DEVELOPING AND EVALUATING A METHOD FOR MULTI-OVULATION AND EMBRYO TRANSFER (MOET) IN THE BISON (BISON BISON)

ANDREW STEPHEN VAN DER WALT

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Supervisor: Prof. J.P.C. Greyling
Co-supervisor: Dr. C. van der Westhuizen

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DECLARATION OF INDEPENDENT WORK

DECLARATION WITH REGARD TO INDEPENDENT WORK

I, ANDREW STEPHEN VAN DER WALT, identity number 7311305059085 and student number 20155980, do hereby declare that this research project submitted to the Technikon Free State for the Degree MAGISTER TECHNOLOGIAE: AGRICULTURE, is my own independent work, and complies with the Code of Academic Integrity, as well as other relevant policies, procedures, rules and regulations of the Technikon Free State, and has not been submitted before to any institution by myself of any other person in fulfilment (or partial fulfilment) of the requirements for the attainment of any qualification.

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LIST OF ABBREVIATIONS

μg	microgram (10^{-6} g)
(IGF-1)	Insulin growth factor – 1
AI	artificial insemination
BSA	Bovine serum albumin
CL	Corpus Luteum
E_2	Estradiol - 17β
FSH	Follicle Stimulating Hormone
GnRH	Gonadotrophin releasing hormone
hCG	Human chorionic gonadotrophin
hMG	human menopaused gonadotrophin
im	intramuscular
IU	International units
kg	kilograms
LH	Luteinizing hormone
mg	milligrams
MJ/kg	mega joule per kilogram
MOET	Multi Ovulation and Embryo Transfer
MR	medium recovery rate
OR	ovulation rate
PGF	Prostaglandin
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PMSG	Pregnant Mare Serum Gonadotrophin
rBST	Recombinant bovine somatotrophin
RR	embryo recovery rate
UO	unfertilised oocytes

