterone is responsible for conditioning and maintaining a proper uterine environment for impending implantation and gestational events. Steroidogenesis within the CL, therefore, plays a vital role in and is essential throughout gestation in the goat and through at least the first trimester of pregnancy in sheep and cattle (Cole and Cupps, 1978). Transformation of the ruptured Graafian follicle into a CL is characterized by the formation of 2 steroidogenic cell types referred to as small and large luteal cells (Donaldson and Hansel, 1965; Deane et al., 1966; O'Shea et al., 1979; Koos and Hansel, 1981). Small luteal cells are of theca cell origin whereas large cells are generated from granulosa cells (Hansel et al., 1987). However, large cells might also derive from theca cell sources via transformed small luteal cell conversion in late-stages of the estrous cycle in cattle (Hansel et al., 1987). Morphometric analysis of CL from cyclic ewes indicates that small luteal cells experience hyperplasia without increasing in size whereas large luteal cells hypertrophy but do not increase in number (Farin et al., 1986). Consequently, CL mass increases throughout the estrous cycle with changes in the proportion of small and large luteal cells being somewhat species-dependent. Dynamic proportional changes do occur in the bovine CL characteristic of the continuous differentiation of small cells into large cells (Hansel et al., 1987). Although this is not considered to be the case in the ovine CL, exogenous LH is known to increase large luteal cell numbers while simultaneously reducing small cell numbers (Farin et al., 1985). LH is the primary pituitary luteotropin (Hansel, 1966; Hoffman et al., 1974) responsible for activating luteal cell progesterone synthesis by means of a membrane bound Ca^{2+}-polyphosphoinositol-protein kinase C second messenger system (Hansel et al., 1987).

In vitro gonadotropin stimulation increases progesterone output by small luteal cells to similar levels measured in the unstimulated large cell populations (Fitz et al., 1982). *In vitro* culture studies further indicate that, in the absence of pituitary LH, large luteal cells secrete the majority of the progesterone produced by the CL compared to a negligible output by small luteal cells (Fitz et al., 1982). This apparent dichotomy between luteal cell types relative to steroidogenesis is a mystery. Small ovine luteal cells are very responsive to exogenous LH, since small cells contain more LH receptors for gonadotropin binding than large cells (Ursely and Leymarie, 1979; Fitz et al., 1982; Rodgers et al., 1983; Hoyer

et al., 1984). After binding LH, progesterone production by small luteal cells escalates in a dose-dependent fashion (Harrison et al., 1987) while LH fails to stimulate further progesterone production by large cells (Fitz et al., 1982). The steroidogenic cellular population increases up to Day 8 in the sheep, at which point circulating progesterone levels remain elevated during the luteal phase until luteolysis occurs (Farin et al., 1986). There appears to be a correlation between the number of LH receptors and progesterone production as luteal tissue develops in the sheep (Niswender et al., 1981). This correlation is characterized by a 40-fold increase in LH receptors and a 6-fold increase in the number of receptors occupied by endogenous LH per small luteal cell between Day 4 and 14 of the ovine estrous cycle (McClellan et al., 1975). Corpora lutea of ewes treated with exogenous gonadotropins have a greater mean cell diameter, and *in vitro* progesterone synthesis by the small luteal cells is elevated (following LH stimulation) compared to normal cycling sheep (Hild-Petito et al., 1987). In contrast, no changes are apparent in the circulating progesterone levels per mg luteal tissue, in basal progesterone production by luteal cells, in the ratio of small to large luteal cells or in the mean diameter of the large cells (Hild-Petito et al., 1987). Prostaglandin $F_{2\alpha}$ receptors have been detected and quantitated on luteal cells in sheep (Fitz et al., 1982) and cattle (Rao et al., 1979). Recently, it has been shown that large luteal cells possess high-affinity binding $PGF_{2\alpha}$ sites, in contrast to the low affinity binding sites detected on small cells (Balapure et al., 1989a). These receptors are responsive to local uterine $PGF_{2\alpha}$ release which triggers luteal regression at the end of the luteal phase. The luteal tissue undergoes degenerative changes and becomes a non-functional ovarian structure (i.e., corpus albicans), allowing for the uninhibited growth of preovulatory follicles in the follicular phase of the next estrous cycle.

Estrous Synchronization

Understanding the physiological and endocrinological interrelationships of the estrous cycle has enabled scientists and livestock producers to manipulate the reproductive activity of domestic ruminants for more than 30 years. The ability to synchronize the onset of estrus and ovulation has proven to be a valuable management tool for increasing livestock reproductive efficiency (e.g., shortening breeding season intervals) and developing strategies for artificial breeding (e.g., artificial insemination, embryo collection/transfer). Methods for synchronizing estrus vary from the simple to the complex. In the late anestrous period of the year, simply introducing a conspecific male to a group of female sheep (Schinckel, 1954; Radford and Watson, 1957) or goats (Ott et al., 1980; Chemineau, 1983) elicits synchronized estrous behavior. During the breeding season, controlling estrus is more complicated and, generally has involved two approaches:

1) regulating follicular development and maturation by using progestogen compounds over an extended period (i.e., 1 to 3 weeks) or 2) inducing luteolysis of the CL with prostaglandins. In the former, a withdrawal of the progestogen treatment causes a reflex pituitary release of gonadotropins and the stimulation of ovarian activity. The prostaglandin approach is based on a similar concept because induced demise of the CL also reduces the negative feedback of progesterone on the pituitary and re-activates robust pituitary gonadotropin secretion. Various exogenous gonadotropin treatments have been used simultaneously with these two strategies to improve the timing of ovulatory events and to stimulate multiple ovulations (i.e., superovulation). The efficacy of using commercially available pharmacological agents for inducing estrus and ovulation under various conditions and for a variety of purposes has been reviewed for both cattle (Mapletoft, 1988) and sheep (Scaramuzzi and Martin, 1984).

The use of exogenous progesterone to prolong and/or mimic the luteal phase and synchronize estrus was first proposed and tested 40 years ago (Dutt and Casida, 1948; O'Mary et al., 1950). These studies and others determined that most ewes given daily progesterone injections (subcutaneously in oil, 10 to 20 mg/injection) for 12 to 16 days demonstrate estrus and ovulate 3 to 4 days after cessation of treatment (Robinson, 1952; Dutt, 1953; Hunter et al., 1955; Robinson, 1961; Hancock and Hovell, 1961; Avery et al., 1962). The daily feeding and then withdrawal of the oral progestogen medroxyprogesterone acetate (6α-methyl-17α-acetoxy-progesterone, MAP) also was demonstrated to be effective (Hogue et al., 1962; Hulet, 1966; Hunt et al., 1971; Manns and Hafs, 1976). These studies determined that the administration of progesterone produces an artificially extended luteal phase and that such treatment does not inhibit uterine $PGF_{2\alpha}$ release (Ginther, 1970). Therefore, the administration of a progestogen for about 2 weeks ensures that the CL experiences complete regression. When the progestogen stimulation is withdrawn from a group of animals which are at random stages of the estrous cycle before treatment, the group responds with synchronized follicular growth, estrus and ovulation. However, the daily administration of steroids is extremely laborious, and the oral delivery approach often results in variable hormone intake (Gordon, 1975).

These problems were incentive for developing simpler procedures which included progesterone-impregnated pessaries (Robinson, 1964; Robinson et al., 1968; Roche, 1976) or implants (Chupin et al., 1972; Boland et al., 1979; Smith et al., 1979; Bretzlaff and Madrid, 1985) which slowly and continuously release hormone intravaginally or subcutaneously. Vaginal pessaries containing 30 mg fluorogestone acetate (9α-fluoro-11ß-hydroxy-17α-acetoxy-progesterone, FGA; Robinson, 1965) or 60 mg MAP (Boland, 1973) have been used commonly in sheep (Colas, 1975; Gordon, 1975; Haresign, 1978;

Armstrong and Evans, 1983; Walker et al., 1986; Torres et al., 1987; Alwan et al., 1988) and goats (Armstrong et al., 1982b, 1983a, 1983b; Nuti et al., 1987; Bretzlaff and Madrid, 1989) and are known to suppress both follicular and luteal activity (Armstrong et al., 1983b). A progesterone-releasing intravaginal device (PRID; 4 to 6.5% progesterone impregnated coil, Roche, 1976, 1978) has been widely applied to the artificial breeding of cattle (Roche et al., 1981). Ear implants containing the specific synthetic hormone norgestomet (11ß-methyl-17α-acetoxyl-19-norprogesterone, Synchro-Mate B, SMB; 3 to 6 mg) also have been used to synchronize estrus effectively in cattle (Mapletoft, 1988) and, to a lesser extent, in sheep (Boland et al., 1979). Estrous synchronization combined with the use of follicle stimulating gonadotropins for ovulation induction has been achieved in deer (*Dama dama*, *Odocoileus virginianus*, *Cervus elaphus*) using MAP-pessaries (Mulley et al., 1988; Waldham et al., 1989) as well as PRIDs (Asher and Smith, 1986; Fisher et al., 1986). Recently, the MAP-pessary approach has been used to effectively synchronize estrus in suni antelope, resulting in both successful natural and artificial breedings (Raphael et al., 1989). A review of the literature suggests that each of these approaches appears to have a broad application and similar effectiveness probably because of the overall similarity in biochemical configuration and mechanisms of action among the various progestogens.

The discovery in the early 1970's of the usefulness of prostaglandins in controlling estrus revolutionized conventional approaches to the artificial breeding of domestic ruminants. A number of independent investigators simultaneously demonstrated the luteolytic effects of $PGF_{2\alpha}$ in cattle (McCracken et al., 1970; Lauderdale, 1972; Liehr et al., 1972; Louis et al., 1972; Rowson et al., 1972). Shortly thereafter, other studies determined that high fertility rates could be achieved after synchronizing estrus with a single i.m. injection of $PGF_{2\alpha}$ given during the luteal phase (Day 8 to 18) (Graves et al., 1974; Lauderdale, 1975; Roche, 1977) and then naturally or artificially breeding cows. At the same time, several investigators began testing the effectiveness of synthetic prostaglandin analogues (e.g., cloprostenol) (Cooper, 1974) which were found equally useful for timing estrus and ovulation (Cooper, 1981; Seguin et al., 1983). The application of prostaglandins to other livestock species was realized rapidly (see review by Inskeep, 1973), especially in sheep (Hawk, 1973; Hughes et al., 1976; Stacy and Gemmell, 1976; Acritopoulou and Haresign, 1980) and goats (Serna et al., 1978; Ogunbiyi et al., 1980). The exact mechanism of action for prostaglandins continues to be elusive. It appears to be associated with the phospholipase C-inositol triphosphate and diacylglycerol second messenger system which causes intracellularly mediated Ca^{2+} reactions that stimulate luteal demise (Davis et al., 1987). The effect of prostaglandins on luteal tissue morphology (Stacy et al., 1976; Betts et al., 1985; Farin et al., 1986) and various luteal enzyme systems

(Rao et al., 1984) clearly demonstrates that there is a degenerative influence of these compounds on the CL. Prostaglandin $F_{2\alpha}$ activates the reduction of 5'-nucleotidase, NAD pyrophosphorylase and galactosyl transferase within 12 to 24 h which reduces luteal blood flow, decreases regulation of redox reactions and inhibits glycoprotein synthesis (Rao et al., 1984). In response to $PGF_{2\alpha}$, the reduction in luteal blood flow (Niswender et al., 1976) can cause a localized metabolic acidosis in the ovarian cortex (Fitz et al., 1984) which increases the binding affinity of $PGF_{2\alpha}$ to luteal receptors (Powell et al., 1975; Balapure et al., 1989a). Luteal cell ultrastructural characteristics reveal that prostaglandin administration is associated with the presence of autophagocytic bodies and the accumulation of lipid together with cell shrinkage and disorganization among cells (Stacy et al., 1976). In combination, a decline in luteal cell structure, function and metabolic integrity occurs followed by a significant reduction in luteal gonadotropin receptors within 24 h of $PGF_{2\alpha}$ administration (Rao et al., 1984). The former decline in receptor integrity is characterized by an uncoupling of regulatory (i.e., binding) and catalytic (i.e., adenyl cyclase) subunits required for the maintenance of normal receptor function (Thomas et al., 1978). After $PGF_{2\alpha}$ treatment, serum progesterone concentrations rapidly decline to <1 ng/ml and CL weight becomes markedly reduced within 24 h in cows (Inskeep, 1973; Harrison et al., 1985) and ewes (Acritopoulou et al., 1977; Deaver et al., 1986).

For $PGF_{2\alpha}$ to be effective in ruminants, ovarian CL must be present, a prerequisite which occurs for approximately 14 days in the cow and goat and for 11 days in the sheep. Therefore, to ensure that there is a site of action for $PGF_{2\alpha}$, a dual injection regimen has been developed in which the compound is administered at a distinct interval which assures that one of the injections coincides with the presence of ovarian CL. By administering $PGF_{2\alpha}$ 11 to 14 days apart in cattle (King and Robertson, 1974) and goats (Ogunbiyi et al., 1980; Greyling and van Niekerk, 1986) and 9 to 11 days apart in sheep (Fairnie et al., 1977; Acritopoulou et al., 1978; Beck et al., 1987), a majority (>80%) of the animals became synchronized into estrus and ovulated simultaneously.

Currently, there is widespread use of prostaglandins for inducing estrus in the domestic livestock industry. For cattle, the $PGF_{2\alpha}$ regimen is used more frequently than serial administration/withdrawal of a progestogen (Cupps et al., 1977; Critser et al., 1980; Greve, 1981; Hasler et al., 1983; Donaldson and Perry, 1983; Monniaux et al., 1983). However, progesterone therapy occasionally has been demonstrated to effectively induce a fertile estrus in postpartum cows (Holtz et al., 1979; Rutter et al., 1985; Odde et al., 1986) or anestrous sheep (Armstrong et al., 1982a; Killian et al., 1985) and deer (Asher and Smith, 1986). In a comparative cattle study, there were no differences in the proportion of animals demonstrating estrus or in the post-synchronization pregnancy rate after $PGF_{2\alpha}$

injection or norgestomet ear implants; however, reproductive efficiency was reduced significantly by the use of PRIDs (Tegegne et al., 1989). In contrast, several studies on sheep have indicated that a dual-$PGF_{2\alpha}$ regimen (i.e., 9 days apart) produced less precise synchronization of estrus and lower subsequent pregnancy rates than a progestogen-pessary treatment (Boland et al., 1978b, 1978c; Hackett et al., 1981b; Acritopoulou-Fourcroy et al., 1982; Henderson et al., 1984). Recently, Beck and coworkers (1987) extended the interval between $PGF_{2\alpha}$ injections to 11 days in sheep and compared to progestogen-treated ewes they found no difference in estrus synchronization efficiency and lamb production after post-synchronization breeding. The combination of progestogen and $PGF_{2\alpha}$ treatments also has been tested and found to be one approach for reducing the length of the progestogen treatment interval. In this strategy, the exogenous progesterone influence is exerted over a minimal interval (7 days) to ensure that any luteal tissue present is sufficiently developed to respond to $PGF_{2\alpha}$ treatment, if not already in a state of regression, while gonadotropin surges are suppressed and ovulation of growing follicles inhibited. Prostaglandin $F_{2\alpha}$ has been used effectively with norgestomet in cattle (Mapletoft, 1988) and with MAP-pessaries in sheep (Fitzgerald et al., 1985) and deer (Haigh et al., 1989).

Induction of Multiple Ovulations

For more than 50 years, exogenous gonadotropins have been used to enhance folliculogenesis and induce a superovulatory response in ruminant species (see reviews: Betteridge, 1977; Anderson, 1978; Greve, 1981; Schiewe, 1983; Smith, 1988). Classically, hormonal preparations consisting of pregnant mares' serum gonadotropin (PMSG: Hammond and Bhattcharya, 1944; Willett et al., 1948; Robinson, 1950; Wallace, 1951; Rowson and Adams, 1957; Hancock and Hovell, 1961) or crude anterior pituitary extracts from horses (HAP: Hammond et al., 1942; Hammond and Bhattcharya, 1944; Folley and Malpress, 1945; Dowling, 1949; Moore and Shelton, 1962, 1964) and sheep (SAP: Casida et al., 1942; Casida et al., 1943; Willett et al., 1948; Umbaugh, 1949; Dziuk et al., 1958) were used to increase ovarian follicular development. The biological properties of the gonadotropins consisted of sufficient FSH and LH activity to stimulate a wave of antral follicle growth, possibly rescuing some follicles from the early stages of atresia (Byskov, 1979; Peters, 1979; Monniaux et al., 1984). Residual exogenous and also endogenous gonadotropins then activate oocyte maturation and ovulation of 1 or more Graafian follicles (Moor et al., 1984). However, the use of exogenous gonadotropins is almost universally associated with excessive variability in ovulatory responsiveness. A portion of this variation has been rectified by altering the purity of the preparations (Boland

et al., 1983; Murphy et al, 1984; Donaldson and Ward, 1986), the dosage used (Lamond, 1974; Smith, 1988; Lerner et al., 1986; Saumande and Chupin, 1986), the timing of administration in the estrous cycle (Phillippo and Rowson, 1975; Lindsell et al., 1986a) and the method of estrous synchronization used concurrently (Saumande et al., 1978; Sreenan et al., 1978; Voss et al., 1983). As discussed below, individual animal variability and lack of a consistent ovarian response (and, thus, embryo production) continues to be the major limitation to the art of ovulation induction (Monniaux et al., 1983; Moor et al., 1984; Draincourt, 1987).

PMSG, also referred to as equine chorionic gonadotropin (eCG; Papkoff, 1981), was discovered by Cole and Hart (1930) and is known to be secreted by specialized trophoblastic cells which invade the maternal endometrium during early gestation (beginning 36 to 40 days post-conception; Allen and Moor, 1972; Allen et al., 1973). PMSG was the preferred superovulatory agent of the 1950's and 1960's because it's biological potency required only a single injection to elicit a multiple ovulatory response (Robinson, 1950; Betteridge, 1977). The half-life of PMSG is 40 to 50 h in cattle (Papkoff, 1978; Licht et al., 1979) and approximately 20 h in sheep (McIntosh et al., 1975). PMSG predominantly consists of a large carbohydrate component (45%) which includes neutral sugars and hexoamines as well as 10% sialic acid (Papkoff, 1981) which undoubtedly contributes to its extended circulating half-life and high molecular weight (60 kDal, Christakos and Bahl, 1979). The amino acid composition closely resembles the equine pituitary gonadotropins, eFSH and eLH, with the alpha subunit of PMSG being nearly identical to eFSH-α (Moore et al., 1979) and the beta subunit similar to eLH-β (Papkoff, 1978). Specific *in vivo* and *in vitro* bioassays indicate that PMSG has both FSH and LH activity (Papkoff, 1981), and its mechanisms of action have been detailed (Dott et al., 1979). Exogenous administration of PMSG results in a predominant FSH-like effect, considering the FSH:LH ratio is 1:12 to 1:30 (Papkoff, 1978) and that eLH has intrinsic FSH activity (Licht et al., 1979). A PMSG dose of 1500 to 3000 IU, 750 to 1250 IU and 1000 to 1500 IU generally has been given to cattle (Laster et al., 1971; Elsden et al., 1974; Booth et al., 1975; Phillippo and Rowson, 1975; Saumande and Chupin, 1977; Boland et al., 1978a; Elsden et al., 1978; Critser et al., 1980; Greve, 1981; Jensen et al., 1982; Yadav et al., 1986b), goats (Moore and Eppleston, 1979; Armstrong et al, 1982b; Armstrong and Evans, 1983; Armstrong et al., 1983a) and sheep (Robinson, 1951; Whyman et al., 1979; Whyman and Moore, 1980; Armstrong and Evans, 1983; Zanwar and Deshpande, 1984; Walker et al., 1986), respectively, to produce a satisfactory multiple ovulatory response. A few attempts have been made to induce multiple ovulations in non-livestock ruminants including the Arabian oryx (*Oryx leucoryx*, 1200 to 2500 IU, 1 to 3

ovulations; Durrant, 1983), gaur (*Bos gaurus*, 2000 to 2500 IU, 0 to 12 ovulations; Stover and Evans, 1984), Soemmering's gazelle (*Gazella soemmeringi*, 1200 IU PMSG, more than 10 preovulatory Graafian follicles; Durrant et al., 1986), water buffalo (*Bubalus bubalis*, 3000 IU, 0 to 8 ovulations; Sharifuddin and Jainudeen, 1984) and white-tailed deer (*Odocoileus virginianus*, 1000 IU, 0 to 7 ovulations; Waldham et al., 1989). In cases in which superovulation is not desirable (i.e., embryo transfer recipients), the administration of a low PMSG dose (i.e., 250 to 500 IU) decreases some of the individual variation often observed in estrus/ovulation onset after MAP-pessary estrous synchronization (goats: Armstrong et al., 1982b; Armstrong and Evans, 1983; sheep: Langford, 1982; Armstrong and Evans, 1983; Chesne et al., 1987; and deer: Asher and Smith, 1987). An excessive PMSG dose (>3000 IU in cattle, >1500 IU in goats and sheep) often results in a hyper-ovarian response characterized by excessive, sometimes cystic follicular development, anovulation and premature luteinization of follicles (Hafez et al., 1963; Van Rensburg, 1964; Henricks et al., 1973; Evans and Robinson, 1980; Monniaux et al., 1983; Saumande and Chupin, 1986).

Pituitary gonadotropins represent another hormone resource for inducing multiple ovulations. Follicle stimulating hormone (FSH) preparations from the anterior pituitary of horses (HAP) and pigs (FSH-P) are conventional sources of exogenous gonadotropins for ruminant species (see reviews, Betteridge, 1977; Smith, 1988). The ease of hormonal extraction and an always abundant supply of porcine pituitaries have resulted in an economic and readily available source of FSH-P gonadotropin. In contrast to the glycoprotein composition of PMSG, HAP and FSH-P preparations consist exclusively of FSH and LH peptides with relatively short half-lives in the circulation (FSH-P, 30 to 110 min, Steelman and Pohley, 1953; Akbar et al., 1974). Compared to FSH-P, HAP is more similar in its gonadotropic composition to PMSG and, therefore, is considered to have a longer half-life (Parlow, 1963; Parlow and Reichert, 1963). Nevertheless, a multiple injection regimen of both of these pituitary gonadotropins is required to produce a superovulatory response. Extracts of HAP have been used in goats (Moore and Eppleston, 1979) and sheep (Moore and Shelton, 1964; Moore, 1970) given at a dosage of 16 to 45 mg once daily over a 3 day period (Trounson and Moore, 1974; Boland et al., 1983; Alwan et al., 1988). Additionally, a daily 3 day treatment, at a HAP total dosage of 100 to 400 mg, has been used effectively in cattle (Hammond and Bhattacharyya, 1944; Dowling, 1949; Moore, 1975; Boland et al., 1983). Although 8 to 15 ovulations have been achieved in HAP-treated sheep (Trounson and Moore, 1974; Boland et al., 1983; Alwan et al., 1988), the unavailability of horse pituitaries has limited the usefulness of this preparation in the United States. Alternatively, FSH-P has been used extensively at a dosage of 16 to 24

mg given over 2 to 3.5 day periods in sheep (Wright et al., 1981; Armstrong and Evans, 1983; Cognie and Torres, 1984; Walker et al., 1986; Chesne et al., 1987; Torres et al., 1987) and goats (Bondioli and Wright, 1981; Armstrong and Evans, 1983; Armstrong et al, 1983b; Nuti et al., 1987) and 28 to 50 mg over 4 to 5 days in cattle (Avery et al., 1962; Elsden et al., 1978; Hasler et al., 1983; Lerner et al., 1986; Walton and Stubbings, 1986; Yadav et al., 1986a, 1986b; Schiewe et al., 1987a; Garcia-Windner et al., 1988). FSH is generally given twice daily by an intramuscular or subcutaneous route. A once daily or single bolus injection regimen has resulted in a more variable response with fewer CL, more large-sized unovulated follicles and a reduced embryo recovery rate in donor cattle (Bellows et al., 1969; Hunt et al., 1971; Wildt et al., 1975; Looney et al., 1981; Hill, 1988) compared to a conventional bi-daily FSH-P approach. Continuous FSH-P infusion in cattle has also been examined (Godke et al., 1980) and, with the advent of implantable, osmotic mini-pumps has been proven effective (Wubishet et al., 1986). Although the latter procedure does not necessarily improve the mean superovulatory response, it may reduce variation in ovarian responsiveness to exogenous gonadotropins.

Comparative evaluations of PMSG and pituitary gonadotropins generally indicate that the most reliable and favorable superovulatory response in ruminant species is achieved using multiple injections of FSH-P. In cattle (Laster, 1973; Elsden et al., 1978), sheep (Armstrong and Evans, 1983; Evans and Armstrong, 1984a; Walker et al., 1986) and goats (Armstrong and Evans, 1983; Armstrong et al., 1983a, 1983b), the use of FSH-P usually results in the recruitment of more ovarian follicles and ovulations than PMSG. One recent cattle study deviated from this norm as demonstrated by a 3-fold increase in the ovulation rate following the use of PMSG compared to FSH-P (Yadav et al, 1986b). Such responses may become more common as investigators continue to purify more biologically potent PMSG preparations with high FSH to LH ratios (Humphrey et al., 1979; Murphy et al., 1984). Improvements also might be made in the chemical composition of FSH-P because LH content is known to vary in this preparation (Murphy et al., 1984; Lindsell et al., 1986b). The use of more purified FSH-P sources has already increased the number of transferable embryos recovered from cattle (Donaldson and Ward, 1986; Donaldson et al., 1986). In general, variability in ovarian response is much greater following PMSG than FSH treatment as indicated by increased numbers of large unovulated follicles following treatment with the former compound (Elsden et al., 1978; Critser et al., 1980; Armstrong et al., 1983a; Monniaux et al., 1984; Yadav et al., 1986b). This problem results from the continued recruitment of more follicles during and immediately after ovulation caused by the long-acting and residual properties of circulating PMSG (Armstrong et al, 1983b). These ancillary follicles can result in pertubations in the endogenous hormonal milieu

which can disrupt estrous behavior, gamete and sperm transport and early development of the pre-implantation embryo (Armstrong et al., 1982b; Jensen et al., 1982). Recently, the use of anti-PMSG antibodies given after a PMSG-induced estrus has been shown to effectively decrease this post-estrus follicular activity (Saumande et al., 1984; Dieleman et al., 1987; Kim et al., 1987). In these studies, more transferable embryos were collected after the artificial breeding of antibody-injected cows and this was attributed to an improved endocrine environment. Overall, a number of comparative studies have reported a more consistent ovulatory response and the recovery of more high quality embryos from FSH-P compared to PMSG-treated animals (Elsden et al., 1978; Critser et al., 1980; Armstrong and Evans, 1983; Armstrong et al., 1983a; Greve et al., 1983) which no doubt is the primary reason for the current preference for FSH-P throughout the livestock industry.

In the early 1980's, another gonadotropin preparation became available primarily for combating human infertility. Human menopausal gonadotropin (hMG) is a FSH and LH preparation extracted from the urine of menopausal women and is considered to have a short-half life (Lauria et al., 1982), although the exact pharmokinetic decay rate is unknown. In menopausal individuals, the production of pituitary gonadotropins is uninhibited due to the lack of negative ovarian estrogen feedback. Each ampule of hMG contains 75 IU FSH and 75 IU LH. Using a twice daily injection schedule given for 5 consecutive days, a total dosage of 2100 IU is effective in cattle (Critser et al., 1982; Lauria et al., 1982) producing a mean of 11 CL from each of 23 (100% ovulated) treated Holstein donors (Lauria et al., 1982). In addition, Lauria and coworkers (1982) determined that hMG reduced ovulatory variability among individuals without decreasing embryo quality (mean of 8 transferable embryos recovered/donor). Subsequently, McGowan et al. (1985) determined that a total dose of about 4200 IU did not increase CL numbers further, but did apparently affect oocyte integrity as fertilization rates were depressed. Compared to FSH-P, hMG produces similar or slightly fewer numbers of ovulations and transferable quality embryos (Alcivar et al., 1984; Murphy et al., 1984; McGowan et al., 1985) and is considered to be an appropriate alternative to FSH-P and PMSG. Unfortunately the escalated use of hMG in humans (Ferraretti et al., 1983; Kemmann et al., 1983; Laufer et al. 1983) has caused commercial costs to be prohibitively expensive and has reduced the practical utility of this product for ruminant species.

Exogenous gonadotropins are foreign proteins capable of eliciting antibody formation in the host female (Jainudeen et al., 1966). This has generated concerns about the long-term effects of repeated gonadotropin treatment as antibodies potentially could cross-react with endogenous hormones and thereby reduce subsequent ovarian responsiveness. Several reports indicate that serial gonadotropin treatments are related to

reduced ovarian function in cattle over time (Christie et al., 1979; Boland and Gordon, 1982; Bastidas and Randel, 1987). However, the records of the commercial cattle embryo transfer industry indicate that there is no difference in ovulation rate within animals after ≤10 repeated FSH-P treatments but fertilization rate and embryo production is decreased (Hasler et al., 1983). Another study determined that the decline in transferable quality embryos with consecutive FSH-P stimulations (i.e., 2 to 10 treatments) was dependent on the individual donor, as pooled data failed to reveal a significant reduction (Donaldson and Perry, 1983). When PMSG was given 5 times to individual cows over resting intervals of more than 60 days between treatments, Moor and coworkers (1984) observed no affect on ovulation rate or viable embryo production. The impact of repeated gonadotropin regimens may be closely correlated to the interval between hormonal treatments (Kanagawa et al.,1981) suggesting that any possible inhibitory antibody response, if present, is time dependent. A survey of various livestock studies failed to reveal a consistent pattern between the number of times donor cows, goats or sheep were superovulated and subsequent embryo production. Interestingly, other data indicate that donor cows with initial high ovulation rates will continue to have high ovulatory responses after subsequent treatments (Schams et al., 1978) suggesting that the effect is, in part, genetically mediated. A similar response has been observed among sheep in that certain genotypes are more or less sensitive to specific exogenous gonadotropin treatments (Torres et al., 1987).

In addition to measuring ovarian activity and embryo recovery, the monitoring of endogenous hormone patterns has been used to assess the effects of various exogenous gonadotropic preparations (PMSG: Lemon and Saumande, 1972; Sreenan et al., 1978; Saumande, 1980; Greve, 1981; Saumande and Lopez-Sebastian, 1982; Armstrong et al., 1983b; Yadav et al., 1986b; FSH-P: Armstrong et al., 1983b; Yadav et al., 1986b; hMG: Lauria et al., 1982). Circulating estrogen concentrations are greater and the magnitude of the LH surge is less in PMSG- compared to FSH-P-treated ewes (Armstrong et al., 1983b; Yadav et al., 1986b), probably as a result of more ancillary, unovulated follicles. In PMSG versus FSH-P injected cows, there are no differences in the mean basal LH or frequency or amplitude of the LH pulses but the LH surge occurs earlier and progesterone levels are elevated for a shorter interval in the PMSG-treated animals (Yadav et al., 1986b). It has been proposed in sheep that PMSG may advance the time of ovulation relative to the onset of estrus by as much as 15 h (Boshoff et al., 1973) which may alter the steroid balance necessary for normal ova transport, fertilization and embryo development. Preovulatory progesterone values of greater than 1 ng/ml in PMSG-treated cattle have been correlated to reduced fertilization and embryo viability (Greve, 1981; Jensen et al., 1982; Greve et al., 1983) In PMSG-treated ewes, if circulating progesterone rises too rapidly,

ova are not retained in the oviduct for a normal duration due to accelerated transport and are released into an asynchronous, potentially hostile, uterine environment which can decrease embryo viability (Whyman and Moore, 1980). Several studies have determined that there is a correlation between CL number and circulating progesterone concentration (Lamond and Gaddy, 1972; Lemon and Saumande, 1972; Sreenan et al., 1978; Saumande, 1980), whereas others have failed to confirm such a relationship (Rajamahendran et al., 1976; Schams et al., 1978; Greve, 1981). The present consensus is that there is an imprecise correlation between CL number and systemic progesterone levels in the cow (Saumande, 1980). However, Saumande and Lopez-Sebastian (1982) have determined that there is a high correlation between total estradiol-17α and the number of follicles induced to ovulate suggesting that this endocrine characteristic might be useful as a preovulatory indicator of ovarian responsiveness to exogenous gonadotropins.

There are considerable data on the temporal relationship of ovulation to gonadotropin-stimulated follicular development. In FSH-P-treated cattle, ovulation begins within 24 h of estrus onset (Maxwell et al., 1978; Yadav et al., 1986a), and 80% of the large follicles (>10 mm in diameter) rupture within 12 h (Yadav et al., 1986a. Likewise in PMSG-injected sheep, the first ovulation occurs within 24 h of the first estrous behavior (Whyman et. al., 1979; Walker et al., 1986) which is slightly earlier than observed after FSH-P treatment (30 to 36 h, Walker et al., 1986). After progestogen-pessary removal in PMSG-injected sheep, the majority (>80%) of ovulations occur within 42 to 66 h compared to 54 to 72 h after FSH-P treatment (Walker et al., 1986). In addition, these same investigators determined that the latter ovulatory interval could be reduced to a 9 hour duration by giving a supplemental injection of gonadotropin-releasing hormone (GnRH, 100 μg, i.v.) 24 h after pessary removal.

The impact of other factors on fertilization rates in gonadotropin-treated cattle has been reviewed recently (Hawk, 1988) and include the age of the donor, number of previous superovulation treatments, influence of exogenous gonadotropins on the follicular population, endogenous hormonal patterns and sperm concentration and site of deposition. These same factors can influence fertilization in other ruminant species, however, the predominant cause of fertilization failure in sheep has been attributed to hormonal treatments (Van Rensburg, 1964; Evans and Robinson, 1980; Whyman and Moore, 1980; Armstrong and Evans, 1983; Hawk et al., 1987). Although exogenous gonadotropins can induce abnormal physiological and endocrinological conditions (especially PMSG), the principal cause of reduced fertilization rates has been related to progestogen and prostaglandin estrous synchronization treatments (Quinlivian and Robinson, 1969; Hawk and Cooper, 1977; Hawk et al., 1981; Pearce and Robinson, 1985; Hawk et al., 1987).

Various investigations have shown that these treatments can cause abnormal sperm transport through the cervix and accelerated sperm mortality within the sheep reproductive tract (Hawk et al., 1981; Hawk, 1983; Evans and Armstrong, 1984b; Pearce and Robinson, 1985; Hawk et al., 1987). However, the detrimental effects of progestogen and prostaglandins on fertility generally are not observed in cattle (Hawk, 1988) and goats (Armstrong et al., 1983a), and inexplicably, have not been observed in occasional sheep studies (Hackett et al., 1981a).

Infertility Associated with Luteal Dysfunction

Inadequate luteal function has been described as the cause of short estrous cycles, but its exact etiology is unknown. It is presently theorized that premature regression of CL occurs because of: 1) inadequate hormonal preparation of the preovulatory follicle; 2) the early release of a luteolysin; and 3) a hypersensitivity of the luteal tissue to a luteolysin. Short luteal phases have been observed routinely during transitional periods from anestrus to estrus in goats (Prasad and Bhattacharyya, 1979; Ott et al., 1980; Camp et al., 1983; Chemineau, 1983) and sheep (Haresign et al., 1975; Oldham and Martin, 1978; Knight et al., 1981; McNeilly et al., 1981; McLeod and Haresign, 1984), and in postpartum cattle (Rutter et al., 1985) and sheep (Land, 1971; Lewis et al., 1981). Frequently these studies note what appears to be structurally abnormal or dysfunctioning CL. For example, following male-induced estrous synchronization/ovulation induction at the end of the anestrous season, CL frequently regress by 4 to 8 days after ovulation in sheep (Oldham and Martin, 1978; Knight et al., 1981) and goats (Ott et al., 1980; Chemineau, 1983). The same type of short cycling pattern is observed in anestrous sheep induced to ovulate with GnRH (McNeilly et al., 1981; McLeod and Haresign, 1984); CL regression generally occurs by Day 4 (Day 0 = onset of ovulation) in such animals (Oldham and Lindsay, 1980; Hunter et al., 1988). Circulating progesterone in short cycling sheep rises to 0.5 to 1.0 ng/ml until Day 3 (Knight et al., 1981; Legan et al., 1985; Southee et al., 1988a), but the abnormal CL are incapable of sustaining normal levels of this hormone despite having an adequate population of LH receptors (McNeilly et al., 1981). By Day 4, these luteal cells also have a reduced ability to produce progesterone *in vitro* and 1 day later these CL contain less progesterone and weigh less than normal CL (McNeilly et al., 1981; Hunter et al., 1988). The relative proportion of small and large luteal cells in abnormal ovine CL is normal through Day 4 (O'Shea et al., 1984). In cattle, poorly developed luteal tissue has been reported following ovulation induction with GnRH 16 to 32 h after prostaglandin treatment compared to the occurrence of normal luteal tissue when GnRH is administered 48 h post-$PGF_{2\alpha}$ (Ohnami et al., 1985). Inadequate gonadotropin-priming of the

preovulatory follicle has been postulated as the cause of luteal dysfunction (McNeilly et al., 1980). However, normal luteal function is not attributable to an increased duration of follicular development in the ewe (McLeod et al., 1982; McLeod and Haresign, 1984) or to a difference in the size or number of granulosa cells in preovulatory follicles (Hunter et al., 1986b).

Progesterone may directly influence the responsiveness of antral follicles to induced changes in gonadotropin secretion (McLeod and Haresign, 1984; Hunter et al., 1986a) or synchronize follicular activity before GnRH-induced pituitary stimulation (Hunter and Southee, 1987). Hysterectomized anestrous ewes pretreated with or without progesterone exhibit extended luteal phases following GnRH-induced ovulation (Southee et al., 1988b), indicating that the uterus plays a role in regressing short-cycle CL. Investigations involving postpartum cows have demonstrated the importance of progesterone priming on normal luteal function (Lamming et al., 1981; Rutter et al., 1985) and the premature luteolytic effect of uterine $PGF_{2\alpha}$ on subnormal CL (Troxel and Kesler, 1984; Copelin et al., 1987). Although CL of cycling cows contains more mass and more LH receptors than postpartum cows, there is no difference in the number of luteal LH receptors between postpartum cows experiencing a short versus normal luteal phase (Rutter et al., 1985). Furthermore, the shortened luteal lifespan of postpartum cows is not attributable to insufficient pre- or post-ovulatory gonadotropic stimulation (Gaverick et al., 1988). The similarity of a short luteal cycle and the positive affect of progesterone pretreatment relative to luteal dysfunction between the anestrous ewe induced to ovulate with GnRH and the postpartum cow suggests that common mechanisms are probably involved. Current evidence suggests that the early release of $PGF_{2\alpha}$ may be responsible for luteal dysfunction in postpartum cows (see review: Gaverick and Smith, 1986) and ewes (Lewis et al., 1981) as indicated by high circulating levels of $PGF_{2\alpha}$. In contrast, hypersensitivity of large luteal cells to a luteolysin is believed to be the cause of early CL regression in the pubertal ewe (Keisler et al., 1983) and GnRH-treated anestrous ewe (Southee et al., 1988b). The fact remains that the exact etiology of premature luteal regression is unknown.

Luteal dysfunction also has been associated with the use of exogenous gonadotropins for ovulation induction (Armstrong et al., 1982a, 1983a, 1983b; Stubbing et al., 1986). A high incidence of premature luteal regression has been reported in goats superovulated with PMSG in conjunction with progestogen pessary (Armstrong et al., 1982b, 1983a) and prostaglandin estrous synchronization (Stubbing et al., 1986) compared to FSH-P-treated conspecifics (Armstrong et al., 1983b). As with the luteal dysfunction of anestrous ewes, serum progesterone rises transiently and declines by Day 4 (Armstrong et al., 1982a; Stubbing et al., 1986). Armstrong and coworkers (1983b) theorize that

abnormal endocrine events associated with multiple ovulation induction may stimulate endogenous $PGF_{2\alpha}$ release from follicles or the uterus, thereby causing the early demise of gonadotropin-induced CL. Other evidence has correlated LH surges of reduced magnitude to short-duration estrous cycles (Camp et al., 1983; Bretzlaff et al., 1988). Abnormal luteal function has also been directly associated with $PGF_{2\alpha}$ (Hansen et al., 1987) and superovulation treatment in cattle (Bouters et al., 1980), as indicated by lower circulating progesterone levels (on Day 10) and the presence of short-lived CL at slaughter (Day 14) compared to normal cycling cows (Bouters et al., 1980). However, it remains unclear whether abnormal CL have an innate short lifespan as a result of inadequate hormonal preparation or increased luteal sensitivity to or early release of a luteolysin.

RECOVERY AND TRANSFER OF EMBRYOS FROM RUMINANT SPECIES

Embryo Collection

Nearly 100 years ago Sir Walter Heape performed the first successful intra-animal embryo transfer using the rabbit (Heape, 1891). This historic event was not attempted in ruminant species until the 1930's and 1940's when the first surgical embryo recoveries were initiated using sheep and goats (Warwick and Berry, 1949). Soon after, successful surgical recovery of oviductal- and uterine-stage embryos was achieved in hormonally-stimulated cows (Umbaugh, 1949; Willett et al., 1951) and ewes (Hunter et al., 1955). Successful recovery of embryos from the reproductive tract is highly dependent on a knowledge of the time kinetics of ovum release, transport and fertilization.

Following ovulation, ova transverse through the oviduct and are fertilized by capacitated spermatozoa in the ampullar-isthmus region. Cleaved cattle, goat and sheep embryos and unfertilized ova pass through the utero-tubal junction (UTJ) and into the uterus 4 days after estrus onset (Hamilton and Laing, 1946). However, simultaneous flushings of the uterus and oviduct indicate that ova can be retained in the oviduct for as long as 5 days (Newcomb and Rowson, 1975), possibly contributing to reduced embryo recovery rates when only the uterus is flushed (Smith and Murphy, 1984). A variety of surgical approaches have been developed, and specific usefulness is highly dependent on the stage of the estrous cycle of the embryo donor (see reviews: Foote and Onuma, 1970; Betteridge, 1977). Early efforts in sheep involved a mid-ventral laparotomy of anesthetized donors that allowed the ovaries, oviducts and uterine horns to be exteriorized for viewing and manipulation (Hunter et al., 1955). Oviductal embryos were recovered by retrograde flushing of 2 to 5 ml of Ringer's solution through a 15 gauge needle inserted in the uterine end of the UTJ and into a 2 mm-bore glass tube placed 3 cm deep into the fimbriated end of the oviduct. Uterine embryos were recovered by antegrade flushing using a similar needle directed toward the UTJ and a collection needle placed in the distal end of the uterine horn, just proximal to a luminal occlusion produced by thumb and finger pressure. These early studies usually produced relatively high embryo recovery rates (58 to 95% of the embryos and ova based on ovarian CL number) (Hunter et al., 1955; Hancock and Hovell, 1961; Moore and Shelton, 1962; Tervit and McDonald, 1969).

The surgical embryo recovery procedures currently used in ruminant species represent a modification of the original approach described by Hunter and coworkers (1955). The collection needle in the antegrade uterine flush technique has been replaced with a glass tube (cattle: Rowson et al., 1969; sheep: Simms et al., 1982) or, more

recently, a Foley catheter using a balloon cuff to occlude distal flow (sheep: Smith and Murphy, 1984). Embryo recovery rates vary from 65 to 75%. A comparable recovery rate of sheep embryos (77%) also recently has been achieved by retrograde perfusion of uterine contents through a cannula secured by a ligature placed adjacent to the UTJ (Chesne et al., 1987). A flexible tubing introduced into the fimbriated end of the oviduct also has been adopted in cattle (Rowson et al., 1969; Drost et al., 1975) and goats (Nuti et al., 1987) for retrograde recovery of oviductal embryos. This approach has been combined with placing a urological catheter in the distal end of the uterine horn to facilitate the simultaneous recovery of both oviductal and uterine stage cattle embryos (Newcomb and Rowson, 1975). Alternatively, the antegrade uterine flush could originate at the cannulated oviduct (cattle: Tervit, 1975) or a retrograde perfusion of uterine contents through the oviductal end (cattle: Baker and Jillella, 1978; Betteridge et al., 1980; sheep: Willadsen et al., 1976; Boundy et al., 1985). The latter three procedures reduce the chance of missing rapidly or slowly transported embryos if only an oviductal or uterine flush is executed alone.

Incentive for developing nonsurgical embryo recovery procedures originated because of concerns about: 1) the tendency for adhesions to form after major surgery, a condition which is potentially detrimental to subsequent fertility; and 2) the time and expense of conducting surgical procedures. It is interesting that the original attempts at nonsurgical embryo recovery in cattle were tested concurrently with the more invasive surgical techniques, yet more than two decades passed before suitable nonsurgical approaches were available routinely. In the initial study, a 2-way catheter (containing supplemental support of a steel stylette) with an inflatable collar was used to penetrate the cervical canal and perfuse each uterine horn under the guidance of rectal palpation (Rowson and Dowling, 1949). Initially, embryo recovery efficiency was poor using a variety of modified devices (Dracy and Petersen, 1950; Dziuk et al., 1958). In these cases, one concern was the inability to prevent donors from becoming pregnant with multiple fetuses. In the early 1970's, the development of 2- and 3-way catheters allowed more effective, transcervical flushing of the uterus, and embryo recovery rates increased to 40% or greater (Sugie et al., 1972; Alexander et al., 1976; Bouters et al., 1976; Drost et al., 1976; Elsden et al., 1976; Rowe et al., 1976; Brand and Drost, 1977; Greve et al., 1977; Lampeter, 1977; Baker and Jillella, 1978; Newcomb et al., 1978; Sreenan, 1978a; Hahn, 1978; Ozil et al., 1979; Rasbech, 1979; Rowe et al., 1980a), as reviewed by Greve (1981). The surge of interest in nonsurgical embryo recovery in large-sized ruminants occurred coincidently with the discovery of the luteolytic effects of prostaglandin $F_2\alpha$ ($PGF_2\alpha$) and its analogues. The availability of prostaglandins also offered an approach for preventing unwanted pregnancies in valuable donor females; it became standard practice to terminate

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any of these potential pregnancies by administering $PGF_2\alpha$ immediately after nonsurgical uterine flushing.

Procedural variations in nonsurgical bovine embryo recovery usually have involved varying the type of catheter system used and the method of medium introduction and recovery. Most laboratories used flexible 2- and 3-way Foley-type catheters (e.g., Foley, Rusch) with an inflatable balloon cuff and a metal or plastic stylette to provide rigidity to the catheter for facilitating insertion through the cervix (Alexander et al., 1976; Bouters et al., 1976; Drost et al., 1976; Elsden et al., 1976; Rowe et al., 1976; Brand and Drost, 1977; Greve et al., 1977; Lampeter, 1977; Baker and Jillella, 1978, Lampeter, 1978; Newcomb et al., 1978; Ozil et al., 1979; Rasbech, 1979; Newcomb, 1980).

For the actual recovery of bovine embryos, usually a flush medium consisting of either modified tissue culture medium (TCM 199: Betteridge and Mitchell, 1974; Drost et al., 1975; Rowe et al., 1976; Betteridge et al., 1980) or modified phosphate buffered saline (PBS: Elsden et al., 1976; Ozil et al., 1979; Betteridge et al., 1980; Newcomb, 1980) supplemented with 1 to 2% heat inactivated bovine serum and antibiotics has been used. The medium is perfused in and out of the uterus using either a gravitational flow procedure (Drost et al., 1976; Elsden et al., 1976; Rowe et al., 1976) or an interchangeable syringe technique (Greve et al., 1977; Rasbech, 1979; Looney et al., 1981). Generally each uterine horn has been flushed separately, however, simultaneous perfusion of the entire uterus by inflating the balloon cuff in the uterine body (i.e., next to the interior cervical os) also is effective (Looney et al., 1981; Schiewe, 1983). Regardless of the type of collection system used, usually over 90% of the flush medium is recovered, and the percentage of embryo and ova nonsurgically recovered from cattle ranges from 50 to 75% (mean, 4.5 to 7.5 embryos/donor) (see review, Greve, 1981).

A comparative study in cattle indicated that there are no differences in recovery efficiency between surgical and nonsurgical embryo collection procedures (Rowe et al., 1976). Although another investigation favored the surgical approach (Baker and Jillella, 1978), accumulated results from the late 1970's validated the effectiveness of nonsurgical embryo recovery, and this technique now is the standard for the commercial embryo transfer industry (Greve et al., 1978; Lampeter, 1978; Halley et al., 1979; Schneider and Hahn, 1979; Schneider et al., 1980). Embryo collection techniques continue to be simplified with the advent of filtration units (Lampeter, 1977; Pugh et al., 1980) which filter the embryos from approximately 90% of the flush medium, thereby reducing the time required to microscopically examine recovered fluids. The anatomy of large-sized ruminants allows these species to be palpated rectally, a characteristic which permits guiding the necessary embryo collection (or transfer) instruments through the cervical

canal. These techniques also have been adapted to several other species of the Bovidae family including the banteng (*Bos javanicus*, W. Rietschel, personal communication), bongo (*Tragelaphus euryceros*, Dresser et al., 1985), eland (*Taurotragus oryx*, Dresser et al., 1982), gaur (*Bos gaurus*, Stover and Evans, 1984; Pope et al., 1988b) and water buffalo (*Bubalus bubalis*, Drost et al., 1986). These procedures do not have practical application to small-sized ruminants because the cervix cannot be manipulated via the rectum. Therefore, surgical embryo collections routinely are performed in species like the sheep and goat and, on occasion, have been used in the white-tailed deer (Waldham et al., 1989) and oryx (*Oryx lecuoryx*, *Oryx dammah*, *Oryx callotis*; Durrant, 1983). However, this strategy imposes fertility risks because of the tendency for abdominal adhesions to form after surgery.

The need to adapt embryo techniques to commercial dairy goats and sheep and to small-sized, rare nondomestic species has served as incentive for developing several novel and atraumatic embryo recovery approaches. To reduce adhesions between the ovaries, oviducts, uterine horns and omentum, a less traumatic surgical approach using laparoscopy has been developed in sheep (McKelvey and Robinson, 1986). In this procedure, the uterus is not exteriorized but rather a laparoscope is inserted through a 1 cm mid-ventral abdominal wall incision to accommodate observation of the reproductive tract. After evaluating ovarian activity, an accessory trocar-cannula is also inserted into the abdominal cavity. The trocar is replaced with a grasping forcep and each uterine horn is secured on separate occasions near the bifurcation. With an additional accessory trocar-cannula, the uterus is bluntly punctured anterior to the forcep. A Foley catheter is inserted through the accessory cannula into the distal end of a uterine horn, followed by repeated perfusion and drainage of the uterus via the catheter. Using this laparoscopic procedure, 50% of the embryos have been recovered without excessively manipulating the reproductive tract. A disadvantage of this technique is that the uterine puncture created by the catheter can not be sutured which can result in endometrial outgrowths and the formation of a fistula (Newcomb and Rowson, 1975; McKelvey and Robinson, 1986; Kraemer, 1989).

Nonsurgical embryo collection procedures are also being developed for small-ruminant species to facilitate embryo recovery without compromising subsequent fertility. The first such technique involved a relatively complicated and time-consuming method for dilating the cervix and passing a Foley catheter in the goat (BonDurant et al., 1985). A more refined approach using a vaginal speculum, a cervical expander and small diameter tubing (≥2.5 mm OD) has recently proven effective for recovering ~90% of all embryos in 15 of 26 does (Nagashima et al., 1987). The most widely-used approach to-date initially was developed for sheep (Coonrod et al., 1986) and has been diagrammed in detail

(Kraemer, 1989). This procedure involves donor sedation and consists of: 1) viewing the cervix via a vaginal speculum; 2) retracting the cervix into the vulvular region by grasping the fornix with laparoscopic forceps; 3) expanding the vulvular space and enhancing visibility of the cervix with a duck-billed speculum; 4) replacing the laparoscopic forceps with Allis tissue forceps; 5) removing the speculum and supporting the external os to allow for single-finger, vaginal palpation of the entire cervix and the uterine body; 6) passing a Verres needle through the cervical canal and into the uterus; and finally 7) repeatedly perfusing and draining sequential low volumes of flush medium using a 3-way stop-cock attached to the Verres needle. This nonsurgical procedure has subsequently been used to successfully recover embryos from angora goats (Bessoudo et al., 1988), suni antelope (*Neotragus moschatus zuluensis*, Raphael et al., 1989), white-tailed deer (Magyar et al., 1988) and the yellow-backed duiker (*Cephalophus sylvicultor*, Pope et al., 1988a). Due to species differences in the size and patency of the cervical canal during the early luteal phase, different sized needles and angiocatheters are necessary for the collection process. As with bovine nonsurgical procedures, technical experience will likely be the primary element necessary to achieve consistently successful embryo recovery from small ruminants.

Embryo Transfer

The pioneering studies of Warwick and Berry in the 1930's and 1940's resulted in the first sheep and goat offspring born following surgical embryo transfer (Warwick and Berry, 1949). Additional reports of successful embryo transfer in sheep followed (Casida et al., 1944; Hunter et al., 1955; Rowson and Adams, 1957; Averill and Rowson, 1958; Moore and Rowson, 1960), and the first bovine calves were born from embryo transfer in the early 1950's (Willett et al., 1951; Willett et al., 1953). Nonsurgical approaches were attempted for over 15 years in cattle (Dowling, 1949; Umbaugh, 1949; Dracy and Petersen, 1950; Rowson, 1951; Dziuk et al., 1958; Avery et al. 1962) before the first offspring were produced in the mid 1960's after either transcervical (Mutter et al., 1964; Rowson and Moor, 1966) or transvaginal (Sugie, 1965) placement of embryos into the uterus. The efficiency of both surgical and nonsurgical bovine embryo transfer procedures was low compared to surgical transfer success rates in sheep (Averill and Rowson, 1958; Moore and Shelton, 1962; Rowson and Moor, 1966; Moor and Rowson, 1966) until the late 1960's when Rowson and coworkers (1969) reported a 91% pregnancy rate using a mid-ventral laparotomy approach similar to that developed for sheep (Hunter et al., 1955). In cattle, the low pregnancy rates associated with nonsurgical embryo transfer were attributed to a greater susceptibility of the uterus to infection during the early luteal phase (see review:

Avery et al., 1962; Christie et al., 1980); however, greater use of aseptic procedures eliminated this problem (Brand et al., 1976; Trounson et al., 1978b).

Conventional surgical embryo transfer in cattle was simplified by the development of a standing flank incision procedure which eliminates the time and expense of performing surgery under general anesthesia (Drost et al., 1975). This approach requires only local anesthesia injected around the incision site and was adopted as the surgical method of choice for commercial operations. Despite the effectiveness of the latter technique, it still potentially impaired subsequent fertility due to possible adhesion formations. With a growing interest in the commercial application of embryo transfer in cattle, nonsurgical embryo transfer procedures greatly improved in the mid to late 1970's (Hahn et al., 1975; Sreenan, 1975; Greve and Rasbech, 1976; Bowen et al., 1978) and eventually replaced surgical transfers. The rapid and atraumatic nature of the nonsurgical technique applied to chute-restrained cattle enhanced the on-farm potential of embryo transfer. By the 1980's, nonsurgical techniques were incorporated into the routine operations of the bovine embryo transfer industry (Greve and Lehn-Jensen, 1979; Halley et al., 1979; Schneider et al., 1980; Wright, 1981), even though this approach slightly reduced pregnancy rates compared to the results from surgical transfers (Sreenan, 1978b).

Based on progress in the bovine embryo industry, surgical and nonsurgical embryo transfers were attempted in several species of nondomestic ruminants. Both approaches were used to produce gaur calves (Stover and Evans, 1984; Pope et al., 1988b) while eland (Dresser et al., 1982), bongo (Dresser et al., 1985) and water buffalo (Drost et al., 1986) offspring were produced after nonsurgical embryo transfer. In the case of the gaur and bongo embryos, offspring resulted from interspecific embryo transfers to a Holstein cow (Stover and Evans, 1984; Pope et al., 1988b) or eland recipient (Dresser et al., 1985), respectively. These investigations confirmed that taxonomically-related recipients could maintain an interspecies pregnancy to term, as previously demonstrated with mouflon sheep and domestic sheep and more commonly between *Bos indicus* and *Bos taurus* cattle (see review: Anderson, 1988). Unfortunately, the concurrent development of nonsurgical procedures for small ruminant species did not occur and embryo transfer success in the goat (Bilton and Moore, 1976; Willadsen et al, 1978; Moore and Eppleston, 1979; Armstrong and Evans, 1983; Armstrong et al., 1983a) and sheep (Armstrong and Evans, 1983; Zanwar and Deshpande, 1984; Bounty et al., 1985; Chesne et al., 1987; Torres et al., 1987) remained strictly dependent on the conventional mid-ventral laparotomy approach until the mid 1980's. A less traumatic, noninvasive transabdominal laparoscopic embryo transfer procedure was developed for the sheep and proved equally effective to conventional laparotomy procedures (Schiewe et al, 1984; McKelvey et al., 1985; Walker

et al., 1985). This laparoscopic approach also has been applied successfully to the suni antelope, a small (~8 kg body weight) nondomestic ruminant (Raphael et al., 1989), thereby confirming the effectiveness of this procedure for nondomestic species.

Specific Factors Influencing Successful Embryo Transfer

A number of factors affect embryo survival following transfer (see reviews: Newcomb, 1976; Wilmut et al., 1985; Sreenan and Diskin, 1987). The reproductive synchrony between the embryo donor and recipient, especially in terms of time of ovulation and endocrine status, is the most critical factor influencing embryo survival after transfer (Lawson et al., 1975). Hunter and coworkers (1955) originally suggested that pregnancy occurs only when the estrous cycle of the recipient ewe is within -16 to +20 h of that of the donor. It later was determined that maximal pregnancy rates were obtained in sheep when donor-recipient synchrony is within ± 12 h (Hancock and Hovell, 1961; Moore and Shelton, 1964). Although exact synchrony provides the optimum condition for pregnancy establishment, high pregnancy rates have resulted when donor and recipient sheep and cattle were as much as 2 days asynchronous (Rowson and Moor, 1966; Rowson et al., 1969; respectively). Asynchronous embryo transfers of more than 2 days retard and accelerate sheep embryonic growth when placed in a less or more developed (< or > 48 h synchrony, respectively) maternal environment, respectively. In each case, the transferred embryo fails to achieve vesicular elongation and implantation (Lawson et al., 1983). Currently, estrous synchronization of ± 24 h is considered to provide acceptable transfer results (see review: Betteridge, 1977; Greve, 1981; Wilmut et al., 1985; Sreenan and Diskin, 1987).

The synchronization between the stage of embryo development and the location of embryo deposition in the reproductive tract (i.e., oviduct or uterus) also influences the utility of embryo transfer. Pregnancy rates in sheep are optimized when 2- to 4-cell embryos are transferred to the oviduct and embryos with 6 or more blastomeres are placed in the uterus (Averill and Rowson, 1958; Moore and Shelton, 1964). Nonsurgical embryo transfer studies in cattle reveal that the exact site of embryo deposition in the uterine horn is not critical (Sreenan, 1975; Tervit et al., 1977; Rowe et al., 1980b; Greve, 1981), although a conflicting report indicates that placement in the tip of the uterine horn compared to the base (near the bifurcation) is advantageous (Christie et al., 1980). In turn, the consensus is that placement of the embryo in the anterior half of the uterus, minimizes trauma to the endometrium. Pregnancy rates are improved when cattle embryos are transferred to the uterine horn ipsilateral to the functional CL (Tervit et al., 1977; Heyman et al., 1978; Christie et al., 1979; Newcomb and Rowson, 1980; Del Campo et al., 1983). Embryonic

signals involved in the maternal recognition of pregnancy in cattle and sheep (see review: Thatcher et al., 1985) must be able to diffuse in the uterine lumen of the ipsilateral horn to inhibit local endometrial synthesis of $PGF_{2\alpha}$ and subsequent luteolysis in sheep (Moor and Rowson, 1966; Niswender and Dziuk, 1966) and cattle (Del Campo et al., 1977). Although pregnancy and luteal function have been maintained following embryo transfer to the contralateral uterine horn of sheep (Moor and Rowson, 1966) and cattle (Tervit et al., 1977; Heyman et al., 1978; Christie et al., 1979), the efficiency of pregnancy maintenance is reduced markedly (Del Campo et al., 1983). The transfer of more than 1 embryo may increase the total number of sheep offspring produced (Moore and Rowson, 1960), but does not improve pregnancy rates (Moore and Rowson, 1960; Quirke and Hanrahan, 1983). Furthermore, the maintenance of twin pregnancies is independent of whether unilateral or bilateral embryo transfer procedures have been used in cattle (Anderson et al., 1979) and sheep (Torres and Sevellec, 1987), indicating that, under space limiting condition, migration of embryos occurs. Embryo migration between the uterine cornea is possible in cattle, goats and sheep because the bifurcated uterine horns are joined by a common uterine body. Anatomical variations among species, such as a divided cervical canal which eliminates the uterine body (as observed in the oryx [Wildt et al., 1986] and wildebeest [Stover, 1987]), must be considered when contemplating embryo transfer in other ruminant species.

The period when the embryo is *ex situ* (between the donor and recipient) is important in terms of subsequent *in vivo* viability. Initial studies indicated that pre-transfer sheep embryos can survive *in vitro* culture in sheep serum (37°C) for up to 2 h (Averill and Rowson, 1958) and 5.5 h (Hancock and Hovell, 1961) without a significant decline in viability. Later investigations noted that some of these embryos could establish a pregnancy even after 24 to 48 h of culture (Buttle and Hancock, 1964; Tervit and McDonald, 1969). Rowson and co-workers (1969) determined that short-term storage and transfer of cattle embryos in homologous serum adversely affects post-transfer survivability. In contrast, brief (<2.5 h) storage in a chemically-defined tissue culture medium (TCM 199) maintains normal viability that results in high pregnancy rates. *In vitro* culture of sheep embryos in sheep oviductal fluid has been shown to promote development and results in a 55% pregnancy rate after 72 h of culture (Tervit and Rowson, 1974). High pregnancy rates (77%) are also observed in sheep after *in vitro* embryo culture for 24 h in chemically-defined medium (Whitten's medium) supplemented with bovine serum albumin. However, in this study no pregnancies resulted from embryos cultured for 48 h (Peters et al., 1977). In cattle, pre-transfer *in vitro* culture for about 24 h increases early gestational loss of the embryo compared to non-cultured controls (Renard et al., 1980). One

alternative to *in vitro* culture of cattle and sheep embryos has involved *in vivo* culture in the reproductive tract of another species. For example, cow, goat and sheep embryos have been maintained for 3 to 7 days in the ligated rabbit or sheep oviduct, then recovered and used to produce pregnancies after re-transfer into conspecific recipients (cattle: Lawson et al., 1972b; Willadsen et al., 1978; Boland, 1984; goats: Agrawal et al., 1983; sheep: Lawson et al., 1972a; Willadsen et al., 1978). A major problem with this procedure, however, is the difficulty in recovering 100% of the embryos from the "culture" recipient; embryo loss can be substantial (Boland, 1984).

The need for long-term embryo culturing in the transfer process largely has been eliminated by developing embryo freeze-preservation techniques. As discussed in the next section, surplus embryos now can be stored indefinitely until sufficient numbers of suitably synchronized recipient females are available. Conversely, short-term holding of embryos is still required before freshly collected embryos can be transferred. These embryos now can be maintained routinely using a phosphate buffered medium (to effectively reduce pH fluctuations under ambient conditions) supplemented with serum (as a nutrient source). Using this approach, cattle and sheep embryos can be maintained for up to 6 h at room temperature or for 24 h at 5°C (Lindner et al., 1984; Draincourt et al., 1988) without a significant reduction in pregnancy rates.

CRYOBIOLOGY OF EMBRYOS FROM RUMINANT SPECIES

Historical Perspective

The accidental discovery of glycerol's ability to protect spermatozoa during freezing and exposure to subzero temperatures was the foundation for subsequent studies focusing on the cryopreservation of mammalian germ plasm (Polge et al., 1949; Smith and Polge, 1950). A historical review of low temperature biology studies of spermatozoa and ova was reported by Sherman (1964). The rabbit was the first species in which embryo freezing was attempted (Smith, 1952), and was in retrospect, a poor choice because of the low permeability of early-stage embryos to glycerol. This attempt to store rabbit embryos at -79°C was partially successful. Although some embryos apparently developed *in vitro* after thawing, no offspring were produced (Smith, 1952; Ferdows et al., 1958; Smith, 1961). It was also reported that mouse oocytes could be cooled to -10°C, held at -10°C for up to 2 h, fertilized *in vitro* and then yielded live offspring after being transferred to surrogate females (Sherman and Lin, 1958; Sherman, 1963). However, extended periods of exposure to -10°C or brief exposure to lower temperatures proved detrimental to ova viability.

Considerable basic cryobiological research with erythrocytes, tissue culture cells, stem cells and yeast cells laid the foundation for our present conceptual understanding of the thermodynamics of cellular freezing (Lovelock, 1953; Lovelock and Bishop, 1959; Mazur, 1963, 1970; Meryman, 1966; Mazur et al., 1969; Mazur et al., 1972). These data provided the basic information which has permitted rapid progress in freeze-preserving the more complicated multicellular, mammalian embryo. In the early 1970's, the first live mouse offspring were produced following embryo cryopreservation (Whittingham et al., 1972). This study demonstrated that dimethyl sulfoxide (DMSO) was an effective cryoprotectant and that multicellular embryos required slow cooling (0.22 °C/min) and warming (<100°C) to maximize post-thaw viability. Since that milestone in cryobiology, thawed embryos have yielded live young in the cow (Wilmut and Rowson, 1973), rabbit (Whittingham and Adams, 1974), rat (Whittingham, 1975), sheep (Willadsen et al., 1976), goat (Bilton and Moore, 1976), horse (Yamamoto et al., 1982), human (Trounson and Mohr, 1983), eland antelope (Kramer et al., 1983), baboon (Pope et al., 1984), cynomogus monkey (Balmaceda, 1986), marmoset (Summers et al., 1987) and cat (Dresser et al., 1988). Therefore, conventional procedures have been adapted to a variety of species enabling the complete genome of an individual to be freeze-stored in suspended animation in liquid nitrogen (LN_2). To date, only embryos from the pig have been able

to tolerate cooling to subzero or even near-zero temperatures (Polge et al., 1974; Polge and Willadsen, 1978).

The ability to cryopreserve mammalian embryos has revolutionized the domestic livestock embryo transfer industry. Before freezing technology was available, large recipient herds were required to accommodate an unpredictable number of fresh embryos. If an inadequate number of recipients was available, the only recourse was to discard surplus embryos. Freezing technology now allows intensive embryo collection periods to be scheduled which are totally independent of the need to concurrently synchronize estrous cycles in surrogate females. Thawed embryos can be transferred as recipients become available. All evidence suggests that these frozen embryos will remain viable for generations. Mammalian embryos have been freeze-preserved for more than 13 years and produced viable offspring (Wood et al., 1987). The potential influence of background radiation on frozen embryos maintained at -196°C has been shown to be trivial (Whittingham et al., 1977), and a safe storage time for frozen embryos probably exceeds 2000 years (Glenister et al., 1984). Such estimates are particularly important for those charged with sustaining valuable laboratory animal genotypes useful in biomedical research (Whittingham, 1974; Mobraaten, 1986). At least 2 major repositories (at the Jackson Laboratories and the National Institutes of Health) have been developed to maintain thousands of embryos from various mouse strains and genotypes in perpetuity. The potential of embryo cryopreservation is also an asset to the rapidly emerging field of conservation biology and it provides a promising strategy for preserving and managing rare wildlife species (Polge, 1978; Schmidt et al., 1987a). In addition to maintaining genetic diversity and protecting rare animal populations from extinction or catastrophe, frozen embryos can facilitate the movement of germ plasm both intra- and intercontinentally (Bilton and Moore, 1977). The transport of frozen embryos would eliminate the many risks and considerable expenses of shipping live and often dangerous and highly stress-susceptible wild animals. Therefore, the availability of frozen embryo banks used interactively with sperm banks for both captive and free-living animal populations could play a major role in wildlife conservation.

Fundamental Aspects of Embryo Cryobiology

Cryobiology is a science which integrates a variety of disciplines to explain changing cellular events under low temperature conditions. Of pivotal importance to cell survival is the physiochemical relationship of heat and water transport between intra- and extracellular environments. One important consideration is related to the phase changes between the liquid and solid states when aqueous solutions (e.g., physiological saline,

0.9% NaCl) are cooled to low temperatures (Rasmussen and McKenzie, 1968; Cocks and Bower, 1974). At this solution's freezing point (-0.5°C), ice crystal formation is induced and, as water is replaced by ice, the sodium chloride (NaCl) becomes concentrated into a small volume of liquid solution. Continuation of cooling results in additional ice formation and NaCl concentration in the unfrozen liquid gradually increases until the temperature reaches the eutectic point (-21.1°C), the upper limit of salt solubility. Cooling below the eutectic point leads to the solidification of the whole solution as crystalline salt and ice. The addition of a cryoprotective agent such as glycerol or DMSO reduces both the eutectic and freezing points, depending on the solution concentration (Rall et al., 1983). The eutectic points of various salt and cryoprotective solutions have been summarized by Lehn-Jensen (1986). The ability of cryoprotectants to alter the phase change behavior is related to the colligative properties of aqueous solutions.

Because mammalian embryos are composed of approximately 80% water (Leibo, 1986), the fate of intracellular water at subzero temperatures is a primary concern to blastomere survival. The kinetics of cellular water loss during cooling at subzero temperatures is a function of temperature, cooling rate, cell membrane permeability and the cellular surface-to-volume ratio (Mazur, 1963). The relationship of these thermodynamic factors to the freezing and thawing of mouse ova and bovine embryos has been examined in detail (Leibo et al., 1978; Mazur, 1980; Schneider and Mazur,1984). An improved understanding of the physiochemical processes of mouse ova (Leibo et al., 1978), mouse embryo (Rall et al., 1980, 1984; Rall, 1981; Rall and Polge, 1984) and bovine embryo (Lehn-Jensen and Rall, 1983) cryopreservation has been achieved by directly observing physical changes in the embryo and the extracelluar solution using a low temperature light microscope (cryomicroscopy). During freezing, water in the extracellular medium is removed as ice while the cell cytoplasm is simultaneously supercooled. The result is a chemical potential difference (Mazur, 1963) between the concentrated extracellular solution and the dilute intracellular solution which results in the efflux of a transient osmotic pressure gradient (Leibo et al., 1978). Generally speaking, a cell suspension must be cooled slowly enough to allow the cells to dehydrate and eliminate osmotic pressure differences between the intra- and extracellular solution (Lovelock, 1953; Mazur, 1970).

The achievement of sufficient dehydration to allow freezing without cell lysis is closely related to the cooling rate and cryoprotectant effectiveness. The concept of defining an optimum cooling rate for cells is based on the theories that: 1) if the cooling rate is so slow as to allow a constant equilibrium to exist, then the extended exposure to increased solute concentrations (i.e., solution effects and osmotic stress) would result in injury);but 2) if cooling is too rapid, then adequate dehydration is prevented and intracellular

crystalization during cooling and is recrystallized during warming may be lead to injury (Lovelock, 1953; Mazur, 1963, 1970; Leibo et al., 1974). Permeating agents stabilize membrane phospholipids and integral protein macromolecules through the strengthening of hydrophobic forces (Meryman et al., 1977) and act as a solvent for solutes and electrolytes (Dowell et al., 1962). The temperature at which the spontaneous aggregation of water molecules initiates ice nuclei formation (i.e., homogeneous nucleation) in saline alone normally is -10 to -15°C. Adding a 1.0 to 2.0 M glycerol or DMSO solution reduces the onset of ice formation to -38 to -44°C (Mazur, 1970; Leibo et al., 1978; Rall et al., 1983). Furthermore, cryoprotective agents substantially increase the viscosity of intracellular regions, causing water diffusion to cease and the liquid cytoplasm to form a metastable glass upon further rapid cooling (Mazur, 1963; Rall et al., 1984). If the cryoprotectant concentration is sufficiently high, the crystallization of water molecules in the extracellular medium is inhibited completely, and the solution becomes vitrified (i.e., metastable glass). Cryoprotective agents can reduce freezing injury caused by solution effects or intracellular. Caution must be taken, however, to avoid direct cellular damage by osmotic trauma or cryoprotectant toxicity (Mazur, 1977; Ashwood-Smith, 1985; Fahy, 1986), especially when embryos are exposed to very high concentrations, such as in the vitrification approach.

Non-permeating solutes such as sucrose and high molecular weight polymers (i.e., polyvinyl pyrrolidone [PVP], dextran, albumin) can also exert a cryoprotective function. Sucrose is thought to protect by dehydrating cells before cooling thereby reducing the liklihood of intracellular freezing. The cryoprotective action of polymers is poorly understood but it is thought that these compounds stabilize macromolecular interactions in the plasma membrane, and may provide a repair function after thawing. The polymers may reduce the leakage of cytoplasmic solutes, and prevent subsequent cell lysis caused by mechanical damage to blastomere membranes (Edidin and Petit, 1977; Grill et al., 1980; Williams, 1983).

Mazur's models on the thermodynamics of low temperature biology clearly were instrumental in formulating successful embryo freezing strategies for a variety of mammalian species. Conventional procedures adopted in the 1970's for freezing mouse, rat, rabbit, cow, goat and sheep embryos involved slow cooling at 0.2 to 2.0°C/min to ultra low temperatures of less than -60°C before final storage in LN_2 (Whittingham, 1976; Leibo and Mazur, 1978; Maurer, 1978). It also was determined that slow warming (i.e., thawing at room temperature, ~25°C/min) optimized survival, whereas rapid warming (≥ 300°C/min) was detrimental to embryo integrity because of excessive osmotic rehydration (Leibo et al., 1974; Rall, 1981). During most of this decade, embryos generally were

frozen in 1.0 to 2.0 M cryoprotectant solutions containing either DMSO or glycerol. To reduce potential osmotic trauma to embryos, these early studies involved a stepwise addition and removal of cryoprotectant (3 to 6 steps at 5 to 10 min intervals) before freezing and after thawing, respectively. For the actual freezing process, embryos were first cooled from room temperature to -5 to -8°C, equilibrated briefly (~5 min) and then ice was induced to form induced manually in the extracellular solution, a procedure commonly referred to as "seeding". Seeding prevents excessive supercooling of the embryo's cytoplasm which due to uncontrolled spontaneous nucleation as a suspension is cooled to subzero temperatures can result from the release of a latent heat of fusion which normally occurs as a liquid is transformed into a solid (Whittingham, 1977; Leibo and Mazur, 1978).

During the late 1970's, there was a gradual tendency to simplify embryo freezing procedures by phasing out serial dilution steps and plunging embryos into LN_2 at higher temperatures. The first significant modifications in the slow freezing/slow warming procedure for embryos was introduced by Willadsen (1977), Willadsen et al. (1977, 1978) and Whittingham et al.(1979), practices which have evolved into the current standard for farm livestock species (Leibo, 1986). Overall, this approach involves the use of a "short" freezing program with slow cooling stopped at an intermediate subzero temperature (-25 to -40°C) followed by direct transfer of embryos into LN_2. The rapid freezing technique was complemented by rapid embryo thawing in a 37°C water bath, which was thought to reduce the harmful intracellular recrystallization of ice (Whittingham et al., 1979). During rapid freezing, the remaining liquid of the embryo suspension forms a glass that is unstable and will crystalize during slow thawing. In contrast, rapid warming allows the metastable glass that forms between large ice crystals during cooling to return to a liquid state, avoiding any further crystallization (Rall et al., 1984). The stability of the glass phase, however, is influenced by the type of cryoprotectant used. When slow cooling is continued to low temperatures (<-50°C), cattle and mouse embryos preserved in glycerol survive either rapid (360°C/min) or slow (12°C/min) warming, whereas DMSO-treated embryos fail to survive slow warming (Bilton, 1980; Rall and Polge, 1984). Rall and Polge (1984) have contributed this difference to the ability of glycerol to more efficiently interact with or organize water molecules to form a stable glass-like configuration upon rapid freezing.

Progress and Current Status of Embryo Cryopreservation in Ruminant Species

In the past decade, most advances in the freeze/thawing of cattle and sheep embryos have resulted from the use of different cryoprotectants and simplification of techniques, both of which have improved efficiency and post-thaw embryo survival. The specific cryopreservation factors receiving the most attention have included: 1) cryoprotectant; 2)

embryo freezing container; 3) cryoprotectant dilution procedures; 4) cooling rate; and 5) thawing procedure. Perhaps, the most radical change in approach has involved conventional philosophy on an optimal cryoprotectant. Throughout the 1970's, DMSO was the primary cryoprotective agent of choice for freezing mouse (Whittingham et al., 1972; Wilmut, 1972), rat (Whittingham, 1975), rabbit (Whittingham and Adams, 1974), cow (Wilmut and Rowson, 1973; Willadsen et al., 1977), goat (Bilton and Moore, 1976) and sheep (Willadsen et al., 1976; Willadsen, 1977, Willadsen et al., 1977) embryos. Additionally, DMSO was used extensively in the commercial cattle embryo transfer industry (Lehn-Jensen and Greve, 1978; Trounson et al., 1978b; Willadsen et al., 1978; Massip et al., 1979). Studies with mouse embryos, however, suggested that glycerol might be superior to DMSO (Leibo and Mazur 1974). Even so, comparative testing of glycerol for cryopreserving cow embryos did not occur until 1979 (Bilton and Moore, 1979). It now is apparent that when the rapid cooling/rapid warming embryo strategy is used, glycerol significantly improves post-thaw survival compared to DMSO (cattle: Bouyssou and Chupin, 1982; Lehn-Jensen, 1986; mouse: Schiewe et al., 1987b). The reason appears to be related to glycerol's ability to effectively organize water molecules into a more stable glass phase during rapid cooling/thawing which then reduces damaging ice crystal formations during warming (Rall et al., 1984). More recently, DMSO has been shown to have some toxic effects on embryos, a property not yet associated with glycerol (Ashwood-Smith, 1985; Fahy, 1986). Although neither cryoprotectant produces chromosomal abnormalities, DMSO can possibly alter cell differentiation by causing DNA hypomethylation, fragmentation, cell cycle disturbance and activation of genes (Ashwood-Smith, 1985). In the present decade, glycerol gradually has become the cryoprotectant of choice for the cattle embryo transfer industry (Lehn-Jensen, 1980; Lehn-Jensen et al., 1981; Elsden et al.; 1982; Niemann et al., 1982; Kennedy et al., 1983; Seidel et al., 1983; Shea et al., 1983; Massip and Van der Zwalmen, 1984; Leibo, 1984). However, other permeable cryoprotective agents including ethylene glycol (EG) and propylene glycol (PPG) have also been tested and proven effective in the mouse (EG and PPG: Miyamoto and Ishibashi, 1978, 1983; Miyamoto et al., 1979; PPG: Rall and Polge, 1984; Renard and Babinet, 1984), rabbit (PPG: Renard et al., 1984), sheep (PPG: Schiewe et al., 1985b; EG: Heyman et al., 1987), cow (EG: Elsden et al., 1982; PPG: Renard et al., 1981) and human (PPG: Lassalle et al., 1985).

In the 1970's and early 1980's while optimal techniques for embryo freeze-preservation still were being developed, embryos from various species were frozen routinely in glass ampules (mouse: Whittingham et al., 1972; Kasai et al., 1981; Rall and Polge, 1984; rat: Whittingham, 1974 ; rabbit: Whittingham and Adams, 1974 ; sheep:

Willadsen et al., 1976; Willadsen, 1977). As glycerol became more popular, comparative studies testing a variety of embryo freezing containers suggested that the plastic French straw was more appropriate than the ampule for storing embryos. Straws require less space in LN_2 storage tanks than glass ampules, are less likely to break or shatter (Renard and Babinet, 1984) and can be fitted into an embryo transfer device and used to deposit the embryo directly *in utero*. This step avoids the mishandling or loss of embryos after thawing (Leibo, 1984). The straw container, however, is more sensitive to temperature fluctuations than ampules, primarily because of its low thermal mass. One study reported inferior embryo post-thaw survival results using straws compared to ampules (Massip et al., 1979). However, other studies indicate no difference in post-thaw survival of cattle embryos between the 2 types of containers (straw, 45%; ampule, 38%) using a slow cooling rate to -40°C before direct transfer into LN_2 and subsequent thawing in a 37°C water bath (Lehn-Jensen, 1986). It is possible that differences in the handling of embryos in plastic straws among embryo transfer operations may have accounted the variable results, since they are more temperature sensitive. If precautions are taken to avoid excessive supercooling (especially when using straws) during embryo freezing or recrystalization of metastable glass during thawing, then straw containers can be used effectively (Lehn-Jensen, 1986).

Novel approaches for adding and removing cryoprotectant to and from embryos has had a marked effect on embryo handling efficiency. In 1981, Lehn-Jensen first reported that cattle embryos could tolerate a 2-step addition and 4-step removal of glycerol, compared to previous protocols which usually involved a series of 6 pre-freeze and post-thaw equilibration steps. The laborious dilution of cryoprotectant after embryo thawing essentially was eliminated by the development of the "1-step dilution technique" (Leibo, 1986). This procedure was based on the knowledge that placing a thawed embryo into sucrose (a non-permeating solute) creates an osmotic gradient which removes intracellular water and the cryoprotectant. Once dehydrated, the embryonic cytoplasm can re-equilibrate in fresh isotonic medium containing neither cryoprotectant nor sucrose before being transferred *in utero* or cultured *in vitro* (Leibo, 1986). Combined with the use of straws, Leibo (1983, 1984) developed the 1-step straw technique of embryo cryopreservation as an efficient and practical method for thawing, diluting and transferring cattle embryos without removing the embryos from the container in which they were frozen. In these studies, cattle embryos cryopreserved in 1.5 or 2.0 M glycerol were diluted *in situ* with 1.1 or 1.4 M sucrose solutions, respectively. One-step straws (0.25 ml) were prepared using a series of solution columns and 1 cm air space buffers to separate the cryoprotectant solution containing the embryo(s) from the sucrose column before heat sealing and cooling (Leibo,

1986; Rall and Meyer, 1989). After thawing, the straw was shaken in a downward direction to mix the 2 distinct columns and initiate the removal of cryoprotectant from the embryos. The 1-step straw approach has also been applied to the sheep embryos, and, although an initial study suggested that sucrose dilution reduces post-thaw embryo survival compared to conventional stepwise dilution (Merry et al., 1984), more recent results have conclusively demonstrated its effectiveness (Ware and Boland, 1987). The reason for the poor results reported by Merry et al. (1984) probably were related to a low concentration of sucrose (0.25 M) which may not prevent excessive swelling of the embryos during the initial dilution steps. It has been reported that a 1.0 M sucrose solution is optimal, as opposed to 0.25 or 2.0 M, for the dilution of embryos from 1.4 or 2.0 M glycerol solutions (Ware and Boland, 1987). The post-thaw survival results with the 1.0 M sucrose solution were similar to a conventional stepwise dilution procedure for sheep embryos freeze-preserved in 1.4 M glycerol (75% vs 67%, respectively) and were superior when 2.0 M glycerol was used (80% vs 15%, respectively).

A gradually improved understanding of cryo-injury mechanisms has also been incentive for developing rapid embryo freezing procedures that are time and cost-efficient and can be applied to "field" use. These methods recently have been reviewed (Leibo, 1986) and are based on either (1) the combined use of a permeable cryoprotectant and sucrose to enhance pre-freeze dehydration and reduce possible devitrification reactions or (2) the use of high molar concentrations of permeable cryoprotective solutes which avoids ice formation by creating a metastable vitrified solution. Initially, a rapid freezing method was developed for rabbits (Renard et al., 1984) and cattle (Bui-Xuan-Nguyen et al., 1984) involving a 2-step process in which embryos were exposed to a solution containing a mixture of 1.5 to 2.5 M cryoprotectant and 0.5 to 1.0 M sucrose. This resulted in sufficient dehydration to permit the embryos to be placed directly in a -30°C environment for further dehydration (30 to 240 min) before direct transfer into LN_2. More recent studies have demonstrated that the combined use of higher molar concentrations (3.5 M) of permeable cryoprotectants (glycerol or propylene glycol) plus non-permeable sucrose facilitates direct freezing of mouse and cattle embryos in LN_2 vapor (Massip et al, 1984, 1986, 1987). Even without sucrose in the freezing solution, 2.0 M ethylene glycol, glycerol and propylene glycol solutions have yielded good mouse embryo survival rates following direct LN_2 vapor freezing after seeding at -7°C (Miyamoto, 1986; Miyamoto and Ishibashi, 1986).

The most novel cryopreservation procedure developed to-date is referred to as "vitrification" (Fahy et al., 1984) but it has been successfully applied only to mouse (Rall and Fahy, 1985; Rall et al., 1987), rabbit (Smorag et al., 1989) and cattle (Massip et al.,

1986; Rall, personal communication) embryos. The basic concept involves a brief exposure (to avoid toxicity) of embryos to high molar solutions of cryoprotectants that inhibit ice crystal formation after LN_2 exposure. Initial studies involved the use of a complex, high molarity mixture of cryoprotectants (DMSO, polyethylene glycol (PEG), propylene glycol and acetamide; designated as vitrification solution 1 [VS1]). Eventually, an amorphous, glass-like solid is formed devoid of ice crystals in the aqueous solution (Rall and Fahy, 1985). The purpose of including acetamide was to offset any inherent toxicities caused by the high concentrations of DMSO (Rall, 1987). In the initial procedure, embryos were equilibrated in a stepwise fashion in a 5°C environment (i.e., conditions which slowed permeability and reduced osmotic membrane damage; Rall, 1987). Once mixed in the final 100% vitrification solution, embryos were pipetted into straws and directly cooled in LN_2 vapor for ~3 min before final storage in LN_2. More recent studies determined that high molar solutions containing only propylene glycol (VS2) or glycerol (VS3) as the cryoprotective components could be alternatively used for mouse embryos (Rall, 1987). It was determined that VS3a could be effectively used at room temperature, not requiring the safeguard of a cold room to slow permeability and reduce potential toxicity. Acetamide was removed from VS2 and VS3, because DMSO is no longer an additive (Rall, 1987). The VS2-treated embryos are sensitive to prolonged exposure (> 20 min at 0°C). The increased permeability of propylene glycol compared to glycerol results in the intracellular cryoprotectant concentration reaching a toxic level in a shorter period of time. Further vitrification solution modifications (VS3a, VS4a; Rall, personal communication) included removing PEG and increasing the concentration of bovine serum albumin to 6% (w/v) which appears to enhance the formation of the glass-like state (Rall, 1987) while stabilizing plasma membranes during thawing (Grill et al., 1980). The vitrification procedure has been combined with *in situ* sucrose straw dilution (Leibo, 1983) to rapidly remove the highly concentrated intracellular solutes during thawing, thus improving embryo survival rates further (Rall, personal communication). Vitrification is a simple, low-cost embryo freezing technique which eliminates the need for an expensive, programmable LN_2 freezing unit. Because this approach requires only the appropriate high molar cryoprotective solutions and LN_2, it has considerable potential for freezing embryos under field conditions.

The type of embryo warming procedure also appears to affect embryo integrity. Interestingly, most of this effect is exerted on the protective covering of the embryo, the zona pellucida. Normally, conventional rapid cooling/rapid warming (37°C water bath) embryo freezing procedures result in zona pellucida damage in 18 to 37% of cattle embryos (Massip et al., 1979; Lehn-Jensen et al., 1981; Renard et al., 1981; Tervit and Elsden,

1981; Lehn-Jensen, 1986). Cryomicroscopic observations indicate that zona damage is caused by fracture planes which can puncture or crack the zona (Lehn-Jensen and Rall, 1983; Rall et al., 1984; Lehn-Jensen, 1986). However, damage to the zona does not necessarily alter embryonic viability, since at the time of fracture plane formation the cellular mass is shrunken and the perivitelline space is widened which buffers the embryo from mechanical injury. Extracellular glasseous solids are known to fracture at less than -110°C, probably because of expansion and contraction events associated with an increased temperature gradient (Rall and Meyer, 1989). It recently has been determined that the type of thawing conditions used can minimize warming damage to the zona pellucida (Rall and Meyer, 1989). In thawing straw contained cattle ova, slow warming more efficiently prevents zona damage (0%) than rapid warming (24%) which appears related to an overall reduction in thermally-induced mechanical stress. The increased surface area and thin container walls of plastic straws result in a reduced temperature gradient and fewer fracture planes (Rall and Meyer, 1989). Although zona pellucida damage plays a limited role in post-thaw embryo survival (Lehn-Jensen, 1986), this structure is critically important to barring the transmission of pathogens which attempt to infiltrate the embryo *in utero* or *in vitro* (Singh, 1987). Therefore, the ability to maintain an intact-zona following cryopreservation may prove to be an important consideration to the future worldwide transport of genetically valuable embryos.

Implications of Disease Transmission and Embryo Genotype

Successful embryo cryopreservation of ruminant species, especially cattle, has created an international market for the importation (Thibier and Nibart, 1987) and exportation (Mahon and Rawle, 1987) of germ plasm, thereby eliminating the need to transport live animals. One of the most important areas of applied embryo research in the 1980's has been the potential interaction of the embryo with a variety of pathogens. The international transport of embryos has mandated the need to adopt regulatory policies for avoiding disease transmission (Atwell, 1987; Acree and Beal, 1988). Valid concerns that an infectious disease might be transmitted between the donor animal's embryos and the healthy recipient female have resulted in a publication detailing proper embryo handling procedures (International Embryo Transfer Society [IETS] Manual, 1987). To examine the potential of this problem, different fungal, bacterial and viral pathogens have been used to contaminate embryos *in vitro* or embryos have been collected from infected or seropositive donor females. These embryos were then studied via *in vitro* culture or transferred to uninfected recipients. The infectivity of cattle, sheep, goat and pig embryos varies by pathogen and species, as recently reviewed by Singh (1987). Disease transmission via

embryo transfer occurs potentially by: 1) solution; 2) attachment to the zona pellucida; or 3) incorporation within the embryo. A standardized ten-step embryo washing procedure has been recommended to remove possible pathogens present in the embryo flush medium or attached to the external surface of the zona pellucida (IETS Manual, 1987). Fungal and bacterial agents, being large microorganisms, are unlikely to penetrate the zona due to size limitations (Eaglesome et al., 1980) and, therefore, can be eliminated by repeated washing.

Viral pathogens represent the primary focus of embryo disease transmission research, because some small viruses are able to readily infect the embryonic mass (Singh, 1987). Two bovine viruses (infectious bovine rhinotracheitis virus, IBRV, Singh et al., 1982; vesicular stomatitis virus, VSV, Singh and Thomas, 1987) have proven resistant to normal embryo washing procedures, but both are eliminated effectively by exposure (60 to 90 sec) to a 0.25% trypsin solution (pH 7.6 to 7.8) without altering zona integrity. Trypsin treatment, however, does not effectively remove African swine fever virus (ASFV, Singh et al., 1984) and swine vesicular disease virus (SVDV, Singh, 1987) from porcine embryos, which also appear permeable to a variety of viruses (porcine parvovirus, Wrathall and Mengeling, 1979; pseudorabies virus, Bolin et al., 1981; foot-and-mouth disease virus, Singh et al., 1986; hog cholera virus, Singh, 1987; vesicular stomatitis virus, Singh and Thomas, 1987). Although porcine embryos are susceptible to infection under *in vitro* conditions, in the presence of exceedingly high pathogen concentrations the same viruses do not appear to bind to porcine embryos in infected donors (Singh, 1987). Furthermore, embryos collected from seropositive or infected donor cattle (Singh, 1987), sheep (Singh, 1987) and goats (Chemineau et al., 1986; Wolfe et al., 1987) have failed to transmit disease to recipient females or the resulting offspring. Conversely, if zona-free cattle embryos are infected *in vitro* with bluetongue virus (Bowen et al., 1982) or IBRV (Bowen et al., 1985) then embryonic death occurs. However, overall it is important to remember that the embryo resides in an infected donor for only a brief time. Existing data appear to suggest that the possibility of disease transmission is much greater with frozen semen or live animals than with embryo transfer. If the embryo contains an intact zona and is properly washed after collection, it is very likely that disease transmission will be a remote possibility. The zoological community is optimistic that regulations adopted for domestic species will be applicable to related nondomestic species, although more stringent controls may be necessary because of potential species differences. For example, it may be appropriate for import-export regulations to ensure that cryopreserved embryos have a structurally intact zona pellucida after thawing (i.e., without cracks) as an additional safeguard.

As embryo cryopreservation is developed and applied to new mammalian species, special technical modifications will no doubt be necesssary because of species differences in embryo size, permeability characteristics and macromolecular cytoplasmic content (Whittingham, 1977). A comparative cryobiology strategy will likely be needed which will involve cross species research efforts to identify specific cryosensitives like the hypersensitivity of pig embryos to freezing (Polge and Willadsen, 1978) or the inability of early stage (≤8-cell) cattle embryos (Trounson et al., 1976) to survive storage at subzero temperatures. Even within a species (e.g., *Mus musculus domesticus*, laboratory mice) there are reports of genotypic differences that influence embryo survival following cryopreservation (Schmidt et al., 1985, 1987b). Some strains of mice have been more effectively cryopreserved in a glycerol solution than in DMSO, whereas no overt differences are evident in other strains tested (Schmidt et al., 1987b). Perhaps genetically controlled differences in the permeability of the embryos to cryoprotectants result in inadequate permeation before freezing or decrease the efflux of cryoprotectant during dilution. If blastomere production of integral proteins has changed in some mouse genotypes, the membrane permeability to DMSO may be increased making the embryo more susceptible to potential toxicity. Such observations are relevant to the potential use of embryo cryopreservation techniques on nondomestic species. It appears that, not only do potential species differences have to be considered but also that intraspecies genetic variability may have a significant impact on the ability of embryos to survive freezing stress.

CHAPTER I

INFLUENCE OF COMPARATIVE GONADOTROPIN TREATMENT IN SHEEP ON MULTIPLE OVULATIONS, EMBRYO PRODUCTION AND THE OCCURRENCE OF PREMATURE LUTEAL REGRESSION

INTRODUCTION

The success of ovulation induction and embryo transfer depends on consistently producing and recovering multiple, transferable quality embryos from gonadotropin-treated donors. Pituitary-extracted follicle stimulating hormone (FSH-P) or pregnant mares' serum gonadotropin (PMSG) usually are used to "superovulate" sheep (see review: Smith, 1988). Individual ovulatory responses are often quite variable with the use of either gonadotropin. In comparison, FSH-P generally produces the more consistent and acceptable luteal response. Because its long-acting biological effects continually recruit antral follicles throughout the periovulatory interval (Armstrong et al, 1983b), the use of PMSG often results in large numbers of unovulated follicles (Elsden et al., 1978; Critser et al., 1980; Armstrong et al., 1982a; Armstrong and Evans, 1983; Monniaux et al., 1984; Yadav et al., 1986b). These ancillary follicles secrete supplementary hormones (predominantly estrogens) which can alter the endogenous endocrine milieu, thereby disrupting estrous behavior, gamete and sperm transport and early pre-implantation embryo development (Armstrong et al., 1982a; Jensen et al., 1982; Evans and Armstrong; 1984b; Hawk et al., 1987; Hawk, 1988). Concurrently used estrous synchronization treatments can also cause abnormal sperm transport through the cervix in sheep, and thereby reduce fertilization rates (Hawk and Cooper, 1977; Hawk et al., 1981). Each of these pertubations ultimately can affect the recovery of transferable quality embryos.

Inseminating spermatozoa via laparotomy (Trounson and Moore, 1974) or by a laparoscopic approach (Killeen and Caffery, 1982; Armstrong and Evans, 1984; Walker et al., 1985) has successfully circumvented the cervical sperm transport problem (Hawk et al., 1987). Some investigators have tested alternative gonadotropin preparations to improve ovarian response consistency. Horse anterior pituitary (HAP) extract has been found to produce a comparable response to FSH-P treatment in sheep (Moore and Shelton, 1964; Moore, 1970; Boland et al., 1983), but the limited commercial availability of this

preparation restricts practical application. Other gonadotropins have not been tested in sheep, however, a hormone extracted from the urine of menopausal women (i.e., human menopausal gonadotropin, hMG) is used routinely in human fertility programs (Ferraretti et al., 1983; Laufer et al., 1983). This product also induces multiple ovulations in cattle (Critser et al., 1982; Lauria et al., 1982; Alcivar et al., 1984; Murphy et al., 1984; McGowan et al., 1985).

The overall aim of the present study was to examine the influence of estrous synchronization and 3 different gonadotropin treatments on ovarian activity and embryo production, quality and viability in sheep. Specifically, studies were conducted to determine the ovulatory response of sheep to hMG versus FSH-P or PMSG. Treatment effectiveness was determined by analyzing endocrine profiles, ovarian response and embryo production including the assessment of fertilization rate, and embryo quality and biological competence. Because we also observed a high incidence of premature luteal regression, a secondary objective was to determine whether the method of estrous synchronization used would affect luteal function. Lastly, this study had the practical objective of developing an atraumatic laparoscopic procedure for the *in utero* deposition of embryos and semen. Pregnancy rates following transabdominal, laparoscopic embryo transfer were compared to a conventional laparotomy procedure.

MATERIALS AND METHODS

GENERAL METHODS

Animals and facilities. Western crossbred, multiparous white-face ewes, Suffock and Dorset breeding rams and a vasectomized Dorset teaser ram were housed in outdoor, partially sheltered pens (6 m X 25 m) at the National Institutes of Health Animal Center, Poolesville, MD. Ewes between the ages of 2 to 5 years were used as embryo donors and recipients. All sheep were fed a hay-pelleted ration and alfalfa roughage twice daily (0700 h, 1500 h) and provided free access to water. Indoor box stalls (2.5 m X 2.5 m) were used for isolating animals as needed during the course of the study. All surgical procedures were conducted in a fully equipped, on-site, large-animal surgery room.

Semen collection and processing. Semen was recovered by electroejaculation from unanesthetized, restrained rams using a 60-Hz sine wave, AC electrostimulator (P-T Electronics, Boring, OR) connected to a rectal probe (diameter, 2.5 cm; length, 20 cm) with 3 stainless steel electrodes (Howard et al., 1981). To obtain an ejaculate, at least 10

stimulations at 3 V were given with a 3-sec-on/3-sec-off electrical pattern. An additional 10 stimulations with a 1 V increase were used until an ejaculate was collected or until a total of 30 stimulations were administered. If additional semen was needed, the ram was given a 5 min rest before the next series of stimuli (beginning at 4 V). An aliquot of semen was examined immediately with a light microscope (100 X) and 4 random viewing fields for a subjective estimate of spermatozoal motility (0 to 100%) and progressive status (0 to 5: 0 = no movement or forward progression to 5 = rapid, linear forward progression; Pontbriand et al., 1989). Ejaculates with at least a 65% motility and 3.5 progressive status ratings were considered acceptable for artificial insemination (AI). The sperm concentration/ml was calculated by a standard hemacytometer method (Wildt et al., 1983). The ejaculate was placed in a sterile conical test tube and centrifuged (5 min, 1000 X g). The supernatant was discarded and the sperm pellet diluted in sterile, physiological saline to provide 500 X 10^6 motile sperm/ml. The semen sample was maintained at room temperature (21°C) until AI.

Anesthesia and surgical preparation. Feed and water were withheld for 24 h from ewes scheduled for anesthesia and surgery. Atropine sulfate (Med-Tech, Elwood, KS; 0.22 mg/kg, intramuscular [i.m.]) was given as a pre-anesthetic to reduce salivation and stabilize respiration. For each ewe, anesthesia was induced initially with xylazine (Rompun®, Mobay Corp., Shawnee, KS; 0.22 mg/kg, i.m.) followed 5 min later with ketamine hydrochloride (Vetalar®, Parke-Davis, Detroit, MI; 11.0 mg/kg, i.m.). The xylazine-ketamine combination produced a surgical plane of anesthesia which could be maintained with 1 to 2% halothane administered via endotracheal intubation. The abdomen was clipped free of wool and surgically prepared with 3 serial betadine and alcohol scrubs. The ewe was secured in dorsal recumbency on a mechanical surgical table which was positioned at a 45° or 30° angle (head-down) at the onset of either a laparoscopic or laparotomy examination, respectively. Following the surgical procedure, each ewe was returned to an indoor box stall, the intubation tube removed and each animal monitored carefully for recovery. Immediately post-surgery, each ewe was given prophylatic antibiotic (1,500,000 units/day, s.c., Dual-Pen®, TechAmerica, Kansas City, MO) daily for 3 days.

Laparoscopy. For general laparoscopy, a pneumoperitoneum was produced by inserting a 120-mm long Verres needle intra-abdominally in the lower right abdominal quadrant midway between the flank and umbilicus. The needle was attached to an automatic insufflator (Richard Wolf Medical Instruments Corp., Rosemont, IL) by a

flexible gas hose, and approximately 2 l of 100% CO_2 was introduced into the abdominal cavity. A small skin incision (1 cm in length) was made on the midline between the mammary gland and umbilicus, and a 10-mm diameter trocar-cannula was inserted through the peritoneum. The trocar was removed and replaced with a 10-mm, 180° laparoscope (Richard Wolf Medical Instruments Corp.) attached to a high intensity light source via a flexible fiber-optic cable. The Verres needle was used to manipulate the uterine horns, so that the entire surface of each ovary could be examined for the presence of preovulatory follicles and/or corpora lutea (CL). At the conclusion of laparoscopy, all intraabdominally-inserted instruments were removed and the pneumoperitoneum evacuated by hand-pressure. The peritoneal puncture site was sutured with a single, interrupted, 1 gut, absorbable suture and the skin with 0 Dexon, non-absorbable suture using a single interrupted, horizontal mattress knot pattern.