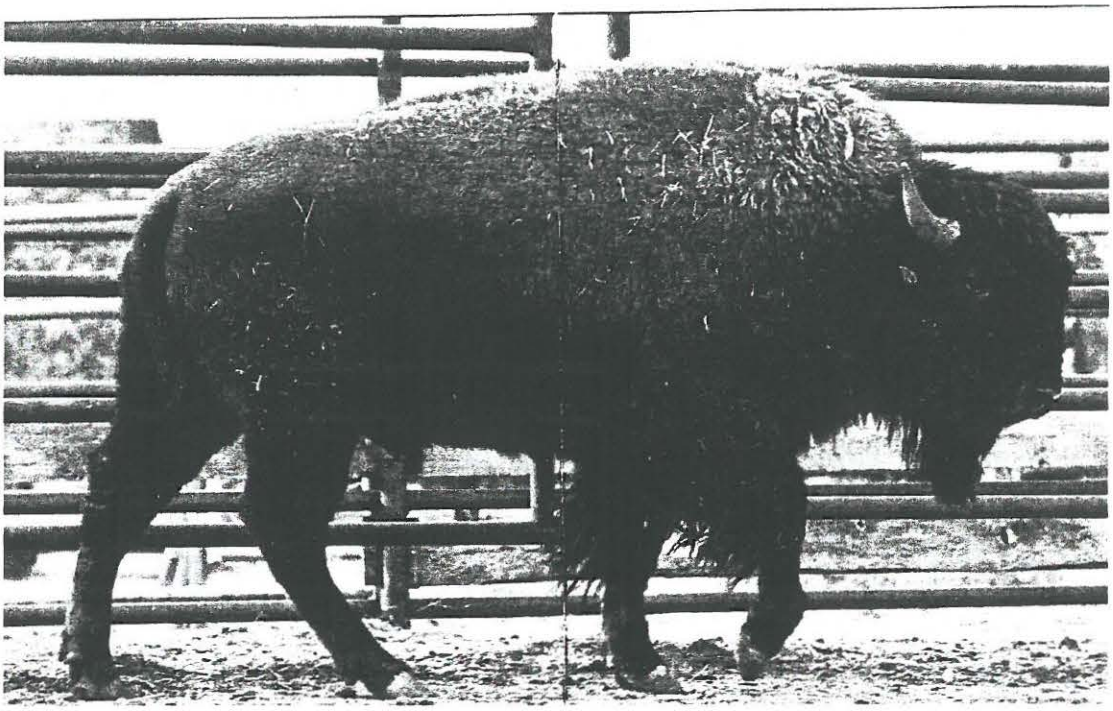

Plate 1 Two-year old bison bull

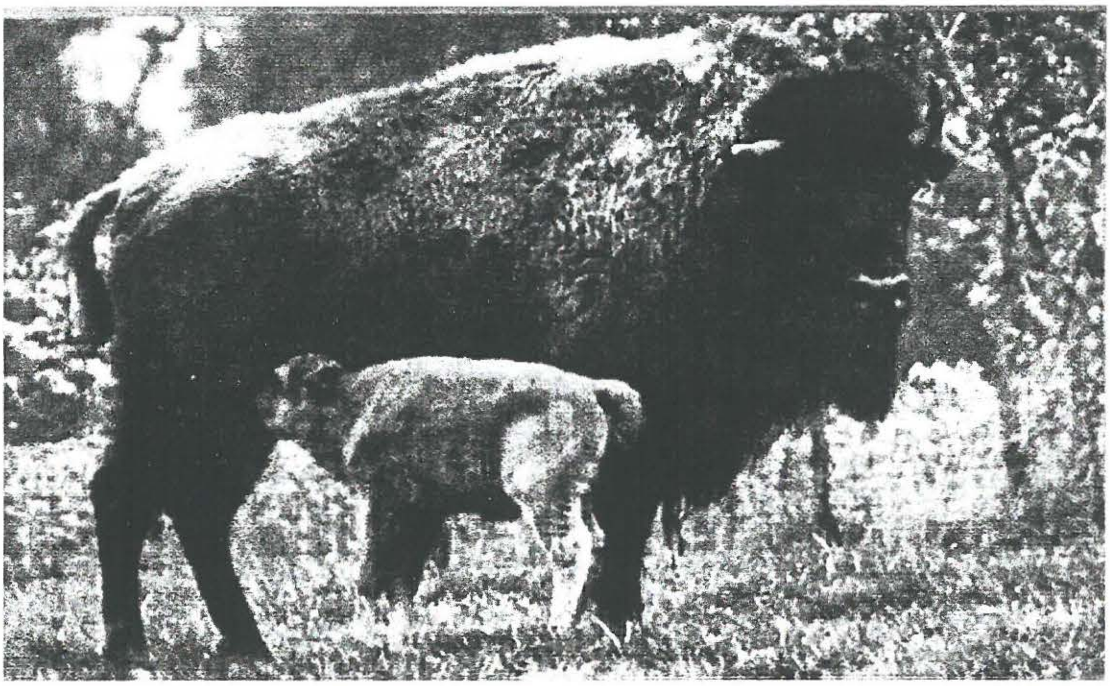

Plate 2 Bison cow with a healthy suckling calf resulting from embryo transfer

CHAPTER 1

INTRODUCTION

The idea of domesticating bison is a goal for some cattlemen, who believe in utilizing the hardiness, long life, foraging habits and feed conversion abilities of the bison in the production of animal protein. It was not until the population of millions of bison, that once roamed the USA ranges, had been reduced to only a few hundred head, that anybody seriously took an active interest in restoring the numbers of these unique animals.

Reproductive studies on the bison have shown that anatomically and physiologically they are very similar to domestic cattle. One notable difference is the apparent seasonality (Table 2.1) of the bison. Bison breeders indicate that bison are seasonally polyestrous, having a cycling season during the late summer and continuous into late autumn. Other bison breeders consider the breeding season to be shorter, ranging from summer to autumn. This observed shorter duration may be partly due to the fact that increased breeding activity is observed only during the early part of the season. Cows that do not conceive early in the season, often do not become pregnant as they are subjected to the stress of winter and the consequent decrease in the quality and quantity of nutrition.