Embryo collection. A sterile mid-ventral laparotomy procedure was used to exteriorize the reproductive tract to allow for examining the ovaries and manipulating the uterus. A 6 cm midline incision was made through the skin and subcutaneous layers immediately distal to the mammary gland. Care was taken to avoid large mid-ventral mammary veins, and an electrical cautery unit was used to coagulate small ruptured vessels and to incise extraneous fat layers. A small puncture was made in the peritoneum, and scissors were used to cut the peritoneal layer along the linea alba. The reproductive tract was grasped, exteriorized and examined for the number, size and general appearance of ovarian follicles and CL. A 10 to 14 French gauge (FG) Foley catheter was inserted through a blunt perforation made into the uterine lumen near the bifurcation of each horn. The balloon cuff of the catheter was inflated with 4 to 7 ml sterile saline to ensure that the flush medium flowed only into the horn lumen and not distally into the uterine body. Hand-pressure was used to occlude the utero-tubal junction to prevent retrograde flow of the flush medium into the oviduct. A total of 40 to 50 ml of modified phosphate buffered saline medium (PB1: Whittingham, 1974; Appendix Table 20) containing 2% heat-inactivated fetal calf serum (FCS, Gibco Laboratories, Grand Island, NY) were flushed through each uterine horn. Using an interchanging syringe (60 ml feeding syringe, Monoject Co., St. Louis, MO) technique, 5 to 10 ml of medium were infused per flush. The infusion syringe was replaced by a collection syringe which recovered the flush medium while the uterine horn was massaged gently. The entire sequence was repeated 5 to 8 times per uterine horn. At the end of the procedure, the catheter was removed and the uterine puncture site sutured with 000 gut, absorbable suture using a Cushing knot pattern. Syringes containing flush medium were decanted immediately into 150 x 15 mm grided

Petri dishes (Quebec Dishes, Veterinary Concepts Inc, Spring Valley,WI) to facilitate embryo searching. At the conclusion of the laparotomy, the peritoneum was sutured with 1 gut, absorbable suture using a simple interrupted knot pattern, the subcutaneous layer with 0 gut using a continuous, subcuticular knot pattern and the skin with 0 Dexon, non-absorbable suture using an interrupted, horizontal mattress knot pattern.

Embryo handling and evaluation. Petri dishes containing flush medium were searched by stereomicroscopy (Wild M-5, Bunton Instruments Co., Rockville, MD) for the presence of embryos and unfertilized ova. Embryos were transferred into 3 ml of holding medium (PB1 medium containing 20% FCS) and maintained at 21°C in a room air atmosphere until subsequent morphological evaluation, quality grading and transfer or cryopreservation. Morphological assessments were performed at 200 to 400X magnification. Unfertilized ova were recognized as an unicellular mass with a smooth vitelline membrane and uniform, granulated cytoplasm, possibly fragmented due to deterioration. Embryos were identified as having multiple blastomeres. The developmental stage of each embryo was recorded as being 2- to 16-cells, morula, late (compact) morula, early blastocyst, blastocyst, expanded blastocyst or hatched blastocyst. An embryo quality grade (QG) of 1, 2, 3 or 4 was assigned to embryos of excellent, good, fair or poor/degenerate quality, respectively (Lindner and Wright, 1983; Schiewe et al., 1987b). The quality ratings were based on the following appearances: QG 1 = normal embryo, spherical shape, symmetrical; QG 2 = embryo with some imperfections such as extruded blastomeres or slightly asymmetrical shape; QG 3 = embryo experiencing partial degeneration or other irregularities including extruded blastomeres or vesiculations; and QG 4 = abnormal embryo with severe blastomere degeneration or lysis.

Radioimmunoassays. Collected sera were assessed for progesterone and estradiol-17β concentration using commercially available ^{125}I-double-antibody radioimmunoassay (RIA) kits (Radioassays Systems Inc., Carson, CA) as previously detailed (Goodrowe et al., 1988). Precipitating solutions containing the first and second antibodies (antisera against rabbit 11α-hydroxyprogesterone-11α-hemisuccinate-human serum albumin and goat anti-rabbit gamma globulin, respectively) were used to detect bound and free unconjugated forms of progesterone. Cross-reactivities to the first antibody were: progesterone, 100%; 20α-dihydroprogesterone, 6.25%; deoxycorticosterone, 3.20%; corticosterone, 0.42%; 17α-hydroxyprogesterone, 0.15%; pregnenolone, 0.06%; androstendione, 0.04%; testosterone, 0.03%; 11-deoxycortisone, pregnenolone sulfate, cholesterol, dihydroepiandrosterone, etiocholanolone, estradiol-17β, estrone, estriol,

estradiol-17α, androsterone, cortisol and aldosterone, <0.01%. Duplicate serum samples (100 µl aliquots) were incubated (37°C) in radiolabelled progesterone and first antibody for 60 min. The second antibody was added, the tubes were shaken and the samples centrifuged for 20 min (2500 X g, 4°C). After decanting the supernatant, the radioactivity in the pellets was measured by gamma spectrometry. The minimum detectable progesterone concentration in this assay was 0.1 ng/ml based on the ED95 level. The standard curve consisted of 0.2 to 40.0 ng/ml aliquots of progesterone. In reference to the standard curve, the amount of progesterone needed to cause 50% inhibition (ED50) was 4.2 ng/ml. Assays were validated for the sheep by confirming parallelism of inhibition curves (Appendix Fig. 18) and performing mass recovery analysis (Y = -0.189 + 1.15X; r = 0.99).

Precipitating solutions containing the first and second antibodies for the estradiol-17β assay (antisera against rabbit 6-keto-estradiol-17β-6-oxime-BSA and goat anti-rabbit gamma globulin, respectively) were used to detect bound and free unconjugated forms of estradiol-17β. The cross-reactivities of the first antibody were: estradiol-17β, 100%; estrone, 20.0%; estriol, 1.51%; estradiol-17α, 0.68%; ethinyl estradiol, testosterone, 5α-dihydrotestosterone, cholesterol, pregnenolone, 17α-hydroxypregnenolone, progesterone, 17α-hydroxyprogesterone, 20α-hydroxyprogesterone, 11-deoxycortisol, cortisol, aldosterone, androstendione, dihydroepiandrosterone and dihydroepiandrosterone sulfate, <0.01%. Serum (100 µl aliquot) was incubated (37°C) in the first antibody and radiolabelled estradiol-17β for 90 min. After adding the precipitating solution, the samples were mixed and centrifuged (2500 X g, 20 min, 4°C), the supernatant discarded and the radioactivity in the pellets quantified by gamma spectrometry. The minimum detectable estradiol-17β concentration was 3.5 pg/ml based on the ED95 level. The standard curve consisted of estradiol-17β at concentrations of 10 to 3000 pg/ml and all samples were measured in duplicate. The amount of estradiol-17β needed to cause 50% inhibition (ED50) was 94.3 pg/ml. Assay validation for the domestic sheep was performed by parallelism of inhibition curves (Appendix Fig. 19) and mass recovery analysis (Y = -8.186 + 1.018X; r = 0.99; P<0.001).

Study 1

Estrous synchronization and induction of ovulation. Exogenous gonadotropins were compared after estrous synchronization of the reproductive cycle of all ewes. This ensured that all ewes were capable of ovulating and facilitated coordinating treatment and surgery schedules. Laparoscopy was performed on each prospective donor ewe 24 h before beginning the estrous synchronization treatment. Ovarian activity was recorded, and

ewes with inactive ovaries (characterized by a complete lack of follicular or luteal tissue) were excluded from the study. Estrous synchronization was accomplished by the intra-vaginal placement of a progestogen (6α-methyl-17α-acetoxyprogesterone, MAP)-impregnated pessary into each ewe for a 12 day period. Each MAP-pessary consisted of a 22 mm diameter polyethylene sponge (Identi-Plugs, JAECE Co., North Tongwanda, NY) attached to a 20 cm long nylon monofilament line (2 ga Vetafil). Each sponge was soaked in a 5 ml solution of ethyl alcohol (95% absolute) and 60 mg MAP (Depo-Provera®, 100 mg/ml, Upjohn Co., Kalamazoo, MI) until fully absorbed. The pessary was allowed to dry while hanging in a ventilation hood for 2 to 3 h and then was refrigerated until used. A 400 IU injection (i.m.) of PMSG (Gestyl®, Diosynth, Chicago, IL) was given 24 h before MAP-pessary withdrawal to enhance ovarian follicular response. A vasectomized, teaser ram with brisket marker paint was placed with the ewes twice daily (early morning, late afternoon) for 2 h. Estrus was determined by observation of a standing mount or by evidence of painted wool over the ewe's rump. Because the estrus synchronizing compound MAP is a progestogen, we considered the possibility that exogenously administered MAP might interfere with the accurate measure of endogenously produced progesterone. However, because circulating progesterone levels began declining consistently in all but 4 of 20 ewes within 6 days after pessary insertion we concluded that this cross-contamination was minimal.

Eight days after the onset of estrus each donor was subjected to a second laparoscopy to confirm the presence of 1 or more CL. One day later, ewes began receiving 1 of 3 exogenous gonadotropin treatments (n = 10 donors/ treatment): 1) a single i.m. injection of 1200 IU PMSG (Gestyl®, Diosynth); 2) a multiple injection (i.m.), decreasing dosage regimen of FSH-P (Schering Veterinary Supplies, Kenilworth, NJ; 5, 4, 4, 3, 3 and 2 mg, respectively; 21 mg total dosage) administered at 12 h intervals; or 3) a multiple injection (i.m.) treatment of hMG (Pergonal®, Serono Labarotories, Randolph, MA; 2, 2, 1, 1, 1, 1 and 1 ampule, respectively; 150 IU/ampule, 1350 IU total dosage) at 12 h intervals. The same lot of each gonadotropin was used throughout the study. Five additional ewes served as controls by receiving multiple saline injections (1 ml, 0.9% NaCl physiological saline; i.m.) at 12 h intervals for 3 days beginning on Day 9. To induce luteal regression, each donor was injected (i.m.) with 15 mg of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, Lutalyse®, Upjohn Co.) 36 h after the initial gonadotropin injection. Twenty-four h later, each ewe was paired for 24 h with 1 of 2 fertile rams for natural mating.

Blood sampling and endocrine analysis. Beginning 2 days before the first laparoscopy, blood samples (10 ml) were collected by venipuncture on a daily basis

(between 0700 and 1000 h) through the day of embryo collection (total, 35 days; 5 randomly selected ewes/gonadotropin treatment group). After a 1 to 2 h clotting period at 4°C, each blood sample was centrifuged at 3000 x g for 10 min and the serum decanted and stored (-20°C) until assayed for progesterone and estradiol-17β.

Artificial insemination, embryo collection and embryo transfer. Each ewe was artificially inseminated with fresh semen 48 to 52 h after the $PGF_{2\alpha}$ injection. Insemination was performed using a laparoscopic procedure developed specifically for intrauterine AI and embryo transfer (ET; Schiewe et al., 1984). A general laparoscopic examination of the ovaries was performed to determine whether ovulation had occurred and to count the number of preovulatory follicles and corpora hemorrhagica (CH). The ovary with the most prominent follicular or luteal development was designated as ipsilateral. A 6 mm diameter, accessory trocar-cannula was inserted through the caudal quadrant of the abdominal wall adjacent to the ipsilateral uterine horn. The accessory trocar was replaced with a forceps device which was used to grasp and secure the uterine bifurcation (for AI) or anterior-third of the uterine horn (for ET). The uterus was elevated vertically to the abdominal wall and a 16 ga, 5.5 cm long Teflon catheter placement unit (Cathlon IV®, Jelco Labarotories, Rariton, NJ) introduced through the abdominal wall at the site directly ventral to the uterine horn. The catheter was inserted gently into the uterine horn with the angle of direction being toward the uterine body for inseminations or the utero-tubal junction for ET. After penetrating the uterine lumen, the stylette needle was withdrawn slightly within the cannula and the latter advanced further. The catheter was considered intraluminal if it passed approximately 1 cm freely without resistance. The stylette then was withdrawn completely and replaced with a 3.5 FG tom-cat catheter (11.5 cm long, Sovereign®, Monoject Co.) attached to a 1 ml syringe containing 0.2 ml of semen (100 x 10^6 motile spermatozoa) for AI or an embryo for ET. Semen was deposited laparoscopically into the uterus of each donor ewe within 30 min to 3 h after ejaculate processing.

Ovarian responsiveness to gonadotropin treatment was confirmed and surgical embryo recovery performed 5 days after AI (Day 6). Control ewes were subjected to laparoscopy to document ovarian status but the uterine horns were not flushed, and these females were permitted to maintain any pregnancy. All recovered embryos were morphologically evaluated and assigned a QG. For individual ewes, embryo recovery rate was calculated by dividing the total number of embryos plus unfertilized ova by the total number of CL present (x 100). The fertilization rate was calculated as the number of embryos recovered divided by the total number of ova plus embryos collected (x 100). A

transferable embryo rate was calculated by dividing the total number of QG 1, 2 and 3 embryos recovered by the total number of embryos recovered (X 100; Schiewe et al., 1987a).

A portion of the QG 1 and 2 embryos was transferred to recipient females following estrus which was artificially-induced by a 12 day MAP-pessary/400 IU PMSG regimen. Estrus was determined by exposure of the recipient ewes to a teaser ram; observations of standing mounts and painted rump wool were considered indicative of estrus. Twelve ewes were subjected to ET either on Day 5 or 6 after induced-estrus (Day 0) using a laparoscopic (n = 6) or a laparotomy approach (n = 6). Two late-morula to blastocyst stage embryos in PB1 medium were placed in a tom-cat catheter and deposited into the anterior one-third of the uterine lumen ipsilateral to the ovary containing the most prominent CL. If both ovaries contained CL, then the uterine horn most easily grasped by the accessory forceps received the embryos. To avoid embryo loss and to identify location within the catheter, 1 blank medium-air space buffer (~0.25 cm) was arranged on each side of the medium aliquot (1 cm) containing the embryos. For pregnancy diagnosis, each recipient was subjected to 2 transrectal probe ultrasound (Sheepreg®, Animark, Aurora, CO) examinations at a 1 week interval beginning 4 to 5 weeks after ET. All ewes were permitted to carry pregnancies to term.

Study 2

Estrous synchronization, induction of ovulation and embryo collection. As detailed in Results, a high incidence of premature luteal regression was observed in all the gonadotropin treatment groups in Study 1. For this reason, we explored the possibility that the one common feature of all groups, the $PGF_{2\alpha}$ estrous synchronization regimen, was somehow contributing to luteal dysfunction. The MAP-pessary synchronization, laparoscopy, laparotomy and embryo recovery/evaluation procedures described for Study 1 in General Methods were applied to a second group of ewes which were scheduled to receive PMSG or FSH-P. Because of the expense of hMG, it was not used in this study. Ten donor ewes were subjected to a pre-treatment laparoscopy to evaluate ovarian activity before insertion of MAP-pessaries. Beginning 36 h before pessary removal, each ewe received either PMSG (n = 5) or FSH-P (n = 5) treatment. At estrus onset, all ewes were allowed to mate naturally and were also laparoscopically-inseminated. Ovaries were examined, and embryos were recovered and evaluated 5 to 6 days after AI as described in Study 1.

Statistical analysis

A completely randomized design with an apriori arrangement of treatments was used to assess the influence of different exogenous gonadotropins and estrous synchronization methods on multiple ovulation and embryo production and the effectiveness of different embryo transfer procedures to produce offspring. All multi-treatment data were subjected to analysis of variance (ANOVA) using a general linear model format and a computerized Statistical Analysis System (SAS, 1984). Differences among treatments were determined using Tukey's HSD procedure (Snedecor and Cochran, 1980). Data expressed as a proportion or percentage were evaluated by Chi square analysis (Snedecor and Cochran, 1980). Fluctuations and differences in endocrine profiles over time were evaluated with a 2-way ANOVA repeated measures program. Experiments involving only 2 treatment groups (Study 1, embryo transfer techniques; Study 2, estrous synchronization methods) were analyzed using an independent Student's *t* test (Snedecor and Cochran, 1980).

RESULTS

In Study 1, among control and gonadotropin groups, pretreatment mean follicle and CL numbers were similar (P>0.10; Table 1) at all observation times (including at the pre-MAP pessary and pre-gonadotropin laparoscopy). On the day of laparoscopic AI, all gonadotropin ewes had more (P<0.05) follicles (> 5mm diameter) than controls, and FSH-P- and hMG-treated ewes had more (P<0.05) CH. On the day of post-gonadotropin embryo collection (Day 6), mean CL number was greater (P<0.01) in FSH-P- and hMG-treated ewes than other groups, and PMSG treatment resulted in more (P<0.01) ovulations than saline alone. Ewes treated with hMG had the fewest number of unovulated follicles on Day 6 but the differences were not significant (P=0.08). Animals given PMSG tended to have the most variable follicular response. Compared to hMG-treated counterparts, these ewes produced about 2-fold more (P>0.05) residual follicles on the day of embryo collection.

Individual progesterone profiles during and immediately after the end of MAP-treatment were similar among the treatment groups (Fig. 1). Over time, circulating progesterone concentrations declined to baseline coincident with or before the withdrawal of each pessary. As expected, serum progesterone rose in all animals following the pretreatment PMSG injection, and individual progesterone profiles were similar (Fig. 1). After $PGF_2\alpha$ and comparative PMSG, FSH-P or hMG treatment, there was no apparent

effect on the temporal demise of existing CL; progesterone profiles in all group females (including controls) rapidly returned to nadir within 24 h of $PGF_{2\alpha}$ administration. Following induced ovulation, individual progesterone patterns were much more variable in PMSG-, FSH-P- and hMG-treated ewes compared to controls (Fig. 1). Figure 2 illustrates mean estradiol-17β and progesterone profiles for the various treatment groups standardized to the day of $PGF_{2\alpha}$ injection (Day 0). Detectable increases in estradiol-17β were measured ~48 to 60 h after the first PMSG, FSH-P or hMG injection. The post-gonadotropin estrogen peak in these sheep occurred 12 to 24 h earlier than in controls. Estrous behavior coincided with peak circulating estradiol-17β; gonadotropin-treated ewes generally demonstrated estrus 24 to 36 h after $PGF_{2\alpha}$ injection whereas controls were in estrus ~48 to 60 h after induced luteolysis. Although temporal progesterone patterns did not differ ($P>0.10$) among the gonadotropin treatment groups, each of these profiles differed ($P<0.05$) from the control. The first detectable rise in progesterone in PMSG-, FSH-P- and hMG-treated females occurred ~24 to 48 h earlier than in naturally ovulating ewes (Fig. 2). Temporal progesterone patterns in the gonadotropin-treated sheep were more variable than that of controls (Fig. 1). In the saline group, progesterone concentration among individual ewes on the day of uterine flushing ranged from 0.8 to 1.8 ng/ml compared to 0.2 to 9.2, 0.2 to 10.1 and 0.2 to 8.0 ng/ml for PMSG-, FSH-P- and hMG-treated females, respectively. Some of this variability was attributed to differences in total CL number, but much of the variation resulted from premature luteal regression in certain ewes. Among the females not experiencing this condition, there was a high correlation between the serum progesterone concentration on the day of uterine flushing and the total number of ovarian CL ($r = 0.84$, $P<0.01$).

On the day of embryo collection, the ovaries of 1 of 5, 4 of 10, 3 of 10 and 6 of 10 control, PMSG-, FSH-P and hMG-treated ewes, respectively, contained abnormal-appearing CL. In contrast to normal, prominent CL which always were at least 3 mm in diameter and bright red in appearance, abnormal CL were less than 2 mm in size, pale in coloration and resembled regressing CL normally observed during the late luteal phase. Ewes either produced all normal-appearing or all regressed CL (Fig. 3). Circulating progesterone profiles of ewes experiencing normal luteal function were markedly different (Fig. 4) from those of ewes with abnormal luteal development. By 72 h after the end of induced estrus, the mean progesterone concentration in sheep with abnormal CL was less ($P<0.05$) than that measured in animals with normal-appearing CL. Within 24 h, serum progesterone in ewes undergoing premature luteal regression had returned to nadir. Retrospective analysis of all endocrine and ovarian data did not identify any unequivocal markers indicative that a given ewe would produce either normal or abnormal CL. There

were more (P<0.05) preovulatory follicles on the day of AI and post-gonadotropin laparoscopies in normal compared to abnormal sheep, but numbers of CH/CL were comparable (P>0.10; Table 2). Some ewes in each treatment group initiated ovulation before AI, but this did not appear to be related to premature luteal regression (data not shown).

No embryos were recovered from donors with regressed CL whereas embryos were recovered from each of the females with normal appearing CL (Table 3). Based on CL number in donors with normal CL, 54% (81/149) of ovulated ova were recovered as unfertilized ova or cleaved embryos (Table 3). The fertilization rate was reduced (P<0.05) in PMSG-treated ewes compared to FSH-P- and hMG-treated sheep. However, the percent of transferable quality embryos recovered was not different (P>0.05) among gonadotropin treatments. Likewise, the mean QG of all recovered embryos was high (1.7 ± 0.2) and not influenced (P>0.05) by treatments. On the day of embryo collection, 77% of the embryos were either morula or blastocysts (Fig. 5). Although there was a wide range of embryo development (from 2-cell stage to hatched blastocysts), the stage of development was not related to gonadotropin treatment.

Embryos from both hMG- and FSH-P-treated donors were capable of producing live offspring after embryo transfer (Table 4). The laparoscopic procedure resulted in pregnancy rates comparable to or greater than those performed by laparotomy. Using the laparoscopic method, penetration of the uterine horn with the Teflon catheter caused no apparent tissue trauma, and its withdrawal after embryo deposition resulted in negligible hemorrhage from the puncture site.

Because some ewes from each treatment group, including 1 control ewe, experienced premature luteal regression, we examined the possibility that the $PGF_{2\alpha}$ given coincident with saline or gonadotropin treatment had a residual lytic effect on the newly formed CL. Study 2 results confirmed this possibility because no premature luteal regression was observed in the 10 ewes treated with PMSG or FSH-P following MAP-pessary estrous synchronization. As observed in Study 1, FSH-P-treated sheep had a greater (P<0.05) mean ovulatory response than PMSG-injected ewes (12.0 ± 3.7 versus 3.2 ± 1.2 CL, respectively) and fewer (P<0.05) residual follicles (1.8 ± 0.8 versus 4.2 ± 1.3 respectively). Similar (P>0.10) high embryo recovery rates (PMSG, 79%; FSH-P, 77%), fertilization rates (PMSG, 97%; FSH-P, 90%) and transferable embryo rates (PMSG, 92%; FSH-P, 86%) were observed between the 2 groups.

DISCUSSION

Intravaginal MAP pessaries were highly effective in synchronizing reproductive activity of randomly cycling ewes, which was in agreement with the results of earlier studies (Boland, 1973; Colas, 1975; Gordon, 1975; Haresign, 1978; Armstrong and Evans, 1983; Walker et al., 1986; Torres et al., 1987; Alwan et al., 1988). Likewise, prostaglandin $F_{2\alpha}$ was luteolytic in cyclic ewes as shown by other workers (Hughes et al., 1976; Stacy and Gemmell, 1976; Acritopoulou and Haresign, 1980). The overall demise of existing CL did not appear to be influenced by the type of gonadotropin preparation used simultaneously to stimulate superovulation. The quantitative and temporal progesterone profiles in the natural cycling and/or gonadotropin-treated ewes were similar to those reported recently by others for naturally cycling ewes (Wheaton et al., 1988) and sheep before and after exogenous PMSG or FSH-P treatment (Armstrong et al., 1983b). The ovarian response to hMG was no different from that observed after FSH-P, demonstrating for the first time that serial hMG is effective in stimulating multiple ovulations in sheep. In this context, our observations agree with those from studies involving hMG treatment in cattle (Alcivar et al., 1984; Murphy et al., 1984; McGowan et al., 1985). Both hMG and FSH-P stimulated an average of 10 ovulations per ewe, which is equal to or greater than the response reported by others using FSH-P (Wright et al., 1981; Armstrong and Evans, 1983; Cognie and Torres, 1984; Evans and Armstrong, 1984a; Walker et al., 1986; Torres et al., 1987). In cattle, hMG apparently reduces ovulatory variability among individual animals (Lauria et al., 1982), an observation we failed to detect in sheep. Nonetheless, there was a strong tendency for the ovaries of ewes treated with hMG to have fewer residual, unovulated follicles post-ovulation, as has been observed in cattle (Lauria et al., 1982; Alcivar et al., 1984).

For ewes producing normal-appearing CL, our embryo recovery rates were within the range reported by others (54 to 79%; Armstrong and Evans, 1983; Smith and Murphy, 1984; Zanwar and Deshpande, 1984; Chesne et al., 1987; Torres et al, 1987). With the exception of the findings of Boundy and coworkers (1985), the ovulatory and embryo recovery responses in our PMSG-treated ewes was considerably less than those of others using a similar estrous synchronization and PMSG injection/dosage schedule (Armstrong and Evans, 1983; Walker et al., 1986). In the past, the variability associated with exogenous gonadotropins has been attributed, in part, to potential impurities in the gonadotropin itself (Humphrey et al., 1979; Murphy et al., 1984; Lindsdell et al., 1986b). The comparatively lower ovulatory response after PMSG was intriguing because, at the time of AI, numbers of preovulatory follicles and CH of these ewes tended to similar to that

of FSH-P- and hMG-treated sheep (Table 1). We considered the possibility that this particular PMSG product (or lot) was low in biopotency. In this context, we used a simple mouse bioassay to compare the Diosynth product to another PMSG preparation purchased from Sigma Chemical Company. Immature C57/BL mice (n = 20 females/treatment) were administered 2.5 IU PMSG followed 48 h later with 10 IU human chorionic gonadotropin and then paired with isogenic males. Uterine tubes were flushed 68 h later and the ovaries fixed for histological sectioning and subsequent hematoxylin-eosin staining. There was no difference in the ovulatory or embryo recovery rate between the Diosynth (29.2 ± 3.5 CL and 22.5 ± 2.3 ova/female, respectively) and Sigma PMSG preparations (32.4 ± 4.1 CL and 25.0 ± 2.5 ova/female, respectively). Therefore, the observation of poor ovulatory response in PMSG-treated sheep could not be explained on the basis of an inherently low product biopotency. It is possible that the long-acting characteristics of PMSG downregulated ovulation of accessory antral follicles while a second follicle cohort continued to be recruited. The latter follicles failed to ovulate and, therefore, were evident 5 days after insemination.

Although temporal progesterone profiles were similar, the first detectable rise in PMSG-treated ewes occurred about 12 h later than in the other 2 hormone-treated groups (Fig. 2). This supports the assertion that ovulation was delayed or inhibited completely in some follicles. It also was possible that PMSG had comparatively higher LH activity than the FSH-P or hMG preparations and PMSG may therefore have prematurely stimulated 1 or more dominant "estrogenic" follicles. It is known that PMSG stimulates preantral follicle development while having a negligible effect on large-sized, antral follicles (Moor et al., 1984). The early ovulation of 1 or more Graafian follicles (present at the onset of gonadotropin treatment) may have inhibited rupture of secondary follicles. Alternatively, the accelerated development of a Graafian follicle may have altered the endogenous endocrine milieu so that the final maturation or ovulation of small antral follicles was disrupted. Follicular-secreted inhibin or excessive estradiol-17β are known to interfere with gonadotropin secretion and receptor competency (Mauleon and Marianna, 1977; Baird and McNeilly, 1981) and this could have interrupted steroidogenesis and LH surge responsiveness causing atresia of secondary follicles. Because of the infrequency of our blood sampling, the estradiol-17β could not be used to test these hypotheses.

Perturbations in periovulatory estradiol and progesterone patterns after PMSG treatment have been associated with a reduction in the rate of oocyte maturation and an alteration in cervical/uterine environments which contributed to poor fertilization (Van Rensburg, 1964; Booth et al., 1975; Armstrong et al., 1982b; Jensen et al, 1982; Moor et al., 1984). Because PMSG can activate sheep oocytes to initiate meiosis prematurely

before the LH surge (Moor et al., 1984), it is likely that the reduced fertilization rates observed in our PMSG-treated ewes were related to the ovulation of aged ova (Hawk, 1988). Abnormal sperm transport frequently has been associated with estrous synchronization (Quinlivan and Robinson, 1969; Hawk and Cooper, 1977; Hawk et al., 1981; Pearce and Robinson, 1985; Hawk et al., 1987). However in the present study, this factor was considered trivial for a couple reasons. First, laparoscopic AI was used which has been shown to be effective in circumventing the sperm transport problem in sheep (Armstrong and Evans, 1984). Second, a very high percentage of ova were fertilized (>90%) after the hMG or FSH-P treatments. Regardless of the gonadotropin source, fertilized embryos developed normally in all treatment groups, resulting in the production of as high a high proportion of transferable quality embryos as that reported by others (Chesne et al., 1987; Torres et al, 1987). The *in vivo* developmental competence of embryos recovered from FSH-P and hMG-treated donors was also confirmed. Laparoscopy proved to be a very effective procedure for depositing embryos into the uterine lumen which agrees with other contemporary findings (McKelvey et al., 1985; Walker et al., 1985). Compared to the conventional laparotomy approach, laparoscopy tended to produce higher pregnancy rates while reducing surgical trauma (i.e., intraperitoneal bleeding, adhesion formations) that could potentially impair subsequent fertility.

To our knowledge, this is the first report of premature luteal regression in sheep treated with exogenous gonadotropins during the breeding season. There were some similarities between this early luteolytic condition and the short luteal phases experienced during the transition from anestrus to estrus in goats (Prasad and Bhattacharyya, 1979; Ott et al., 1980; Chemineau, 1983) and sheep (Oldham and Martin, 1978; Oldham and Lindsay, 1980; Knight et al., 1981) after exposure to a breeding male. Anestrous sheep treated with gonadotropin-releasing hormone frequently experience abnormal luteal function (Haresign et al., 1975; McNeilly et al., 1981; McLeod and Haresign, 1984; Hunter et al., 1988). Short luteal phases also are commonly observed in postpartum sheep (Lewis et al., 1981) and cattle (Rutter et al., 1985). Progesterone priming (related to secretions from short-lived CL of initial ovulations) often restores normal luteal function in seasonally anestrous and postpartum ewes (Oldham and Martin, 1978; Lewis et al., 1981). In cycling goats, premature luteal regression consistently occurs after MAP or $PGF_{2\alpha}$ treatment when 1000 IU PMSG is given concurrently (Armstrong et al., 1982a, 1983a, 1983b; Stubbing et al., 1986). Luteal dysfunction in superovulated goats, however, is associated infrequently with FSH-P treatment (Armstrong et al., 1983b). In our sheep study, premature luteal regression occurred regardless of the type of gonadotropin

treatment, but it was clearly associated with $PGF_{2\alpha}$ administration. Synchronizing estrus with MAP eliminated the occurrence of abnormal CL.

Armstrong and coworkers (1983b) theorize that abnormal endocrine events associated with multiple ovulation induction might stimulate endogenous $PGF_{2\alpha}$ release from follicles or the uterus causing early luteolysis. This assertion could not be substantiated in our study since even 1 non-hormonally-treated, control ewe experienced luteal dysfunction. Furthermore, unlike the goat, premature luteal regression in sheep was an all-or-none phenomenon. In the goat, it is not unusual to observe normal and regressed CL co-existing on the same ovary (Camp et al., 1983; Armstrong, personal communication). These abnormal sheep CL became dysfunctional ~48 to 72 h after ovulation based on the rapidly decreasing serum progesterone concentration which declined to baseline within 3 to 4 days. A similar temporal demise occurs in goats (Armstrong et al., 1982a; Stubbing et al., 1986). Both MAP (Armstrong et al., 1982b, 1983a) and $PGF_{2\alpha}$ (Stubbing et al., 1986) treatments are associated with early CL demise in goats. However, pretreating goats with progesterone for 0 to 2 days before PMSG injection prevents luteal dysfunction (Armstrong et al., 1987). It is difficult to explain how $PGF_{2\alpha}$ treatment influences subsequent luteal development, considering that it is rapidly metabolized endogenously within seconds to minutes (Zubay, 1988). Conversely, it is odd that progestogen pretreatment would be advantageous at a time when endogenous progesterone is being secreted by luteal tissue of $PGF_{2\alpha}$-treated animals. It could be that the rapid demise of the CL (and progesterone secretion) following $PGF_{2\alpha}$ results in a short, inadequate interval for follicular maturation. In contrast, when MAP pessaries are used, the removal of the sponge results in a more attenuated decline in circulating progesterone, causing a delay in the onset of estrus and ovulation. This delay may provide additional time for follicular development which may facilitate follicle maturation, perhaps allowing granulosa/theca cells to be more capable of transforming into normal luteal cells. The exact etiology of premature luteal regression remains unknown but this condition appears to result from: 1) inadequate hormonal preparation of the preovulatory follicle; 2) the early release of a luteolysin; and/or 3) a hypersensitivity of the luteal tissue to a luteolysin. A more comprehensive analysis of premature luteal regression in hormone-treated ewes and the influence of estrous synchronization treatment are being addressed in parallel studies (Schiewe et al., 1990b).

Fortunately, the alternative use of progestogens, in association with exogenous gonadotropins, appears to counteract abnormal luteal development. The consistency of producing a large number of transferable embryos was enhanced when short-acting FSH-P or hMG were used to induce a superovulatory response. Although hMG was highly

effective in eliciting multiple ovulations in sheep, its current practical utility is rather limited. During the course of the studies, hMG began to be used extensively as a gonadotropic source for combating human infertility. Because of: 1) the apparent expense of preparation; and 2) its popularity as a product for enhancing human ovarian activity for natural conception, AI or *in vitro* fertilization, hMG presently is not very cost-effective for use with livestock, including sheep. Although hMG is an excellent alterative to FSH-P, its cost-effectiveness probably is impractical for use on a routine basis. Nonetheless, considering potential species differences that may exist in applying embryo techniques to nondomestic wildlife (Schiewe et al., 1990a), the utility of a variety of gonadotropins and releasing hormones warrants testing. In the event that embryo transfer is necessary to enhance propagation of a highly endangered species, gonadotropin costs would be of secondary importance, and hMG might be particularly useful for producing transferable quality embryos.

TABLE 1. Ovarian response of ewes to different gonadotropin treatments[a]

Treatment	No. of ewes	Time of Observation: Pre-MAP pessary CL	Pre-MAP pessary Follicles[b]	Pre-gonadotropin CL	Pre-gonadotropin Follicles[b]	Laparoscopic AI CH	Laparoscopic AI Follicles[b]	Post-gonadotropin CL	Post-gonadotropin Follicles[b]
Control	5	1.2±0.2	1.6±0.4	1.2±0.2	1.2±0.2	0.2±0.1[c]	1.4±0.2[c]	1.4±0.2[c]	2.6±1.2
PMSG/$PGF_{2\alpha}$	10	1.0±0.3	2.6±1.1	1.6±0.2	1.6±0.2	1.0±0.6[cd]	4.9±0.6[d]	3.1±1.1[d]	5.6±3.3
FSH-P/$PGF_{2\alpha}$	10	0.8±0.3	2.6±0.6	1.8±0.2	2.9±1.0	3.1±1.2[d]	6.6±1.1[d]	9.9±1.8[e]	4.2±1.4
hMG/$PGF_{2\alpha}$	10	1.3±0.2	2.3±0.8	1.8±0.2	2.7±0.8	2.3±0.7[d]	7.5±1.8[d]	10.0±1.9[e]	2.2±1.0

[a] Values are expressed as means ± SEM.
[b] Follicles ≥ 5 mm in diameter.
[c,d,e] Mean (± SEM) column values with different superscripts differ ($P<0.01$).

Figure 1. Individual serum progesterone profiles of control and gonadotropin-treated ewes (*-denotes day[s] of hormone injection) following progestagen-pessary (MAP) and $PGF_{2\alpha}$ (PG) estrous synchronization treatments. A time line of scheduled treatments is provided, indicating when artificial insemination (AI), and embryo collections (EC) were performed.

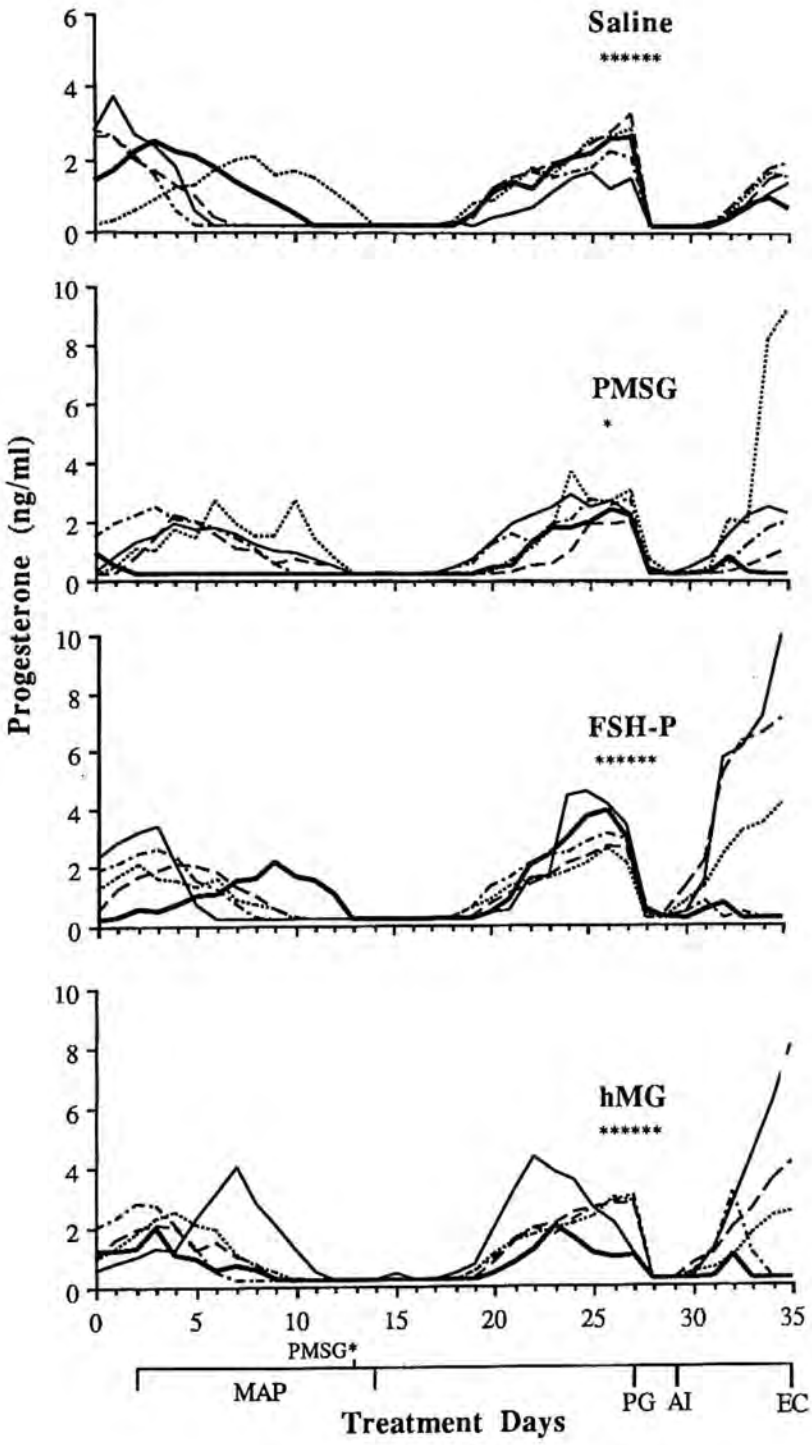


Figure 2. Mean (±SEM) temporal serum estradiol-17β and progesterone profiles of control and gonadotropin-treated ewes. Data are standardized to the day of $PGF_{2\alpha}$ treatment (Day 0). Day 8 represents the day of uterine flushing. PMSG was administered on Day -1.5, FSH-P on Days -1.5 to +1.5 and hMG on Days -1.5 to +1.5.

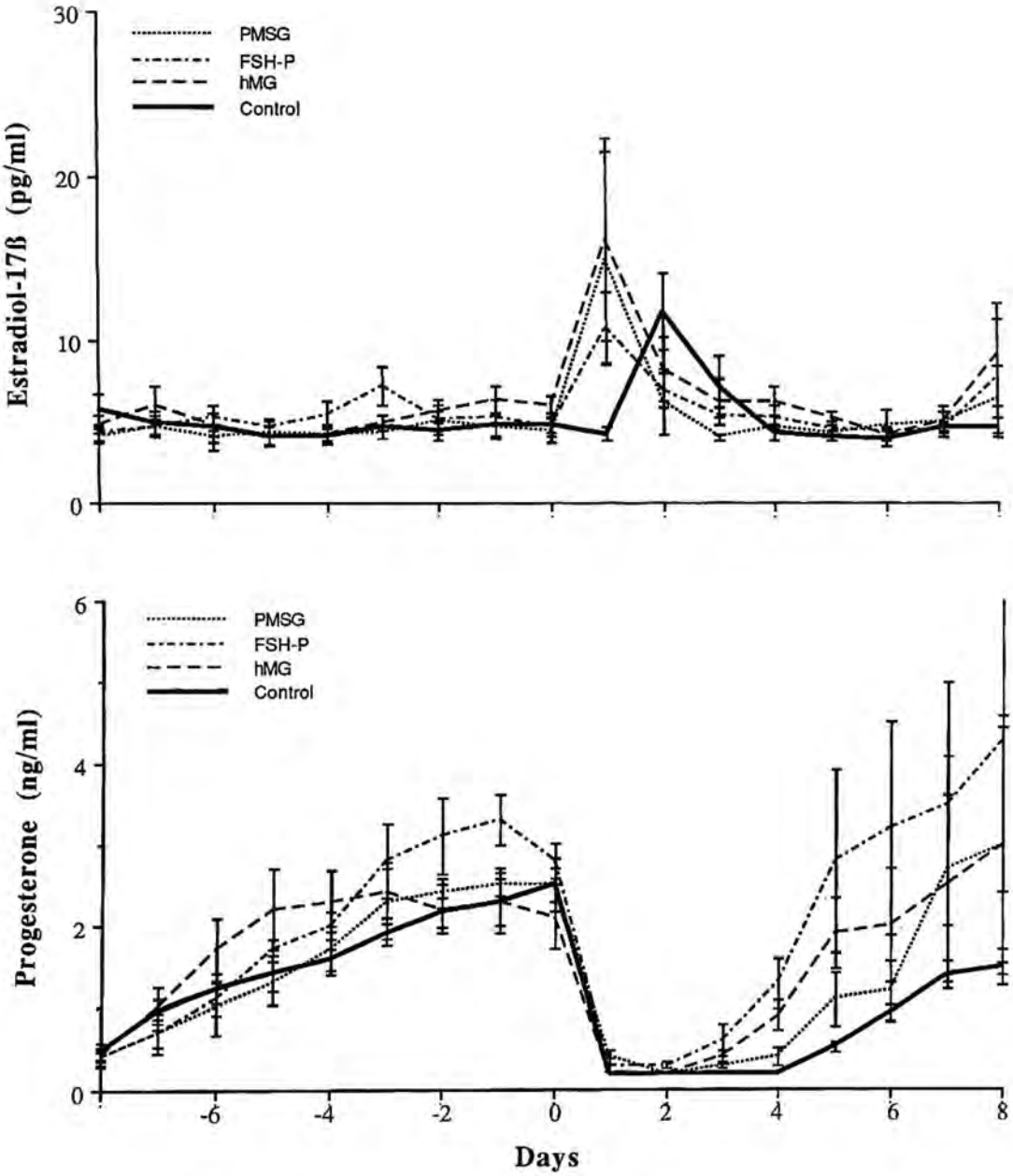
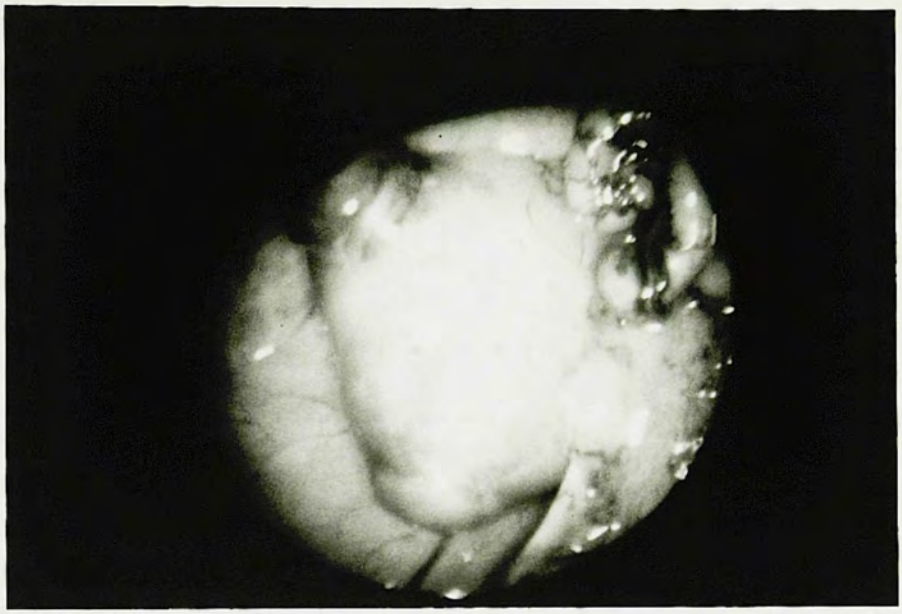


Figure 3. Sheep ovaries with normal CL (top panel) versus abnormal, prematurely regressing CL (bottom panel) on Day 6.



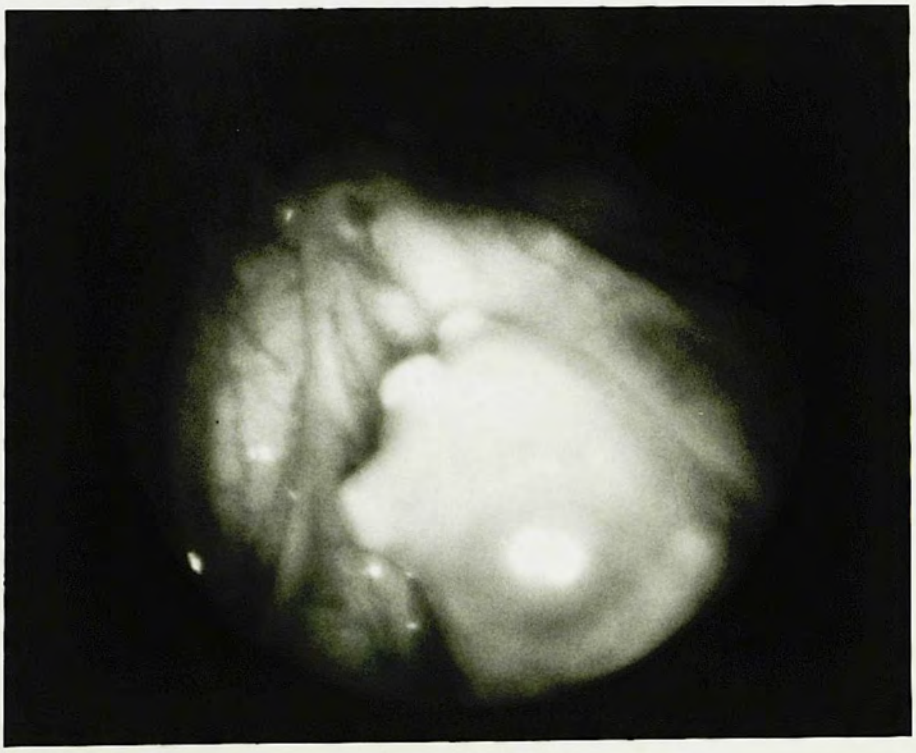


Figure 4. Mean (±SEM) temporal serum progesterone profiles of gonadotropin-treated ewes experiencing either a normal luteal phase (n = 9) or premature luteal regression (n = 6). Data are normalized from the day after $PGF_{2\alpha}$ injection (Day -1) to embryo collection (Day 6). Statistical differences ($P<0.05$) are indicated by an asterisk (*).

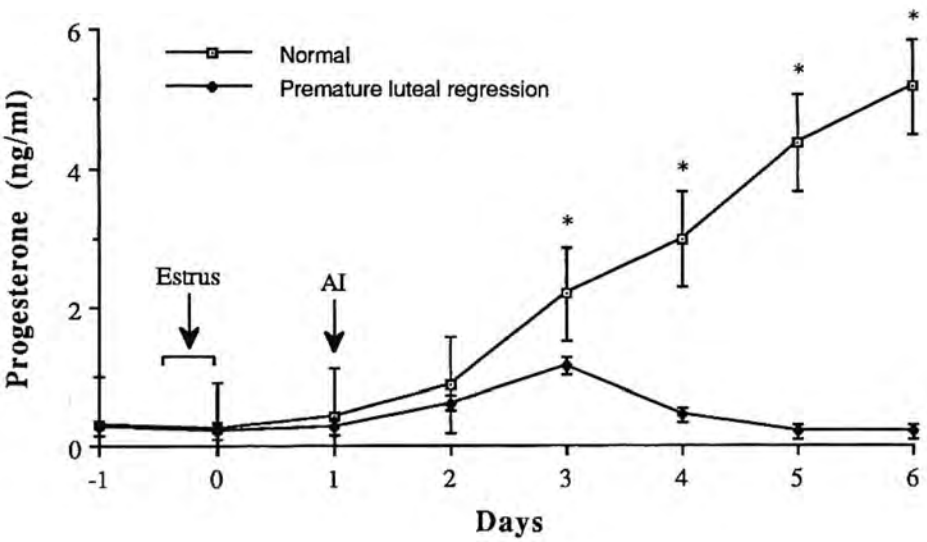


TABLE 2. Ovarian status of ewes experiencing either a normal or abnormal luteal phase following gonadotropin-PGF$_{2\alpha}$ treatment

Luteal condition	No. of ewes	Time of Observation: Pre-gonadotropin CL	Pre-gonadotropin Follicles[a]	Laparoscopic AI CH	Laparoscopic AI Follicles[a]	Post-gonadotropin CL	Post-gonadotropin Follicles[a]
Normal	17	1.7±0.2	2.4±0.7	2.2±0.6	9.5±1.7[b]	8.5±1.7	5.6±2.0[b]
Abnormal	13	1.9±0.2	2.2±0.7	1.5±0.6	5.7±1.2[c]	6.5±1.2	2.1±0.8[c]

[a] Follicles ≥ 5 mm in diameter.
[b,c] Mean (±SEM) column values with different superscripts differ (P<0.05).