Anatomically, bison have small ovaries and reproductive tracts, which could be compared to that of first-calf beef heifers. Consequently, follicles and CL's are smaller and more difficult to identify by rectal palpation.

Problems associated with the use of reproductive techniques, such as superovulation and embryo transfer in the bison, are not the technologies as such, but the handling and management of the animals. Most bison are not amenable to domestication and are managed as wild animals. The bison therefore responds accordingly and are often dangerous when handled. The stress encountered during these procedures on the animal is also a factor that could affect the response of the bison to superstimulation.

A compounding problem with the few animals still left in the early 1900's, hints that the bison can experience a serious inbreeding problem. This problem has led to early abortions and birth defects in many cases. A need has therefore arisen to increase the number of bison in order to create various bloodlines. One of the more economical and feasible ways to increase the bison numbers and achieve this goal is by making use of reproductive technologies such as embryo transfer, *in vitro* fertilization and artificial insemination (AI). Embryo transfer has the advantage of accelerating the rate of genetic progress and increasing the number of offspring produced. This study thus looks at the possibility of using accelerated breeding techniques generally implemented in domestic cattle to help increase the bison numbers and potential meat production.

CHAPTER 2

LITERATURE REVIEW

2.1 BISON REPRODUCTION

2.1.1 Introduction

Domestic cattle (*Bos taurus, Bos indicus*) and North American "buffalo" (*Bison bison*), the African wild buffalo (*Syncerus caffer*), and the water buffalo (*Bubalus bubalis*) are in the family Bovidae, but belong to different genera and have different chromosome numbers (Appendix II). Many aspects of reproduction are similar in cattle and bison, but indiscriminate extrapolation of reproductive phenomena of efficiency in cattle and the bison must be avoided.

The world population of approximately 50 000 domestic bison, *Bison bison,* has been broadly classified into the wood bison and the plain bison. The two types differ in their body conformation, but are reproductively similar (2n = 60 chromosomes) (Jainudeen & Hafez, 2000).

Bison are unique in their response to current reproductive technologies used in domestic cattle. Unlike other exotic or wild ruminants, the bison do respond to protocols used in domestic cattle, when administered under favourable conditions. Therefore, bison being a ruminant and being reproductively similar to cattle, conditions influencing Multi Ovulation Embryo Transfer (MOET) will be compared with those of bovine in this review.

Currently limited studies have been carried out in the field of applied reproduction in the bison (*Bison bison*).

Superovulatory treatment of female animals aim at producing the maximum number of viable oocytes during a single oestrous period. Results from superstimulatory trials and embryo recovery studies in farm animals have demonstrated a large variation with respect to individual and group responses (Elsden, Nelson & Seidel, 1978; Boland, Goulding & Roche, 1991; Goulding, Williams, Roche & Boland, 1991).

Due to the almost non-existence of literature available on MOET techniques in other species of the bovidae family, this literature review will be concentrated on the domestic cow. As a starting point, developments on MOET technique on the domestic cow may be useful in providing valuable information and limits with relevance to the bison.

The objective of this review is an attempt at giving background regarding the status quo, and to substantiate the most important factors that could effect superstimulation and embryo production in the bison.

2.1.2 Puberty

The bison attains puberty at a later age than cattle - it first exhibits oestrus between 21 and 24 months. First conception occurs at an average bodyweight of 250 to 275kg, which is usually attained at 24 to 36 months of age (Jainudeen & Hafez, 2000). The testes of the bison descend into the scrotum at 2 to 4 months of age, although they may be present in the scrotum at birth in some animals. Spermatogenesis commences at 12 to 15 months, although the ejaculate only contains viable spermatozoa at an age of 24 months (Table 2.1) (Jainudeen & Hafez, 2000).