TABLE 3. Influence of luteal status on embryo recovery following different gonadotropin treatments

Treatment	Total no. embryos/ total no. CL: normal CL (%)	Total no. embryos/ total no. CL: regressed CL (%)	Recovered embryo condition (%): Fertilization rate	Recovered embryo condition (%): Transferable embryo rate
Control	-----	-----	----	---
PGF$_{2\alpha}$/PMSG	11/23 (48)	0/7 (0)	55[a]	84
PGF$_{2\alpha}$/FSH-P	41/75 (55)	0/23 (0)	95[b]	80
PGF$_{2\alpha}$/hMG	29/51 (57)	0/49 (0)	86[b]	84

[a,b] Mean percentages with different superscripts differ (P<0.05).

Figure 5. Cumulative analysis of developmental stage and quality grade (QG) of embryos recovered from gonadotropin-treated ewes with normal CL 5 days after *in utero* AI. QG 1 = excellent to QG 4 = poor/degenerate.

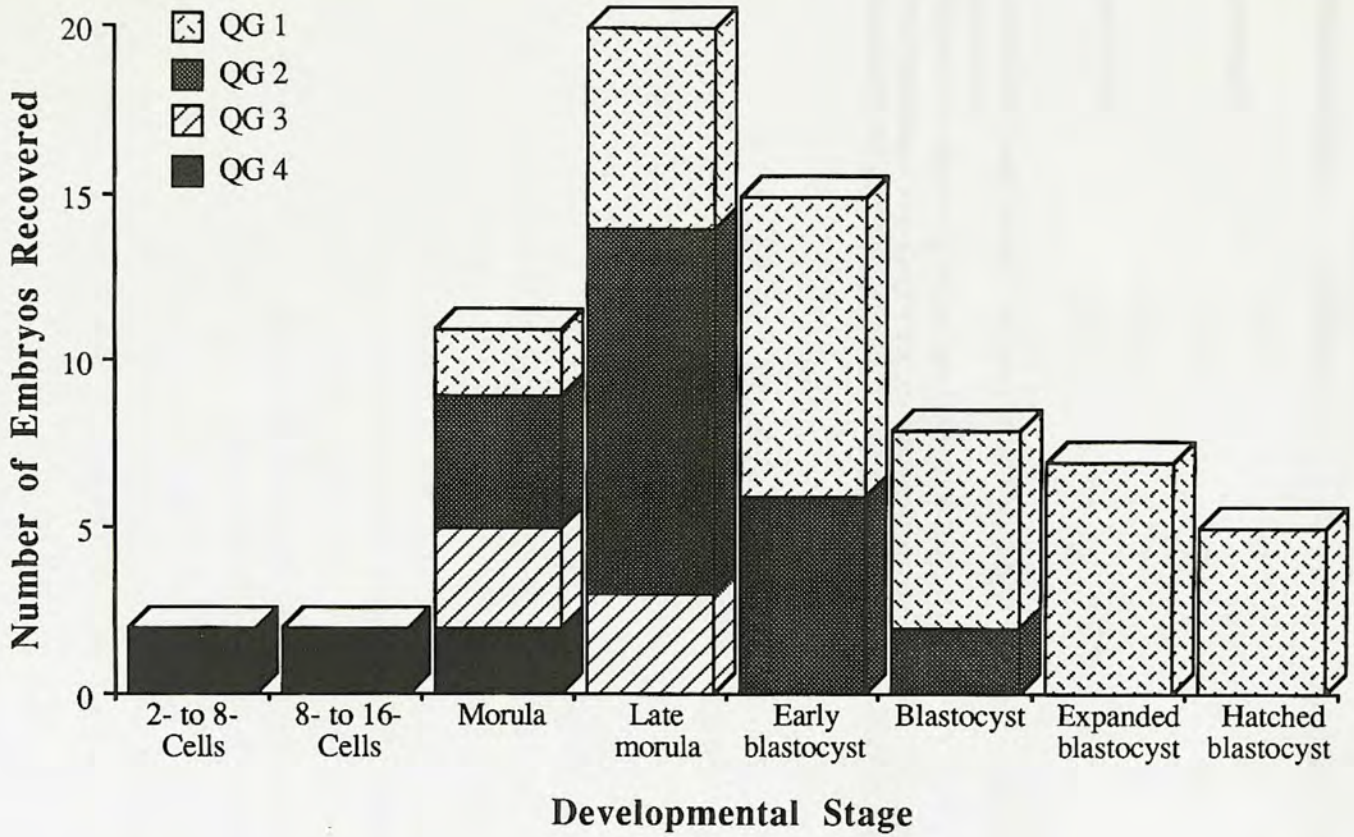


TABLE 4. Effectiveness of fresh ovine embryo transfer by either a laparotomy or a laparoscopic surgical approach

Embryo transfer by	Donor gonadotropin treatment[a]	No. of recipient ewes		No. of pregnant ewes	Overall pregnancy rate[b]
Laparotomy	hMG	4	(n=6)	1	16.6%
	FSH-P	2		0[c]	
Laparoscopy	hMG	2	(n=6)	1	50.0%
	FSH-P	4		2	

[a] Limited recipient availability precluded the comparative use of embryos from PMSG-treated donors.

[b] Pregnancies resulting in the production of live offspring.

[c] Fetal resorption was diagnosed by laparoscopy in one ewe at 60 days gestation after initial ultrasound pregnancy confirmation on Day 33 and 40 of gestation.

CHAPTER II

PREMATURE LUTEAL REGRESSION IN SUPEROVULATED SHEEP: RELATIONSHIP TO ESTROUS SYNCHRONIZATION METHOD, CIRCULATING HORMONES, LUTEINIZING HORMONE/PROSTAGLANDIN $F_{2\alpha}$ RECEPTORS AND LUTEAL PROGESTERONE CONTENT

INTRODUCTION

Premature demise of the corpus luteum (CL) after exogenous hormone stimulation represents a poorly understood cause of embryo recovery failure in goats and sheep (Armstrong et al., 1982b, 1983a, 1983b; Stubbing et al., 1986; Schiewe et al., 1990c). Luteal dysfunction in these species is generally characterized by a transient rise and fall in circulating progesterone concentrations within 4 days of ovulation (Armstrong et al., 1982b; Stubbing et al., 1986; Southee et al., 1988a). A high incidence of premature luteal regression has been reported in goats treated with progestogen pessaries (Armstrong et al.,1982b, 1983a) or prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and pregnant mares' serum gonadotropin (PMSG; Armstrong et al., 1983b; Stubbing et al., 1986). This same condition also occurs as goats and sheep approach transitional periods of anestrous. Short luteal phases have been associated with hormone- (McLeod et al., 1982; McLeod and Haresign, 1984; Bretzlaff et al., 1988; Southee et al., 1988a) and male-induced (Oldham and Martin, 1978; Knight et al., 1981; Oldham et al., 1985; Pearce et al., 1987) ovulations in anestrous sheep. Armstrong and coworkers (1983b) theorize that abnormal endocrine events associated with superovulation might stimulate endogenous $PGF_{2\alpha}$ release from ovarian follicles or the uterus causing early luteolysis. The preovulatory luteinizing hormone (LH) surge in short-cycling goats might also be reduced in magnitude compared to that measured in females exhibiting normal length cycles (Camp et al., 1983; Bretzlaff et al., 1988). Luteal tissue develops poorly in cattle induced to ovulate with GnRH 16 to 32 h after $PGF_{2\alpha}$ treatment; however, luteal tissue develops normally when GnRH is administered 48 h post-$PGF_{2\alpha}$ (Ohnami et al., 1985) suggesting that there may be a relationship between the onset of the LH surge and luteal competency. It remains unclear whether abnormal CL have an inherently short lifespan as a result of inadequate preovulatory follicular development, premature luteolysin release or hypersensitivity to luteolysis due to decreased luteotropin stimulation.

Ovine CL consist of large and small steroidogenic cell populations which have been described morphometrically (O'Shea et al., 1984; Rodgers et al., 1984; Farin et al., 1986) and biochemically (Fitz et al., 1982; Hoyer and Niswender, 1985; Balapure et al., 1989a). Large and small luteal cells are believed to develop from follicular granulosa and theca interna cells, respectively (McClellan et al., 1975; O'Shea et al., 1980). Small luteal cells contain the majority of the LH receptors (Fitz et al., 1982) and have low affinity binding sites for $PGF_{2\alpha}$ (Balapure et al., 1989a). Although LH binding to small luteal cells is responsible for stimulating adenylate cyclase activity and increasing steroidogenesis (Fitz et al., 1982; Rodgers et al., 1983; Hoyer and Niswender, 1985; Harrison et al., 1987), the functional significance of low affinity $PGF_{2\alpha}$ receptors is unclear. It is possible that in luteolysis these sites play a complementary role to high affinity $PGF_{2\alpha}$ binding sites found on large luteal cells (Fitz et al., 1982; Balapure et al., 1989a) by binding excess $PGF_{2\alpha}$ released by the uterus during the onset of luteal regression (McCracken et al., 1984). Alternatively, the low affinity $PGF_{2\alpha}$ receptor may actually be a binding site for some other eicosanoid which is structurally similar to $PGF_{2\alpha}$ (Balapure et al., 1989a). A progressive increase in luteal weight throughout the estrous cycle of naturally cycling ewes is characterized by hyperplasia of small luteal cells and hypertrophy of large cells (Farin et al., 1986). It appears that mean cell diameter and LH-stimulated steroidogenesis by small luteal cells are increased in cyclic ewes after superovulation (Hild-Petito et al., 1987). Other changes in luteal components (e.g., luteal cell size ratio and receptor populations) may offer insight into the causes for abnormal, short luteal phases when progesterone production is inhibited within 3 to 4 days after induced ovulation in anestrous (Southee et al., 1988a), postpartum (Braden et al., 1989) and superovulated ewes (Schiewe et al., 1990c).

The aim of this study was to characterize the etiology of premature luteal regression in gonadotropin-treated ewes by examining periovulatory endocrine patterns and luteal tissue development during the early luteal phase. We were particularly interested in determining whether the type of estrous synchronization approach used in concert with ovulation induction contributes to luteal competency. This was studied by determining the relationship of circulating levels of steroids and gonadotropins, LH and $PGF_{2\alpha}$ luteal cell receptor populations, luteal cell morphometrics and steroidogenesis to the occurrence of luteal dysfunction in estrous synchronized, superovulated sheep.

MATERIALS AND METHODS

Animals and facilities. Western crossbred, multiparous, white-face ewes (2 to 5 years of age), Dorset breeding rams and a vasectomized Dorset teaser ram were housed in outdoor, partially sheltered pens (6 m X 25 m) at the National Institutes of Health Animal Center, Poolesville, MD. All sheep were fed a hay-pelleted ration and alfalfa roughage twice daily (0700 h, 1500 h) and provided free access to water. Indoor box stalls (2.5 m X 2.5 m) were used for isolating animals as needed during the course of the study. All surgical procedures were conducted in a fully equipped, on-site large-animal surgery room.

Estrous synchronization and induction of ovulation. Sixteen donors were assigned to either a 6α-methyl-17α-acetoxyprogesterone-pessary (MAP, 60 mg Depo-Provera® [Upjohn Co., Kalamazoo, MI], 12 day intra-vaginal insertion interval; n = 8) or $PGF_{2\alpha}$ (Lutalyse® [Upjohn Co.], 10 mg, i.m., 10 day apart; n = 8) estrous synchronization treatment as described previously (Schiewe et al., 1990c). Each female was given FSH-P (Schering Veterinary Supplies, Kenilworth, NJ) twice daily over a 3 day period (5, 4, 4, 3, 3 and 2 mg, respectively; i.m.) beginning 36 h pre-MAP removal or at the time of the second $PGF_{2\alpha}$ injection. Donors were placed with 1 of 4 brisket-painted Dorset rams 24 h after MAP removal or $PGF_{2\alpha}$ injection for natural mating. Four naturally cycling, nonhormonally-treated ewes served as controls. Estrous behavior was monitored in all ewes for at least 30 min twice daily (0700 and 1800 h) by checking for mounting activity or by observing painted wool on the female's rump.

Enucleation of luteal material and embryo collection. A laparoscopic examination was performed on Day 0, that is 48 to 52 h post-MAP removal or $PGF_{2\alpha}$ (near the expected time of ovulation) to document ovarian activity and to perform an *in utero* insemination (Schiewe et al., 1990c). Control ewes were subjected to laparoscopy and a sham-AI 24 h after the onset of a natural estrus (also designated Day 0). On Day 3 and 6, each ewe was anesthetized and subjected to a laparotomy procedure (Schiewe et al., 1990c) to remove all luteal tissue from the left and right ovary, respectively. Each CL was removed by gentle dissection-enucleation with a blunt-tipped dental tool to separate the luteal tissue from the ovarian cortex. Care was taken to minimize the amount of residual connective tissue retained on each CL. Because these enucleation sites were extensively vascularized, each was sutured closed using 000' gut. Enucleated CL from each donor were placed together in sterile Hank's balanced salt solution (HBSS, Hanks and Wallace, 1949; Appendix Table 21) and stored at 4°C until further prepared for hormone receptor

analysis. On Day 6, each uterine horn also was exteriorized and flushed (Schiewe et al., 1990c) for embryos/ova.

Luteal tissue preparation. Within 1 to 3 h of removal from the ovary, connective and extraneous vascular tissue were excised from enucleated luteal tissue and the CL from each ovary weighed using an analytical balance (Mettler AE100, Fisher Co., Springfield, NJ). A sterile 100 ml specimen cup containing 10 ml HBSS was weighed initially, the luteal tissue added, the sample re-weighed and the luteal weight extrapolated. Each CL was sliced into 0.5 mm sections using a Stadie-Riggs hand microtome (Thomas Scientific, Swedesboro, NJ) and divided into 3 weighed portions to accommodate separate analyses of LH receptors, $PGF_{2\alpha}$ receptors and progesterone content. Tissue designated for LH receptor and progesterone content analysis was placed into 1 to 3 ml transport storage tubes (Fisher Co.), frozen directly in liquid nitrogen (LN_2) vapor and stored in LN_2. The remaining luteal tissue, designated for $PGF_{2\alpha}$ receptor analysis, required additional pre-freeze processing to prepare suspensions of predominantly isolated cells, as described by Balapure et al. (1989b). A suspension of single cells was prepared from the 0.5 mm slices by dissociating tissue in Ca^{2+}- and Mg^{2+}-free HBSS containing 2000 U collagenase (Worthington Type IV) per g tissue per flask, 0.1% bovine serum albumin (BSA, Fraction V, Sigma Chemical Co., St. Louis, MO) and 0.2% deoxyribonuclease (Sigma Chemical Co.). Luteal suspensions from Day 3 and 6 CL were incubated in a 37°C metabolic shaker bath for 30 and 60 min, respectively. Dissociated cells were washed twice in tissue culture medium 199 (TCM 199, Morgan et al., 1950; Morton, 1970; Appendix Table 22) using refrigerated (4°C) centrifugation at 3000 x g for 10 min to eliminate residual collagenase. The final cellular pellet was resuspended in TCM 199 containing 0.3% BSA. The preparation was stored overnight (4°C) and then frozen within 12 h after processing. The total number of viable cells in each cell suspension was counted with a hemacytometer immediately before freezing. In addition, the proportion of small (10 to 20 μm in diameter) and large (≥20 μm in diameter) luteal cells in the tissue suspension was assessed on the basis of characteristic cellular differences described by others (Rodgers and O'Shea, 1982; Farin et al., 1986; Hild-Petito et al., 1987). Although some cells may have been damaged during the dissociation process (particularly large cells which appear to be more fragile; T. Fitz, personal communication), the procedures used for estimating cell numbers were consistently applied to all treatment groups. Cells were aliquoted into cryovials in 0.5 ml TCM 199 and then 0.5 ml of 15% dimethyl sulfoxide (DMSO)-TCM 199 solution was added (4°C) and allowed to equilibrate for 10 min. Luteal cells were frozen in a programmable freezing unit (Planer Products Ltd., Model 204; TS Scientific, Perkasie,

PA) by cooling at 4°C per min to -38°C and then 10°C per min to -110°C before storage in LN_2. It was determined previously that frozen-thawed luteal cells maintained viability and possessed $PGF_{2\alpha}$ receptors indistinguishable from cells of the same lot before freezing (Balapure et al., 1989a).

Blood sampling and endocrine analysis. Blood samples (10 ml) were obtained by jugular venipuncture from all ewes beginning 1 day before gonadotropin treatment or at the onset of detected estrus for controls. Daily blood sampling (between 0700 to 0900 h) continued for 9 to 13 consecutive days. To precisely characterize preovulatory estradiol-17β, follicle stimulating hormone (FSH) and LH dynamics in estrus synchronized animals, frequent blood samples were collected near the time of the anticipated preovulatory LH surge. To accommodate frequent blood sampling, ewes were moved from the normal outdoor pen environment to indoor-isolation stalls at the time of pessary removal or the $PGF_{2\alpha}$ injection. An indwelling catheter with an obturator (Becton-Dickinson, Rutherford, NJ) to prevent clotting was inserted into a jugular vein (Brown et al., 1987). Beginning 12 h after pessary removal or $PGF_{2\alpha}$ injection, blood samples (5 ml/sample) were collected at 2 h intervals for 24 h. All blood samples were processed for serum collection and storage (Schiewe et al., 1990c), and serum estradiol-17β, FSH and LH and progesterone concentrations were determined by specific radioimmunoassay (RIA) procedures. Because a previous study had detected no differences in estradiol-17β concentrations in superovulated sheep with normal or abnormal luteal phases (Schiewe et al.,1990c), this hormone was measured only during the 24 h serial bleeding window to characterize the preovulatory estrogen surge.

Steroid concentrations were determined in unextracted sera using commercially available ^{125}I-double-antibody RIA kits (Radioassays Systems Inc., Carson, CA) as detailed and validated for sheep (Schiewe et al., 1990c). For the estradiol-17β assay, precipitating solutions containing the first and second antibodies (antisera against rabbit 6-keto-estradiol-17β-6-oxime-BSA and goat anti-rabbit gamma globulin, respectively) were used to detect bound and free unconjugated forms of estradiol-17β. Duplicate serum samples (100 µl aliquot) were incubated (37°C) with the first antibody and radiolabelled estradiol-17β for 90 min. The second antibody was added and the samples centrifuged at 2500 x g for 20 min at 4°C. The supernatants were discarded and radioactivity in the pellets quantified by gamma spectrometry. The minimum detectable estradiol-17β concentration (i.e., assay sensitivity) was 3.5 pg/ml based on the level at which 95% maximum binding occurred (ED95). The amount of estradiol-17β needed to cause 50% binding inhibition (ED50) was 94.3 pg/ml.

The progesterone assay involved incubating serum (100 µl, 37°C) with radiolabelled progesterone and first antibody (antiserum against rabbit 11α-hydroxyprogesterone-11α-hemisuccinate-human serum albumin) for 60 min. The second antibody (goat anti-rabbit gamma globulin) was added and the samples centrifuged for 20 min (2500 X g, 4°C). After decanting the supernatant, the radioactivity in the pellets was measured by gamma spectrometry. The assay sensitivity (determined at the ED95 level) was 0.1 ng/ml and the amount of progesterone needed to cause 50% inhibition (ED50) was 4.2 ng/ml.

Serum FSH and LH were analyzed by validated homologous double-antibody RIAs (Niswender et al., 1969; Brown et al., 1987). Purified ovine FSH (LER-1976-A2) and LH (LER-1374-A) were used as the labelled ligands and NIH-FSH-S8 and NIH-LH-S18 as the standards. Cross-reactivities of the antibodies with other pituitary hormones (thyroid-stimulating hormone, prolactin or growth hormone) were <2%. Duplicate aliquots of serum (100 µl) or standards (LH: range, 0.03125 to 4.0 ng/tube; FSH: range, 1.56 to 200 ng/tube, respectively) were incubated at 4°C with first antibody for 48 h and then labelled hormone for 24 h. The second antibody was added 24 h following addition of labelled hormone (final volume = 500 µl) and incubated for an additional 3 d after which 1 ml of cold PBS was added and the antibody bound hormone was separated from free hormone by centrifugation (2500 X g, 30 min). The amount of LH and FSH needed to cause 50% inhibition (ED50) was 0.4 and 13 ng/tube, respectively. The minimal level of detectable FSH and LH (based on the ED95 level) were 10.9 and 0.5 ng/ml for 100 µl serum, respectively. Intra- and interassay variability for all assays was less than 10%.

Progesterone content of luteal tissue. The progesterone content of luteal tissue was determined by the ^{125}I-double antibody RIA procedure described for serum (Schiewe et al., 1990c). The wet weight of blot dried tissue slices was determined with an analytical balance immediately before being homogenized with a Polytron at medium speed using 3 3-sec pulses. To extract progesterone, homogenates were placed in absolute (95%) ethanol for 24 h. The homogenate was then centrifuged (2500 X g, 10 min) and the pellet was separated from the supernatant which was stored at -20°C. Before subsequent determination of progesterone content, an aliquot of the organic phase was dried in a 12 X 75 mm test tube and reconstituted in assay buffer.

LH receptor and $PGF_{2\alpha}$ receptor analysis. The number of LH receptors was determined by a modification of the procedure described by Diekman et al. (1978). The total number of unoccupied LH receptors was determined using Scatchard analysis of

saturation curves. Thawed tissue slices were homogenized as described above and suspended in 25 mM Tris HCl (pH 7.35) buffer containing 1 mM $CaCl_2$, 1 mM NaN_3 and 0.1% gelatin (1 ml/100 mg wet weight tissue equivalents). Aliquots (100 µl) of crude luteal tissue homogenates (2 mg tissue weight) were incubated in Tris buffer with ^{125}I-hCG (9.5 Ci/mmol; 100 µl) ranging from 10,000 to 2.1 X 10^6 cpm in a final volume of 500 µl. Parallel incubations were conducted using 1 µg hCG (Sigma Chemical Co.) to determine the NSB. Samples were incubated in a metabolic shaker bath at 25°C for 16 h. Following incubation, 3 ml of Tris buffer were added to all tubes containing tissue homogenates. The tubes were centrifuged at 7,000 X g (4°C) for 15 min and the supernatant decanted. The radioactivity of each pellet was measured in a TM Analytic gamma counter having an ^{125}I detection efficiency of 82%. The LH receptor binding affinity (K_d) was 5.54 ± 2.13 X 10^{-11} M. The affinity of binding sites was determined by the slope of the line-of-best fit calculated in the Scatchard analysis by LIGAND (Munson and Rodbard, 1980) for a particular receptor type.

Prostaglandin $F_{2\alpha}$ receptor analyses were performed with thawed, luteal cell preparations by previously validated techniques (Balapure et al., 1989a, 1989b). Duplicate aliquots containing 50,000 large luteal cells (100 µl) were incubated with 50 nCi [^{3}H] $PGF_{2\alpha}$ (195 Ci/mmol; 100 µl) and 1 of 15 unlabelled $PGF_{2\alpha}$ buffer concentrations (0 to 16 µM) in a total volume of 210 µl HBSS-BSA buffer (pH, 5.75) at 30°C for 45 min in a metabolic shaker bath. Ice-cold buffer (pH, 7.35) containing 25 mM Tris, 1 mM $CaCl_2$, 0.1% BSA (Tris-Ca-BSA) was added (1.5 ml) to terminate the incubation and the incubates were applied to glass microfiber filters on a Yeda filtration manifold. The filters were washed twice with 1.5 ml of Tris-Ca-BSA buffer, placed in vials containing 10 ml scintillation fluid and equilibrated for ~16 h at 4°C. Bound radioactivity was determined with a Tracor Mark III liquid scintillation, beta counter having a tritium detection efficiency of 44%. Binding data were converted to Scatchard plots using LIGAND (Munson and Rodbard, 1980). The number of high and low affinity $PGF_{2\alpha}$ receptors was quantitated by Scatchard analysis (binding affinity: K_d = 12.09 ± 1.20 nM and K_d = 870 ± 160 µM, respectively).

Statistical Analysis

A completely randomized design with an apriori arrangement of treatments was used to assess the periovulatory hormonal patterns and luteal characteristics of ewes synchronized into estrus using MAP-pessaries versus $PGF_{2\alpha}$. Analysis of variance (ANOVA) with a general linear model format was used to assess statistical variations in ovulation rate, luteal weight, luteal cell morphometrics, receptor populations and

progesterone content of luteal cells. These analyses were facilitated with the aid of a computerized software package (Systat: Wilkinson, 1987). Differences among treatments were determined by Tukey's HSD procedure (Snedecor and Cochran, 1980). Pooled data involving the evaluation of 2 groups (i.e., ewes with normal luteal function versus those experiencing premature luteal regression) were analyzed by an independent Student's *t* test (Snedecor and Cochran, 1980). Temporal profiles and differences in hormone concentrations over time were evaluated with a 2-way ANOVA, repeated measures program. A hormonal surge was defined as a pattern in which the hormone concentration increased more than 3 standard deviations (SD) above basal levels. Results are expressed as means ± SEM.

RESULTS

The mean ovulatory response of natural cycling control ewes was lower ($P<0.01$) than that observed in sheep treated with FSH-P following MAP-pessary or $PGF_{2\alpha}$ estrous synchronization (Table 5). Therefore, the total amount of luteal tissue mass (by weight) was increased ($P<0.05$) in FSH-P-treated compared to naturally cycling ewes on Day 3. However, there were 2 types of CL observed in this study: 1) normal CL which on Day 3 and 6 were bright red and prominently raised above the ovarian surface (Schiewe et al., 1990c); and 2) abnormal CL which appeared to be undergoing premature luteal regression. These latter sites were pale and pink to white in appearance (Schiewe et al., 1990c). The differences in gross coloration between CL types usually was evident by Day 3. By Day 6, these regressing CL could be classified as corpora albicans (CA) because of the distinct lack of vascularity (i.e., white color) and viable luteal cells upon tissue dissociation. Consistent with the premature demise of CL, no embryos/ova were recovered from these donors on Day 6, while embryos/ova were recovered from all donors with normal appearing luteal tissue (mean, 7.7 ± 2.2 embryos/ewe). The incidence of premature luteal regression was related to treatment and occurred in none of the controls, 1 of 8 MAP synchronized females and 7 of 8 $PGF_{2\alpha}$ treated ewes ($P<0.05$). Because a high proportion of ewes treated with PGF2a experienced early luteal regression, the mean total mass of luteal tissue on Day 6 was less ($P<0.05$) than that observed in the MAP-synchronized group (Table 5). On Day 3, the average weight of CL undergoing premature luteolysis was not different from that of normal-appearing CL (Table 6). However by Day 6, the latter were 5.4-fold greater ($P<0.01$) in average mass than the former. In ewes with normal appearing CL, mean CL weight was similar among the groups (Table 6) suggesting that

estrous synchronization and gonadotropin treatment had a negligible effect on individual CL mass. Regardless of treatment, there was a highly positive correlation between CL number and weight on Day 3 ($r = 0.88$, $P<0.001$) but no correlation on Day 6 ($r = 0.30$, $P>0.10$) because of rapidly declining CL function in half the hormone-treated ewes. However, in ewes with normal appearing CL on Day 6, the correlation between CL number and mass remained high ($r = 0.81$, $P<0.001$).

Individual endocrine profiles depicting temporal changes in steroid and gonadotropin concentrations for each ewe throughout the course of treatment are presented in the Appendix (Fig. 22 to 31). Figures 6 to 8 illustrate the mean circulating steroid and gonadotropin profiles for the controls and estrous-synchronized ewes demonstrating normal luteal function or premature luteal regression, respectively. Preovulatory estradiol-17β surges were detected in 14 of 16 hormone-stimulated ewes, but the treatment had no effect ($P>0.05$) on the preovulatory estradiol-17β surge. Generally, serum estradiol-17β levels increased more than 8- to 10-fold from basal levels (4 to 6 pg/ml) to a peak concentration (mean, 59.3 ± 10.9 pg/ml) which occurred by 20 to 36 h (mean, 25.4 ± 1.5 h; duration of surge 6 to 12 h) after MAP removal or $PGF_{2\alpha}$ injection. After ovulation, mean serum LH profiles from control and FSH-P-treated ewes were similar ($P>0.10$). There were no differences ($P>0.10$) in peripheral FSH or LH concentrations or temporal patterns from ewes receiving estrous synchronization treatments. Regardless of whether FSH-P-treated ewes eventually possessed normal or regressed CL, the FSH and LH patterns were similar ($P>0.10$; Fig. 7 and 8). Preovulatory FSH surges were detected in 13 of 16 hormone-treated ewes. Serum FSH patterns increased from a baseline concentration of 50 to 100 ng/ml to a mean peak of 237.0 ± 14.4 ng/ml (range, 180 to 330 ng/ml) which occurred at an average of 26.5 ± 1.1 h after MAP removal or $PGF_{2\alpha}$ injection. Although sporadic, secondary FSH surges were observed among individual ewes during the next 9 days; however, peripheral concentrations generally remained below 150 ng/ml. Across treatment groups, a preovulatory surge of LH was detected in 11 of 16 ewes and was sustained for ~6 to 10 h. Circulating LH increased from baseline concentrations of 3 to 7 ng/ml to a mean peak of 72.4 ± 4.2 ng/ml (range, 60 to 85 ng/ml) which occurred 28.8 ± 1.1 h after MAP removal or $PGF_{2\alpha}$ injection. There was little fluctuation in circulating LH concentrations after ovulation (remaining below 10 ng/ml) and no difference ($P>0.10$) among treatment groups.

Compared to naturally cycling ewes, sheep treated with FSH-P (regardless of estrous synchronization approach) began secreting progesterone 48 to 72 h before controls. In sheep with normal CL, mean circulating progesterone concentrations (Fig. 7) were higher ($P<0.05$) than control levels from Day 2 to Day 6 with peak progesterone

production (mean, 4.3 ± 0.6 ng/ml) being an average of 3-fold greater (P<0.05). There was no apparent difference between the circulating progesterone patterns from MAP-synchronized ewes producing normal CL and the 1 $PGF_{2\alpha}$-injected ewe demonstrating normal CL function. Likewise, the mean progesterone profiles for $PGF_{2\alpha}$-treated sheep experiencing premature luteal regression appeared similar to the pattern produced by the one MAP-synchronized ewe with the same condition. The most marked difference observed was in the post-ovulatory progesterone profile for control and normal luteal function ewes compared to sheep undergoing early luteolysis. Although mean progesterone levels were higher (P<0.05) on Day 2 in FSH-P-treated (mean, 0.9 ± 0.2 ng/ml) compared to control ewes (0.2 ng/ml), progesterone levels steadily declined thereafter in sheep experiencing premature luteal regression (Fig. 8). Mean circulating progesterone concentration in the latter ewes was not different (P>0.10) than controls on Day 3 and was reduced (P<0.05) to baseline (≤0.2 ng/ml) by Day 4. Progesterone production continued to be elevated in control and FSH-P-treated ewes with normal luteal function through Day 6 (Fig. 6 and 7).

Viability analysis of the thawed luteal cells revealed that ~80% of the dissociated cells survived freeze-preservation. There was apparent differences in the viability of luteal cell types among treatments before or after freezing. On Day 3, control sheep and ewes with normal CL or premature luteal regression had similar numbers of large luteal cells (Table 7). However, FSH-P-treated ewes with normal CL had more (P<0.05) small and total luteal cells than controls or sheep with early regressing CL. On Day 6, luteal cells were so atrophied in the premature regression group that quantitating large versus small cells was impossible. However, as on Day 3, more (P<0.05) small and total luteal cells (~3-fold increase) were observed on Day 6 in the gonadotropin-treated ewes with normal CL compared to controls. For both groups, the number of large cells remained constant between Days 3 and 6 while small cell numbers increased (P<0.05) by ~1.7-fold.

On Day 3 and 6, the normal CL of ewes treated with FSH-P had more progesterone/mg of luteal tissue than control sheep or females experiencing premature luteal regression (Table 8). Because the luteal progesterone content between MAP and $PGF_{2\alpha}$ treatments was different on either Day 3 or 6, it appeared that progesterone steroidogenesis was compromised by ~60 h of ovulation in ewes with short luteal phases. In the analysis of luteal cell receptor populations, no differences (P>0.05) between normal and regressed CL were detected in LH receptor number on Day 3 (Table 8). However by Day 6, the LH receptor population was 4- to 5-fold lower (P<0.05) in ewes with regressed CL compared to control and FSH-P-treated animals with normal CL. Evaluation of $PGF_{2\alpha}$ receptors revealed no differences in the number of high affinity receptors on Day 3 or 6 (although

poor cell viability in the premature luteal regression group prevented analysis). There was no difference ($P>0.10$) in low affinity $PGF_{2\alpha}$ receptor numbers between controls and ewes with normal CL on Days 3 and 6 (Table 8). In contrast, the population of low affinity $PGF_{2\alpha}$ receptors in sheep with abnormal CL was reduced ($P<0.05$) on Day 3 compared to control ewes. Neither LH nor $PGF_{2\alpha}$ receptor population affinities were influenced by exogenous hormone treatment, as demonstrated by the similarity of Scatchard analysis binding plots for representative hormone treated or naturally cycling ewes (Fig. 9 and 10, respectively).

DISCUSSION

Inducing ovarian activity in sheep using FSH-P in conjunction with either a MAP or $PGF_{2\alpha}$ estrous synchronization treatment produced multiple ovulations similar to rates reported by others (Smith, 1988; Schiewe et al., 1990c). The present investigation affirmed that 1) $PGF_{2\alpha}$ estrous synchronization was associated with a high incidence of premature luteal regression in FSH-treated sheep and 2) this anomaly occurred much less frequently when cycles are synchronized with MAP. In a recent study (Schiewe et al., 1990c), we determined that premature demise of multiple CL was not associated exclusively with a particular exogenous gonadotropin, as regression occurred in $PGF_{2\alpha}$-treated ewes injected with pregnant mares' serum gonadotropin (PMSG, 40%), FSH-P (30%) or human menopausal gonadotropin (hMG, 60%). Premature luteolysis also was observed in 1 of 5 natural cycling ewes given $PGF_{2\alpha}$. Short luteal phases were not exhibited in sheep pretreated with MAP-pessaries and induced to ovulate by either PMSG or FSH-P in the comparative gonadotropin study (Schiewe et al., 1990c). We concluded, therefore, that exogenous $PGF_{2\alpha}$ may have a residual detrimental effect on subsequent luteal integrity after induced estrus (Schiewe et al., 1990c). This observation also generated a practical problem since no embryos were recovered from ewes with regressed CL at 5 days after AI.

Luteal dysfunction has been observed frequently in PMSG/$PGF_{2\alpha}$-treated goats (Armstrong et al., 1983b; Stubbing et al., 1986) and may have contributed to reduced fertility of estrus-synchronized sheep treated with $PGF_{2\alpha}$ as opposed to progestogen pessaries (Boland et al., 1978b; Hackett et al., 1981b; Acritopoulou-Fourcroy et al., 1982). We detected abnormally short luteal phases characterized by a transient rise and fall in circulating progesterone within 4 days of ovulation. Similar findings of early luteal regression have been observed in anestrous ewes induced to ovulate with GnRH (McLeod

et al., 1982; McLeod and Haresign, 1984; Hunter et al., 1988; Southee et al., 1988a) or ram exposure (Oldham and Martin, 1978; Knight et al., 1981; Oldham et al., 1985; Pearce et al., 1987). Because luteal development is related to major cellular transformations of the post-ovulatory follicle, the factors normally influencing follicular maturation (i.e., granulosa cell numbers and gonadotropin receptor populations) may contribute to luteal competency (Armstrong et al., 1982b). A premature or abbreviated LH surge (Camp et al., 1983; Pearce et al., 1987; Bretzlaff et al., 1988) has been associated with inadequate follicular development before ovulation induction leading to possible abnormal luteal function (McNatty et al., 1982; McLeod et al., 1982; Legan et al., 1985). Progestogens act at the level of the hypothalamo-pituitary axis to inhibit estrogen positive feedback and delay the preovulatory gonadotropin surge (McLeod et al., 1982; McLeod and Haresign, 1984). Therefore, MAP preovulatory treatment may ensure that sufficient time is available for follicular cells to acquire the maturity needed for optimal luteinization and functional development (Armstrong et al., 1982b). Progestogen priming before ovulation induction eliminates premature luteolysis in anestrous ewes (Oldham et al., 1980; McLeod and Haresign, 1984; Southee et al., 1988a). In our study, estrous synchronization with MAP pessaries usually was associated with normal CL formation and luteal phases. Nonetheless, the common occurrence of premature luteolysis in $PGF_2\alpha$-treated ewes could not be attributed to differences in temporal hormonal profiles during the periovulatory period. This suggests two possibilities. First, the similarity in preovulatory gonadotropin and estradiol-17β profiles may indicate that folliculogenic events are not associated with premature CL demise. Alternatively, circulating hormones may be inadequate markers for these events or the blood sampling protocol used may have been in sufficiently rigorous to detect subtle endocrine differences between animal groups.

Circulating progesterone profiles clearly indicated that premature CL demise occurred 2 to 3 days after ovulation which was similar to endocrine results in similarly treated goats (Armstrong et al., 1983b). However, the accelerated, post-ovulatory production of progesterone in hormonally-injected sheep compared to natural cycling controls was unrelated to the production of normal or abnormal CL. Circulating progesterone concentration in ewes with normal CL was correlated highly with CL number, which was in agreement with similar findings in cattle (Saumande, 1980) and sheep (Schiewe et al., 1990c). Therefore, progesterone monitoring served as an informative index of the extent and normalcy of luteal development.

Dynamic changes in luteal cell morphology occurred consistently over time between groups. Control and gonadotropin-treated ewes with normal CL experienced cyclic changes in the number of cells calculated after dissociation similar to previous observations

in naturally cycling sheep (Farin et al., 1986). The number of small luteal cells increased between Days 3 and 6 without a corresponding change in the large luteal cell population. Additionally, the large-to-small luteal cell ratio (~1:4) was within the normal range reported by others for natural cycling (Farin et al., 1986; Harrison et al., 1987) and PMSG-treated ewes (Hild-Petito et al., 1987). Compared to controls, the increased population of small luteal cells in gonadotropin-treated ewes with normal CL may have accounted for the observed increase in total luteal weight. Corpora lutea from ewes undergoing premature luteolysis already were experiencing a sharp reduction in both large and small luteal cells by Day 3, but the primary loss was with the smaller cell type. Although total luteal mass was greater in sheep with regressing CL than in controls, these CL did not produce more small luteal cells or progesterone (in circulation or in tissue). Basal progesterone secretion by large and small luteal cells *in vitro* was not different between gonadotropin-treated and natural cycling ewes, but LH- and dbcAMP-stimulated progesterone production by small luteal cells significantly increased in ewes injected with PMSG (Hild-Petito et al., 1987). Therefore, it is likely that the elevated serum progesterone concentrations and progesterone content/mg luteal tissue in gonadotropin-treated sheep was due to an increased small cell population and also a corresponding enhancement of steroidogenesis. Although large luteal cells have a higher level of basal progesterone secretion (Fitz et al., 1982; Hoyer et al., 1984; Harrison et al., 1987; Hild-Petito et al., 1987), it is the small luteal cells which contain most of the LH receptors and, therefore, are most responsive to gonadotropin stimulation (Fitz et al., 1982). The enhanced steroidogenic potential of the small luteal cells in this study, however, could not be attributed to increased LH binding, because the number of LH receptors/mg tissue was no different among groups. A recent evaluation of short-lived CL in postpartum ewes (Braden et al., 1989) also determined that the reduced steroidogenic competency of luteal cells is not caused by changes in LH receptor numbers. Therefore, it is most logical to presume that reduced progesterone production on Day 3 in gonadotropin-treated ewes with regressing CL was caused by a declining steroidogenic, small luteal cell population and/or an insensitivity to luteotropic stimuli.

The rapid loss of luteal competency in ewes experiencing premature, short luteal phases was similar to the normal demise of CL caused by increased pulsatile release of $PGF_{2\alpha}$ from the uterus (McCracken et al., 1984). The occurrence of premature luteal regression at ~Day 4 after the LH surge was consistent with the time frame when natural cyclic ewes are susceptible to the luteolytic effects of exogenous prostaglandin treatment (Acritopoulou and Haresign, 1980). In addition, it generally is agreed that in postpartum animals the exposure of GnRH-induced CL to high endogenous levels of $PGF_{2\alpha}$ is, in part, responsible for premature luteolysis (Lewis et al., 1981; Troxel et al., 1984; Copelin

et al., 1987). Treating anestrous ewes with GnRH (with or without progestogen pretreatment) sustains luteal integrity by removing the causative luteolytic source, the uterus (Southee et al., 1988b). It appears, therefore, that some factor (probably $PGF_{2\alpha}$) of uterine origin is responsible for inducing premature luteal regression. Premature luteolysis also could be due to an increased sensitivity of the CL to tonic secretion of a luteolysin, as recently suggested for the postpartum ewe (Braden et al., 1989). In the latter study, early luteolysis during the postpartum period could not be attributed to increased numbers of prostaglandin receptors or binding affinity to $PGF_{2\alpha}$ by the large luteal cells. In the present study, we identified 2 classes of $PGF_{2\alpha}$ binding sites (high and low affinity) in ovine luteal tissue which supported and verified recently published data (Balapure et al., 1989a, 1989b). Differences in $PGF_{2\alpha}$ receptor number/luteal cell were not detected between control and gonadotropin-treated ewes producing normal luteal tissue. However, hormone-treated ewes experiencing premature luteolysis had fewer low affinity $PGF_{2\alpha}$ receptors (Day 3), which are the binding sites predominantly located on small luteal cells (Balapure et al., 1989a). The reduction in small luteal cell populations of ewes with regressing CL on Day 3 was not attributed to premature luteolysis, but instead to some inherent developmental changes since the presence of degenerate/non-viable small luteal cells was minimal in luteal cell suspensions. The possibility exists that the low affinity $PGF_{2\alpha}$ binding site has a high affinity for some other eicosanoid, perhaps a luteotropin such as PGE_2 (Balapure et al., 1989a). The onset of premature luteal demise in superovulated ewes of this study occurred at a time when large luteal cells acquire high affinity $PGF_{2\alpha}$ binding sites (Fitz et al., 1982). A significant difference with regards to the population of high affinity receptors did not exist among treatments. In comparison of only FSH-P-treated ewes, there was a tendency ($P<0.10$) for fewer high affinity binding sites to be present on the luteal cells recovered from regressed CL ewes on Day 3. If both high and low affinity receptors are actually reduced, it is possible that this difference is due to a downregulation of $PGF_{2\alpha}$ binding sites stimulated by the production of and binding to endogenous $PGF_{2\alpha}$ released before enucleation. The possibility exists that luteal cells became hypersensitive to a luteolysin caused by functional changes in CL integrity.

A consensus of scientific opinion indicates that preovulatory progesterone priming is critical to normal luteal development in the sheep, especially in previously acyclic ewes (Cognie et al., 1982; McLeod and Haresign, 1984; Pearce et al., 1985; Southee et al., 1988a). An intriguing, yet confounding factor in our study was that ewes were under the influence of endogenous progesterone immediately before $PGF_{2\alpha}$. Despite this "priming" effect, a high incidence of premature luteal regression occurred. The possibility has been considered that exogenous and endogenous progestogens may exert different effects on

preovulatory follicular maturation and subsequent luteal development. We do know that there was an initial period of post-ovulatory luteal development and at least transient steroidogenic capabilities demonstrated by all sheep which experienced premature luteal regression. However, the inability of luteal cells to continue secreting progesterone was unrelated to lack of LH stimulation at the cellular level because pituitary gonadotropin secretion and cellular LH receptor numbers were similar (Day 3) between ewes with normal or abnormal luteal function. However, a possible relationship may exist between the number of low affinity $PGF_{2\alpha}$ receptors present on small luteal cells and luteal competency. Additional studies are necessary to delineate the cause-effect relationship of exogenous $PGF_{2\alpha}$ to premature luteal regression at the level of the small luteal cell during the periovulatory phase. It is at this time that morphological features (e.g., microfilament and microtubule infrastructure) are established and intercellular channels (e.g., Ca^{2+} channels) are formed by microvilli between luteal cells (Rodgers et al., 1985). This event facilitates cellular communications believed to be involved in steroidogenesis and perhaps necessary for $PGF_{2\alpha}$ to exert a luteolytic effect.

Although effective for inducing luteolysis and estrus in the ewe, $PGF_{2\alpha}$'s influence on subsequent luteal development must be considered critically. Our data would suggest that, under the conditions described, exogenous $PGF_{2\alpha}$ is contraindicated because of an increased incidence of premature luteal regression. Hypotheses that the cause of early luteolysis was associated with abnormal preovulatory endocrine events and/or inadequate follicular maturation (i.e., functionality and population of granulosa and thecal cells) were not substantiated by these results or a recent report in our laboratory (Schiewe et al., 1990c). The latter study determined that preovulatory follicular events (i.e., antral follicle numbers, time of ovulation) in gonadotropin-treated ewes with regressed CL were not different from sheep with normal CL. The exact cause of luteal dysfunction remains unknown, however, the problem appears to occur at the cellular level and likely involves the uterine release of a luteolysin on Day 3 to 4 of the induced estrous cycle. In the periovulatory period, changes in the small luteal population and low affinity $PGF_{2\alpha}$ binding sites may be responsible for the transient normalcy of CL function. Additional studies are necessary to determine the influence of $PGF_{2\alpha}$ on development, maturation and transformation of follicular theca and granulosa cells to luteal cells. Our understanding of the etiology of premature luteal regression also may be improved if we can resolve how progestogen pretreatment inhibits the abnormal physiological changes associated with early luteolysis.

TABLE 5. Ovarian response and luteal status of individual ewes to exogenous FSH and estrous synchronization treatment

Treatment[a]	Sheep ID	Ovarian status (CL No.)[b] left	right	total	Luteal condition[c]	Luteal weight (g)[d] Day 3	Day 6
Control/Sham	1	1	1	2	+	0.51	0.67
	2	2	0	2	+	0.15	0.51
	3	1	1	2	+	0.20	0.46
	4	2	0	2	+	0.36	0.46
		1.5	0.5	2.0±0.0[e]	4/4+	0.3±0.1[e]	0.5±0.1[e]
MAP/FSH-P	5	6	6	12	+	0.99	2.80
	6	8	8	16	+	1.45	4.10
	7	4	3	7	+	0.50	0.92
	8	8	6	14	+	0.90	1.90
	9	9	7	16	+	0.80	3.20
	10	8	8	16	+	1.70	2.50
	11	5	10	15	+	1.30	1.70
	12	3	3	6	-	0.40	0.11
		6.4	6.4	12.8±1.5[f]	7/8+	1.0±0.2[f]	2.2±0.5[f]
$PGF_{2\alpha}$/FSH-P	13	3	6	9	-	0.70	0.07
	14	6	4	10	-	0.72	0.39
	15	2	0	2	+	0.55	0.73
	16	17	8	25	-	2.40	0.79
	17	8	6	14	-	1.10	0.48
	18	6	0	6	-	0.51	0.27
	19	2	2	4	-	0.39	0.22
	20	1	2	3	-	0.43	0.29
		5.6	3.6	9.2±2.7[f]	1/8+	0.9±.3[f]	0.5±.1[e]

[a] Hormonal treatments: MAP - medroxyprogesterone acetate; $PGF_{2\alpha}$ - prostaglandin $F_{2\alpha}$; FSH-P - follicle stimulating hormone-pituitary extract.

[b] Based on observations on Day 3.

[c] + = normal luteal tissue; - = abnormal luteal tissue (premature luteal regression) determined on Day 3 and confirmed on Day 6.

[d] Total luteal mass from the left (Day 3) or right (Day 6) ovary/ewe.

[e,f] Mean column values (±SEM) with different superscripts differ ($P<0.05$).

TABLE 6. Influence of hormonal treatment and luteal condition on luteal tissue development

Luteal condition[a]	No. of ewes	Mean CL weight/ewe (mg) Day 3	Day 6
Normal luteal function			
Control	4	305.0 ± 81.7 (n = 4 CL)	525.0 ± 49.8 (n = 4 CL)
MAP/FSH-P	7	163.6 ± 22.7 (n = 49 CL)	363.2 ± 45.5 (n = 49 CL)
$PGF_{2\alpha}$/FSH-P	1	550.0 (n = 1 CL)	730.0 (n = 1 CL)
Overall mean		242.9 ± 44.0[b]	450.6 ± 42.7[c]
Premature luteal regression			
MAP/FSH-P	1	133.3 (n = 3 CL)	33.0 (n = 3 CL)
$PGF_{2\alpha}$/FSH-P	7	203.9 ± 40.39 (n = 43 CL)	90.4 ± 15.3 (n = 31 CL)
Overall mean		228.4 ± 42.7[b]	83.7 ± 14.8[d]

[a] Hormonal treatments: MAP - medroxyprogesterone acetate; $PGF_{2\alpha}$ - prostaglandin $F_{2\alpha}$; FSH-P - follicle stimulating hormone-pituitary extract.

[b,c,d] Mean (± SEM) values with different superscripts differ ($P<0.05$).

Figure 6. Mean (±SEM) temporal progesterone, FSH and LH profiles for control ewes beginning at the onset of detected estrus (Day 0).

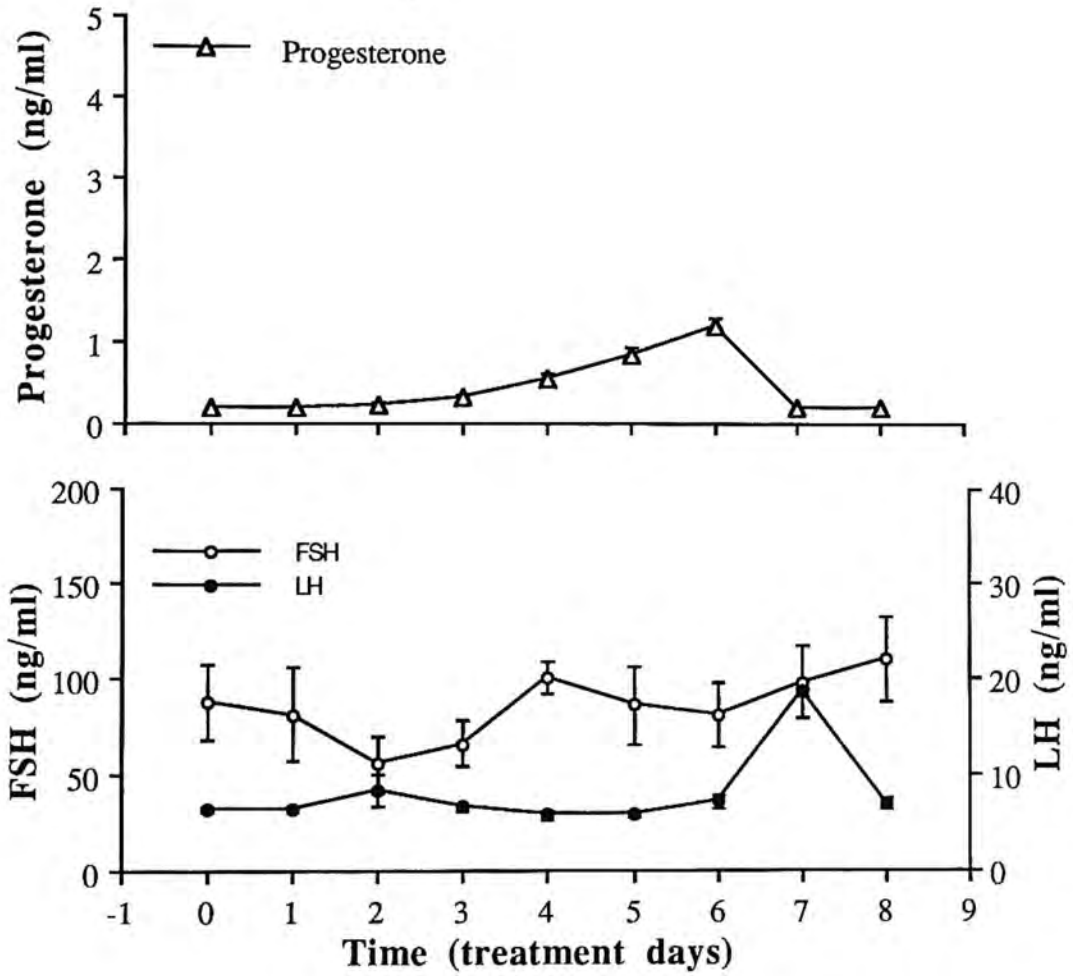


Figure 7. Mean (±SEM) temporal estradiol-17β, progesterone, FSH and LH profiles for FSH-P-treated ewes with normal CL beginning 12 h after estrous synchronization treatment. The time of estrus, artifical insemination (AI) and CL enucleation (CLX) are indicated.