Table 2.1 Reproductive characteristics of cattle and bison bulls

Characteristic	Cattle	Bison
Age at puberty (months)*	10 to 12	10 to 15
Sexual season	none	Spring, Summer
Duration of seminiferous epithelial cycle (days)	13.5	13.5
Semen:		
Volume (ml)	4 to 10	2 to 5
Concentration (10^9/ml)	0.8 to 2.0	0.3 to 1.5
Bull to cow ratio	1.30	1.30

* Defined as age of first ejaculate containing 50 million sperm with 10% progressive motility (cattle) or age at first collection (buffalo)

2.1.3 Oestrous cycles

Breeding Season

Seasonal calving patterns reported have been attributed to ambient temperature, photoperiod and feed supply. The photoperiodic effect in both bison and cattle on oestrous cyclicity is similar. Bison calving in summer or fall resume ovarian cyclicity earlier than those in winter or spring (Ahmad, Chaudhry & Khan, 1981). Decreasing day length and cooler ambient temperatures also favour cyclicity. During summer when ambient temperature and photoperiod are at their maximum, prolactin levels are highest (Kaker, Razdan & Galhotra *et al.*, 1982) and plasma progesterone levels are lowest (Rao & Pandey, 1982). High ambient temperatures may also contribute to this seasonality by depressing the male libido (Jainudeen & Hafez, 2000).

2.1.4 Cyclic changes

Many cyclic changes in the ovaries, tubular genitalia and hormonal secretions in the bison are comparable to those of cattle (Figure 2.1). Assessment of ovarian activity is based on combinations of daily oestrous detection with a vasectomized male, rectal palpation or laparoscopic observation of the ovaries or plasma progesterone assay. Progesterone levels in plasma and milk, as in cattle, reflect the endocrine activity of the *corpus luteum* (CL) but levels are generally lower in the bison (Table 2.2). Exogenous $PGF_{2\alpha}$ will cause regression of the cyclic CL. $PGF_{2\alpha}$ of uterine origin, as in cattle, is the luteolysin in the bison (Jainudeen & Hafez, 2000).

2.1.5 Oestrus and ovulation

The length of the oestrous cycle in the bison is 19 to 22 days and oestrus lasts 12 to 30 hours. Overt signs of oestrus are less intense than in cattle. Acceptance of the male is the most reliable sign of oestrus in the bison, like e.g. in the water buffalo. Less than a third of bison in oestrus are detected by homosexual behaviour.

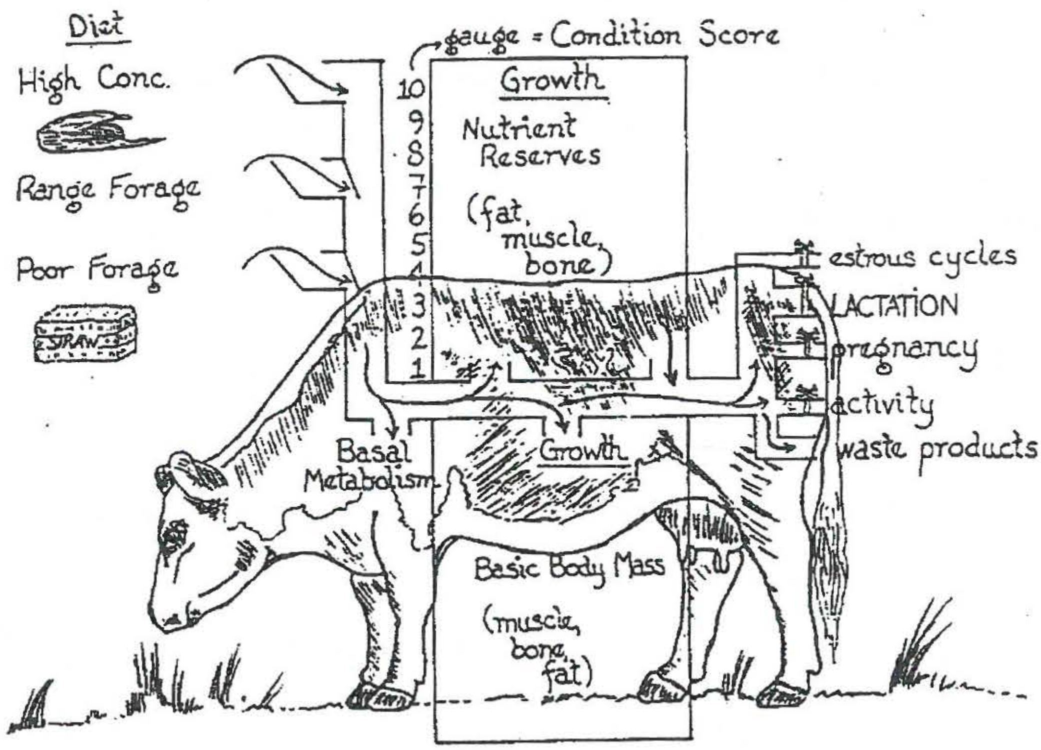

Figure 2.1 **Partitioning of nutrients in beef cows fed diets differing in quality (Short & Adams, 1988)**

Sexual receptivity of the male can be determined as in cattle by a vasectomized male or an androgenized female fitted with a chin ball mating device or the use of heat-mount detectors. The efficiency of these oestrous detection aids, however, may be reduced because of the wallowing habits of bison. Oestrus commences towards late evening, with peak sexual activity occurring between 18:00 and 6:00. Matings could continue until late morning, but cease during later daylight hours. Ovulation, as in cattle, occurs 15 to 18 hours after the onset of oestrus. Ovulation is preceded by a surge of LH at the onset of oestrus and a single ovum is shed during one oestrous cycle (Kaker, Razdan & Galhotra, 1980).

Table 2.2 Female reproductive characteristics of cattle and bison (Short & Adams, 1988)

Parameter	Cattle mean (range)	Bison mean (range)
Sexual season	Polyestrous	Polyestrous
Mean age at puberty (range-months)*	15 (10 to 24)	18 (15 to 30)
Oestrous cycle:		
Mean length (range-days)	21 (14 to 29)	21 (18 to 22)
Mean oestrous duration (range-hours)	18 (12 to 30)	21 (17 to 24)
Ovulation (hours):		
Type	Spontaneous	Spontaneous
Time from onset of oestrus (range-hours)	30 (18 to 48)	32 (18 to 45)
Number of ova shed	1	1
Mean life span of CL (range-days)	16	16
Fertilizable life of ova (hours)	20 to 24	Unknown
Mean time entry of embryo into uterus	6-7 days	
(range-hours after ovulation)	90 (64 to 96)	Unknown
Mean gestation length (range-days)	280 (278 to 293)	280 (278 to 293)
Mean age at first calving (range-months)	30 (24 to 36)	42 (36 to 56)
Mean postpartum interval (range-days):		
Uterine involution (range)	45 (32 to 50)	35 (16 to 60)
First ovulation (range)	30 (10 to 110)	30 (10 to 110)
Mean calving interval (range-months)	13 (12 to 14)	13 (12 to 14)

2.1.6 Breeding

Male sexual behaviour is similar but less intense than in the bovine bull and libido is suppressed during the hotter part of the day. Sniffing of the vulva or female urine and the Flehmen reaction precedes mounting of the oestrous female. Artificial insemination in bison is similar to that of cattle but is not commonly practiced due to the difficulty of detecting oestrus and the handling of the animal (Lamming, Darwash & Back, 1989).

2.1.7 Ovarian function

Both progesterone and estrogen levels in the bison cow blood plasma decrease immediately following parturition. No clearly defined pattern of either LH or FSH secretion occurs during the first 5 days postpartum, irrespective of whether the cow is suckled (Carruthers & Hafs, 1980; Peters, Lamming & Fisher, 1981). During this anovulatory phase of the puerperium, large ovarian follicles secrete levels of estradiol -17β but most cows neither exhibit oestrus nor ovulate (Rawlings, Wier, Todd, Manns & Hyland, 1980).

During the transition from acyclicity to cyclicity GnRH release increases the frequency of plasma LH episodes leading to follicular activity and estradiol secretion. Estradiol in turn enhances the pituitary responsiveness to GnRH. The episodic release of LH may be a prerequisite for the onset of cyclic ovarian activity (Figure 2.2) in the bison cow (Lamming, Wathes & Peters, 1981). In about 50% of cows, a transient priming of progesterone for up to

10 days precedes ovulation (Lamming, Darwash & Back, 1989). The interval from parturition to first ovulation shows considerable variability (Table 2.2).