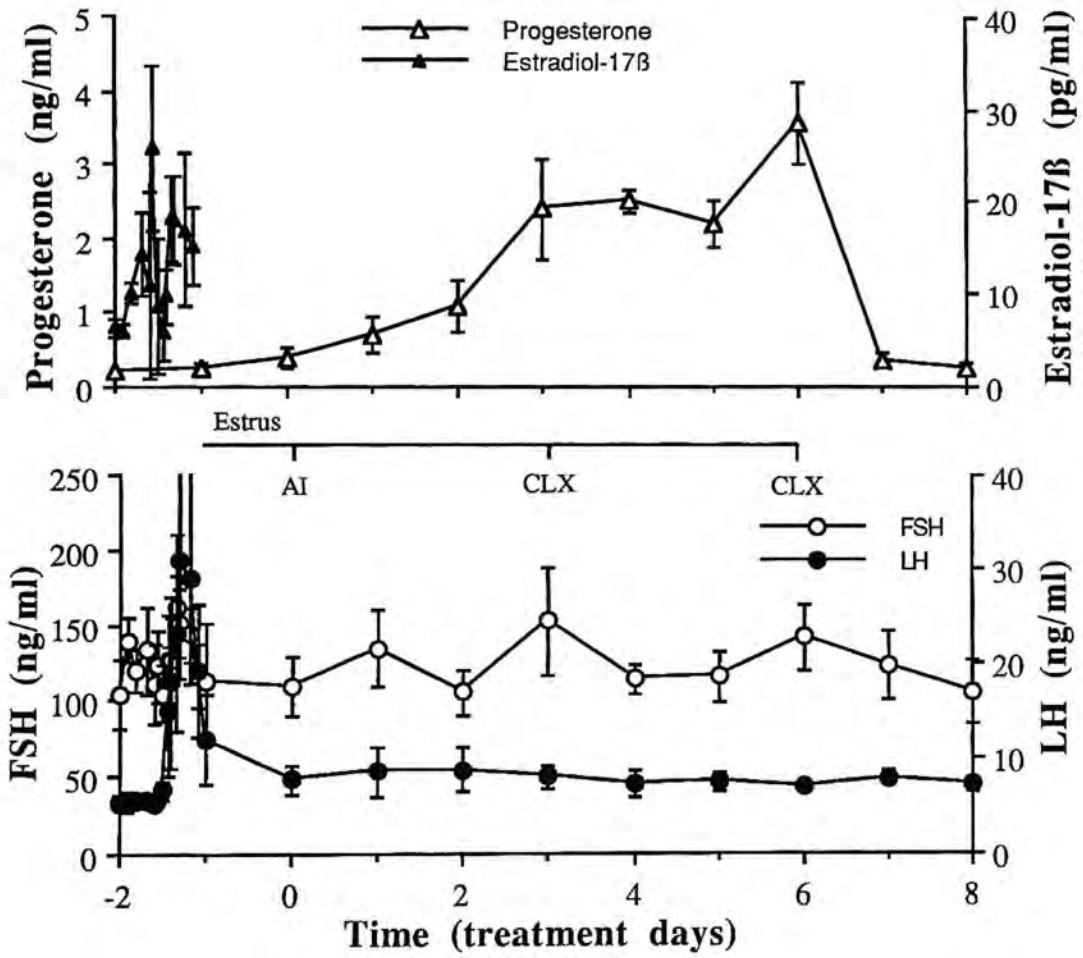


Figure 8. Mean (±SEM) temporal estradiol-17β, progesterone, FSH and LH profiles for FSH-P-treated ewes with prematurely regressed CL beginning 12 h after estrous synchronization treatment. The time of estrus, artifical insemination (AI) and CL enucleation (CLX) are indicated.

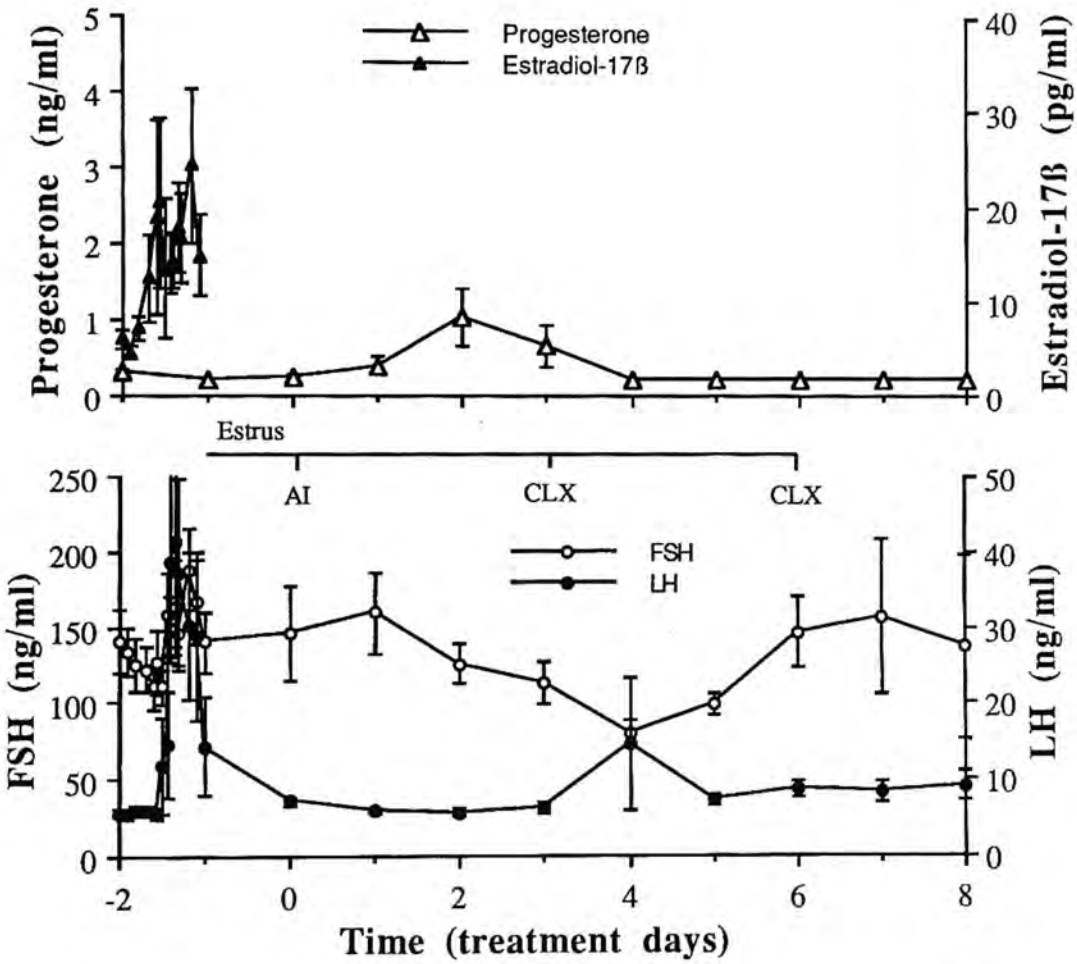


TABLE 7. Influence of gonadotropin treatment and CL condition on cell sizes of ovine luteal tissue dissociates on Day 3 and 6 of the estrous cycle

Group	No. of ewes	Dissociates (extrapolated to cells/entire CL) Day 3 luteal cell population (X 10^6) large	small	total	Day 6 luteal cell population (X 10^6) large	small	total
Control/sham	4	2.2±0.4	5.1±2.0[a]	7.2±1.6[b]	3.01±1.1	9.25±0.8[a]	12.3±0.4[a]
Normal CL	8	4.2±1.5	13.5±3.3[b]	17.8±4.7[a]	4.38±1.7	25.69±5.3[b]	30.1±6.7[b]
Premature luteal regression	8	2.9±1.0	6.6±1.6[ab]	9.6±2.4[ab]	ND	ND	ND

[a,b] Mean (± SEM) column values with different superscripts differ ($P<0.05$).
ND = non-detectable.

TABLE 8. Effect of luteal status and hormonal treatment on the steroid content and receptor populations of luteal tissue

Corpus luteum condition	No. of ewes	Progesterone content µg/mg luteal tissue		LH receptor fmol/mg luteal tissue		$PGF_{2\alpha}$ receptor (bound [M]/50,000 luteal cells) high affinity (X 10^{-10})		low affinity (X 10^{-9})	
		Day 3	Day 6	Day 3	Day 6	Day 3	Day 6	Day 3	Day 6
Control	4	0.09[b] ±0.02	0.09[b] ±0.01	1.74 ±1.28	3.43[a] ±1.77	2.26 ±0.69	4.13 ±1.16	1.68[a] ±0.23	2.65 ±0.95
Normal	8	0.17[a] ±0.02	0.36[a] ±0.06	0.93 ±0.16	3.33[a] ±0.70	3.09 ±1.12	2.45 ±0.65	1.55[ab] ±0.43	2.77 ±1.00
Premature luteal regression	8	0.12[ab] ±0.03	0.06[b] ±0.01	1.98 ±0.94	0.72[b] ±0.14	1.43 ±0.53	------ ------	0.98[b] ± 0.23	------ ------

[a,b] Mean column values (± SEM) with different superscripts differ ($P<0.05$).

Figure 9. Repesentative Scatchard analysis plots for LH receptor binding are illustrated for a control (top panel) and MAP/FSH-P-treated (bottom panel) ewe with normal CL on Day 6.

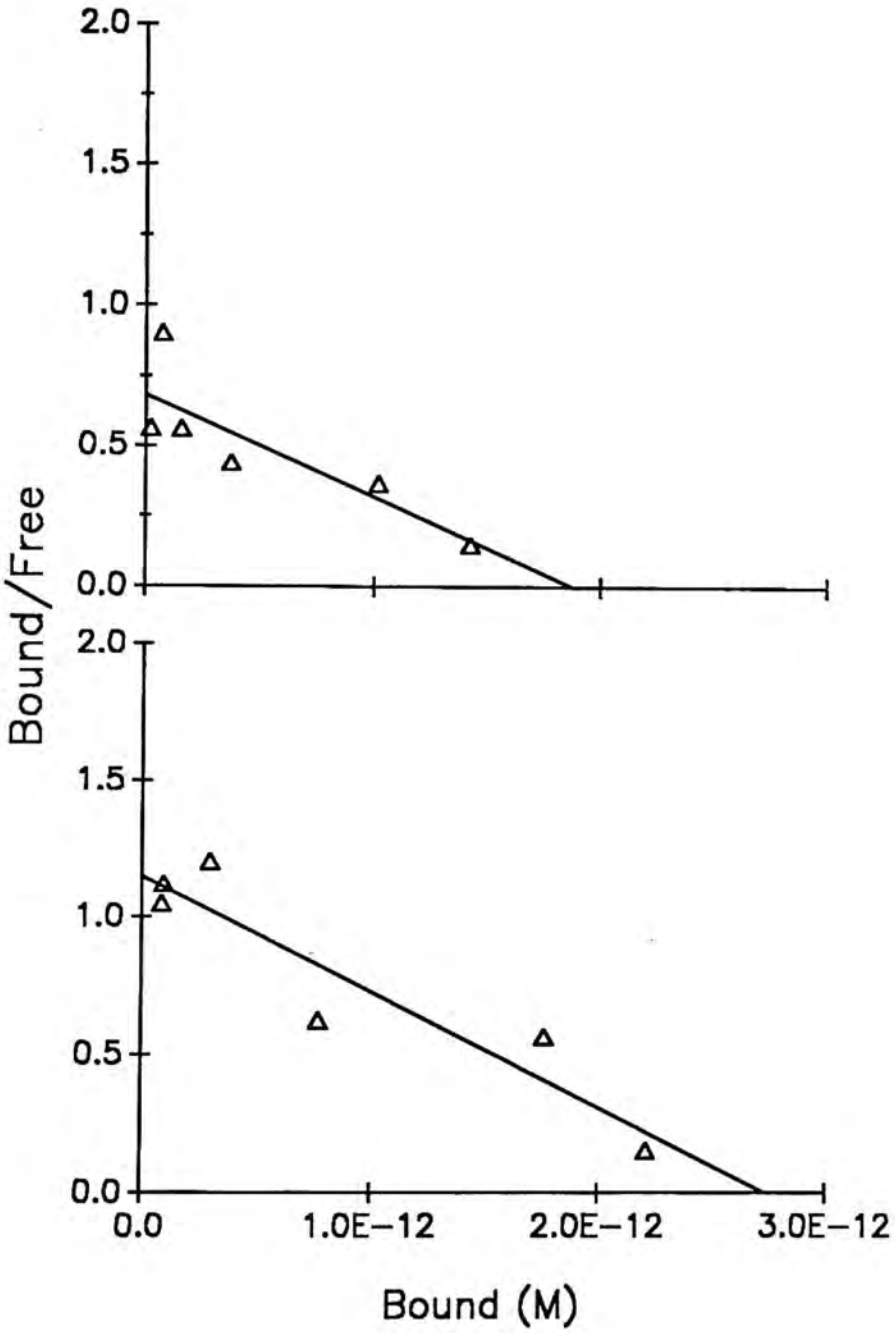
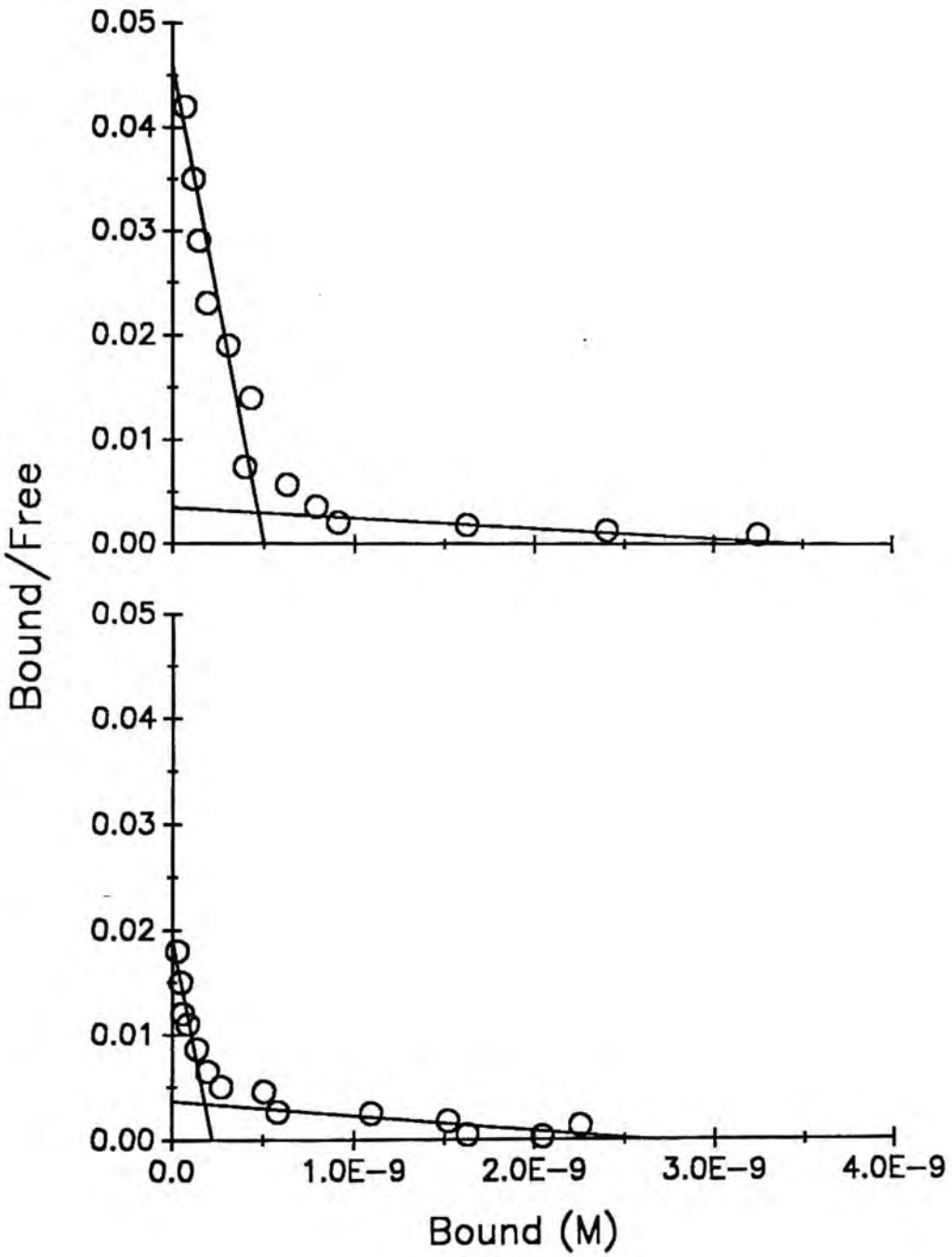

2.0
1.5
1.0
0.5
0.0
Bound/Free
2.0
1.5
1.0
0.5
0.0
0.0
1.0E−12
2.0E−12
3.0E−12
Bound (M)

Figure 10. Repesentative Scatchard analysis plots for $PGF_2\alpha$ receptor binding are illustrated for a control (top panel) and MAP/FSH-P treated (bottom panel) ewe with normal CL on Day 6. The binding patterns reveal the presence of both high affinity (vertically directed slope) and low affinity (horizontally directed slopes) binding sites.


Bound/Free
0.05
0.04
0.03
0.02
0.01
0.00
0.0
1.0E−9
2.0E−9
3.0E−9
4.0E−9
Bound (M)

CHAPTER III

OVINE EMBRYO CRYOPRESERVATION: ANALYSIS OF CRYOPROTECTANT, COOLING RATE AND *IN SITU* STRAW DILUTION USING CONVENTIONAL FREEZING OR VITRIFICATION

INTRODUCTION

Sheep embryos were first cryopreserved in a 1.5 M dimethyl sulfoxide (DMSO) solution using a slow cooling/slow warming procedure (Willadsen et al., 1976). Shortly thereafter, a rapid cooling/rapid warming technique was found to be effective (Willadsen, 1977; Willadsen et al., 1977). In both cases potential osmotic stresses before and after freezing were reduced by the gradual equilibration of embryos in cryoprotective solutions using multiple steps. Time and labor were decreased when it was shown that cattle embryos could survive a 2-step pre-freeze equilibration and 4-step post-thaw cryoprotectant dilution (each equilibration interval requiring ~10 min; Lehn-Jensen et al., 1981). Further technical simplification was made possible when Leibo (1983) reported that thawed cattle embryos could be diluted out of the cryoprotectant solution in a single step by placing embryos into a sucrose solution. The embryos osmotically dehydrate as the cryoprotectant leaves the cells and once equilibrium has been restored, are rehydrated in normal isotonic medium.

The type of cryoprotectants used has changed over time with glycerol replacing DMSO for cattle embryo cryopreservation (Bouyssou and Chupin, 1982; Lehn-Jensen, 1986). Glycerol and other glycols also are effective for sustaining the post-thaw viability of mouse embryos using conventional freezing procedures (Miyamoto and Ishibashi, 1978, 1983; Miyamoto et al., 1979; Rall and Polge, 1984). Particularly noteworthy was the observation that propylene glycol (1, 2-propanediol) forms a more stable glass phase than glycerol (Renard and Babinet, 1984). This is important because it may reduce the chance of embryonic or zona pellucida damage caused by ice crystal formations occurring during warming and cooling. The utility of glycerol (Ware and Boland, 1987) and ethylene glycol (Heyman et al., 1987) for freezing sheep embryos has received only minimal attention.

In the 1980's, straw containers became the preferred form of storage vessel for cattle embryos. These finding were based on comparative studies with glass ampules (Massip et al., 1979; Lehn-Jensen, 1986) and proven effectiveness in the commercial embryo transfer industry (Elsden et al., 1982; Seidel et al., 1983; Shea et al., 1983; Massip et al., 1984). An *in situ* sucrose dilution procedure, referred to as the 1-step® straw was developed to facilitate thawing and directly placing the embryo into the cow's uterus in a single convenient step (Leibo, 1983, 1984, 1986). Leibo calculated that a 0.7 to 1.4 M sucrose solution was needed to completely remove intracellular cryoprotectants from embryos previously equilibrated in 1.0 to 1.4 M glycerol solutions. Initial efforts to freeze sheep embryos in a 1-step straw containing 0.25 M sucrose suggested that the survival rate of thawed embryos was reduced significantly when compared to multi-step dilution (Merry et al., 1984). However, 1.0 M sucrose recently was reported to be effective for diluting cryopreserved sheep embryos in straws (Ware and Boland, 1987).

New approaches have been developed to cryopreserve embryos rapidly. Each procedure involves the partial dehydration of embryos before subzero cooling. Using a 2-step process, cattle embryos survive exposure to 1.5 to 2.5 M cryoprotectant/0.5 to 1.0 M sucrose solution mixture and -30°C for 30 to 240 min before being transferred directly into liquid nitrogen (LN_2; Bui-Xuan-Nguyen et al., 1984). More recent studies have demonstrated that the combined use of high molar concentrations (3.5 M) of permeable cryoprotectants (glycerol or propylene glycol) plus non-permeable sucrose facilitates direct freezing of mouse and cattle embryos in LN_2 vapor (Massip et al., 1984, 1986, 1987). Even without sucrose in the freezing solution, 2.0 M ethylene glycol, glycerol and propylene glycol solutions sustain high mouse embryo survival rates following direct LN_2 vapor freezing after seeding at -7°C (Miyamoto, 1986; Miyamoto and Ishibashi, 1986). The most novel cryopreservation procedure developed recently is "vitrification" (Fahy et al., 1984) which uses high concentrations of cryoprotectants (4.0 to 6.5 M) to prevent ice crystal formation during cooling. To-date, vitrification has been applied successfully to mouse (Massip et al., 1984; Rall and Fahy, 1985; Rall et al., 1987), rabbit (Smorag et al., 1989) and cattle (Massip et al., 1986, 1987; Rall, unpublished data) embryos.

The general aim of this project was to assess the sensitivity of the pre-implantation sheep embryo to cryopreservation by examining the influence of cryoprotectant, cooling rate and different approaches to cryopreservation (freezing versus vitrification). This was accomplished by: 1) comparing the *in vitro* and *in vivo* survival of sheep embryos after cryopreservation in glass ampules using glycerol or propylene glycol; 2) determining the highest subzero temperature that slow cooling (0.3°C/min) can be interrupted by rapid cooling (direct transfer to LN_2) without altering the viability of cryopreserved embryos; and

3) comparing the efficacy of cryopreservation by conventional freezing methods and "1-step" straws using glycerol or propylene glycol or to vitrification in a glycerol-based solution.

MATERIALS AND METHODS

Study 1

Embryo collection, handling and cryopreservation. Seventy late morula- to blastocyst-stage embryos were surgically recovered from adult, donor ewes pre-treated with either progestogen (MAP, Depo-Provera®, Upjohn Co., Kalamazoo, MI; 60 mg) intravaginal pessaries for 12 days or prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, Lutalyse®, Upjohn Co.; 15 mg, i.m., 12 days apart) (Schiewe et al., 1990c). Ovarian follicular growth was induced by i.m. injection of follicle stimulating hormone-pituitary extract (FSH-P, Schering Veterinary Supplies, Kenilworth, NJ; dosage: 5, 4, 4, 3, 3, 2 mg at 12 h intervals) beginning 36 h before $PGF_{2\alpha}$ injection or MAP-pessary removal (Schiewe et al., 1990b). Donor ewes were both naturally mated and artificially inseminated 6 days before flushing the uterus for embryos (Schiewe et al., 1990c).

Embryos were quality graded (QG) within 30 min of recovery by rating morphological appearance as excellent to poor (1 to 4 scale, respectively; Schiewe et al, 1987b, 1990c). In brief, this classification system consisted of 4 quality grades (QG) s: QG 1 = normal embryo, spherical shape, symmetrical; QG 2 = embryo with minor imperfections such as some extruded blastomeres or slightly asymmetrical shape; QG 3 = embryo experiencing partial degeneration or other irregularities including extruded blastomeres or vesiculations; and QG 4 = abnormal embryo with severe blastomere degeneration or lysis. After grading, embryos were maintained for 1 to 3 h in PB1 medium (Whittingham, 1974; Appendix Table 20) containing 20% fetal calf serum (FCS) at room temperature (~21°C). Embryos were equilibrated in 0.75 M and 1.5 M solutions of glycerol (Sigma Chemical Co., St. Louis, MO; n = 35) or propylene glycol (Sigma Chemical Co.; n = 35) in PB1 and frozen individually in 0.2 ml of fresh 1.5 M glycerol or propylene glycol in glass ampules. Ampules were prepared from 6 X 50 mm test tubes (Kimble Co., Owens, IL) as follows: 1) tubes were washed in 7X soap (Linbro®, Flow Laboratories, McLean, VA), rinsed in distilled water and air dried; 2) tubes were heated in a propane flame and pulled to produce a fine tapered neck (<0.25 mm diameter; Willadsen, 1977); and 3) each ampule was dry-heat sterilized. After the embryo-cryoprotectant solution was added to the vial, the ampule neck was heat-sealed using a propane flame.

The ampules were frozen in a Planer Products R204 LN_2 vapor freezing unit (TS Scientific, Quakerstown, PA) as follows: 1°C/min to -5°C; seeded at the meniscus with a forceps pre-cooled in LN_2; equilibrated for 10 min; 0.3°C/min to -15, -20, -25, -30 or -35°C (n = 10 embryos/treatment) and then plunged into LN_2 for storage. The temperature of a replicate glass tube containing 0.2 ml of cryoprotective solution with a 26 ga thermocouple (BAT-12, Bailey Scientific, Sattlebrook, NJ) was used to precisely detect subzero temperatures. After 3 to 8 months of storage in LN_2, ampules were thawed rapidly (>300°C/min) for 20 sec in a 37°C water bath, the embryo was aspirated from the ampule using a glass pipette and then placed into fresh 1.5 M cryoprotective solution for 5 to 10 min. Embryos were transferred into a 0.75 M sucrose-PB1 solution for 10 min to remove intracellular glycerol or propylene glycol and then washed twice in PB1 medium to eliminate residual sucrose and permit the blastomeres to rehydrate. After 10 min equilibration in PB1 medium, embryos were evaluated morphologically and assigned another QG rating. Furthermore, zona pellucida integrity was examined carefully for the presence of distinct cracks or fracture damage (Schiewe et al., 1987b). Following post-thaw evaluations, 50 embryos (n = 25/cryoprotectant treatment) were assigned subsequently to *in vitro* culture analysis (Experiment 1). An additional 20 embryos (n = 10/cryoprotectant treatment) that were plunged at -35°C were assigned to an embryo transfer trial (Experiment 2).

In vitro culture (Experiment 1). Thawed embryos were placed in 3 ml of Whitten's medium (Whitten, 1959; Appendix Table 23), supplemented with 1.5 mg/ml bovine serum albumin (BSA) in 10 X 35 mm Petri dishes (Falcon, Becton-Dickinson Labware, Lincoln Park, NJ) and cultured at 37°C in a humidified atmosphere of 5% CO_2 air. After 24 h, embryos were quality graded and assigned a fluorescence viability grade (VG). The VG rating was based on the fluorescence intensity of the cellular mass of each embryo on a graded scale (5 = very bright, uniform fluorescence to 0 = no fluorescence; Schiewe et al., 1985a, 1987b). This assay measures the metabolic activity and membrane integrity of blastomeres as determined by the intracellular illumination produced by the enzymatic conversion of fluorescein diactetate to photoreactive fluorescein (Mohr and Trounson, 1980). Embryos were exposed to a 2.5 µg fluorescein diactetate (Sigma Chemical Co.)/ml PB1 solution for 1 min, washed in fresh PB1 medium and then evaluated using a fluorescence microscope (Leitz Orthoplan, Bunton Instruments Co., Rockville, MD). Embryos with a VG rating of 3.0 or more were considered to have survived the freeze/thawing procedure.

Embryo transfer (Experiment 2). Estrus was synchronized in recipient females using a MAP-pessary/PMSG regimen (Schiewe et al., 1990c). On Day 6 of the recipient's synchronized estrous cycle, frozen embryos were thawed (37°C), diluted from the cryoprotectant and graded as described above. Within 30 min of thawing 2 embryos were transferred to each of 10 recipients by transabdominal laparoscopy (Schiewe et al., 1984). Pregnancy was diagnosed by rectal ultrasound 28 to 35 days and again 42 to 49 days after embryo transfer.

Study 2

Embryo cryopreservation and *in vitro* culture. Embryo donor ewes were synchronized with MAP-pessaries and treated with FSH-P (as described in Study 1). Embryos were collected, maintained in PB1 holding medium for 1 to 3 h at room temperature (21°C) and those with a QG of 1 or 2 were assigned randomly to the cryopreservation treatments. Some embryos were equilibrated in 0.75 M and 1.5 M glycerol (n = 30) or propylene glycol (n = 30) in PB1 at 20°C for 10 min each while others were assigned to vitrification (n = 15). Embryos equilibrated with glycerol and propylene glycol were divided equally into either ampule or 1-step® straw treatments (n = 15 embryos/cryoprotectant/container). Embryos frozen in ampules were processed as described for Study 1. For the 1-step® straw treatment, embryos were individually aspirated into a 1 cm column of fresh 1.5 M cryoprotectant (approximately 25 µl) within a 0.25 ml plastic insemination straw (Veterinary Concepts, Inc., Spring Valley, WI). The embryo-cryoprotectant column was buffered bilaterally by a 0.5 cm air space. A 6.8 cm column of 1.0 M sucrose-PB1 solution was positioned adjacent to 1 air space and a 1.0 cm column of 1.5 M cryoprotective solution next to the opposite air space (Leibo, 1983; Rall and Meyer, 1989; Fig. 11). Both ends of each straw were hermetically-sealed using an impulse heat sealer (Veterinary Concepts, Inc.) which melted and crimped the opposing edges against a heated electrode.

All 1.5 M glycerol- and propylene glycol-treated embryos were frozen using the equipment and conditions described in Study 1. Ampule and straw containers were plunged into LN_2 at -35°C. The 1-step® straws were seeded at the meniscus of the cryoprotectant- and sucrose-medium columns on either side of the air space buffers surrounding the cryoprotectant-embryo column. Ampules were seeded as described in Study 1.

Embryos designated for vitrification were first rinsed in PB1 containing 6% BSA for 5 to 10 min at 20°C (Rall, 1987). Embryos were transferred into a solution of 1.625 M glycerol-PB1/BSA solution (designated 25% VS3a) and allowed to equilibrate for 20 min.

During this time, the straw container was prepared. First, a 7.5 cm column of sucrose was injected into the straw using a 27 ga, 3.8 cm long needle attached to a 1 ml syringe until it wetted the plug. Then, a 1 cm column of VS3a solution (6.5 M glycerol plus 6% BSA in PB1) was placed adjacent to the sucrose column but separated by a 0.5 cm air space. Care was exercised to prevent wetting the wall of the air space with either solution. After equilibration in 25% VS3a, embryos were transferred in a ~3 µl volume into 2 ml of 4.2 M glycerol-PB1/BSA (65% VS3a) and gently mixed by swirling with the transfer pipette. The pipette was rinsed in 65% VS3a and then, after 1.5 min of exposure, the embryos were transferred into the 100% VS3a column in the straw. The straw was heat-sealed at both ends and, after 1 min of exposure to the 100% VS3a solution, plunged directly into cold N_2 vapor in a "dry" LN_2 shipping tank (MVE, Model BD5, Cryo-Associates, Rockville, MD). After 3 min, the straws were transferred into LN_2 for storage. Embryos cryopreserved by all methods were stored (-196°C) for 1 week to 3 months.

Ampules were thawed and embryos diluted using 0.75 M sucrose-PB1 medium as described for Study 1. Frozen 1-step® glycerol and propylene glycol straws were removed from LN_2 and placed vertically (plug end up) in room air for 2 min. The outer surface of the straw was wiped dry and, then while holding the unplugged end, the straw was shaken in a downward arc 3 to 5 times to mix the sucrose and cryoprotectant solutions. The straw was placed in a vertical position (plug end down) in a 37°C water bath for 3 min and then transferred into a room temperature (20°C) water bath for another 2 min (vertical position, plug end up). Straws containing vitrified embryos were removed from LN_2, held horizontally for 10 sec in room air and placed (plug end up) in a room temperature water bath. After 10 sec, these straws were wiped dry, shaken and incubated in water baths as described above. The heat seal was cut from the unplugged end and also between the wet and dry sections of the plug. The wet plug was pushed into the straw with a fine cannula (<1 mm diameter) to eject the fluid contents into a Petri dish. The shrunken embryos were collected and transferred into 2 ml of PB1 medium for 10 min to allow for osmotic rehydration. Embryos were placed into individual wells of a 24-well tissue culture plate (Falcon 3024, Becton-Dickinson Labware) each containing 1 ml aliquots of culture medium (Dulbecco's modified Eagles Medium, DMEM; Dulbecco and Freeman, 1959; Appendix Table 24) supplemented with 8 mg/ml BSA, 10% FCS, 4.5% glucose and 1% antibiotic-antimycotic (Gibco Laboratories, Grand Island, NY). Embryos were cultured in a 5% CO_2 humidified air incubator at 37°C for 1 week. About half of the culture medium was replaced daily to reduce metabolic by-products which potentially could inhibit *in vitro* embryo development. Morphological evaluations, including stage of development and QG rating were recorded at 24 h intervals from the start of *in vitro* culture. During the culture

interval, particular emphasis was placed on observations of embryonic degeneration, blastocyst hatching, blastocoelic expansion, trophoblastic attachment to and outgrowth from the plastic culture surface and possible monolayering of embryonic stem cells on the culture surface. Embryos were referred to as trophoblastic outgrowths (TBO) because the trophectoderm was viable and growing whereas inner cell mass development was not apparent. After 1 week of culture, many TBO began shedding embryonic stem cells from the expanding vesicle to form a monolayer of cells on the culture plate surface.

Embryo transfer. Twenty-one late morula- to blastocyst-stage embryos cryopreserved by the glycerol, propylene glycol or VS3a 1-step® straw method (n = 7 embryos/treatment) were transferred into recipient ewes by the procedures described above. Pregnancy was diagnosed by rectal ultrasound as described in Study 1.

RESULTS

Study 1

Cryoprotectant treatment did not influence post-thaw embryo survival based on overall mean QG and VG assessments (Table 9). Overall embryo integrity, especially the viability rating, depended on the temperature at which slow cooling was terminated and rapid cooling initiated (i.e., the plunge temperature). Although overall QG ratings were similar ($P>0.10$) among plunge treatment groups at 24 h of culture, the mean VG rating was higher ($P<0.05$) for the -35°C compared to the -15, -20 and -25°C treatment groups. Additionally, the proportion of embryos meeting the survival criterion (VG≥3.0) was greater ($P<0.05$) for embryos plunged at -30 or -35°C (19 of 20) compared to those plunged at warmer temperatures (18 of 30 embryos plunged at -25°C or higher).

Embryo quality was similar for all treatment groups before freezing and immediately after thawing and dilution (Fig. 12). Measurable effects of cryoprotectant were detected only after 24 h of culture. At that time, there was no significant interaction of glycerol and plunge treatment on the QG of embryos (Fig. 12). However, when propylene glycol was used the QG of embryos plunged at -15 and -20°C was lower after 24 h of culture than those plunged at -30 or -35°C. The VG values of glycerol-treated embryos plunged at -30 and -35°C were higher ($P<0.05$) than those plunged at -20 and -25°C (Table 10). Furthermore, VG values were higher ($P<0.05$) when propylene glycol-treated embryos were plunged from -30°C or lower compared to -15 or -25°C. The most significant effect of cryoprotectant was on post-thaw zona pellucida integrity. Damage to the zona pellucida varied from small hairline fractures to fragmentation and loss of zona

sections which created a channel between the extracellular medium and the perivitelline space (Fig. 13). This damage was much more prevalent in embryos exposed to glycerol (40%) than propylene glycol (4%) (Table 10). The occurrence of freeze-induced zona damage did not depend on plunge temperature (Table 10).

Embryos frozen in either the glycerol or propylene glycol solution were capable of resulting in the birth of live-born lambs after transfer to recipient ewes. Pregnancy was confirmed by ultrasound in 1 of 5 and 4 of 5 recipients receiving glycerol- or propylene glycol-treated embryos, respectively. However, 1 pregnant recipient receiving a propylene glycol-treated embryo failed to deliver an offspring, likely due to fetal death and resorption.

Study 2

Neither the type of cryoprotectant (glycerol versus propylene glycol) nor freezing container (straw versus ampule) affected the QG ratings of sheep embryos immediately post-thawing or at 24 or 48 h of culture ($P>0.10$, Table 11). After a total of 48 h culture, most embryos still were "good" or higher quality. The embryo QG's for vitrified embryos were no different ($P>0.10$) from that measured using the conventional freezing approaches.

The results supported the finding of Study 1 that the use of a specific cryoprotectant appeared related to the incidence of zona pellucida damage. Within the ampule treatment group, glycerol-treated embryos had a higher ($P<0.05$) likelihood of zona pellucida damage. However, zona pellucida integrity also depended on the type of freezing container. Fourteen zonae of the 45 (31%) embryos frozen in ampules were damaged compared to only 2 of 45 (4%) embryos cryopreserved in straws ($P<0.05$; Table 11).

Dilution of embryos within the straw freezing container simplified handling procedures. Regardless of the cryoprotectant used, thawed embryos diluted by using the "1-step" approach were less ($P<0.05$) likely to degenerate and more likely to develop *in vitro* than embryos diluted after recovery from ampules. This translated into more ($P<0.05$) expanded blastocysts (at 48 h of culture), hatched blastocysts (between 48 to 96 h of culture) and embryos with trophoblastic vesicles and outgrowths (between 96 and 168 h of culture) in the straw compared to ampule treatments. Compared to the *in situ* sucrose straw approach, vitrification resulted in a similar number of embryos reaching the expanding blastocyst stage within 48 h culture. But after prolonged culture, vitrified embryos more closely mimicked the poorer results of the ampule treatment (Table 11).

All hatching blastocysts continued to develop and expand *in vitro* for up to 1 week and formed large vesicles (0.5 to 1 mm in diameter) which usually attached to the culture dish surface (trophoblastic outgrowths). After 1 week of culture, embryos cryopreserved

in straws using propylene glycol had the the highest incidence ($P<0.05$) of development to embryonic cell monolayers compared to all other treatments.

Post-thaw QG of embryos transferred to recipient ewes was not different regardless of the cryopreservation method (mean, 1.6 ± 0.2) and was comparable to that reported for embryos in the *in vitro* culture studies (Table 11). Only 1 of 21 (4%) embryos transferred had a damaged zona pellucida. Live-born offspring were produced in each of the 3 treatment groups (Table 12) and 3 of 7 thawed embryos previously frozen in propylene glycol and straw containers resulted in lambs. Furthermore, 2 lambs were produced after cryopreserving embryos by vitrification and 1 lamb following glycerol/1-step straw treatment.

DISCUSSION

These are the first results demonstrating the *in vitro* and *in vivo* development of sheep embryos cryopreserved using either propylene glycol cryoprotectant or vitrification. In Study 1, the efficacy of glycerol or propylene glycol for sustaining sheep embryo viability after cryopreservation in ampule containers was similar to that reported by Willadsen and coworkers (1977) using DMSO and ampules. In those studies, DMSO was diluted from the thawed embryos by a time-consuming, 6-step dilution procedure. Later studies (Merry et al., 1984; Heyman et al., 1987; Ware and Boland, 1987) demonstrated that glycerol or ethylene glycol-frozen sheep embryos survive a 3-step dilution process without compromising post-thaw survivability. Our results indicated that sheep embryos withstand pre-freeze equilibration in glycerol or propylene glycol using an accelerated 2-step approach. The ability of sheep embryos to equilibrate rapidly without experiencing blastomere lysis is comparable to that of glycerol-treated cattle embryos (Lehn-Jensen et al., 1981; Lehn-Jensen, 1986). This suggests that, under the described conditions, the embryos of both species respond similarly osmotically during equilibration in cryoprotectant.

Unlike cattle embryos which survive rapid cooling (plunging into LN_2) beginning at ~-20°C (Lehn-Jensen, 1986), sheep embryos were sensitive to plunge temperatures warmer than -30°C. However, some sheep embryos survived following rapid cooling at -15°C, especially after exposure to glycerol, but the viability was less than observed at temperatures of -30 to -35°C. One role of cryoprotectants is to reduce potentially damaging intracellular ice-crystal formation during rapid cooling at subzero temperatures greater than -40°C. This probably results from the interaction of cryoprotectant with structural and

cryoprotectant with structural and biochemical components within cells and the binding of all water (Doebler and Rinfret, 1962; Boutron, 1986). Cryoprotectants also act on a colligative basis to increase the viscosity of the various intracellular compartments, which results in the liquid cytoplasm forming a metastable glass during rapid cooling (Mazur, 1963; Rall et al., 1984). The variation among species in embryo tolerance to rapid cooling (early plunge temperatures) may be related to inherent biochemical differences within the blastomeres. This variability in biochemical constituency could affect the stability of the intracellular glass phase as the embryo passes through the various stages of rapid cooling or warming. It is likely that the type and concentration of cryoprotectant also plays a critical role in dictating the point at which embryos can be rapidly cooled. In the present study, there was a tendency for glycerol-treated embryos to have a higher rate of post-thaw viability at the warmer plunge temperature. It is tempting to interpret this finding as reflecting a more efficient ability of glycerol to readily form a metastable glass phase than propylene glycol. However, the potential early colligative advantage of glycerol was transient as propylene glycol-treated embryos experienced a similar or greater post-thaw viability when rapid freezing was initiated at -30 to -35°C. Actual cryophysical data using erythrocytes demonstrates that propylene glycol results in fewer intracellular ice crystals than glycerol (Boutron, 1984; Boutron and Arnaud, 1984).

A reduction in zona pellucida damage occurred using propylene glycol compared to glycerol for sheep embryos cryopreserved in ampules. Similar levels of zona damage have been reported after rapid thawing of DMSO- and glycerol-treated sheep (Willadsen et al., 1976) and cattle (see review: Lehn-Jensen, 1986) embryos. Cryomicroscopic observations indicate that the damage is caused by fracture planes which can puncture or crack the zona (Lehn-Jensen and Rall, 1983; Rall et al., 1984; Lehn-Jensen, 1986). Extracellular glasseous solids can fracture at less than -110°C probably because of expansion and contraction events associated with an increased temperature gradient (Rall and Meyer, 1989). Therefore, the increased incidence of zona damage observed in glycerol-treated embryos contained in glass ampules likely was related to a decreased efficiency to dissipate abrupt temperature fluctuations. A higher rate of zona integrity was retained when embryos were frozen in plastic straws as opposed to glass ampules. A similar phenomenon has been observed in cattle embryos (Massip et al., 1979) and unfertilized ova (Rall and Meyer, 1989). In thawing cattle ova, slow warming more efficiently prevents zona damage than rapid warming which appears related to an overall reduction in thermally-induced mechanical stress. The increased surface area and thin container walls of plastic straws result in a reduced temperature gradient and fewer fracture planes (Rall and Meyer, 1989). The reduction in zona damage of propylene glycol-treated embryos probably was related to

the cryoprotectant's ability to more effectively organize water molecules into a stable glass phase during rapid cooling/warming events (Renard and Babinet, 1984). Phase diagrams correlating cryophysical changes to temperature and cooling rates indicate that equimolar solutions of glycerol form more ice compared to propylene glycol at the -35°C plunge temperature (Boutron, 1984, 1986). These fundamental differences in the cryoprotectants may have altered crystals morphology. Structural differences in the glasseous solid could have made glycerol solutions more brittle and less tolerant of temperature gradient-induced expansion and contraction during rapid cooling and warming. In the context of the present results, then, it was logical to expect the observed result that the combined use of glycerol, thin-walled plastic containers and a slow post-thaw warming protocol most effectively sustained the integrity of the sheep embryo zona pellucida.

The effect of cryoprotectant and storage container on embryo survival paralleled the influence of these same factors on zona pellucida integrity. Using the described criterion for post-thaw survival, the glycerol-ampule treatment resulted in overall lower post-thaw embryo viability as well as the highest incidence of zona damage. However, it is unlikely that survival was directly affected by the same physical factors causing disruption of the zona. At the time of fracture plane formations, the cellular mass is shrunken (to ~40% of normal size) and the perivitelline space is expanded (Schneider and Mazur, 1984). These structural changes buffer the embryo from actual mechanical injury induced by extracellular freeze fracture formations. Therefore, we speculate that the comparatively lower embryo survival in the glycerol-ampule treatment group was unrelated to the concurrent increase in zona damage.

Overall post-thaw survival of embryos frozen in glycerol was not different from that using propylene glycol, and live offspring resulted following both treatments. The data from Table 11 indicated that, using the 1-step straw approach, propylene glycol leads to slightly higher embryo development *in vitro* and *in vivo* when compared to glycerol. Recent permeability studies indicate that glycerol equilibrates in the sheep morula more slowly than propylene glycol (Szell et al., 1989). Therefore, if larger scale studies demonstrate that glycerol-diluted sheep embryos do have reduced developmental potential, the cause could be related to either inadequate intracellular equilibration prior to rapid cooling or osmotic stress caused by incomplete removal of glycerol from the blastomeres during sucrose dilution. Until detailed studies are conducted, our data suggest that there is no major difference between glycerol or propylene glycol in combination with the *in situ* sucrose straw dilution approach. It also is interesting to note that ethylene glycol, a cryoprotectant even more permeable than propylene glycol, has been reported to be effective for cryopreserving sheep embryos (Heyman et al., 1987). These investigators

reported that a low (0.25 M) concentration of sucrose is efficacious for diluting embryos by the 1-step method.

For conventional embryo freezing procedures, the 1-step straw with *in situ* sucrose dilution simplifies the thawing and diluting process. However for vitrification, 1-step *in situ* dilution offers an added benefit by allowing for rapid dilution of a highly concentrated cryoprotective solution potentially injurious to embryos at temperatures above 0°C. Another vitrification solution, designated VS2, contains the highly permeable cryoprotectant propylene glycol but is toxic to mouse embryos at 0°C after only 20 min of exposure (Rall, 1987). Therefore, VS3a (used in the present study) generally is considered safer for embryos maintained at ambient temperature (Rall et al., 1987) because glycerol permeates relatively slowly. The VS3a mixture also contains albumin (a high molecular weight polymer) which stabilizes the plasma membrane, especially after thawing (Grill et al., 1980). Polymers are known to reduce the leakage of cytoplasmic constituents and water and prevent subsequent cell lysis caused by mechanical damage to cell membranes (Edidin and Petit, 1977; Grill et al., 1980; Williams, 1983). Because the glycerol in VS3a does not fully permeate into blastomeres before vitrification, glycerol permeation will resume after thawing. Therefore, immediate *in situ* sucrose dilution reduces potential injury due to toxic exposure and osmotic stresses caused by increased influx of glycerol. In the present study, most thawed vitrified sheep embryos exhibit normal cell membranes, cytoplasmic inclusions and quality grades of 1 or 2 (excellent/good) for up to 24 h of culture. Therefore, we speculate that the embryo degeneration associated with vitrification probably was more related to the well-know cytotoxic effects of the solutions than to osmotic or mechanical damage to the blastomeres. Although most vitrified embryos developed to expanded blastocyst *in vitro*, fewer hatched or formed trophoblastic vesicles, outgrowths or monolayers compared to other straw treatments. It has been demonstrated that the embryonic inner cell mass (fetal precursor tissue) is more sensitive to certain chemical toxins than the surrounding trophoblast (Snow, 1973). Therefore, it is possible that the inner cell mass (ICM) of these embryos was selectively damaged by the concentrated vitrification solution which could have interferred with further development.

Two vitrified embryos transferred to recipient ewes resulted in live-born offspring which complemented the previous live births produced with vitrified mouse (Rall et al., 1987), rabbit (Smorag et al., 1989) and cattle (Massip et al., 1986, 1987; Rall, unpublished data) embryos. Although the VS3a medium was used to successfully cryopreserve sheep embryos, the overall vitrification procedure requires refinement to improve post-thaw viability rates to those achieved by the conventional 1-step straw approach. Because it is a simple, low cost embryo cryopreservation technique, vitrification

deserves considerably more attention as a biotechnique for preserving germ plasm. Because this procedure eliminates the need for an expensive, programmable LN_2 freezing unit and requires only the appropriate high molar cryoprotectant solution and LN_2 vapor, vitrification has considerable potential for cryopreserving embryos under field conditions. Such applications will be extremely useful for embryo preservation studies involving less developed countries or wildlife species.

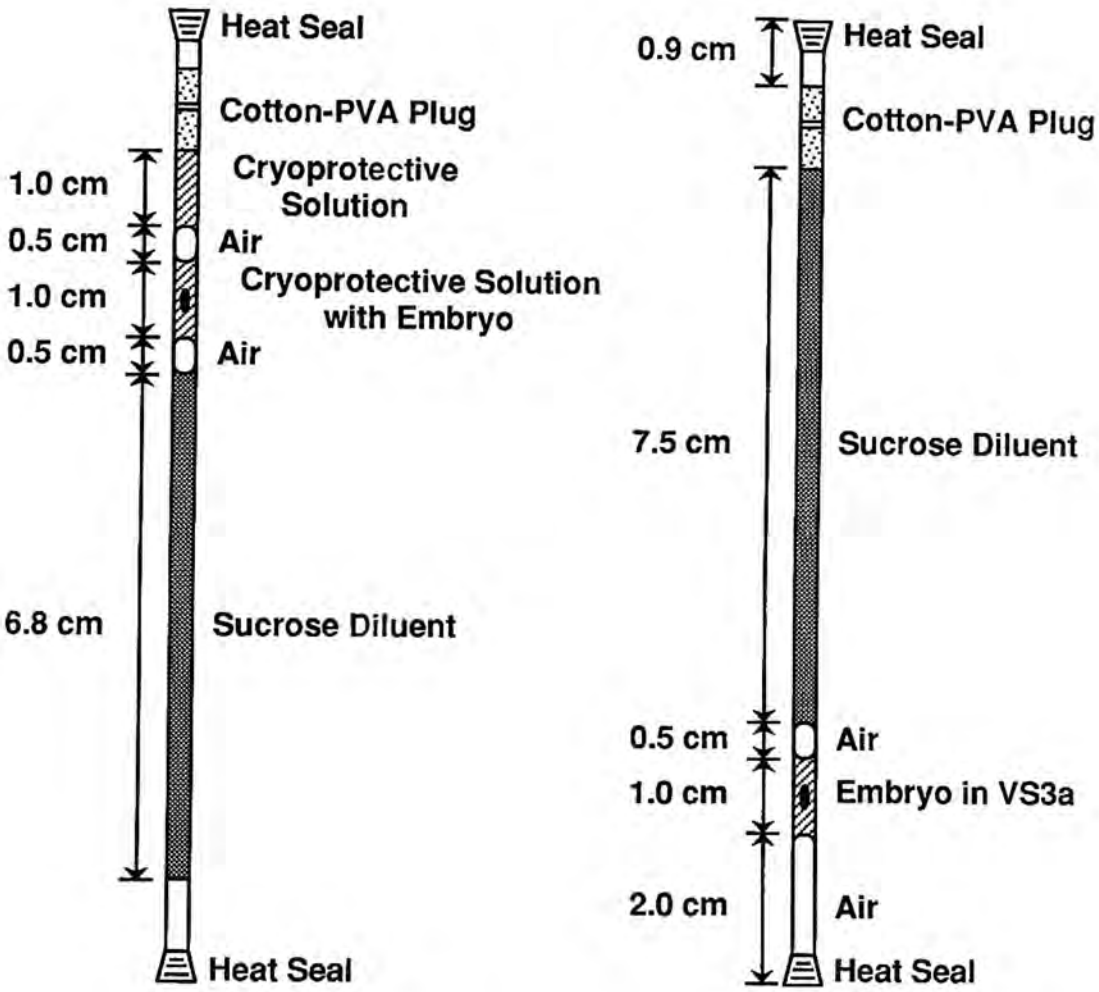
TABLE 9. Influence of cryoprotectant and plunge temperatures on the post-thaw quality, viability and zona pellucida integrity of cryopreserved sheep embryos

Evaluation trait	Cryoprotectant		Plunge temperature (°C)				
	Propylene glycol	Glycerol	-15	-20	-25	-30	-35
Quality grade:							
Pre-freeze	1.6±0.1	1.5±0.1	1.8±0.4	1.6±0.4	1.6±0.2	1.6±0.4	1.4±0.2
Post-thaw	2.0±0.2	2.1±0.2	1.8±0.4	2.0±0.3	2.2±0.4	2.2±0.5	1.8±0.4
24 h *in vitro* culture	2.4±0.2	2.5±0.2	2.6±0.5	2.8±0.4	2.2±0.4	2.2±0.4	2.0±0.5
FDA viability grade:							
24 h *in vitro* culture	2.9±0.2	3.1±0.3	2.7±0.5^{bc}	2.5±0.4^{c}	2.3±0.7^{c}	3.6±0.5^{ab}	4.0±0.4^{a}
Number of embryos with zona pellucida damage/number of treated embryos (%)	1/25 (4)^{a}	10/25 (40)^{b}	1/10 (10)	3/10 (30)	3/10 (30)	2/10 (20)	2/10 (20)

Quality and viability grades are expressed as means ± SEM.

^{a,b,c} Row values with different superscripts within primary treatment divisions differ ($P<0.05$).

Figure 11. Descriptive illustration of how the solutions are arranged in the conventional freezing (left) and vitrified (right) one-step straws.


Heat Seal
Cotton-PVA Plug
1.0 cm
Cryoprotective Solution
0.5 cm
Air
1.0 cm
Cryoprotective Solution with Embryo
0.5 cm
Air
6.8 cm
Sucrose Diluent
Heat Seal
0.9 cm
Heat Seal
Cotton-PVA Plug
7.5 cm
Sucrose Diluent
0.5 cm
Air
1.0 cm
Embryo in VS3a
2.0 cm
Air
Heat Seal

0.25 cc Straw

TABLE 10. Influence of plunge temperature on post-thaw viability and zona pellucida integrity of sheep embryos cryopreserved in glycerol or propylene glycol solutions

Evaluation trait	Plunge temperature (°C)	Glycerol	Propylene glycol
FDA viability grade[a]	-15	3.0±.6[bc]	2.4±.7[d]
	-20	2.4±.4[c]	2.6±.5[cd]
	-25	2.2± 7[c]	2.4±.6[d]
	-30	3.3±.5[bc]	4.0±.5[bc]
	-35	3.8±.4[b]	4.2±.4[b]
Proportion of embryos with zona pellucida damage (%)	-15	1/5 (20)	0/5 (0)
	-20	3/5 (60)	0/5 (0)
	-25	2/5 (40)	1/5 (20)
	-30	2/5 (40)	0/5 (0)
	-35	2/5 (40)	0/5 (0)

[a] Viability grades are expressed as means ± SEM after 24 h *in vitro* culture.
[b,c,d] Column values with different superscripts within evaluation traits differ ($P<0.05$).

Figure 12. Influence of glycerol and propylene glycol cryoprotectants on the maintenance of embryo quality (1 = excellent to 4 = poor/degenerate) following rapid cooling at 5 different subzero plunge temperatures.

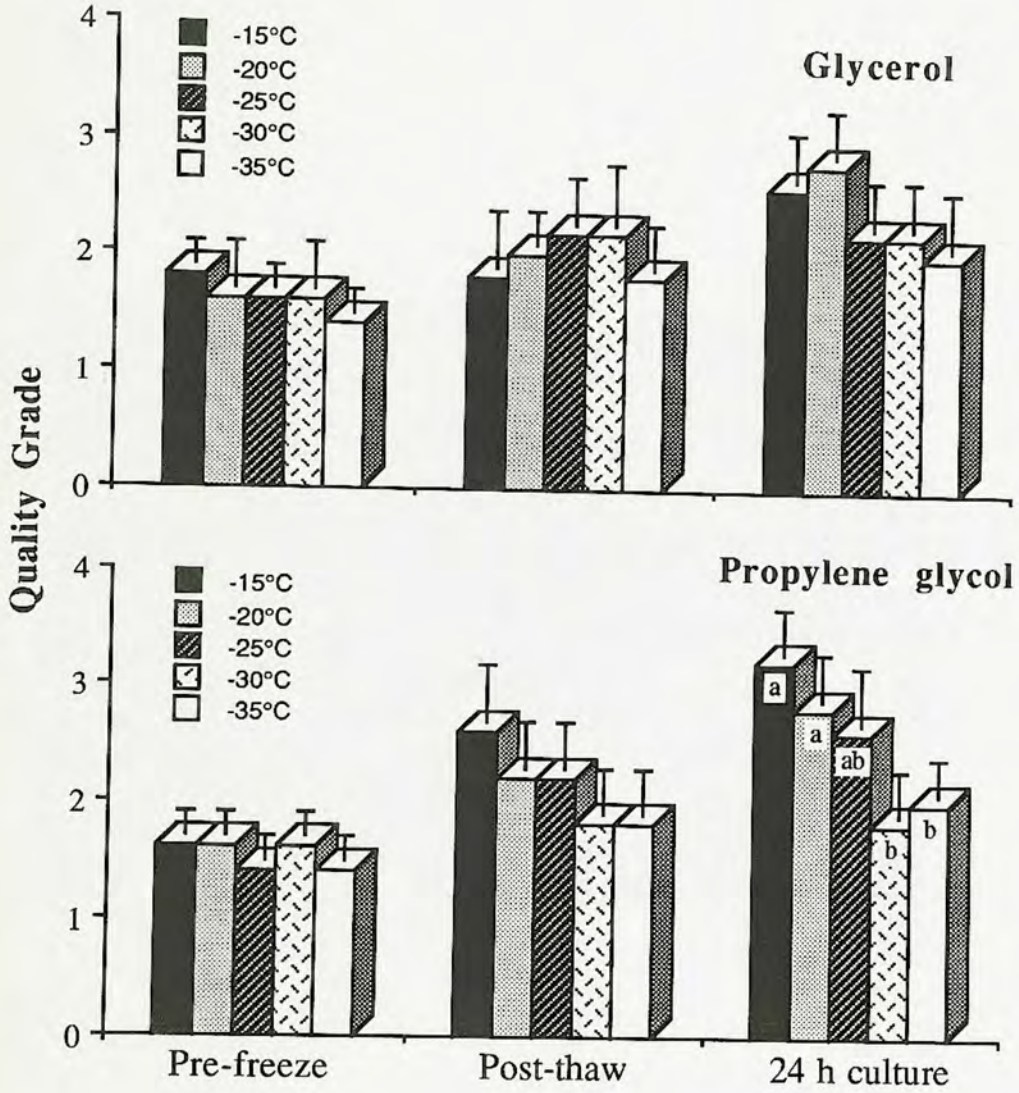

Glycerol
Propylene glycol
-15°C
-20°C
-25°C
-30°C
-35°C
Quality Grade
0
1
2
3
4
a
a
ab
b
b
Pre-freeze
Post-thaw
24 h culture

Figure 13. Fracture damage to a section of the zona pellucida of this cryopreserved sheep embryo occurred during the thawing process, however viability of the embryonic mass is not consequently altered.

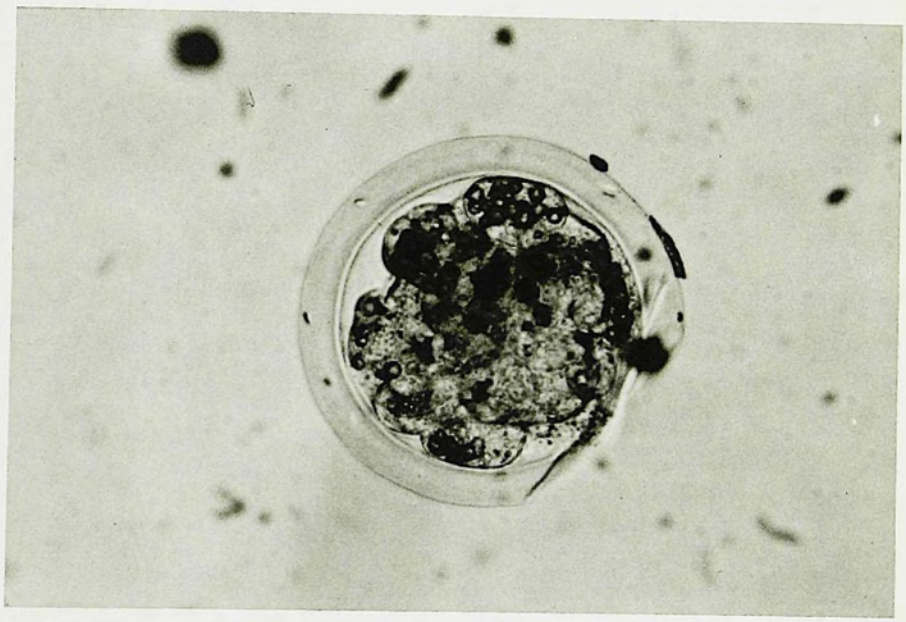


TABLE 11. Zona pellucida integrity and post-thaw viability of sheep embryos cryopreserved in glycerol versus propylene glycol and straws versus ampules compared to vitrification

Observation	Glycerol-straw	Propylene glycol-straw	Vitrification solution 3a-straw	Glycerol-ampule	Propylene glycol-ampule
Quality grade:					
0 h	2.0±0.3	1.8±0.3	1.8±0.2	2.3±0.3	2.2±0.3
24 h	2.0±0.3	1.9±0.3	1.9±0.2	2.5±0.3	2.3±0.4
48 h	2.2±0.3	2.0±0.3	2.3±0.4	2.8±0.4	2.6±0.4
Zona pellucida damage (%)	1/15 (6.7)^{bc}	1/15 (6.7)^{bc}	0/15 (0)^{c}	7/15 (46.7)^{a}	2/15 (13.3)^{b}
In vitro development:					
at 48 h					
No. of degenerate	4/15 (26.7)^{a}	4/15 (26.7)^{a}	6/15 (40.0)^{ab}	8/15 (53.4)^{b}	7/15 (46.7)^{b}
No. of expanded blastocysts	11/15 (73.4)^{a}	11/15 (73.4)^{a}	9/15 (60.0)^{ab}	7/15 (46.7)^{b}	8/15 (53.4)^{b}
from 48 to 96 h					
No. of hatched blastocysts	9/15 (60.0)^{ab}	11/15 (73.4)^{a}	6/15 (40.0)^{b}	6/15 (40.0)^{b}	7/15 (46.7)^{b}
from 96 to 168 h					
No. of vesicles	9/15 (60.0)^{ab}	11/15 (73.4)^{a}	6/15 (40.0)^{b}	6/15 (40.0)^{b}	7/15 (46.7)^{b}
No. forming trophoblastic outgrowths	8/15 (53.4)^{ab}	10/15 (66.7)^{a}	4/15 (26.7)^{b}	4/15 (26.7)^{b}	6/15 (40.0)^{b}
No. forming monolayers	3/15 (20.0)^{ab}	6/15 (40.0)^{a}	2/15 (13.4)^{b}	2/15 (13.4)^{b}	3/15 (20.0)^{ab}

Means are averages ± SEM.

^{a,b,c} Row values with different superscripts differ ($P<0.05$).

TABLE 12. Post-thaw embryo quality grade, pregnancy rate and number of lambs born after cryopreserving embryos in straws using glycerol, propylene glycol or a vitrification approach

Assessment	Glycerol	Propylene glycol	Vitrification solution 3a
No. of recipients[a]	7	7	7
Quality grade: 0 h	2.0±0.4	1.8±0.4	1.8±0.4
Zona pellucida damage (%)	1/7 (14.3)	1/7 (14.3)	0/7 (0.0)
No. pregnant	2	3	2
No. live offspring born	1	3	2

Values are expressed as percentages, means (± SEM) or proportions.
[a] One frozen-thawed embryo transferred/recipient.

CHAPTER IV

ESTROUS SYNCHRONIZATION, OVULATION INDUCTION AND EMBRYO CRYOPRESERVATION IN THE SCIMITAR-HORNED ORYX, BONGO, ELAND AND GREATER KUDU

INTRODUCTION

Ensuring the existence of free-ranging species and maintaining the genetic diversity of captive animal populations are worldwide concerns of conservation biologists. Artificial breeding and cryopreservation strategies could be useful for enhancing reproductive efficiency, conserving valuable germ plasm and maintaining a diverse gene pool for the future. Ovulation induction and embryo transfer procedures developed in conventional livestock species (see reviews: Foote and Onuma, 1970; Seidel, 1981; Sreenan, 1983, 1988; Smith, 1988) have been applied to a few nondomestic Artiodactyla including the eland (Dresser et al., 1982), oryx (Durrant, 1983), gaur (Stover and Evans, 1984), bongo (Dresser et al., 1985), yellow-backed duiker (Pope et al., 1988a) and suni antelope (Raphael et al., 1989). In each of these studies, ovarian activity was induced in at least some of the females using exogenous hormones. Births have been recorded from the transfer of eland (Dresser et al., 1982), bongo (Dresser et al., 1985), gaur (Stover and Evans, 1984; Pope et al., 1988b) and suni (Raphael et al., 1989) embryos, but these events usually have been "one-time" accomplishments. There is a need for more basic research, especially into physiological factors affecting successful embryo recovery and transfer. Reproductive biotechniques may not be adapted easily to wildlife taxa. These species are wild and often excitable and difficult to manage under conventional research conditions. There also is evidence that even closely-related species demonstrate many species-specific reproductive mechanisms (Wildt et al., 1988; Wildt, 1990b). Even within-species genotype plays a major role in dictating reproductive events (Bindon et al., 1986; Schmidt et al., 1985, 1987b). Genetic differences among and within species illustrate the many challenges to be anticipated as embryo techniques are applied to nondomestic ruminants.

The scimitar-horned oryx (*Oryx dammah*) is indigenous to the arid regions of Northern Africa where wild populations are threatened because of uncontrolled hunting, warfare and excessive grazing by domestic livestock (Nowak and Paradiso, 1983;

Grzimek, 1984). Observations of captive animals reveal that the oryx has a nonseasonal polyestrous reproductive cycle, achieves sexual maturity at ~11 months of age and has a gestation of 242 to 256 days (Knowles and Oliver, 1975). Few details are known about the reproductive physiology of the oryx, although urinary monitoring and steroid conjugate analysis suggest that the estrous cycle averages 20 to 24 days in duration (Durrant, 1983). This paper describes two studies, the first conducted to test the hypothesis that ovulation induction and embryo cryopreservation procedures developed in a domestic sheep model (Schiewe et al., 1990c, 1990d) could be applied effectively to the scimitar-horned oryx. A second study was performed to determine whether these embryo-related techniques could be applied to bongo (*Tragelaphus euryceros*), eland (*Taurotragus oryx*) and greater kudu (*Tragelaphus strepsiceros*), 3 related antelope species. These large-sized, nondomestic ruminants are indigenous to Central and Southern Africa (Grzimek, 1984) and, like the oryx, little information is available on their reproductive physiology. Limited data suggests that they are polyestrous with an average estrous cycle and gestation of 21 to 23 days and a gestation length of 7 to 9 months in length, respectively (Nowak and Paradiso, 1983).

Specific experiments were designed to: 1) evaluate the comparative effectiveness of progestogen-impregnated vaginal pessaries versus prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) for estrous synchronization (oryx only) and of exogenous pregnant mares' serum gonadotropin (PMSG) and follicle stimulating hormone-pituitary extract (FSH-P) to induce multiple ovulations; 2) determine the feasibility of collecting uterine-stage embryos using a nonsurgical approach; 3) evaluate the influence of treatment confinement and physical manipulations (anesthesia, rectal palpation, uterine catheterization/flushing) on animal well-being; and 4) examine the efficacy of the cryoprotectants dimethyl sulfoxide, glycerol and propylene glycol for cryopreserving embryos while evaluating the ability of thawed embryos to result in live-offspring after embryo transfer. Overall, these studies were designed to determine the applicability of artificial breeding strategies while simultaneously developing a database on the reproductive physiology and endocrinology of these species.

MATERIALS AND METHODS

GENERAL METHODS

Animals and facilities. A herd of 24 scimitar-horned oryx maintained on 12 hectares of pasture at the Conservation and Research Center (CRC), National Zoological Park, Front Royal, VA, were used as embryo donors and recipients. Two mature, fertile breeding males were housed in areas isolated from the females except during periods of

breeding. Four to 8 weeks before scheduled embryo collection or transfer, oryx were captured from the field and relocated to a 750 m^2 corral containing a barn with adjoining solid-sided stalls to facilitate animal containment and darting. At this time, 1 or 2 males also were translocated to adjacent pens in visual and olfactory proximity to females. All animals were fed a grain concentrate and alfalfa hay diet twice daily and allowed free access to water.

Eighteen multiparous scimitar-horned oryx served as embryo donors on 1 to 3 occasions per year. Four to 6 females were assigned to various treatments simultaneously during each embryo collection phase. Three oryx from the CRC herd and females from the Detroit Zoological Park (n = 3), the Metro-Miami Zoo (n = 5) and Kings Dominion (n = 5) served as embryo recipients. To determine the effect of herd isolation, confinement and frequent manipulations on animal weight, 6 CRC donor females were weighed weekly for 8 consecutive weeks during the combined estrous synchronization, gonadotropin treatment, rectal palpation and embryo recovery interval.

For the antelope species, donor females were maintained at either the National Zoological Park (Washington, DC; bongo, n = 3), Milwaukee Zoological Gardens (Milwaukee, WI; bongo, n = 1), Metro-Miami Zoo (Miami, FL; eland, n = 3), Kings Dominion (Doswell, VA; eland, n = 4) or the Detroit Zoological Park (Royal Oak, MI; greater kudu, n = 2). During the course of this study, all females were handled and treated within the normal captive surroundings (i.e., zoo exhibit areas).

Anesthesia and surgical preparation. Rectal palpation, medroxyprogesterone acetate (MAP)-pessary treatments, blood sampling and embryo collection/transfer procedures required animal anesthesia. Feed and water were withheld 48 and 24 h before all anesthetic procedures, respectively. The anesthesia regimen involved the combined intramuscular (i.m.) administration of xylazine (Rompun®, Haver-Mobay Corp., Shawnee, KS) and etorphine (M99, Lemmon Co, Sellersville, PA) at a dosage of 0.2 mg/kg and 0.025 mg/kg for oryx, 0.07 mg/kg and 0.008 mg/kg for bongo, 0.2 mg/kg and 0.009 mg/kg for eland and 0.08 mg/kg and 0.008 mg/kg for greater kudu, respectively, delivered by a carbon dioxide (CO_2)-air pistol or rifle dart. A surgical plane of anesthesia was maintained with ketamine hydrochloride (Vetalar®, Parke-Davis, Detroit, MI; 50 to 100 mg), as needed, by intravenous (i.v.) supplementation. At the conclusion of the immobilization interval, anesthesia was reversed by i.v. administration of diprenorphine (M50/50, Lemmon Co.) and yohimbine (Lance Co., Boulder, CO) antagonists. The dosage administered was the same for each drug, but varied by species (0.05, 0.016, 0.018 and 0.016 mg/kg for oryx,

bongo, eland and greater kudu, respectively). Each animal was confined in the barn until alert and fully recovered.

Embryo collection,handling and evaluation. A Foley catheter (16 to 22 FG, 30 ml balloon cuff, Bardex, Veterinary Concepts, Inc., Spring Valley, WI) with stainless steel stylette (to provide catheter rigidity) was inserted intracervically into each uterine horn with assistance *per rectum*. The catheter was secured *in utero* by inflating the catheter cuff with 10 to 20 ml of sterile water. The stylette was removed and a 3-way Y-junction connector system (Veterinary Concepts, Inc.) was attached to the catheter and an embryo filtering device (VCI®, Veterinary Concepts, Inc.) on opposite ends. Each uterine horn was flushed separately with 250 to 500 ml of Dulbecco's phosphate buffered saline (Veterinary Concepts, Inc.) containing 2% heat-inactivated fetal calf serum (FCS, Gibco Laboratories, Grand Island, NY) and 1% antibiotic-antimycotic (Gibco Laboratories). This was accomplished by repeatedly infusing 20 to 60 ml of flush medium via the remaining syringe-fitted connector end. Flush medium was recovered through the catheter, connector system and filtering device, with the residual medium collected into a 1 l flask. To reduce infection risk, the uterus was infused with 50 ml of a 2% Nitrofurazone solution (A.J. Buck Co., Baltimore, MD) immediately post-flushing. An injection of $PGF_2\alpha$ (25 mg. i.m.) was given to induce corpora lutea (CL) demise and eliminate the risk of a multiple offspring pregnancy caused by unrecovered embryos. Unfiltered flush medium was decanted and the filter thoroughly rinsed into 100 x 15 mm Petri dishes.

Dishes were searched by stereomicroscopy (Wild M-5, Bunton Instruments Co., Rockville, MD) for the presence of embryos and unfertilized ova (UFO). Embryos were transferred into 3 ml of holding medium (PB1 medium containing 20% FCS; Whittingham, 1974) and maintained in 20°C room air until subsequent evaluation, quality grading and transfer or cryopreservation. Morphological assessments were performed at 200 to 400X. Unfertilized ova were recognized as an unicellular mass with a smooth vitelline membrane and uniform, granulated cytoplasm which may have been fragmented due to deterioration. Embryos were identified as having multiple blastomeres. The developmental stage of each embryo was recorded as being 2- to 16-cells, morula, late (compact) morula, early blastocyst, blastocyst, expanded blastocyst or hatched blastocyst. The integrity of the zona pellucida was evaluated in frozen-thawed embryos to assess the extent of damage, especially cracks caused by cryopreservation and thawing. An embryo quality grade (QG) of 1, 2, 3 or 4 was assigned to embryos of excellent, good, fair or poor/degenerate quality, respectively (Lindner and Wright, 1983; Schiewe et al., 1987b). The quality ratings were based on the following appearances: QG 1 = normal embryo, spherical shape, symmetrical;

QG 2 = embryo with some imperfections such as extruded blastomeres or slightly asymmetrical shape; QG 3 = embryo experiencing partial degeneration or other irregularities including extruded blastomeres or vesiculations; and QG 4 = abnormal embryo with severe blastomere degeneration or lysis.

Radioimmunoassays. Hormone concentrations were determined in unextracted sera using commercially available ^{125}I-double-antibody radioimmunoassay (RIA) kits (Radioassays Systems Inc., Carson, CA) as previously detailed (Schiewe et al., 1990b, 1990c). The first and second antibodies (in the form of precipitating solutions) for the progesterone assay were rabbit 11α-hydroxyprogesterone-11α-hemisuccinate-human serum albumin and goat anti-rabbit gamma globulin, respectively. Cross-reactivities to the first antibody were: progesterone, 100%; 20α-dihydroprogesterone, 6.25%; deoxycorticosterone, 3.20%; corticosterone, 0.42%; 17α-hydroxyprogesterone, 0.15%; pregnenolone, 0.06%; androstendione, 0.04%; testosterone, 0.03%; 11-deoxycortisone, pregnenolone sulfate, cholesterol, dihydroepiandrosterone, etiocholanolone, estradiol-17β, estrone, estriol, estradiol-17α, androsterone, cortisol and aldosterone, <0.01%. Serum (100 µl aliquots) was incubated (37°C) in radiolabelled progesterone and first antibody for 60 min. The second antibody then was added, tubes shaken and the samples centrifuged for 20 min (2500 X g, 4°C). After decanting the supernatant, the radioactivity in the pellets was measured by gamma spectrometry. The standard curve consisted of 0.2 to 40.0 ng/ml aliquots of progesterone, and all samples were assessed in duplicate. For the standard curve, the amount of progesterone from each species needed to cause 50% inhibition (ED_{50}) was 3.6 ng/ml. The minimum detectable progesterone concentration in this assay was 0.2 ng/ml, based on the ED_{95} level. The assay was validated for each species by examining parallelism of inhibition curves (Appendix Fig. 20) and by performing mass recovery analysis (oryx: $Y=-0.969 + 1.34X$, $r=0.97$; bongo: $Y=-1.23 + 1.48X$, $r=0.97$; eland: $Y=-1.05 + 1.32X$, $r=0.98$).

Serum cortisol levels were measured using a commercially available ^{125}I-double-antibody RIA kit (RIANEN™, New England Nuclear, N. Billerica, MA). A combined first and second antibody was used consisting of a rabbit cortisol antibody pre-reacted with an antiserum to rabbit gamma globulin. The cross-reactivities to the rabbit cortisol antibody included: cortisol, 100%; cortisol 21-glucuronide, 100%; prednisolone, 62.8%; corticosterone, 14.7%; 11-deoxycortisol, 4.3%; 17α-hydroxyprogesterone, 3.5%; 11-deoxycorticosterone, 1.9%; prednisone, 1.8%; cortisone, 1.6%; progesterone, 1.6%; dexamethasone, 0.6%; androstendione, 0.4%; dihydrotestosterone, 0.4%; testosterone, 0.2%; aldosterone, 0.2%; spironolactone, 0.2%; tetrahydrocortisol, 0.1%; tetrahydro-11-

deoxycortisol, 0.07%, tetrahydrocortisone, pregnenolone, cholesterol, diethylstilbesterol, metyrapone and estradiol-17β, <0.02%. Duplicate serum samples (10 µl) were incubated for 30 min with the combined first and second antibodies. Samples then were centrifuged at 4°C for 10 min (2500 X g), the supernatant decanted and the radioactivity of the remaining pellets measured by gamma spectrometry. The standard curve consisted of cortisol added at concentrations of 10 to 500 ng/ml. In this assay, an ED50 level was reached by adding 90.2 ng/ml of cortisol from each of the species. The minimum detectable cortisol concentration was 6.2 ng/ml, based on the ED95 level. The assay was validated for each species by confirming parallelism of inhibition curves (Appendix Fig. 21) and performing mass recovery analysis ($r \geq 0.99$; oryx, $Y=-19.1 + 1.14X$; bongo, $Y=-16.9 +1.29X$; eland, $Y=2.0 + 0.91X$; greater kudu, $Y=-7.5 + 1.07X$).

Study 1, Trial 1

Estrous synchronization, induction of ovulation and embryo collection. For synchronization of estrus, donor females were assigned randomly either to a 60 mg MAP (Depo-Provera®, Upjohn Co., Kalamazoo, MI) vaginal pessary treatment (Schiewe et al., 1990c) involving a 16 day insertion interval (n = 17) or to a 25 mg and 35 mg $PGF_2\alpha$ (Lutalyse®, Upjohn Co.) treatment (n = 16) administered 12 days apart, respectively. Likewise, estrous synchronization of recipient females was achieved either by MAP-pessary (n = 12) or $PGF_2\alpha$ (25 mg and 25 mg, 12 days apart, respectively; n = 12) treatment. To induce follicular development, donors randomly received either PMSG (Equitech, Inc., Atlanta, GA) as a single injection (2000 IU, i.m.; n = 16) or FSH-P (Schering Veterinary Supplies, Kenilworth, NJ) b.i.d. for 5 days (5 mg i.m. injections, 50 mg total; n = 17). Supplemental PMSG (500 to 2000 IU) was given 48 h later to PMSG treated donors with inactive ovaries as determined by rectal palpation. Administration of PMSG and FSH-P was initiated 72 h and 48 h before removal of MAP-pessaries or the second $PGF_2\alpha$ injection, respectively, as part of a 2 X 2 factorial design. Donors were introduced to a breeding male (2 or 3 females per male) 24 h after the final MAP or $PGF_2\alpha$ estrous synchronization treatments. Observations for behavioral estrus were made at least twice daily (0700 h and 1700 h) for 2 to 3 consecutive days with each animal being observed for 4 to 12 h/day. Recipient females received a single 500 to 1000 IU PMSG injection (i.m.) 48 h before pessary removal or $PGF_2\alpha$ injection. Because vasectomized teaser males were not available to detect estrus in recipients, the onset of estrus was estimated to occur 72 h after MAP-pessary removal or the second $PGF_2\alpha$ injection.

Rectal palpations of the reproductive tract were performed immediately before onset of estrous synchronization treatment, at the time of MAP pessary withdrawal or on the day

of the second $PGF_2\alpha$ injection and during the embryo collection procedure. At these 3 times, all surfaces of each ovary were palpated to determine the number and estimated size of preovulatory follicles and CL. Nonsurgical embryo recovery was performed 8 or 9 days after MAP-pessary removal or the second $PGF_2\alpha$ injection. Embryos were washed and maintained for 1 to 3 h at room temperature (20°C) in PB1 medium containing 20% FCS and then assessed for developmental stage and quality grade before freezing.

Embryo cryopreservation and transfer. Because facility limitations restricted the number of oryx available for use at one time, recipient females were not synchronized concurrently for fresh embryo transfers. Therefore, recovered oryx embryos were used to study their sensitivity to freezing and thawing. Embryos were diluted at 10 min intervals in 0.75 M and 1.5 M PB1 solutions containing either dimethyl sulfoxide (Sigma Chemical Co., St. Louis, MO; n = 10), glycerol (Sigma Chemical Co.; n = 10) or propylene glycol (Sigma Chemical Co.; n = 10) before being placed individually into 6 X 50 mm ampules containing 0.2 ml of a 1.5 M cryoprotective solution (Schiewe et al., 1990d). Embryos were cooled in a programmable liquid nitrogen (LN_2) vapor freezing unit (Planer Products Ltd., Model R204) as follows: 1°C/min to -6°C, seeded at the meniscus with a forceps precooled in LN_2, equilibrated for 10 min, 0.3°C/min to -35°C and finally plunged into LN_2 for storage. Embryos were thawed in a 37°C water bath for 20 sec, pipetted from the ampule, placed in fresh 1.5 M cryoprotectant for 5 min, diluted in 0.75 M sucrose-PB1 solution for 10 min and equilibrated in fresh PB1 medium for 10 min (Schiewe et al., 1990d). Each embryo was evaluated and quality graded before *in vitro* culture or transfer. Embryos were placed in a 35 X 10 mm Petri dish (Falcon, Becton-Dickinson Labware) containing 3 ml of culture medium (Dulbecco's modified Eagles Medium, DMEM; Dulbecco and Freeman, 1959; Appendix Table 24) with 8 mg/ml BSA, 10% FCS and 1% antibiotic-antimycotic (Gibco Laboratories, Grand Island, NY) to facilitate *in vitro* analysis (Schiewe et al., 1990d). Embryos were cultured in a 5% CO_2 humidified air incubator (37°C) for 3 days and evaluated for the occurrence of blastocyst expansion and hatching or degeneration. For *in vivo* analysis, ovaries of recipient females were palpated *per rectum* to confirm the presence of a prominent CL before embryo thawing and transfer. If the first embryo thawed was of QG 3 or 4, then a second embryo was thawed from the same cryoprotectant treatment group. One or 2 embryos were placed into a 0.25 ml plastic straw and the straw loaded into a 53 cm disposable embryo transfer device (Veterinary Concepts, Inc.). Preliminary data revealed that the oryx has a small, bifurcated cervical canal that was difficult to penetrate during the luteal phase. After the ovary containing the prominent CL was identified, the ipsilateral cervical canal was widened by performing a sham transfer,

that is, inserting a "blank" embryo transfer device through the canal to the interior os and maintaining it in that position for 5 to 10 min. The instrument was removed and replaced with the embryo transfer device containing a thawed embryo which was expelled into the anterior half of the uterine horn (total transfers = 8). For 1 recipient, transcervical insertion of the transfer device was not possible so embryos were inserted *in utero* using a transabdominal laparoscopic transfer technique, similar to that described for sheep (Schiewe et al., 1984). For this procedure, the anesthetized oryx was intubated and maintained on 2% halothane gas for approximately 45 min, the interval predicted necessary to confirm the presence of CL and thaw and introduce the embryos into the uterine lumen. The anesthetized animal was placed in a recumbent, head-down position on a surgical table and prepared for surgery. After insufflation of the abdominal cavity with 3 to 4 l of 100% CO_2 using a Verres needle, a 2 cm midline skin incision was made approximately 6 cm proximal to the umbilicus. A 10 mm diameter trocar-cannula was inserted through the peritoneum, the trocar replaced by a 10 mm laparoscope and a fiber optic light source attached to provide intraabdominal viewing of the reproductive tract (Roberts, 1968). After evaluating the ovaries and confirming the presence of a CL, an accessory 5 mm trocar-cannula was inserted and the trocar replaced by a Palmer forcep to grasp the ipsilateral uterine horn during the embryo transfer procedure (Schiewe et al., 1984). The embryo transfer process was facilitated by introducing a 16 ga, 5.5 cm long Teflon catheter placement unit (Cathlon IV®, Jelco Laboratories, Rariton, NJ) through the abdominal wall into the uterine horn. The stylette needle was replaced by a 3.5 FG tom-cat catheter (11.5 cm long, Monoject, St. Louis, MO) containing the embryos which were deposited into the uterine lumen.

Blood sampling and endocrine analysis. Blood samples were collected at times coinciding with anesthesia required for estrous synchronization treatment and/or rectal palpation. At least 10 females per estrous synchronization and gonadotropin treatment had a blood sample collected (10 ml) at the onset of progestogen-pessary or $PGF_{2\alpha}$ treatment (i.e., before any drug or hormone treatment) and during embryo recovery. Serum progesterone concentrations were characterized and correlated to ovarian response after gonadotropin treatment. Serial blood samples were collected at 15 min intervals for ~1.5 h from anesthetized donors (n = 20) during nonsurgical embryo collection to monitor adrenal activity by measuring serum cortisol concentration. Temporal cortisol profiles in these animals were compared to 2 other groups: 1) To evaluate the influence of anesthesia alone, 4 randomly cycling oryx were anesthetized and serially bled (as described above) with no rectal palpation or uterine catheterization/flushing; 2) To monitor maximal adrenal

responsiveness, 4 randomly cycling oryx were anesthetized (as above) and challenged with an exogenous bolus of synthetic adrenocorticotropin (ACTH, Cortrosyn®, Organon, West Orange, NJ; 75 units i.m. per female) given immediately after the first blood sample. These females also were serially bled as described above and blood sera were collected, stored (-80°C) and analyzed for progesterone and cortisol.

Study 1, Trial 2

The often poor ovulatory response to PMSG in Trial 1 was incentive to compare PMSG dose and 2 different PMSG batch preparations (i.e., lots). Twenty mature oryx were subjected to estrous synchronization using 25 mg Lutalyse® administered i.m. twice 12 days apart. On Day 10 (Day 0 = first $PGF_2\alpha$ injection) of treatment, donors were given either a single PMSG (Equitech, Inc.) injection (Group I, 2500 IU, Lot #501; Group II, 5000 IU, Lot #501; Group III, 2500 IU, Lot #503; n = 5 females/group) or multiple FSH-P injections (Group IV, 7, 7, 6, 6, 5, 5, 4, 4, 3 and 3 mg b.i.d. for 5 days, respectively) through Day 13. The PMSG source used for Groups 1 and 2 (Lot #501) represented the same preparation used in Study 1, Trial 1; Lot #503 (Group III) was analyzed by the commercial supplier (Equitech, Inc.) which reported that this preparation was approximately 1.5-fold more bioactively potent than Lot #501 based on a rat testicular bioassay (Papkoff, 1981). Donors were placed with a breeding male for natural mating (as in Trial 1) and estrous behavior checked twice daily (0700 h, 1700 h) over a 30 min interval. Embryos were nonsurgically recovered from anesthetized donors 8 to 9 days after the second $PGF_2\alpha$ injection. Ovarian status was determined by rectal palpation, the number of preovulatory follicles and CL/ovary recorded, and embryos were evaluated morphologically and assigned a quality grade.

Study 2

The estrous cycle of each female was synchronized by testing the utility of a dual-$PGF_2\alpha$ regimen consisting of 500 µg Estrumate® (Haver-Mobay Corp., Shawnee, KS) administered (i.m.) twice 12 days apart (Day 0 and Day 12, respectively). Estrumate®, was substituted for Lutalyse® (Study 1) because this product was more concentrated allowing for a reduced fluid volume (2 ml versus 5 ml, respectively) when administered by dart. Estrumate and Lutalyse products are considered equally effective for inducing luteolysis in cattle (Donaldson, 1984). To induce ovulation, females were injected (i.m.) with either 2500 IU PMSG (bongo, n = 5; eland, n = 5; greater kudu, n = 1; Lot #501) on Day 10 of treatment or FSH-P (bongo, n = 5; eland, n = 5; greater kudu, n = 1) twice daily for 5 d (decreasing dosage regimen: 7, 7, 6, 6, 5, 5, 4, 4, 3 and 3 mg, respectively)

beginning on Day 9. A conspecific breeding male was introduced on Day 12 after the second $PGF_{2\alpha}$ injection, except for eland at Kings Dominion in which no male was available. Seven days after the onset of estrus, donor females were anesthetized and the ovaries palpated *per rectum* to determine the number of ovarian follicles and CL. Nonsurgical embryo collection was attempted only in ovulating females. Embryos were evaluated morphologically and quality graded.

To provide baseline information on post-ovulation progesterone concentration and adrenal responsiveness to anesthesia and uterine catheterization/flushing, serial blood samples were collected at 15 min intervals as described for the oryx (Study 1, Trial 1). Sera were collected, stored (-80°C) and evaluated by radioimmunoassay for progesterone and cortisol.

Statistical analysis

A completely randomized design with a 2 X 2 factorial arrangement of treatments was used to assess the influence of different exogenous gonadotropins and estrous synchronization methods on multiple ovulation and embryo production in the oryx. Conversely, an apriori gonadotropin treatment design was used to assess the bongo, eland and greater kudu data. Analyses of primary treatment effects and treatment interactions were performed using analysis of variance (ANOVA) with a general linear model format and a computerized Statistical Analysis System (SAS, 1984). Differences among treatments were determined using Tukey's HSD procedure (Snedecor and Cochran, 1980). Data expressed as a proportion or percentage were evaluated by Chi square analysis (Snedecor and Cochran, 1980). Fluctuations and differences in endocrine profiles over time were evaluated using a 2-way ANOVA repeated measures program.

RESULTS

Study 1, Trial 1

The scimitar-horned oryx was determined to have a bifurcated cervix leading to separate uterine horns without an uterine body. This finding initially was based on rectal palpation and transcervical catheter passage attempts and later was confirmed by necropsy examination of an adult female at the Detroit Zoological Park (N. Richter, unpublished data). Palpation indicated that the oryx cervix consisted of 4 annular rings with the bifurcation generally beginning between the first and second ring. One female was an exception and displayed a double cervix with dual cervical os.

Within individual oryx, there often was a marked difference in ovarian size which ranged from estimates of ~1 to 24 cm^3. In only one case was more than 1 CL detected on the ovary of an oryx palpated before gonadotropin treatment. The difference in gonad size and the site of ovulations did not favor consistently ($P>0.10$) the left or right ovary.

Both MAP and $PGF_{2\alpha}$ proved equally effective ($P>0.10$) for synchronizing estrus, and overall, 25 of 33 (76%) synchronized/gonadotropin-treated oryx ovulated (Table 13). In all cases, the exogenous gonadotropin was ineffective in increasing the size or causing ovulation on the small inactive ovary (~1 cm^3) which was detected in 6 of 30 oryx. Of the 25 ovulatory females which had ≥2 CL, 57% had 2 to 5 CL and 19% had more than 11 CL. Mean number of CL/ovulating female tended to be higher ($P<0.10$) in the FSH-P compared to the PMSG treatment groups. When the data were analyzed within synchronization treatment, FSH-P produced a greater ($P<0.05$) ovulatory response than PMSG following $PGF_{2\alpha}$; however, the difference was not significant ($P>0.10$) using MAP. There was a wide variation in serum progesterone concentrations among individual ovulatory animals within treatments (Table 13). There was no effect ($P>0.10$) of estrous synchronization or gonadotropin treatment on circulating progesterone and the correlation with CL number was low and nonsignificant ($r = 0.39$, $P>0.10$).

Overt signs of estrus, including mounting, were observed in 15 of 33 (45%) of the donors. Males typically sniffed the genital region and freshly voided urine of estrual females and then demonstrated a Flehmen response. The male repeatedly struck the flank/hind leg region with a foreleg to test female receptivity before attempting a mount. Females were observed to be sexually receptive for as short as 30 min ($n = 12$) and as long as 12 h ($n = 1$). Copulation was observed on 8 occasions and consisted of 3 to 5 repeated mounts by the male followed by a strong ejaculatory thrust lasting less than 15 sec. Males achieved penetration only once/estrus. Female to female mating behavior was not observed.

Embryos and/or unfertilized ova were recovered in 53% of the flush attempts (mean, 4 embryos or ova/female; range, 1 to 16 embryos/female), but only from females with at least 1 prominent CL. There was no indication that retrograde flow of medium through the oviducts occurred during uterine flushing as medium recovery was excellent; generally 90 to 100% of infused PBS was recovered. Overall, 32 of 37 (92%) recovered ova were fertilized and 28 (87.5%) of these were rated with a QG of 1 or 2 (Table 14, Fig. 14).

A high proportion of late morula- to blastocyst-stage embryos (60%) maintained an excellent or good QG after thawing (Table 15). A higher ($P<0.05$) proportion of dimethyl sulfoxide- (70%) and glycerol-treated (60%) embryos maintained their prefreeze QG

compared to the propylene glycol counterparts (33%) as indicated by the number of QG 1 or 2 embryos after thawing. Post-thaw zona pellucida damage occurred in 10 of 30 embryos and was unaffected (P>0.10) by cryoprotectant treatment (Table 14). Two of 3 thawed embryos (1 dimethyl sulfoxide- and 1 glycerol-treated) cultured *in vitro* developed to the expanded and hatched blastocyst stages (Fig. 15). The transfer of thawed embryos into synchronized recipients failed to produce any offspring (Table 16). Only 3 of 9 recipients produced a prominent CL, and only 5 of 9 females had serum progesterone concentrations >1.0 ng/ml on the day of embryo transfer (Table 16). The difficulty in passing the embryo transfer device through the cervix also may have contributed to pregnancy failure as the degree of difficulty was rated as moderate to very difficult in 5 of 9 oryx (Table 16). Passage of a transfer device was not possible in recipient #9 (Table 16) and despite a laparoscopic embryo transfer, this female did not become pregnant.

Mean serum cortisol concentration across all treatment groups was 35.3 ± 3.4 ng/ml (~12 to 15 min after initial anesthetic injection). Although there was a tendency for serum cortisol to be elevated during the initial rectal palpation/uterine flushing period (Fig. 16) compared to nonmanipulated controls, the difference was nonsignificant (P>0.10). By the 30 min sample period, circulating cortisol concentrations in manipulated and nonmanipulated oryx were parallel. At the end of the sampling interval mean cortisol level was 22.4 ± 3.6 and 16.4 ± 2.0 ng/ml in the manipulated and nonmanipulated females which was similar (P>0.10) to the first post-anesthetic value. In contrast, mean cortisol concentrations in nonmanipulated, ACTH-treated oryx increased 3-fold within 30 min to peak values of 113.5 ± 5.6 ng/ml which was sustained for the entire sampling period.

Although increased adrenal responsiveness to anesthesia/reproductive tract manipulation was not reflected in the acute serum cortisol profiles, there was evidence of chronic stress in some oryx. After confinement to the corral, females frequently refused to eat and 12 of 14 animals lost an average of 5 kg during the 30 to 60 day trial period. Two oryx lost as much as 0.4 kg/day. Irrespective of the acute effects of treatment on body weight, all oryx donors and recipients subsequently bred naturally and produced live-offspring. During the course of this study, oryx were anesthetized on a total of 172 occasions without complications or injuries to animals or personnel.

Study 1, Trial 2

The number of palpable CL or ovarian follicles was not influenced (P>0.10) by doubling the PMSG dose or by using a PMSG lot with higher reported biopotency (Table 17). Ovarian response to FSH-P tended to be less than that observed in Trial 1 with mean CL numbers and preovulatory follicles being similar (P>0.10) among all 4 gonadotropin

treatment groups (Table 17). Seventeen of 20 (85%) donors ovulated, but only degenerate embryos and unfertilized ova were recovered during the course of this study. Embryos/ova were recovered from 15 of 17 ovulating females with only 2 donors exhibiting any evidence of fertilization. In the latter females, degenerate 2- to 8-cell embryos accompanied unfertilized ova.

Study 2

Reproductive tract and ovarian size was proportional to species body size; the larger eland and bongo had the largest reproductive organs. The greater kudu reproductive organs on the average palpated smaller than that of the scimitar-horned oryx. The eland, bongo and greater kudu all displayed a bicornuate uterus consisting of 2 symmetrical uterine horns, a uterine body and a single-channeled cervix. Unlike the scimitar-horned oryx, there was little within animal variation in ovarian size. In this study a total of 29 anesthetic episodes were performed (eland, n = 10; bongo, n = 17; greater kudu, n = 2) without complications or injuries to animals or personnel.

Using the described $PGF_2\alpha$-gonadotropin treatments, 8 of 10 eland and all of the bongo and both greater kudu donors ovulated (Table 18). In the eland and bongo, ovarian responsiveness was enhanced ($P<0.05$) by FSH-P compared to PMSG treatment. Preliminary data did not reveal a difference in the greater kudu. The short time interval between repeated gonadotropin treatment of bongo (60 days) as well as scimitar-horned oryx (≥90 days) females did not inhibit ovarian responsiveness (Table 19). Some females had high ovulatory responses (7 to 16 CL) after the third or fourth consecutive treatment while others consistently produced 1 to 4 CL each time. Although serum progesterone concentrations varied markedly on the day of embryo recovery (Table 18), mean concentrations were similar ($P>0.10$) between bongo (5.5 ± 1.8 ng/ml) and eland (4.1 ± 3.1 ng/ml) and were no different from the overall mean measured in the oryx (Table 13). For the eland and bongo, the correlation between total CL number and circulating progesterone concentration on the day of embryo recovery was high ($r = 0.99$ and 0.97, respectively) and significant ($P<0.01$).

Insertion of the catheter through the cervix and into the uterus was not difficult for any of the 3 species. Embryos and/or ova were recovered from 6 of 10 eland (range, 7-15), 9 of 10 ovulating bongo (range, 1-8) and both of the greater kudu. Based on the number of CL palpated, the recovery rate of ova was 69, 88 and 100% for eland, bongo and greater kudu, respectively, compared to 60% for scimitar-horned oryx in Study 1, Trial 1 and 2.

Evaluation of temporal cortisol profiles in the 3 species indicated little change in adrenal activity in the bongo or greater kudu over time (Fig. 17). In the eland, circulating cortisol rose from 15.3 ± 1.7 ng/ml to a mean peak of 28.1 ± 10.6 ng/ml 60 min later ($P<0.05$), an observation likely related to the general difficulty of maintaining anesthesia in this species. Using the described anesthetic regimen, eland constantly required ketamine-HCl supplementation to sustain a surgical plane of anesthesia and avoid movement during palpation/uterine flushing. Likewise the elevated mean cortisol concentration of 26.0 ± 7.7 ng/ml in the bongo at 75 min probably was related to increased alertness associated with recovery. Interestingly, baseline cortisol concentrations at the first post-anesthetic blood sampling in all 3 species was only half that detected in scimitar-horned oryx (Fig. 17).