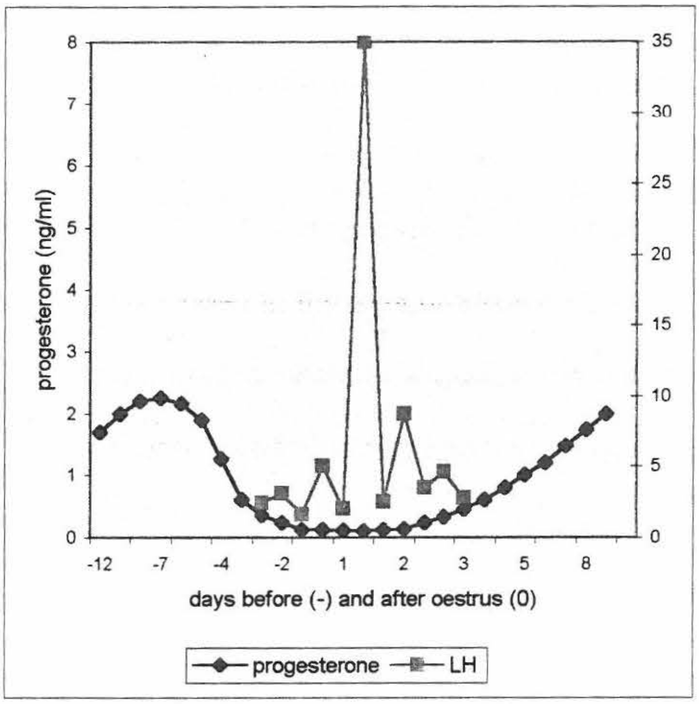

Figure 2.2 **Plasma progesterone levels during the oestrous cycle and the surge of LH at oestrus in the bison**

Multiparous cows ovulate earlier than primiparous cows. Suckling and the plane of nutrition delays the time of the first postpartum ovulation in bison cows. The incidence of first postpartum ovulation without oestrous behaviour, is relatively high, thus the first oestrus may not necessarily reflect the resumption of ovarian cyclicity. Generally, oestrus in the bison is observed for the first time at about 35 days postpartum. The conception rate is also generally lower at first postpartum oestrus than at subsequent oestrous periods. Bison cows are bred after 50 days postpartum and should conceive by 80 days to maintain a calving interval of 12 months (Jainudeen & Hafez, 2000).

2.1.8 Reproductive performance

Due to the fact that no economic necessity currently exists to keep breeding records of pasture managed bison under extreme conditions, many farmers use only the number of calvings and calves born or weaned as criteria for reproductive efficiency. Reproductive efficiency of the bison, however, can be measured by similar criteria used for cattle (Table 2.2). Conception rates of 50 to 60% are obtained in cattle with chilled semen, compared to 25 to 45% with frozen semen, while over 60% is obtained with natural service. The pregnancy rate following a restricted breeding season of 2 to 3 months in bison herds varies between 30 and 75%, depending on the nutritional and lactational status of the females at mating. Calf crops as high as 80% have been reported in some bison herds (Jainudeen & Hafez, 2000).

The calving interval is a fertility index widely used at ranching level. Under range conditions, a bison generally produces two calves in two years. The calving interval is influenced by the individual dam, the year and season of conception and parity (Lundstrom, Abeygunawardena, De Silva & Perera, 1982).

2.1.9 Increasing reproductive performance

Delayed age at first calving, problems related to oestrous detection and days open in the female, and loss of libido in the male are the major constraints to increasing reproductive rates in the bison (Jainudeen & Hafez, 2000). Improvements in nutritional status could increase growth rates and advance the onset of puberty. Similarly, management practices such as early weaning, induction of oestrus with prostaglandins and improved nutrition advanced the resumption of postpartum ovarian activity and reduce the days open in the bison. The marked seasonal fluctuations in libido and semen quality in the male may be overcome by providing cooling facilities for bison during the hot season. Induction of oestrus with synthetic analogues of $PGF_{2\alpha}$ and fixed-time insemination with frozen semen may prove useful in restricting the mating season so that calvings occur when water and forage are abundant (Jainudeen, 1984).

2.2 NUTRITIONAL STATUS AND BODY CONDITION SCORE

2.2.1 Nutrition

Reproductive rate has a major influence on the production efficiency of cattle (or the bison for that matter), and nutritional management is one of the main limiting or controlling factors affecting reproduction. Shortage of energy as such, is one of the most common problems associated with cattle (or bison) reproduction management. In order to effectively manage energy nutrition, a better understanding is essential regarding the variable affects the various phases and controls the mechanisms of reproduction (Short & Adams, 1988).

Limited energy intake in terms of dietary quality or quantity will delay puberty, lengthen the post partum anoestrous period and cause anoestrus in cyclic cows or heifers. Effects include changes in gonadotropic hormone secretion from the pituitary, production of progesterone during both the oestrous cycle and pregnancy, differential sensitivity of the pituitary – hypothalamus axis to steroids and releasing hormones and changes in ovarian activity as measured by hormone secretion, follicular development and ovulation. This variation in ovarian response seems to be related to differences in the degree of energy limitation, body condition score and whether body weight is maintained. Varying blood glucose levels, are the specific source through which energy manifests its effects on reproduction (Short & Adams, 1988). Nutritional management of cattle and thus also the bison, can have a profound effect on reproduction and thus production efficiency via its effect on puberty in the heifers (Boland & O'Callaghan, 1999; O'Callaghan & Boland, 1999), post partum anoestrous interval and maintenance of oestrous cycles (Weaver, 1987).

It is important to understand the general strategy that a cow employs in the partitioning nutrients (energy) for various body functions. Nutritional effects have been observed and measured using energy as a variable. The primary advantage of beef cattle and even to a greater extend bison in agricultural production is their ability to utilize low quality (low energy and protein content) roughage as a feed source for maintenance and production. However, problems can occur as diet quality (especially energy) has been shown to have a direct effect on reproduction levels, as shown in Figure 2.1 (Short & Adams, 1988).

The priority in the utilization of available energy by an animal can be set out as follows:

1. Basal metabolism
2. Body activity (walking, etc.)
3. Body growth
4. Energy reserves
5. Pregnancy
6. Lactation
7. Additional energy reserves
8. Oestrous activity and initiation of pregnancy.
9. Excess reserves (fat deposition).

The priority of these body functions can vary somewhat in terms of absolute and relative importance, as physiological conditions such as lactation, pregnancy, growth environment and genotype change (Short & Adams, 1988).

Nutrient partitioning for production and reproduction are thus the result of complex interactions between diet quantity and quality, nutrient reserves and the demand for growth (basic body weight), metabolism and other body functions. Reproduction is controlled by a complex combination of hormonal and neural mechanisms and it is important to comprehend that not only does nutrition affect reproduction, but to understand how these effects are mediated through the mechanisms controlling reproduction. Figure 2.3 summarizes some of these mechanisms involved in reproduction (Short & Adams, 1988).