DISCUSSION

This study demonstrates the comparative responsiveness of 4 African antelope species to estrous synchronization/exogenous gonadotropin treatments. Compared to results usually obtained with domestic sheep and cattle, the overall utility of these treatments for the purpose of embryo transfer was relatively inefficient. Basic animal handling procedures were not problematic, and it was relatively easy to adapt existing methods for the darting, safe anesthesia and effective uterine catherization/flushing. The primary limitations to embryo recovery and transfer were: 1) a lack of estrous behavior which potentially contributed to a low incidence of mating activity and, thus, poor fertilization rates; and 2) a resilience of the ovaries to conventional exogenous gonadotropin regimens. Compared to results in sheep (Cognie and Torres, 1984; Alwan et al., 1988; Schiewe et al., 1990c) and cattle (Hasler et al., 1983; Monniaux et al., 1983; Walton and Stubbings, 1986; Schiewe et al., 1987a), there was a much lower incidence of "superovulation". Overall, of the 75 scimitar-horned oryx, eland, bongo and of greater kudu used in Studies 1 and 2, fewer than 20% produced more than 5 CL after gonadotropin treatment. The gonadotropin dosages used in these species were similar or greater to those commonly used in cattle (Elsden et al., 1978; Yadav et al., 1986; Walton and Stubbings, 1986; Schiewe et al., 1987a). Although oryx and greater kudu females were less than half the body weight of domestic cattle, a majority did not superovulate. The absence of multiple ovulations could not be attributed to ovarian hyperstimulation because large numbers of unovulated follicles generally were not present; only 1 oryx female had cystic-like ovaries at the time of uterine flushing.

Although more than 75% of the females were induced hormonally to ovulate, the precise synchrony of estrus was difficult to evaluate because of a low incidence of overt estrual behavior or confirmed mounting by a male. Actual breeding behavior in the scimitar-horned oryx more closely resembled that of sheep and goats (Hulet et al., 1975) than cattle and was characterized by the Laufschlag display (leg kick; Pfeifer, 1985) to test female receptivity. In the absence of a breeding male, dominant female oryx reportedly will mount subordinate females and exhibit Flehmen (Pfeifer, 1985). We observed neither of these behaviors in hormone-treated females. Detailed behavioral observations were made only for the scimitar-horned oryx, however, the other species were observed periodically for estrus during periods of anticipated mating behavior. Nonetheless, copulatory behavior was not observed in any of the eland, bongo or 2 greater kudu. Some females may have exhibited mating activity at night, but this speculation probably could be dismissed because a high proportion of unfertilized ova were recovered post-uterine flushing. The cause for the low intensity of estrus is unknown. Although some females and males may have been stressed as a result of being moved to new corral locations for study, other animals were manipulated in their native exhibit and also failed to mate. For all species, it appeared that the increased human interference associated with moving animals, giving hormone injections and introducing males increased animal activity. Some oryx females constantly paced along the enclosure fence, and all experienced a significant weight loss indicative of chronic stress.

Interestingly, compared to published cortisol concentrations in cattle (Alam and Dobson, 1986) and sheep (De Silvia et al., 1986), the serial cortisol profiles measured in females during anesthesia, rectal palpation and uterine flushing did not suggest hyperadrenal activity in any of the 4 species studied. Peripheral cortisol concentrations in ewes and cows ranged from 2 to 30 ng/ml which was within the range measured in these nondomestic bovids. However, it is worth noting that circulating cortisol was elevated only 3-fold in oryx following ACTH which was considerably less than the 5- to 10-fold increase observed in comparably-treated cattle and sheep (Matteri and Moberg, 1982; Moberg, 1985; Armstrong, 1986). Increased ACTH and corticosteroid levels associated with various stressors are known to interfere with ovarian responsiveness to exogenous hormones and manifestation of estrus in cattle and sheep (Stoebel and Moberg, 1982; Moberg, 1984, 1985). If indeed this occurred in the present study, then blood concentrations of cortisol were a poor index of stress. Regardless, the extensive manipulations imposed during the course of this project had no discernible effect on the ability of these animals to eventually reproduce naturally.

The effectiveness of estrous synchronization in the scimitar-horned oryx was assessed by comparing a progestogen-pessary to a $PGF_2\alpha$ regimen, whereas ovarian activity was stimulated by comparing the use of FSH-P to PMSG in all species. The progestogen or $PGF_2\alpha$ were equally effective for synchronizing ovarian activity, which was comparable to previous findings involving $PGF_2\alpha$–gonadotropin-treated cattle (Voss et al., 1983) and sheep (Schiewe et al., 1990c). Furthermore, the efficacy of Estrumate to stimulate luteal regression in gonadotropin-treated eland, bongo and greater kudu was confirmed, and the results were similar to those involving cattle (Lindsell et al., 1986a; Yadav et al., 1986a). In theory, PMSG would be preferable for inducing ovulation in wild hoofstock because of its long half-life (120 to 360 h; Schams et al., 1978; Yadav et al., 1986b) which would eliminate the need for multiple injections of the shorter-acting FSH-P. The single injection protocol also perhaps would lessen potential "manipulatory" stress. However, in agreement with earlier studies in cattle (Elsden et al., 1978; Monniaux et al., 1983) and sheep (Schiewe et al., 1990c), the multi-injection FSH-P regimen generally resulted in more CL in the oryx, eland and bongo than after PMSG. The number of CL in these scimitar-horned oryx was higher than that observed in Arabian, fringe-eared or scimitar-horned oryx (Durrant, 1983) treated similarly. The CL number in FSH-P-treated eland and bongo was within the range reported by Dresser and associates (1982; 1985) for these same species. Like these investigators, we also observed a high rate of ovulatory variability among individuals, especially after PMSG, which also agreed with cattle results (Betteridge, 1977; Greve, 1981). Interestingly, a high proportion of oryx females (~30%) failed to respond to either exogenous PMSG or FSH-P, even when pre-treatment palpation demonstrated that these animals were cycling. Repeated gonadotropin treatments over time did not result in reduced ovulatory responses in oryx or bongo females. Cattle results generally indicate that there is no decline in ovarian responsiveness after 10 or fewer repeated treatments using exogenous FSH (Donaldson and Perry, 1983, Hasler et al., 1983), although there is some individual variability (Donaldson and Perry, 1983). The impact of repeated gonadotropin regimens may be correlated closely to the interval between treatments (Kanagawa et al.,1981) suggesting that any possible inhibitory antibody response, if present, occurs in a time-dependent fashion. When PMSG is given 5 times to individual cows at more than 60 day intervals, Moor and coworkers (1984) observed no affect on ovulation rate or viable embryo production. Therefore, even if ovulation numbers are low in gonadotropin-treated antelope, existing cattle data and the present results suggest that females could be repeatedly stimulated without adversely affecting natural ovulation ability or fertility.

Because such a high proportion of oryx failed to respond to PMSG, we speculated that either an inadequate dosage was being used or perhaps the commercial lot tested had a low biopotency. Neither a 2-fold dose increase nor a 1.5-fold increase in PMSG bioactivity increased the number of CL or unovulated follicles on the day of uterine flushing. Increasing the PMSG dose similarly in cattle, does not necessarily increase CL number (Saumande and Chupin, 1986) but the number of unovulated follicles increases (Betteridge, 1977; Schiewe, 1983). In the scimitar-horned oryx, providing more exogenous gonadotropin or a more biopotent PMSG source did not increase ovarian responsiveness. Unlike conventional livestock, these antelope species usually reacted differently to conventional exogenous gonadotropins. This suggests two possibilities which warrant further attention. First, the potential ovarian activity elicited by the exogenous gonadotropins was being suppressed by a simultaneous stress response. Second, it is possible that these species may produce different isoforms of pituitary gonadotropins, endogenous hormones so different that the ovaries do not recognize gonadotropic preparations that stimulate extensive follicular growth in other species (Ulloa-Aquirre and Chappel, 1982; Keel et al., 1987). In either case, further studies of the basic reproductive physiology of these species is necessary to delineate the causes of reduced ovarian responsiveness to exogenous gonadotropins.

It was possible to nonsurgically catheterize the cervix and uterus of all species. The bifurcated cervical lumen (without a uterine body) of the oryx was similar to recent similar observations in the wildebeest (Stover, 1987) and made catheter passage more difficult than the other species. However, once the Foley catheter was secured in the uterine horn, multiple embryos and/or ova could be recovered. In the initial study, there was a high fertilization rate in oryx donors which was similar to or greater than fertilization rates in cattle using optimal AI procedures (Schiewe et al., 1987a). Conversely, there was a low fertilization rate in eland, bongo and greater kudu donors, as well as oryx in a second experiment. This may have resulted from the lack of copulatory activity or possible abnormal sperm transport as has been demonstrated in $PGF_2\alpha$-treated/superovulated sheep (Hawk et al., 1987). Our ability to produce oryx embryos in Study 1 and the ability by Dresser et al. (1982, 1985) to produce eland and bongo embryos after $PGF_2\alpha$-induced estrus and natural mating indicate the unlikelihood of a sperm transport problem. It is more likely possible that the change in daily routine, combined with hormonal injections and immobilizations were stressful, causing inhibition of estrous behavior and mating activity and, thus, poor fertilization rate.

Transferable quality oryx embryos appeared to survive cryopreservation based on post-thaw assessments and *in vitro* culture. Dimethyl sulfoxide and glycerol were more

effective cryoprotectants than propylene glycol and provided a higher degree of protection for scimitar-horned oryx embryos during subzero cooling and warming events, as previously described for cattle embryos (Lehn-Jensen, 1986). These findings contradict the excellent results reported for frozen/thawed mouse (Renard and Babinet, 1984; Rall and Polge, 1984) and sheep (Schiewe et al., 1990d) embryos cryopreserved in propylene glycol. One advantage of this particular cryoprotectant is its ability to reduce the incidence of zona pellucida damage after thawing mouse (Renard and Babinet, 1984) and sheep (Schiewe et al., 1990d) embryos. However, for oryx embryos, there was a similar percentage of thawed embryos with some damage to the zona regardless of treatment. The relatively high incidence of zona damage (33%) could, in part, be attributed to the use of glass storage containers which are known to increase damage compared to plastic straw containers (Schiewe et al., 1990d). It appears that species differences may exist in terms of propylene glycol's colligative properties (i.e., organized binding of water molecules) and ability to form a metastable glass upon rapid cooling at -35°C and rapid warming. Unfortunately, even excellent and good quality thawed embryos failed to produce a live offspring after transfer to a surrogate female. This lack of success no doubt was related in part to some technical difficulties in penetrating the cervical canal with the embryo transfer device. However, poor ovulatory response of the recipient also likely contributed as few of these females produced at least 1 prominent CL, again the result of ovulatory resistance to exogenous gonadotropin or to stress. It is important to note, that although no offspring resulted, the transabdominal laparoscopic embryo transfer procedure could be performed as safely in the scimitar-horned oryx as originally described for the domestic sheep (Schiewe et al., 1984). This procedure recently has been used to produce a live suni antelope calf (*Neotragus moschatus zuluensis*; Raphael et al., 1989).

We have demonstrated the feasibility of inducing multiple ovulations and nonsurgically recovering embryos/ova in 4 antelope species. However, consistent observations of a low incidence of overt estrous behavior and variable responsiveness to conventional gonadotropins preclude present techniques from being routinely effective for either generating large numbers of embryos or offspring after embryo transfer. Our findings may be related, in part, to natural resilience to the exogenous gonadotropins tested or to chronic stress associated with confinement when individuals are subjected to isolation, repeated darting for drug delivery and restraint. Although embryo transfer holds considerable promise for increasing population size rapidly, enhancing the distribution of genes from outstanding parents or preserving genetic diversity via cryopreservation, it is apparent from these studies that much more work is required to make this technology practical. Furthermore, our results affirm our previous assertion that mammals have

evolved a vast array of reproductive strategies (Wildt, 1990a, 1990b) and that this physiological diversity will complicate applying biotechnology to conservation efforts.

TABLE 13. Palpation and endocrine assessments of gonadotropin-treated scimitar-horned oryx donors on the day of embryo collection

Treatment[b]	Total no. of females	Ovulation Data[a]: No. of females with ≥1 CL (%)	No. of CL X̄±SEM	No. of CL range	Serum progesterone (ng/ml) X̄±SEM	Serum progesterone (ng/ml) range
Synchronization						
MAP	17	14 (82)	3.6±0.9	1-11	4.7±1.8	0.7-21.3
$PGF_2\alpha$	16	11 (69)	3.9±1.5	1-16	2.9±0.8	0.5-7.6
Gonadotropin						
PMSG	17	12 (71)	2.7±0.8[d]	1-11	4.6±1.8	0.6-21.3
FSH-P	16	13 (81)	4.6±1.6[c]	1-16	3.5±1.0	0.5-10.1
Interaction						
MAP-PMSG	10	7 (70)	3.3±1.4[cd]	1-11	5.9±2.8	0.7-21.3
MAP-FSH-P	7	7 (100)	3.9±1.3[c]	1-11	3.1±1.8	1.0-10.1
$PGF_2\alpha$-PMSG	7	5 (71)	1.8±0.2[d]	1-2	2.4±1.1	0.6-5.6
$PGF_2\alpha$-FSH-P	9	6 (67)	5.7±2.7[c]	1-16	3.8±1.2	0.5-7.6
Overall	33	25 (76)	3.7±0.8	1-16	4.1±1.4	0.5-21.3

[a] Progesterone values indicated that one FSH-P and one PMSG treated female (both MAP-treated) had ovulated, although luteal tissue was not confirmed at rectal palpation. These individuals were not included in the calculation of mean CL response.

[b] MAP = medroxyprogesterone acetate; $PGF_2\alpha$ = prostaglandin $F_2\alpha$; PMSG - pregnant mares' serum gonadotropin; follicle stimulating hormone-pituitary extract (FSH-P).

[c,d] Mean column values (± SEM) within the gonadotropin subtreatment groups ($P<0.10$) and the interaction treatment groups ($P<0.05$) with different superscripts differ .

TABLE 14. Nonsurgical embryo recovery results from ovulating scimitar-horned oryx following estrous synchronization and ovulation induction

Treatment interaction	Total no. of donors flushed	No. of females in which ova/embryos were recovered	Embryos recovered per donor female		
			no.	stage[a]	OG
MAP-PMSG	6	4	4	2 M, 2 Bl	1-2
			2	1 dM, 1 UFO	4
			1	1 M	1
			1	1 8-c	3
MAP-FSH-P	4	1	2	2 M,	2,3
$PGF_2\alpha$-PMSG	4	2	1	1 UFO	
			1	1 d8-16-c	4
$PGF_2\alpha$-FSH-P	5	3	16	16 M-BL	1-2
			8	8 BL	1-2
			1	1 UFO	
Overall	19	10	37	32 normal 2 degenerated 3 unfertilized	

[a] UFO= unfertilized ova, d= degenerate, 8-c= 8-cell, 16-c= 16-cell, M= morula and BL= blastocyst.

Figure 14. These transferable quality late morula to early blastocyst stage embryos are representatives of a group of 16 which were nonsurgically recovered from a gonadotropin-treated scimitar-horned oryx female (200X).

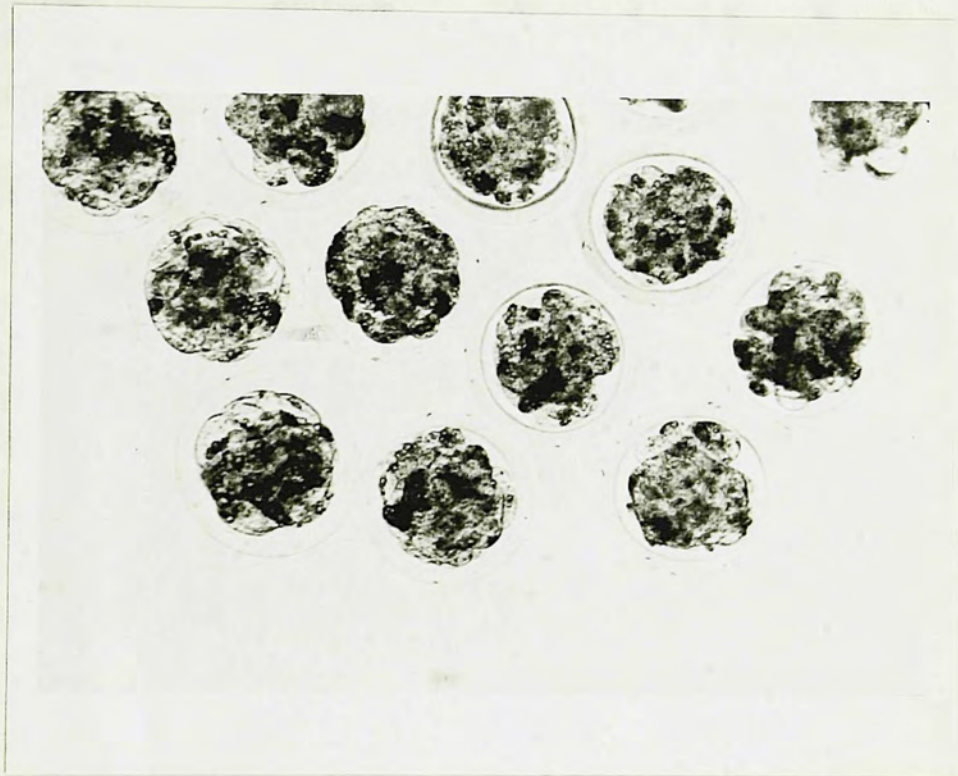


TABLE 15. Influence of cryoprotectant on post-thaw maintenance of quality grade and zona pellucida integrity for cryopreserved scimitar-horned oryx embryos

Cryoprotectant	Period of QG assessment	Quality grade (no.) 1	2	3	4	Embryos with zona damage[a] (%)
Dimethyl sulfoxide	pre-freeze	5	5	0	0	
	post-thaw	3	4	2	1	30
Glycerol	pre-freeze	4	6	0	0	
	post-thaw	3	4	1	2	40
Propylene glycol	pre-freeze	5	4	1	0	
	post-thaw	1	3	4	2	30
Total	pre-freeze	14	15	1	0	
	post-thaw	7	11	7	5	33

[a] Cracked or fractured zona pellucida.

TABLE 16. Response of scimitar-horned oryx recipients to transfer of thawed embryos

Recipient location	ID	Prominent CL[a]	Serum progesterone (ng/ml)	Post-thaw embryo QG	Difficulty of transfer[b]	Result[c]
Kings Dominion						
	1	no	0.6	2	3	NP
	2	no	5.5	3	2	NP
	3	no	3.3	2	2	NP
Detroit Zoological Park						
	4	no	0.3	3,4[d]	1	NP
	5	yes	7.9	2	2	NP
Metro-Miami Zoo						
	6	no	0.7	1	1	NP
	7	yes	1.5	1,2[d]	1	NP
	8	yes	4.4	1	3	NP
	9	no	0.1	1,3,4[d]	1[e]	NP

[a] Palpable, prominent luteal tissue raised above the ovarian surface.
[b] 1= no difficulty in passage of ET rod through cervix; 2= moderate difficulty; 3= very difficult passage.
[c] No pregnancy (NP) based on term gestation.
[d] Multiple embryos transferred.
[e] Laparoscopic embryo transfer.

Figure 15. Two frozen/thawed oryx expanded blastocysts (200X) after 24 h *in vitro* culture.

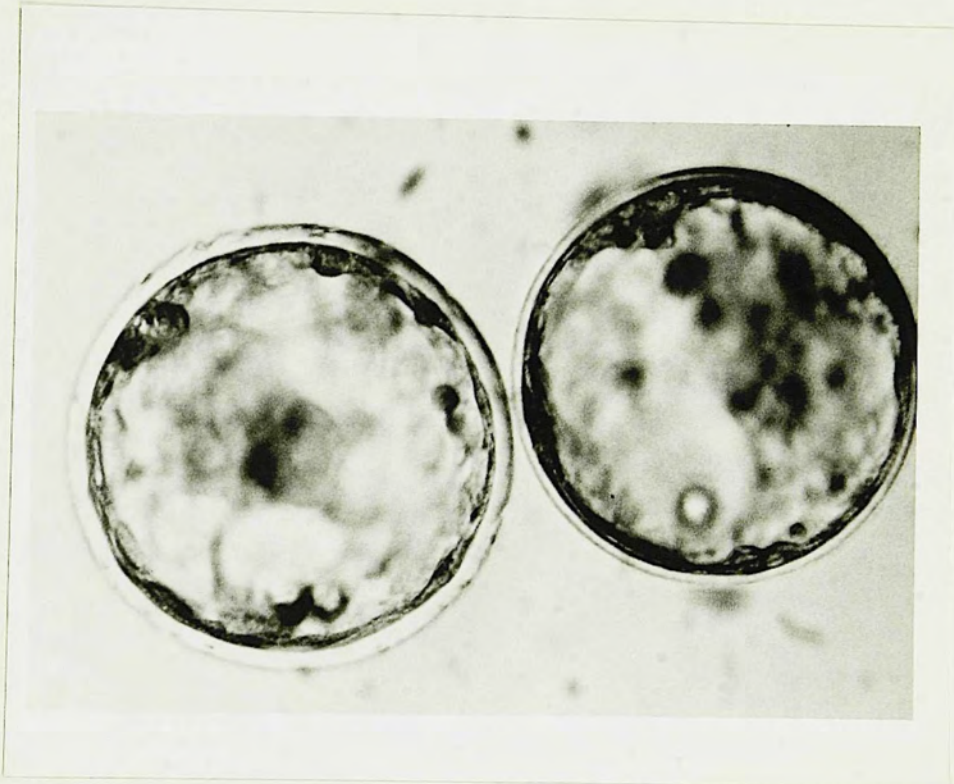


Figure 16. Mean (±SEM) cortisol concentrations in control, ACTH-treated and donor/recipient oryx based on serial samples collected at 15 min intervals.

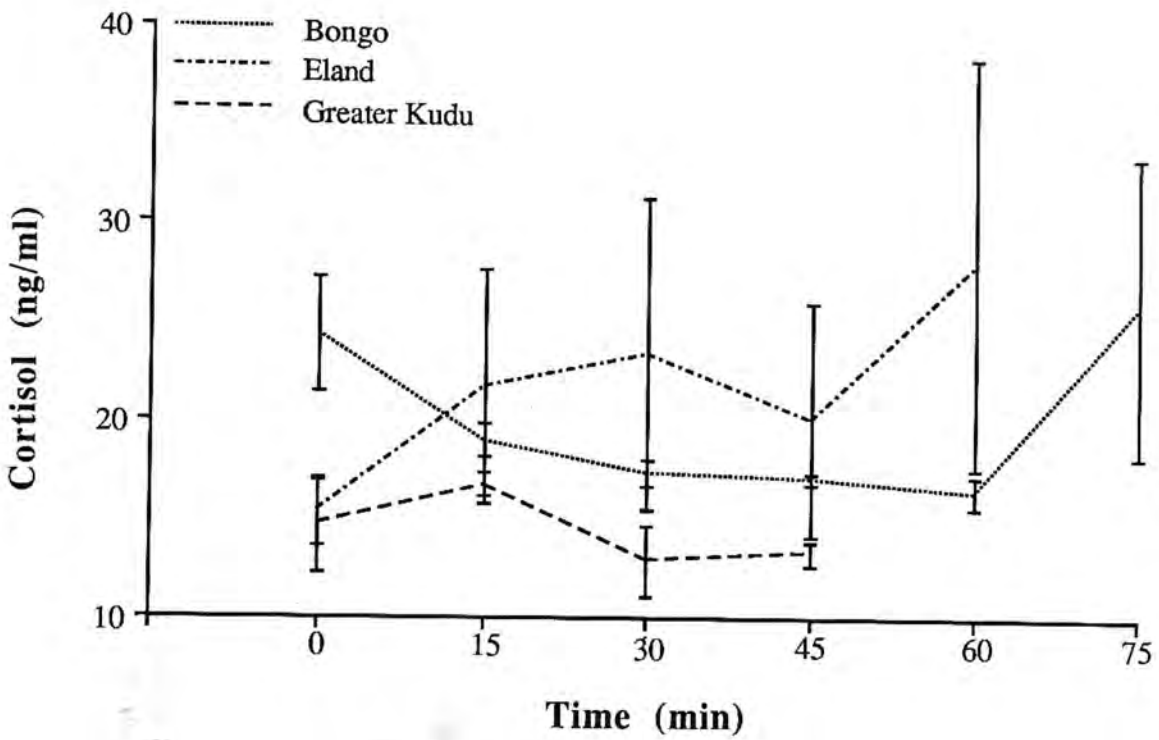

Bongo
Eland
Greater Kudu
Cortisol (ng/ml)
40
30
20
10
0
15
30
45
60
75
Time (min)

TABLE 17. Influence of gonadotropin and PMSG dosage and biopotency on ovulation induction and embryo recovery in the scimitar-horned oryx

Treatment	Gonadotropin type	Gonadotropin lot	Total dosage	No. of females ovulated/treated	Day of embryo recovery: No. of CL	Day of embryo recovery: No. of preovulatory follicles[a]	Day of embryo recovery: No. of embryos or ova/ovulating female[b]
I	FSH-P	RV1	50 mg	5/5	2.6±0.7	2.4±1.5	1.2±0.6
II	PMSG	501	2500 IU	3/5	1.6±0.7	1.4±0.5	0.8±0.5
III	PMSG	501	5000 IU	5/5	2.0±0.5	1.8±0.8	1.2±0.4
IV	PMSG	503	2500 IU	4/5	1.6±0.5	1.8±0.4	1.2±0.4

Values are expressed as means ± SEM.

[a] ≥5 mm in diameter.

[b] Only degenerate quality (QG 4) embryos were recovered.

TABLE 18. Palpation and endocrine assessments of gonadotropin-treated eland, bongo and greater kudu donors on the day of embryo collection

Species/ treatment[a]	Total no. of females	No. of females with ≥1 CL (%)	Ovulation Data: No. of CL X̄±SEM	No. of CL range	Serum progesterone (ng/ml) X̄±SEM	Serum progesterone (ng/ml) range
Eland						
PMSG	5	3 (60)	1.0±0.6[b]	1-3	1.2±0.2	0.7-1.5
FSH-P	5	5 (100)	10.6±4.6[c]	1-25	7.1±6.5	0.7-19.4
Bongo						
PMSG	5	5 (100)	1.4±0.3[b]	1-2	2.4±1.0	1.0-5.3
FSH-P	5	5 (100)	4.6±1.8[c]	1-8	8.6±2.6	2.4-13.7
Greater kudu						
PMSG	1	1 (100)	1.0	---	1.1	------
FSH-P	1	1 (100)	1.0	---	1.9	------

[a] PMSG = pregnant mares serum gonadotropin; FSH-P = follicle stimulating hormone-pituitary extract.

[b,c] Mean column values (± SEM) within the gonadotropin subtreatment groups with different superscripts differ ($P<0.05$).

Table 19. Influence of repeated gonadotropin treatment within a 1 year period on ovulation rates in scimitar-horned oryx and bongo

Species / Donor ID	Interval between treatments (days)	Hormonal treatment[a]	Ovarian response: number of CL	Ovarian response: number of follicles
Oryx				
1	0	FSH-P/MAP	1	1
	120	FSH-P/$PGF_{2\alpha}$	0	0
	90	PMSG/MAP	0	10[b]
	120	FSH-P/$PGF_{2\alpha}$	2	2
2	0	FSH-P/MAP	2	0
	120	FSH-P/$PGF_{2\alpha}$	0	0
	90	PMSG/MAP	11	1
	120	FSH-P/$PGF_{2\alpha}$	16	0
3	0	FSH-P/$PGF_{2\alpha}$	0	0
	90	PMSG/MAP	4	3
	120	FSH-P/$PGF_{2\alpha}$	3	0
4	0	FSH-P/$PGF_{2\alpha}$	2	2
	90	PMSG/MAP	3	3
	120	PMSG/$PGF_{2\alpha}$	2	2
Bongo				
1	0	PMSG/$PGF_{2\alpha}$	1	3
	60	FSH-P/$PGF_{2\alpha}$	3	1
	60	PMSG/$PGF_{2\alpha}$	1	2
2	0	FSH-P/$PGF_{2\alpha}$	2	5
	60	PMSG/$PGF_{2\alpha}$	2	3
	60	FSH-P/$PGF_{2\alpha}$	7	1
3	0	FSH-P/$PGF_{2\alpha}$	1	5
	60	PMSG/$PGF_{2\alpha}$	1	2
	60	FSH-P/$PGF_{2\alpha}$	8	0

[a] PMSG = pregnant mares serum gonadotropin; FSH-P = follicle stimulating hormone-pituitary extract; MAP = medroxyprogesterone acetate; $PGF_{2\alpha}$ = prostaglandin $F_{2\alpha}$.

[b] Cystic ovarian condition caused by apparent hyperstimulation.

Figure 17. Mean (±SEM) cortisol concentrations in bongo (n=10), eland (n=10) and greater kudu (n=2) based on serial blood samples collected at 15 min intervals.

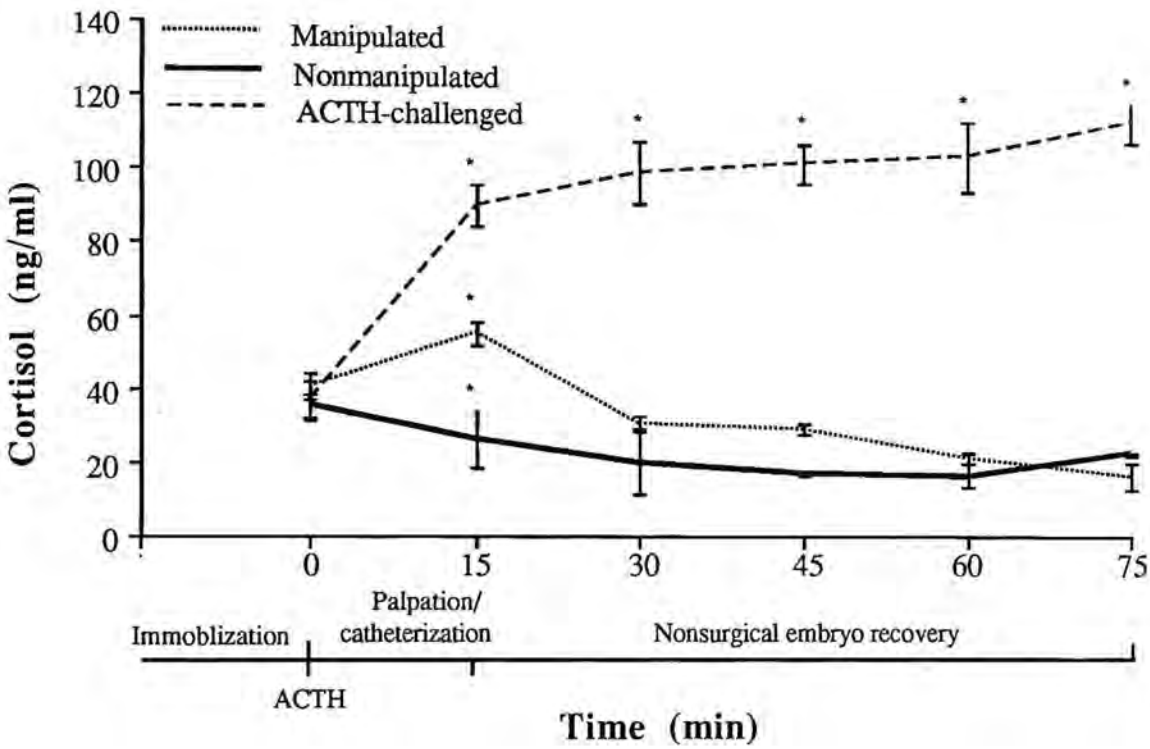

Manipulated
Nonmanipulated
ACTH-challenged
Cortisol (ng/ml)
140
120
100
80
60
40
20
0
0
15
30
45
60
75
Palpation/
catheterization
Immoblization
Nonsurgical embryo recovery
ACTH
Time (min)

Table 20. Composition of modified Phosphate Buffered Medium (PB1)*

Composition		g/l
Inorganic salts:		
	NaCl	8.0
	KCl	0.2
	$CaCl_2$	0.12
	KH_2PO_4	0.2
	$MgCl_2 \cdot 6H_2O$	0.1
	Na_2HPO_4	1.15
Other components:		
	Na pyruvate	0.0036
	Glucose	1.0
	Bovine serum albumin (fraction V)	3.0
	Phenol red	0.01
	Penicillin	100 U/ml
	Distilled H_2O	up to 1000 ml

*Whittingham, 1974

Table 21. Composition of Hanks Balanced Salt Solution (HBSS)*

Composition	g/l
Inorganic salts:	
NaCl	8.0
KCl	0.4
$CaCl_2 \cdot 2H_2O$	0.186
KH_2PO_4	0.06
$MgSO_4 \cdot 7H_2O$	0.2
Na_2HPO_4	0.048
$NaHCO_3$	0.35
Other components:	
Glucose	100
Hepes	4.7
Bovine serum albumin (fraction V)	10.0
Phenol red	0.01
Distilled H_2O up to 1000 ml	

*Hanks and Wallace, 1949

Table 22. Composition of Tissue Culture Medium 199 (TCM 199)*

	Compostion	g/l
Inorganic salts:		
	NaCl	6.8
	KCl	0.4
	$CaCl_2 \cdot 2H_2O$	0.265
	$MgSO_4 \cdot 7H_2O$	0.2
	$Na_2H_2PO_4 \cdot H_2O$	0.14
	$NaHCO_3$	2.2
	$Fe(NO_3)_3 \cdot 9H_2O)$	0.72
Amino acids:		
	L-alanine	0.025
	L-arginine HCl	0.07
	L-aspartic Acid	0.03
	L-cysteine $HCl \cdot H_2O$	0.0001
	L-cystine	0.02
	L-glutamic Acid	0.067
	L-glutamine	0.1
	Glycine	0.05
	L-histidine $HCl \cdot H_2O$	0.022
	Hydroxy L-proline	0.01
	L-isoleucine	0.02
	L-leucine	0.06
	L-lysine HCl	0.07
	L-methionine	0.015
	L-phenylalanine	0.025
	L-proline	0.04
	L-serine	0.025
	L-threonine	0.03
	L-tryptophan	0.01
	L-tyrosine	0.04
	L-valine	0.025
Vitamins:		
	p-Aminobenzoic acid	0.00005
	Ascorbic acid	0.00005
	Biotin	0.00001
	Calciferol	0.0001
	D·Ca·pantothenate	0.00001
	Cholesterol	0.0002
	Choline Cl	0.0005
	Folic acid	0.00001
	I-Inositol	0.00005
	Menadione	0.00001
	Nicotinamide	0.000025
	Nicotinic acid	0.000025
	Pyridoxal HCl	0.000025
	Pyridoxine HCl	0.000025
	DL_2-tocopherolphosphate Na_2	0.00001

Compostion	g/l
Vitamins: (continued)	
Riboflavin	0.00001
Thaimine HCl	0.00001
Tween 80	0.005
Vitamin A·acetate	0.00014
Other components:	
Adenine sulfate·$2H_2O$	0.01
Adenosine-5-monophosphoric acid·$2H_2O$	0.0002
Adenosine-5-triphosphate $2Na·4H_2O$	0.001
Deoxyribose	0.0005
Glutathione (reduced)	0.00005
Guanine HCl	0.0003
Hypoxanthine	0.0003
D-ribose	0.0005
Na acetate·$3H_2O$	0.083
Thymine	0.0003
Uracil	0.0003
Xanthine	0.0003
Glucose	1.0
Hepes	5.9574
Bovine serum albumin (fraction V)	3.0
Phenol red	0.01
Antibiotic-antimycotic	1% by volume
Distilled H_2O	up to 1000 ml

*Morton, 1970

Table 23. Composition of Whitten's Medium*

Composition	g/l
Inorganic salts:	
NaCl	6.4
KCl	0.356
Ca lactate	0.527
KH_2PO_4	0.162
$MgSO_4 \cdot 7H_2O$	0.294
$NaHCO_3$	0.19
Na pyruvate	0.025
Dextrose	1.0
Bovine serum albumin (fraction V)	3.0
Phenol red	0.01
Penicillin G	0.075
Streptomycin	0.05
Distilled H_2O	up to 1000 ml

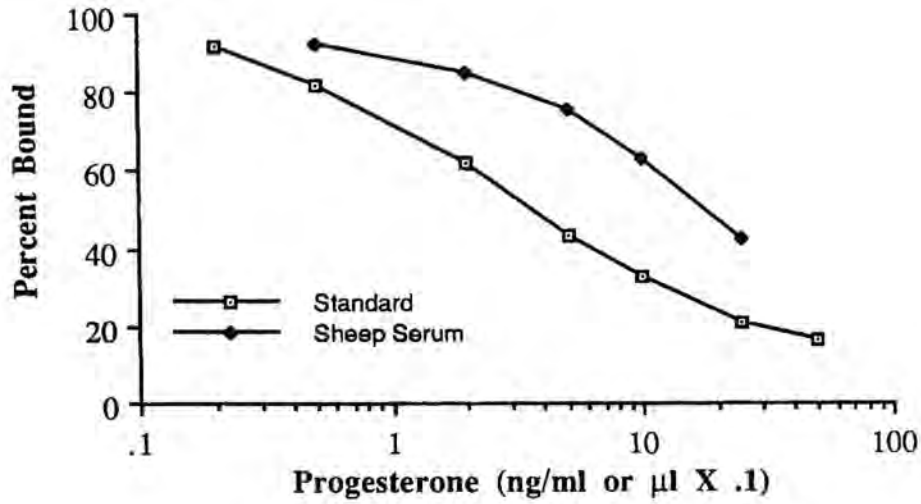
*Whitten, 1959

Table 24. Composition of Dulbecco's Modified Eagles Medium (DMEM)*

	Composition	g/l
Inorganic salts:		
	NaCl	8.0
	KCl	0.4
	$CaCl_2 \cdot 2H_2O$	0.186
	KH_2PO_4	0.06
	$MgSO_4 \cdot 7H_2O$	0.2
	Na_2HPO_4	0.048
	$NaHCO_3$	0.35
Amino acids:		
	L-arginine HCl	0.084
	L-cystine	0.048
	Glycine	0.03
	L-histidine $HCl \cdot H_2O$	0.042
	L-isoleucine	0.1048
	L-leucine	0.1048
	L-lysine HCl	0.1462
	L-methionine	0.03
	L-phenylalanine	0.066
	L-serine	0.042
	L-threonine	0.0952
	L.tryptophan	0.016
	L-tyrosine	0.072
	L-valine	0.0936
Vitamins:		
	D·Ca·pantothenate	0.004
	Choline Cl	0.004
	Folic acid	0.004
	I-inositol	0.007
	Nicotinamide	0.004
	Pyridoxal HCl	0.004
	Riboflavin	0.0004
	Thaimine HCl	0.004
Other components:		
	Na pyruvate	0.11
	Glucose	4.5
	Hepes	5.9575
	Bovine serum albumin (fraction V)	8.0
	Phenol red	0.01
	Fetal calf serum	10% by volume
	Antibiotic-antimycotic	1% by volume
	Distilled H_2O	up to 1000 ml

*Dulbecco and Freeman, 1959

Figure 18. Validation results of progesterone radioimmunoassay to sheep serum with the top panel displaying parallelism of the inhibition curves and the bottom panel exhibiting mass recovery analysis.


Percent Bound
100
80
60
40
20
0
Standard
Sheep Serum
.1
1
10
100
Progesterone (ng/ml or µl X .1)

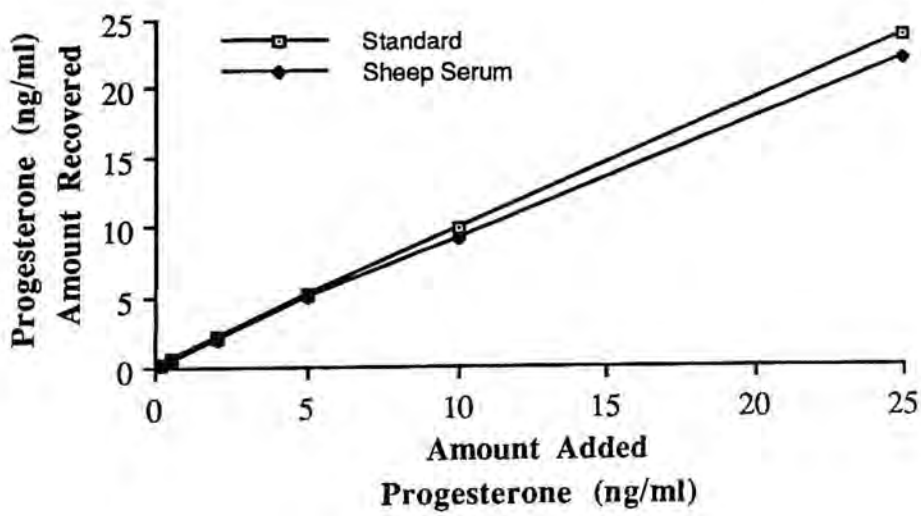
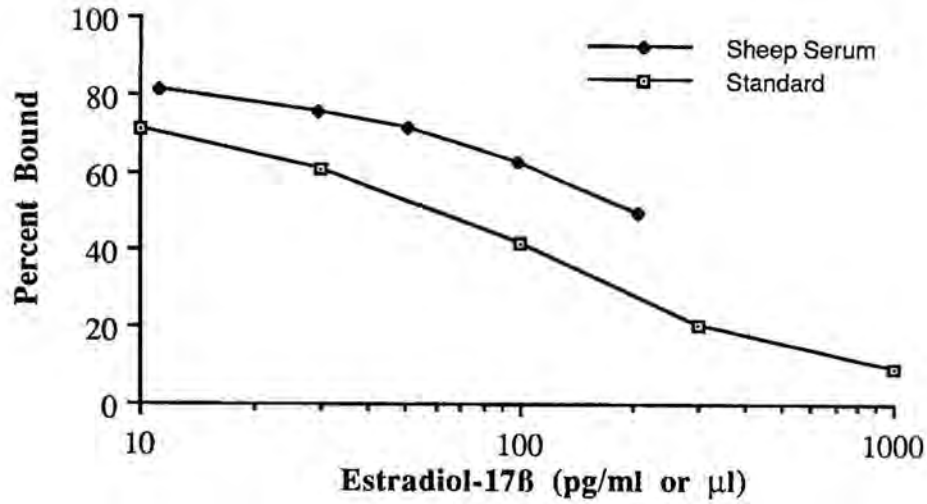

Progesterone (ng/ml)
Amount Recovered
25
20
15
10
5
0
Standard
Sheep Serum
0
5
10
15
20
25
Amount Added
Progesterone (ng/ml)

Figure 19. Validation results of estradiol-17β radioimmunoassay to sheep serum with the top panel displaying parallelism of the inhibition curves and the bottom panel exhibiting mass recovery analysis.


100
80
60
40
20
0
Percent Bound
Sheep Serum
Standard
10
100
1000
Estradiol-17ß (pg/ml or µl)

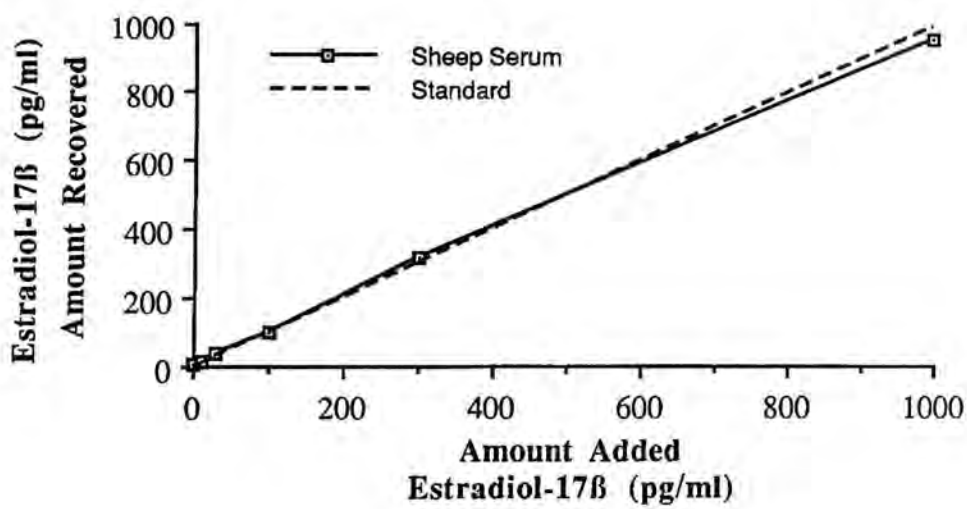
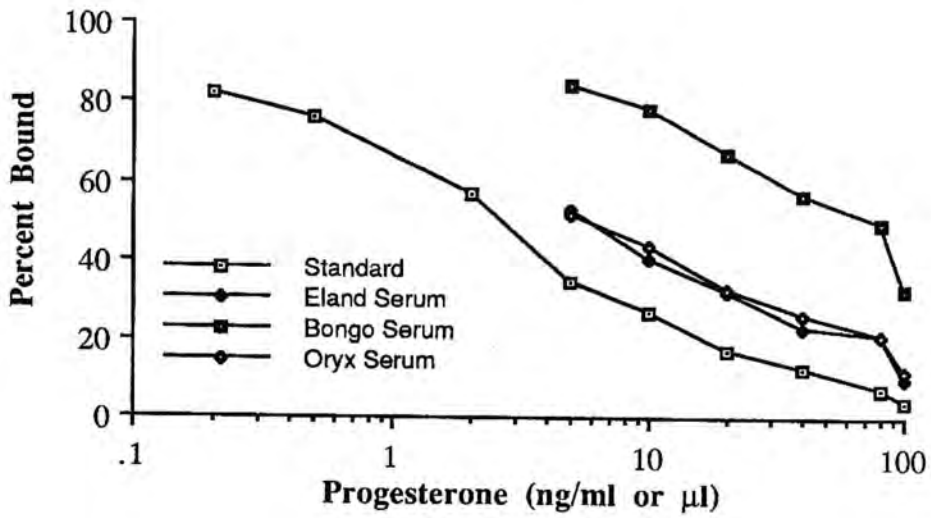

1000
800
600
400
200
0
Estradiol-17ß (pg/ml)
Amount Recovered
Sheep Serum
Standard
0
200
400
600
800
1000
Amount Added
Estradiol-17ß (pg/ml)

Figure 20. Validation results of progesterone radioimmunoassay to scimitar-horned oryx, bongo and eland serum. The top panel displays parallelism of the inhibition curves and the bottom panel exhibits mass recovery analysis.


100
80
60
40
20
0
Percent Bound
Standard
Eland Serum
Bongo Serum
Oryx Serum
.1
1
10
100
Progesterone (ng/ml or µl)

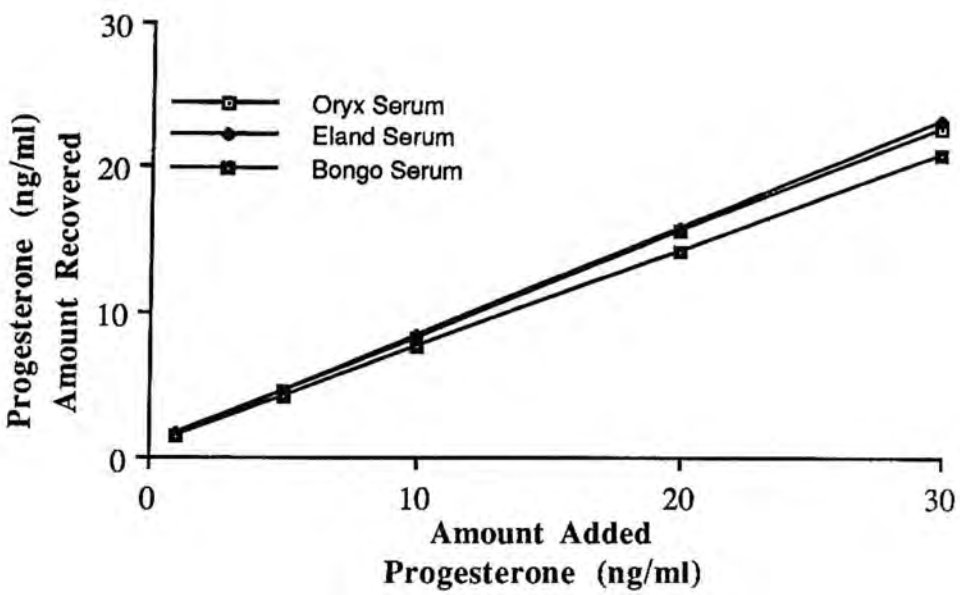
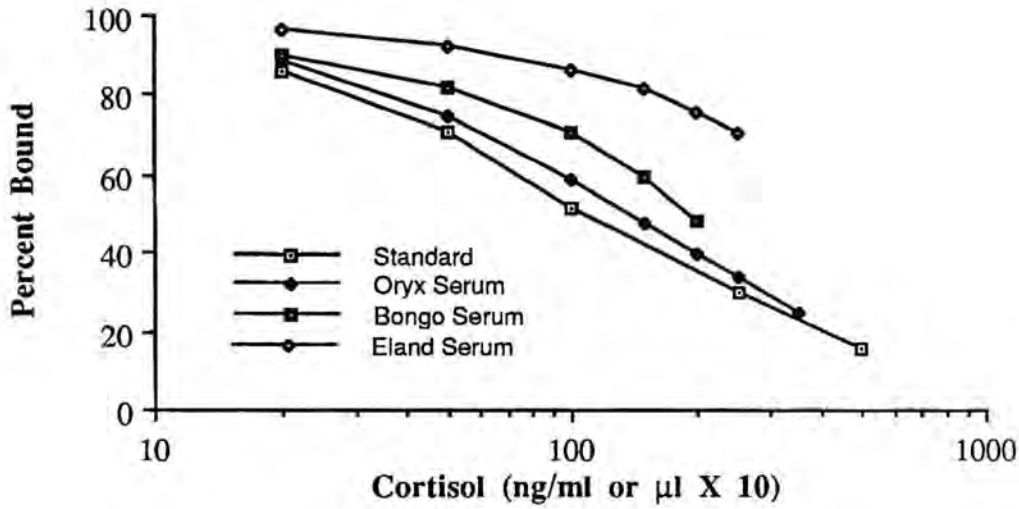

30
20
10
0
Progesterone (ng/ml)
Amount Recovered
Oryx Serum
Eland Serum
Bongo Serum
0
10
20
30
Amount Added
Progesterone (ng/ml)

Figure 21. Validation results of cortisol radioimmunoassay to scimitar-horned oryx, bongo and eland serum. The top panel displays parallelism of the inhibition curves and the bottom panel exhibits mass recovery analysis.


100
80
60
40
20
0
Percent Bound
Standard
Oryx Serum
Bongo Serum
Eland Serum
10
100
1000
Cortisol (ng/ml or µl X 10)

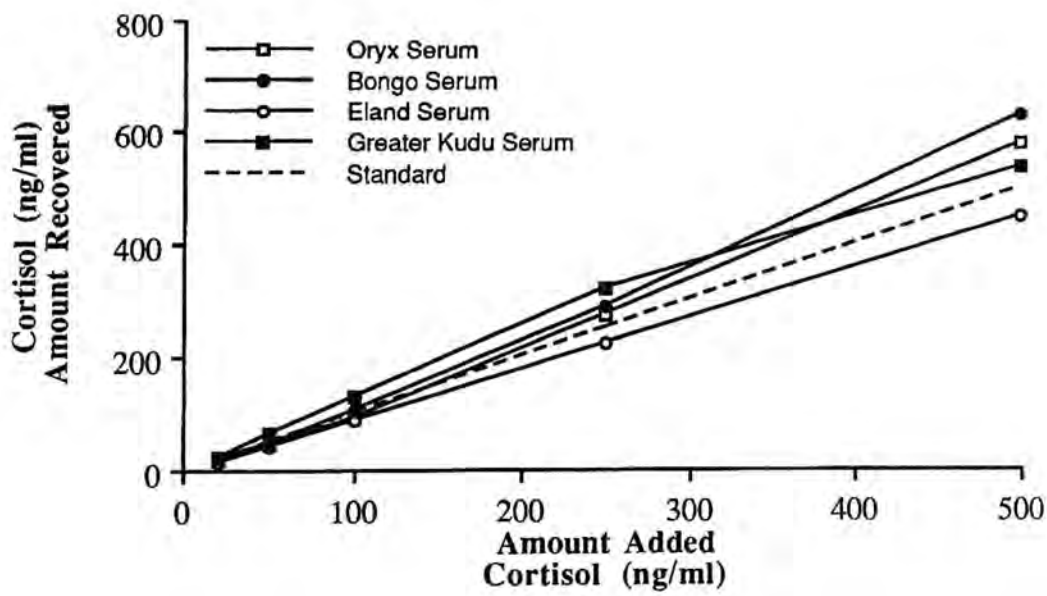
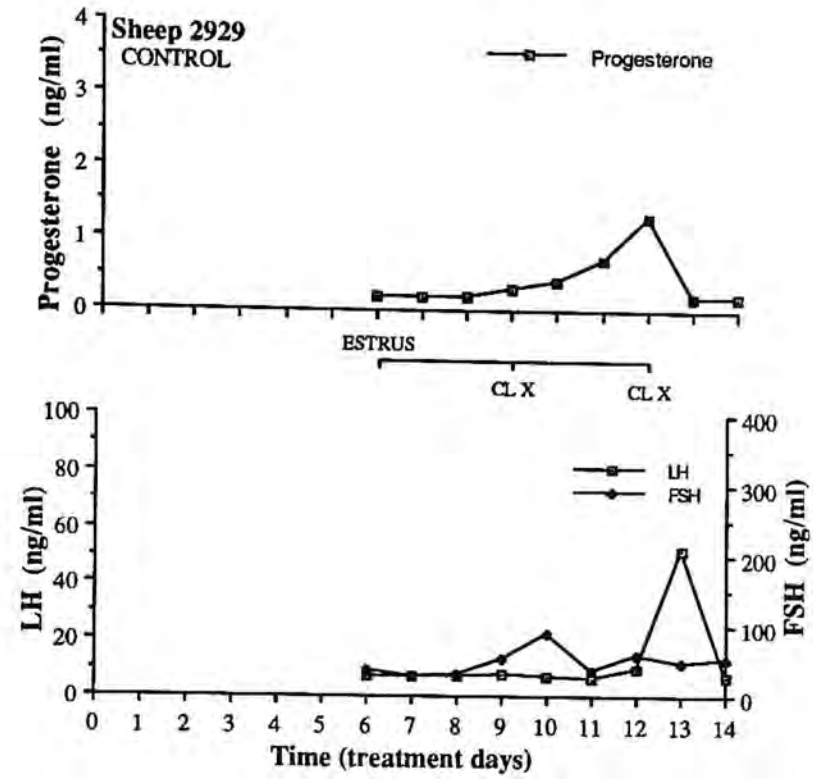

800
600
400
200
0
Cortisol (ng/ml)
Amount Recovered
Oryx Serum
Bongo Serum
Eland Serum
Greater Kudu Serum
Standard
0
100
200
300
400
500
Amount Added
Cortisol (ng/ml)

Figure 22A. Temporal endocrine profiles of individual control ewes after the onset of estrus are displayed on the double template graphs. The timing of estrus and CL removal (CLX) are indicated between the graphs.


Sheep 2929
CONTROL
Progesterone
Progesterone (ng/ml)
ESTRUS
CL X
CL X
LH
FSH
LH (ng/ml)
FSH (ng/ml)
Time (treatment days)

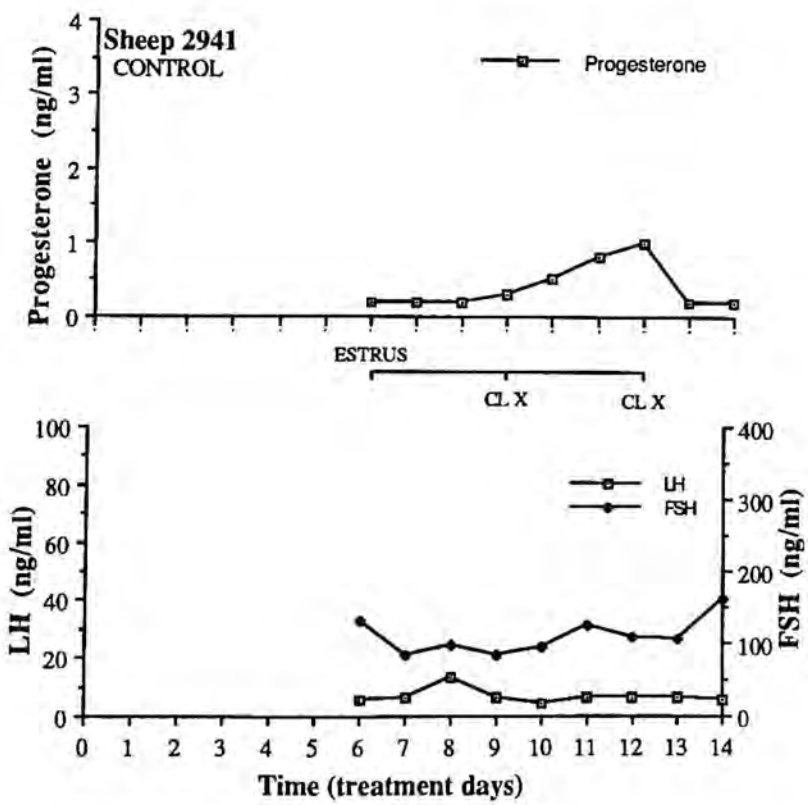
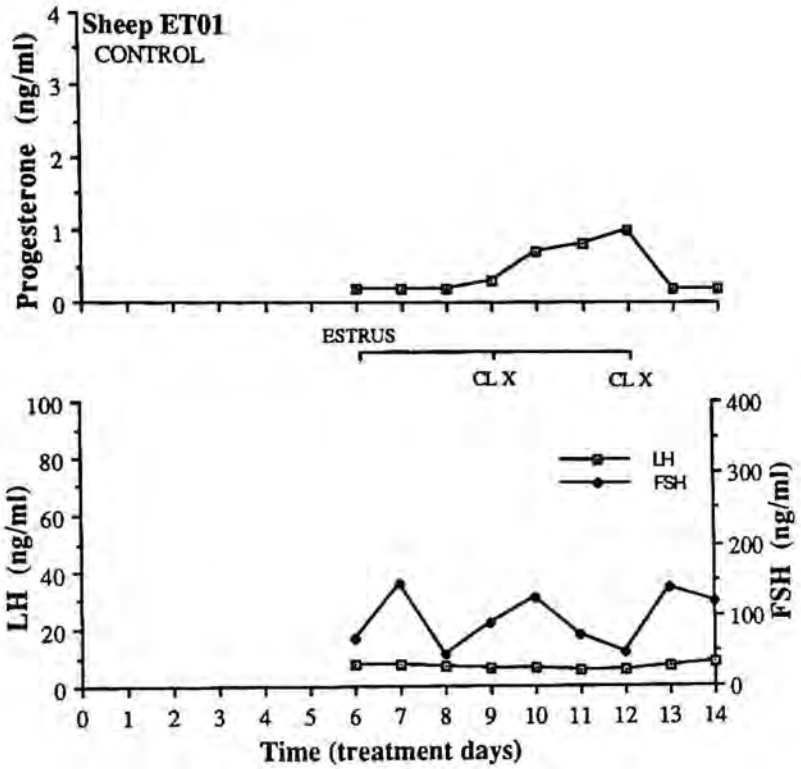

Sheep 2941
CONTROL
Progesterone
Progesterone (ng/ml)
ESTRUS
CL X
CL X
LH
FSH
LH (ng/ml)
FSH (ng/ml)
Time (treatment days)

Figure 22B. Temporal endocrine profiles of individual control ewes after the onset of estrus are displayed on the double template graphs. The timing of estrus and CL removal (CLX) are indicated between the graphs.


Sheep ET01
CONTROL
Progesterone (ng/ml)
ESTRUS
CL X
CL X
LH (ng/ml)
FSH (ng/ml)
LH
FSH
Time (treatment days)

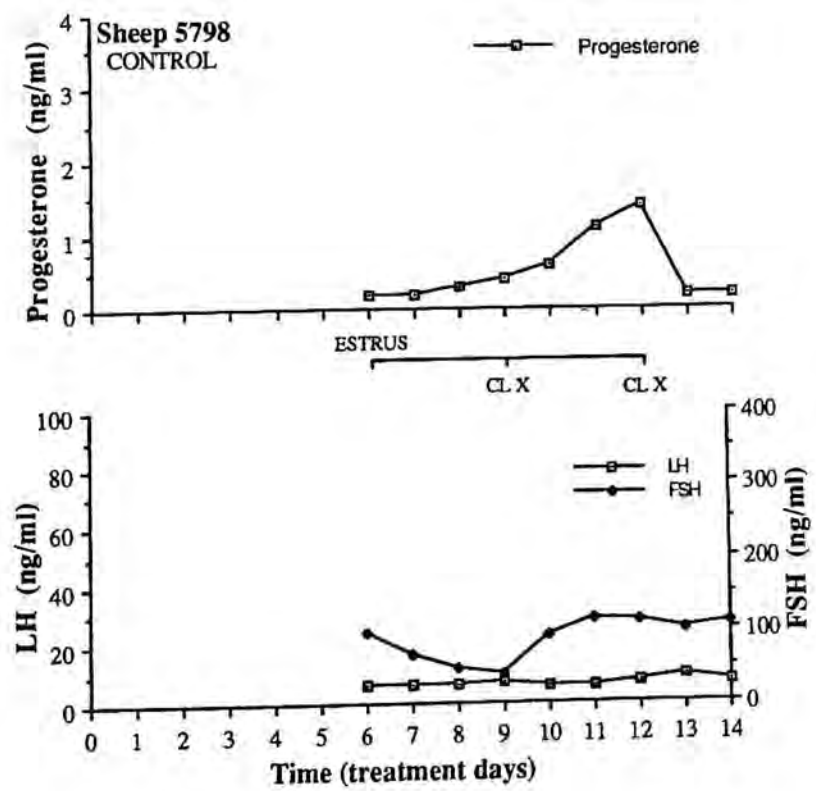
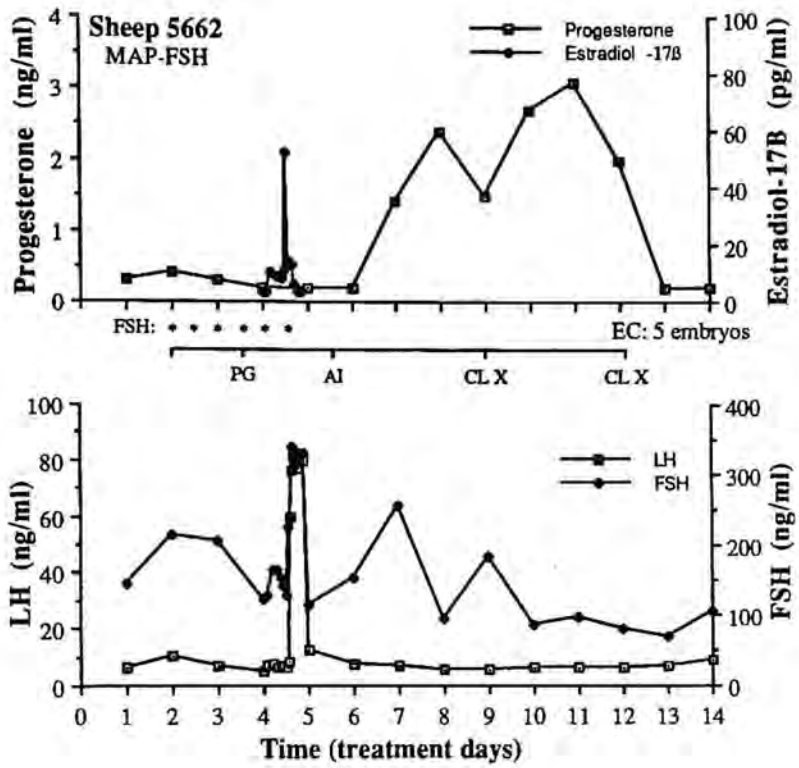

Sheep 5798
CONTROL
Progesterone
Progesterone (ng/ml)
ESTRUS
CL X
CL X
LH (ng/ml)
FSH (ng/ml)
LH
FSH
Time (treatment days)

Figure 23A. Temporal endocrine profiles throughout the periovulatory and luteal phases of individual MAP-treated ewes are exhibited on the double template graphs. The timing of hormonal treatments (FSH, MAP), onset of estrus, artificial insemination (AI), CL removal (CLX) and embryo collection (EC) are indicated on the double template graphs for each donor.


Sheep 5662
MAP-FSH
Progesterone
Estradiol -17β
Progesterone (ng/ml)
Estradiol-17B (pg/ml)
FSH: * * * * * *
EC: 5 embryos
PG
AI
CL X
CL X
LH
FSH
LH (ng/ml)
FSH (ng/ml)
Time (treatment days)

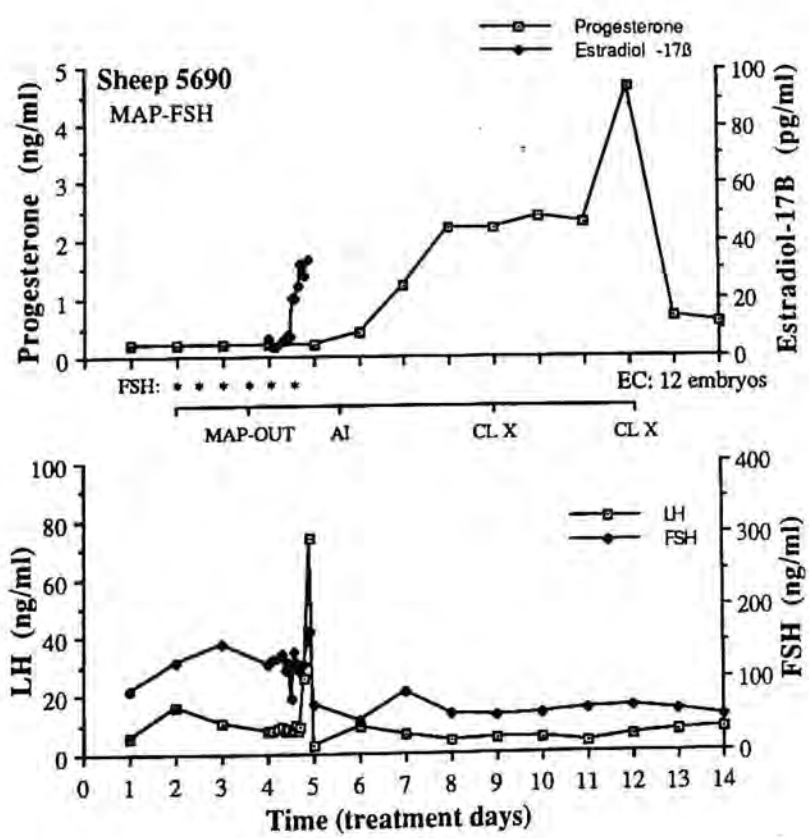
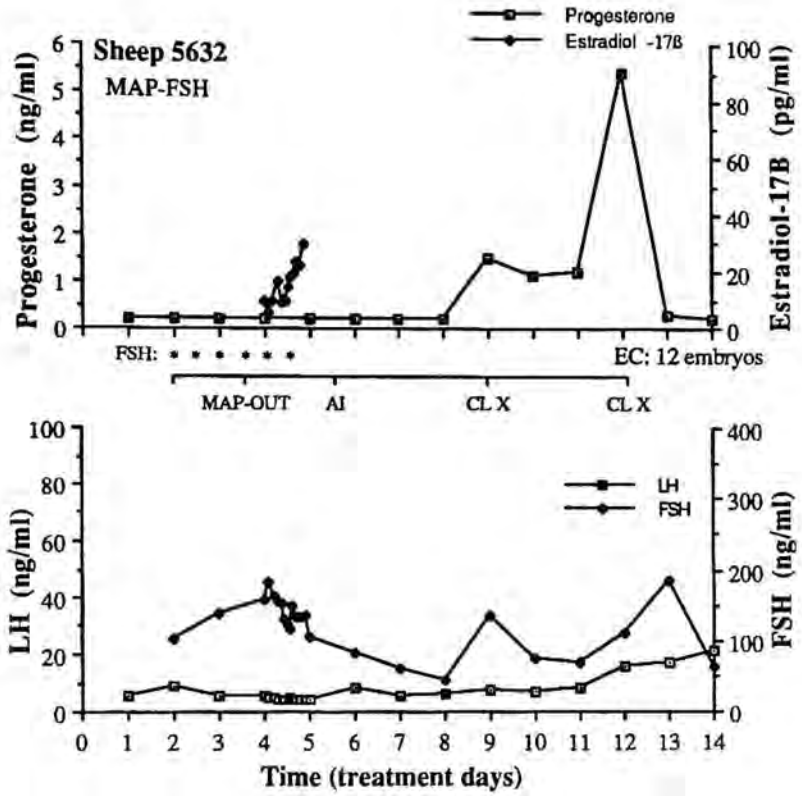

Sheep 5690
MAP-FSH
Progesterone
Estradiol -17β
Progesterone (ng/ml)
Estradiol-17B (pg/ml)
FSH: * * * * * *
EC: 12 embryos
MAP-OUT
AI
CL X
CL X
LH
FSH
LH (ng/ml)
FSH (ng/ml)
Time (treatment days)

Figure 23B. Temporal endocrine profiles throughout the periovulatory and luteal phases of individual MAP-treated ewes are exhibited on the double template graphs. The timing of hormonal treatments (FSH, MAP), onset of estrus, artificial insemination (AI), CL removal (CLX) and embryo collection (EC) are indicated on the double template graphs for each donor.


Sheep 5632
MAP-FSH
Progesterone
Estradiol -17ß
Progesterone (ng/ml)
Estradiol-17B (pg/ml)
FSH:
EC: 12 embryos
MAP-OUT
AI
CL X
CL X
LH
FSH
LH (ng/ml)
FSH (ng/ml)
Time (treatment days)

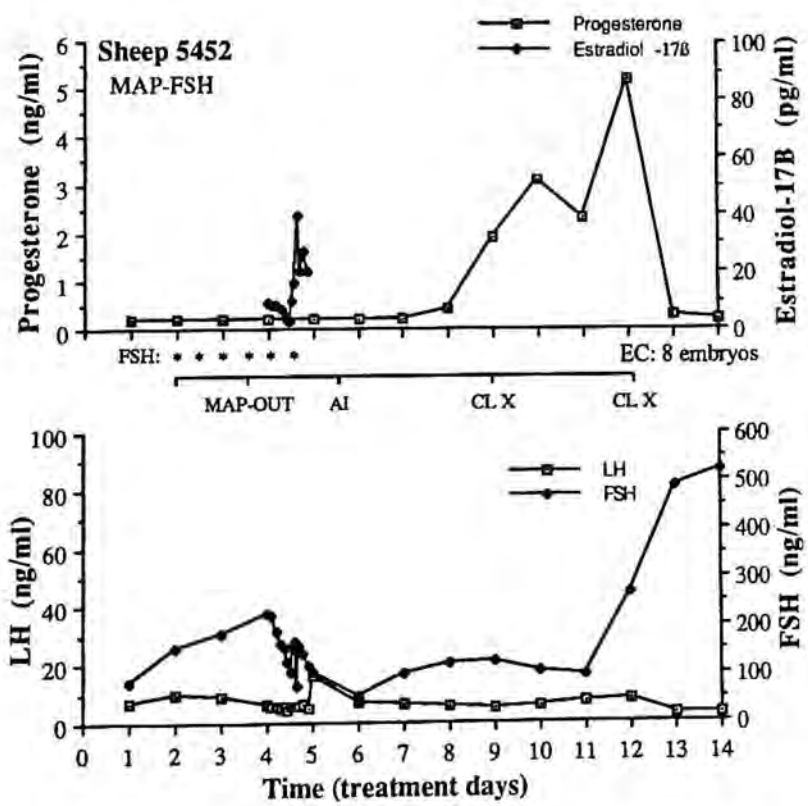
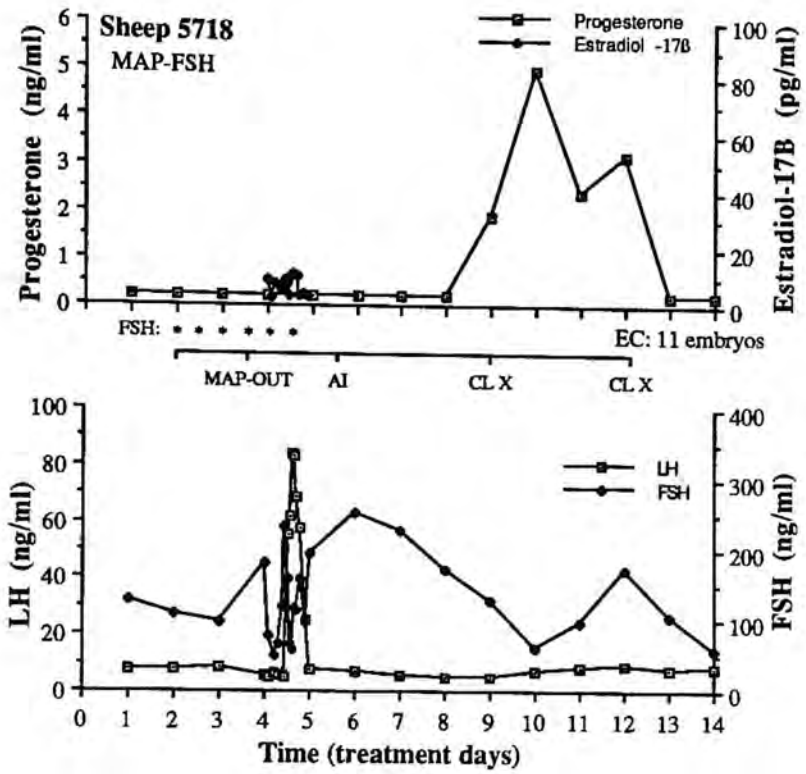

Sheep 5452
MAP-FSH
Progesterone
Estradiol -17ß
Progesterone (ng/ml)
Estradiol-17B (pg/ml)
FSH:
EC: 8 embryos
MAP-OUT
AI
CL X
CL X
LH
FSH
LH (ng/ml)
FSH (ng/ml)
Time (treatment days)

Figure 23C. Temporal endocrine profiles throughout the periovulatory and luteal phases of individual MAP-treated ewes are exhibited on the double template graphs. The timing of hormonal treatments (FSH, MAP), onset of estrus, artificial insemination (AI), CL removal (CLX) and embryo collection (EC) are indicated on the double template graphs for each donor.


Sheep 5718
MAP-FSH
Progesterone
Estradiol -17β
Progesterone (ng/ml)
Estradiol-17B (pg/ml)
FSH:
EC: 11 embryos
MAP-OUT
AI
CL X
CL X
LH
FSH
LH (ng/ml)
FSH (ng/ml)
Time (treatment days)

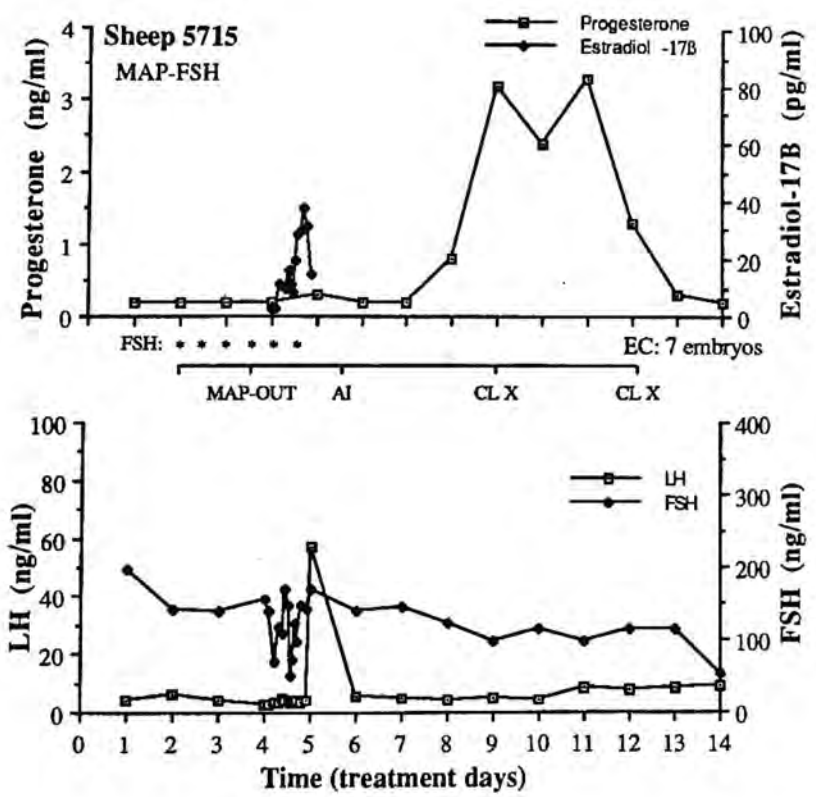
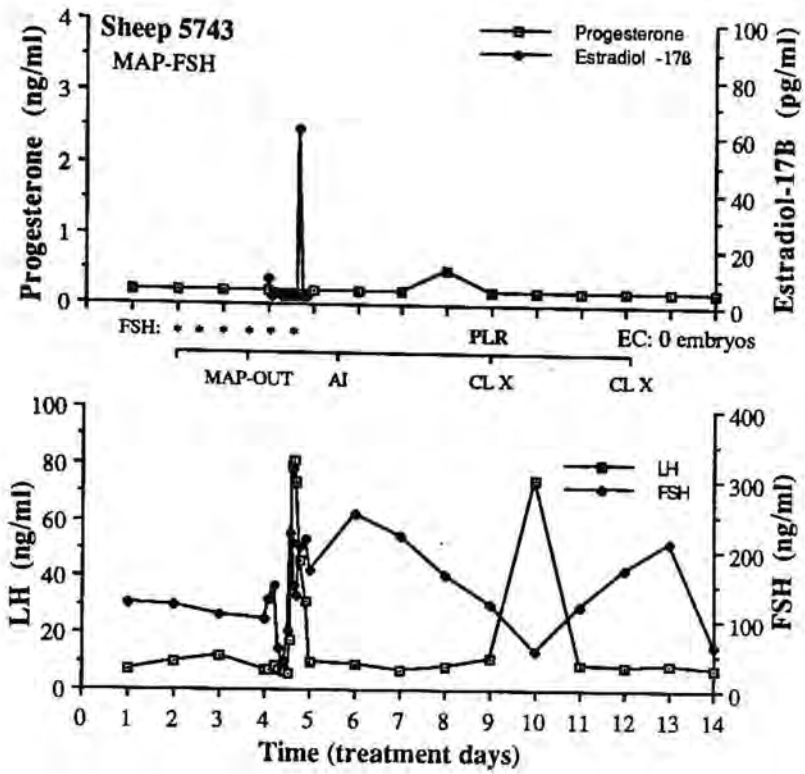

Sheep 5715
MAP-FSH
Progesterone
Estradiol -17β
Progesterone (ng/ml)
Estradiol-17B (pg/ml)
FSH:
EC: 7 embryos
MAP-OUT
AI
CL X
CL X
LH
FSH
LH (ng/ml)
FSH (ng/ml)
Time (treatment days)

Figure 23D. Temporal endocrine profiles throughout the periovulatory and luteal phases of individual MAP-treated ewes are exhibited on the double template graphs. The timing of hormonal treatments (FSH, MAP), onset of estrus, artificial insemination (AI), CL removal (CLX) and embryo collection (EC) are indicated on the double template graphs for each donor.


Sheep 5743
MAP-FSH
Progesterone
Estradiol -17ß
Progesterone (ng/ml)
Estradiol-17B (pg/ml)
FSH: * * * * * *
PLR
EC: 0 embryos
MAP-OUT
AI
CL X
CL X
LH
FSH
LH (ng/ml)
FSH (ng/ml)
Time (treatment days)

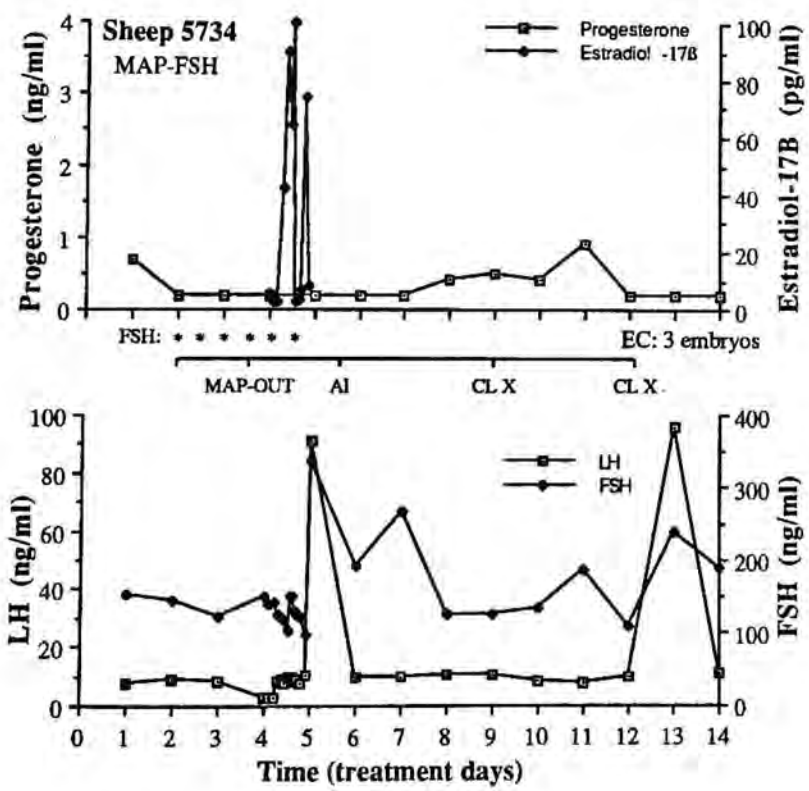
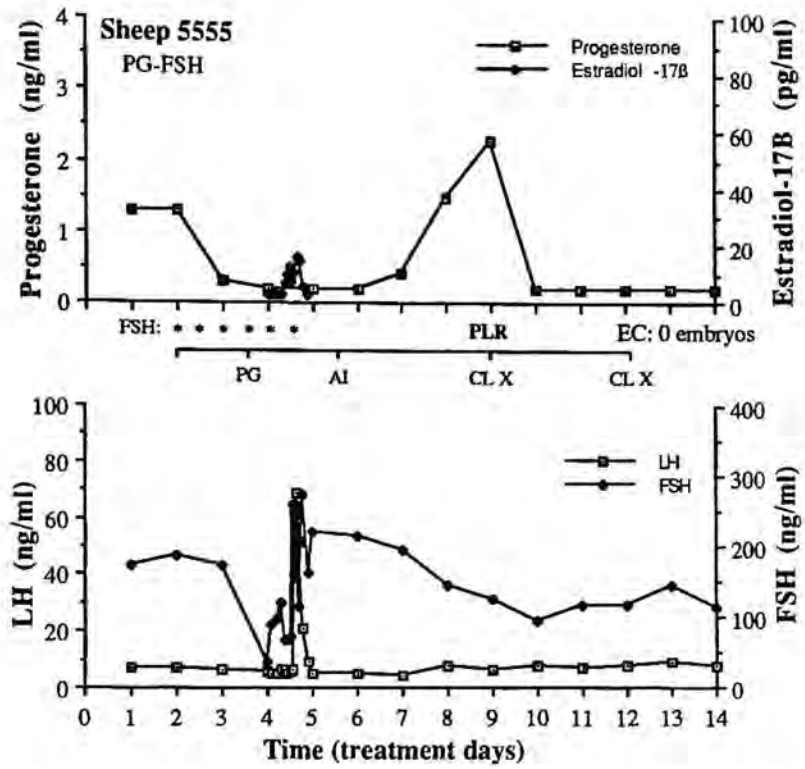

Sheep 5734
MAP-FSH
Progesterone
Estradiol -17ß
Progesterone (ng/ml)
Estradiol-17B (pg/ml)
FSH: * * * * * *
EC: 3 embryos
MAP-OUT
AI
CL X
CL X
LH
FSH
LH (ng/ml)
FSH (ng/ml)
Time (treatment days)

Figure 24A. Temporal endocrine profiles throughout the periovulatory and luteal phases of individual $PGF_2\alpha$-treated ewes are exhibited on the double template graphs. The timing of hormonal treatments (FSH, PG), onset of estrus, artificial insemination (AI), CL removal (CLX) and embryo collection (EC) are indicated on the double template graphs for each donor.


Sheep 5555
PG-FSH
Progesterone
Estradiol -17β
Progesterone (ng/ml)
Estradiol-17B (pg/ml)
FSH: * * * * * *
PLR
EC: 0 embryos
PG
AI
CL X
CL X
LH
FSH
LH (ng/ml)
FSH (ng/ml)
Time (treatment days)

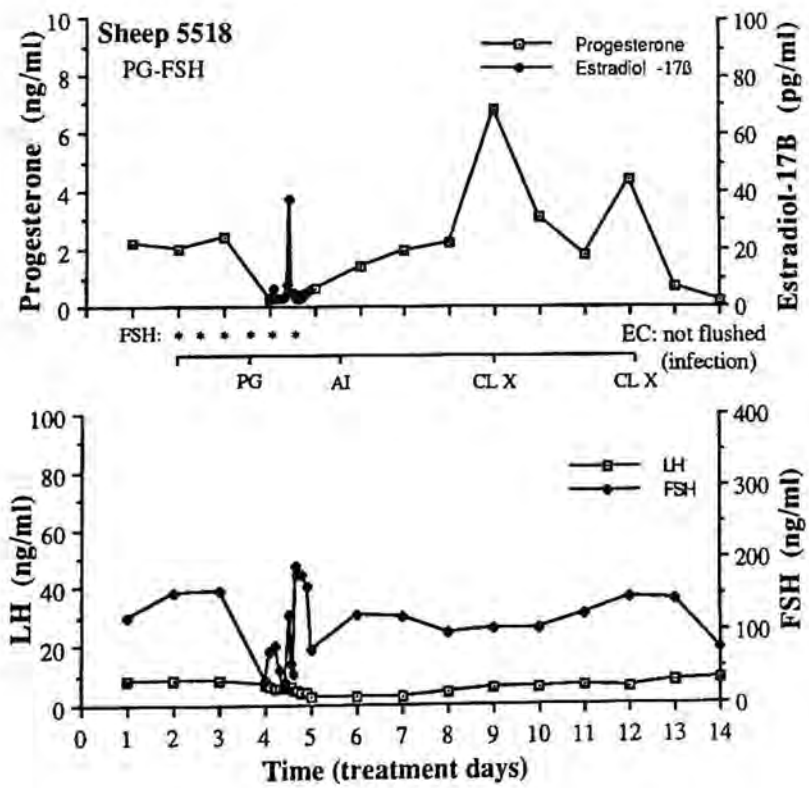
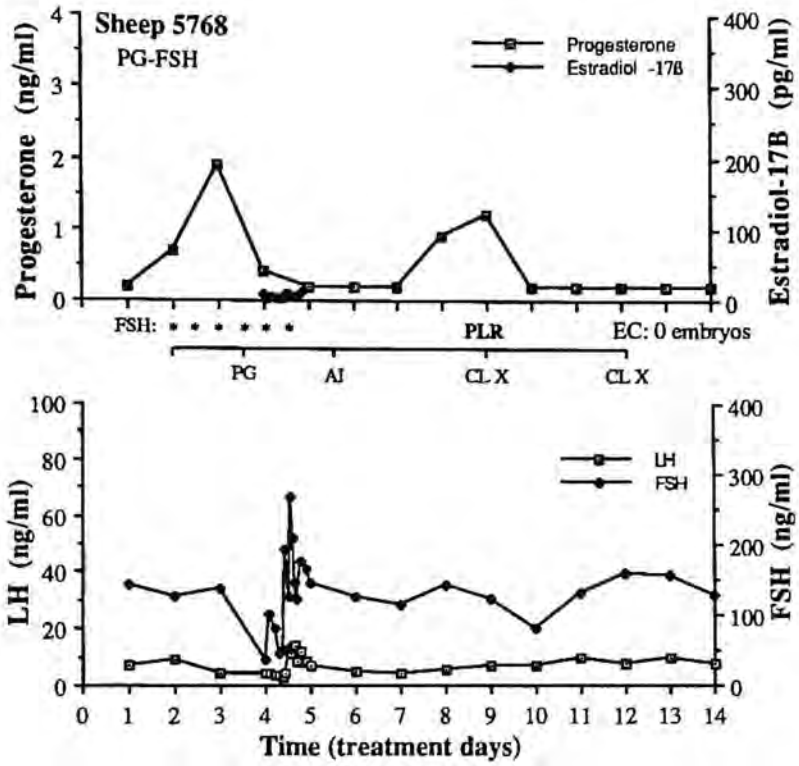

Sheep 5518
PG-FSH
Progesterone
Estradiol -17β
Progesterone (ng/ml)
Estradiol-17B (pg/ml)
FSH: * * * * * *
EC: not flushed
(infection)
PG
AI
CL X
CL X
LH
FSH
LH (ng/ml)
FSH (ng/ml)
Time (treatment days)

Figure 24B. Temporal endocrine profiles throughout the periovulatory and luteal phases of individual $PGF_2\alpha$-treated ewes are exhibited on the double template graphs. The timing of hormonal treatments (FSH, PG), onset of estrus, artificial insemination (AI), CL removal (CLX) and embryo collection (EC) are indicated on the double template graphs for each donor.


Sheep 5768
PG-FSH
Progesterone
Estradiol -17ß
Progesterone (ng/ml)
Estradiol-17B (pg/ml)
FSH:
PLR
EC: 0 embryos
PG
AI
CL X
CL X
LH
FSH
LH (ng/ml)
FSH (ng/ml)
Time (treatment days)

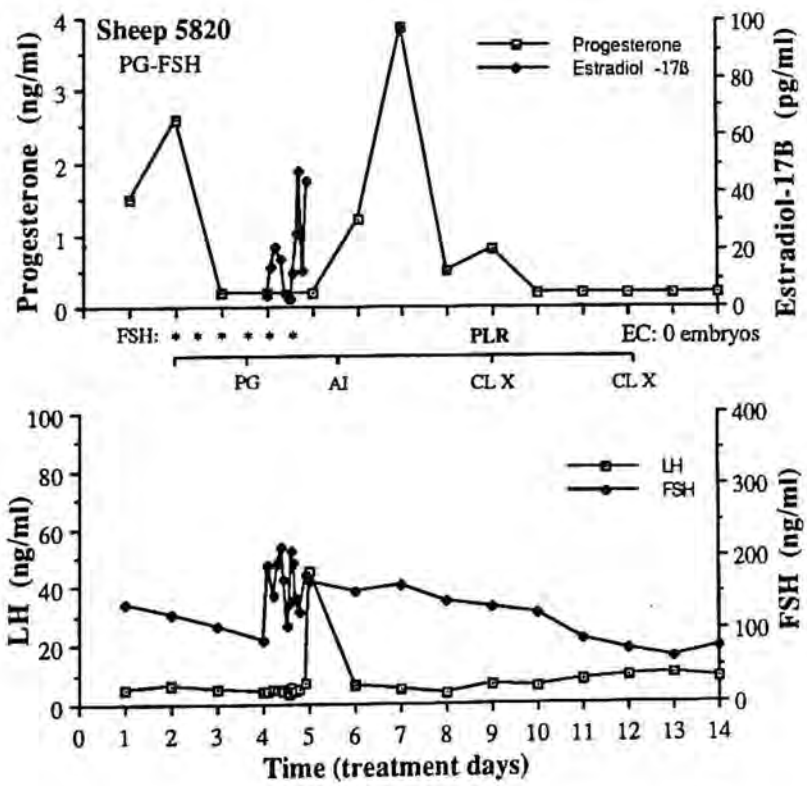
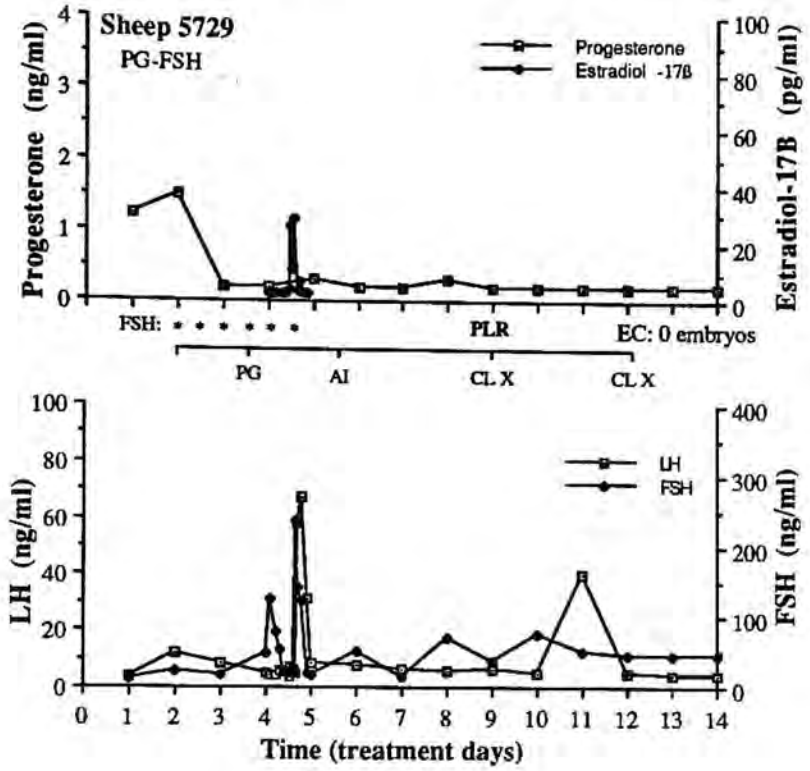

Sheep 5820
PG-FSH
Progesterone
Estradiol -17ß
Progesterone (ng/ml)
Estradiol-17B (pg/ml)
FSH:
PLR
EC: 0 embryos
PG
AI
CL X
CL X
LH
FSH
LH (ng/ml)
FSH (ng/ml)
Time (treatment days)

Figure 24C. Temporal endocrine profiles throughout the periovulatory and luteal phases of individual $PGF_2\alpha$-treated ewes are exhibited on the double template graphs. The timing of hormonal treatments (FSH, PG), onset of estrus, artificial insemination (AI), CL removal (CLX) and embryo collection (EC) are indicated on the double template graphs for each donor.


Sheep 5729
PG-FSH
Progesterone
Estradiol -17β
Progesterone (ng/ml)
Estradiol-17B (pg/ml)
FSH:
PLR
EC: 0 embryos
PG
AI
CL X
CL X
LH
FSH
LH (ng/ml)
FSH (ng/ml)
Time (treatment days)

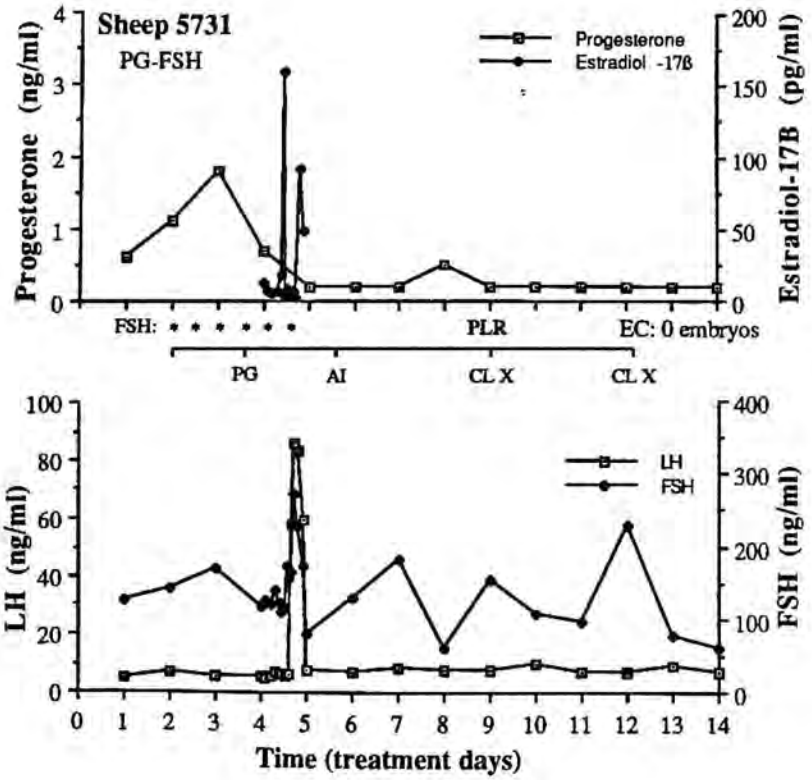
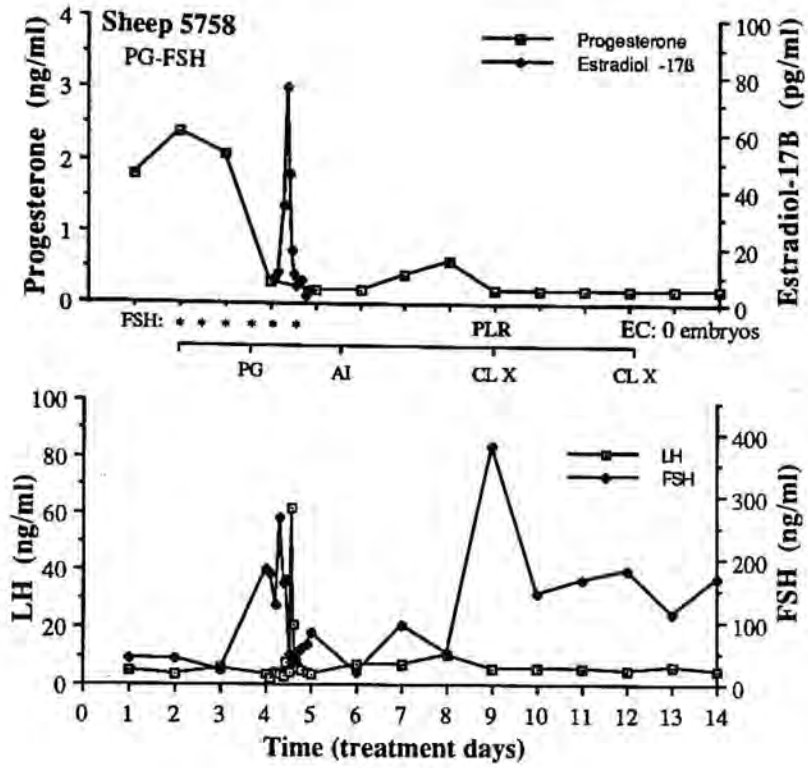
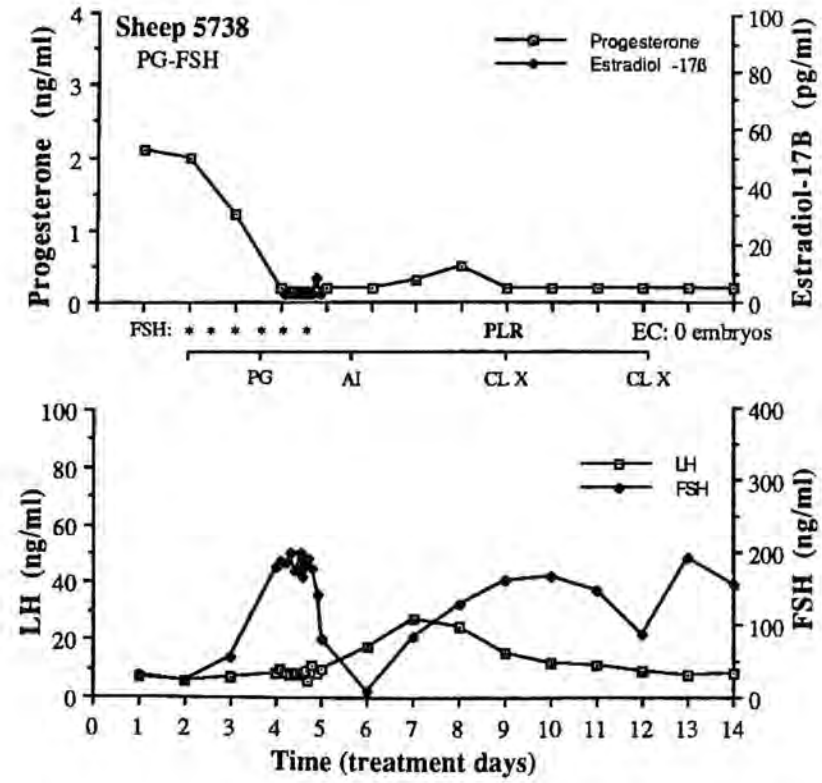

Sheep 5731
PG-FSH
Progesterone
Estradiol -17β
Progesterone (ng/ml)
Estradiol-17B (pg/ml)
FSH:
PLR
EC: 0 embryos
PG
AI
CL X
CL X
LH
FSH
LH (ng/ml)
FSH (ng/ml)
Time (treatment days)

Figure 24D. Temporal endocrine profiles throughout the periovulatory and luteal phases of individual $PGF_2\alpha$-treated ewes are exhibited on the double template graphs. The timing of hormonal treatments (FSH, PG), onset of estrus, artificial insemination (AI), CL removal (CLX) and embryo collection (EC) are indicated on the double template graphs for each donor.


Sheep 5758
PG-FSH
Progesterone
Estradiol -17ß
Progesterone (ng/ml)
Estradiol-17B (pg/ml)
FSH: * * * * * *
PLR
EC: 0 embryos
PG
AI
CL X
CL X
LH
FSH
LH (ng/ml)
FSH (ng/ml)
Time (treatment days)


Sheep 5738
PG-FSH
Progesterone
Estradiol -17ß
Progesterone (ng/ml)
Estradiol-17B (pg/ml)
FSH: * * * * * *
PLR
EC: 0 embryos
PG
AI
CL X
CL X
LH
FSH
LH (ng/ml)
FSH (ng/ml)
Time (treatment days)

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Acritopoulou, S.W., J.P. Haresign, J.P. Foster and G.E. Lamming. Plasma progesterone and LH concentrations in ewes after injection of an analogue of prostaglandin $F_{2\alpha}$. J. Reprod. Fert. 49: 337-340, 1977.

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