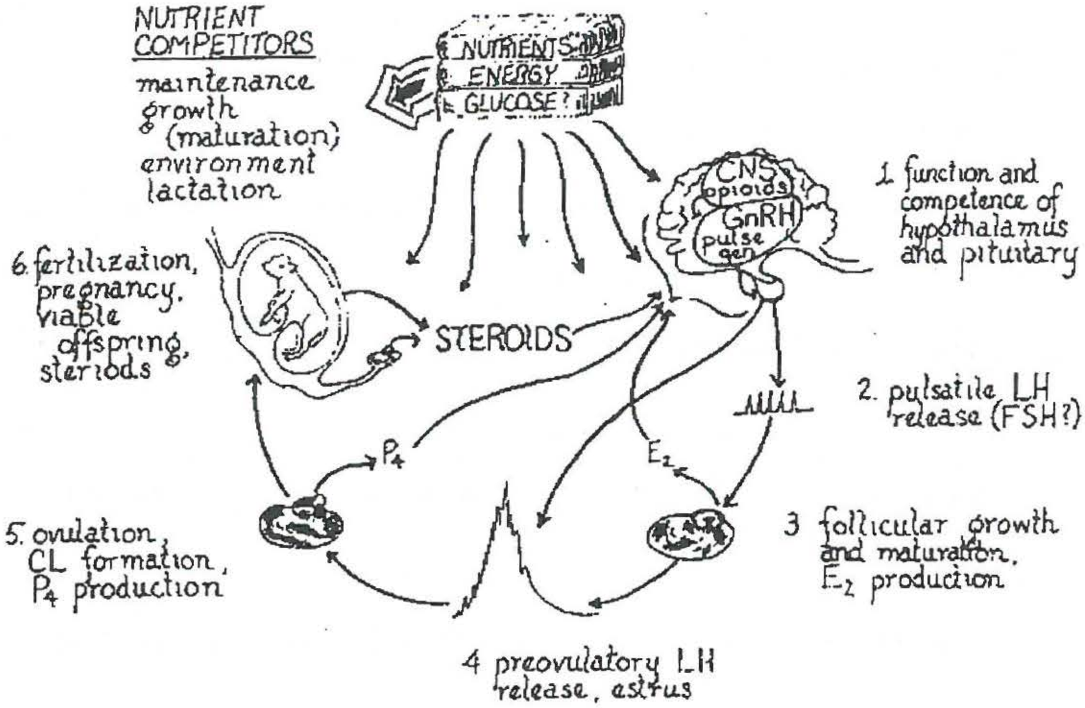

Figure 2.3 **Schematic presentation of the interrelationships that exists between the neural-hormonal control of reproduction in the cow and the modulation that nutrition has on this milieu (Short & Adams, 1988)**

2.2.2 Effect of restricted energy intake on the oestrous cycle

The effects of energy restriction during the oestrous cycle are observed both at the ovarian and pituitary-hypothalamic levels. Several reports of lower serum progesterone (P4) concentrations with under-feeding have been recorded (Beal, Short, Staigmiller, Bellows, Kaltenbach & Dunn 1978; Ferguson & Chalupa, 1989). Evidence of a direct ovarian effect has also been reported. It was noted that incubated CL tissue from underfed heifers synthesize less progesterone (P4), although Staigmiller and England (1982), found no effect following underfeeding, on the ability of incubated follicles to produce estradiol (E2). Experiments with superovulated cows have been inconclusive in their determination of the effect of nutritional status on the ovarian response. Murphy, Enright, Crowe, McConnell, Spicer, Boland and Roche (1991), observed an increase in follicular response to a superovulation treatment in underfed cows, with no effect on ovulation rate. A decrease in ovulation rate was recorded by Dufour Adelakoun and Matton (1981) in underfed cows, with an increased variation in ovulation rate. The effect of feed restriction on LH release is contrary to that for P4, in that a greater concentration of LH is observed in the serum either at the natural oestrus or following the injection of GnRH. This increased release of LH may be partially due to a decrease in the negative feedback of P4 on the hypothalamus (Beal *et al.*, 1978). Imakawa, Kittok and Kinder (1983) fed three levels of energy for 150 days and found that energy restriction decreased the incidence of heifers demonstrating oestrus and anoestrous heifers did not respond to a progestin-oestrogen treatment for the induction of oestrus. This decreased oestrous response was at least, partially due to a decrease in LH secretion and heifers that demonstrate anoestrus due to energy restrictions, may do so because of a hypersensitivity of the hypothalamus to the oestrogen feedback.

Murphy *et al.* (1991) reported of the first work relating to the effect of level of nutrition on reproduction. Here it was reported that a low dietary intake delays follicular development and ovulation, and that both nutritional level and lactation reduces the serum glucose concentrations. Weaver (1987) also examined differences in follicular development, but did so at a specific time post partum (30 days). In this situation, no differences were recorded in follicular development, plasma oestradiol (E2) concentrations or ovulation response to FSH and GnRH treatments. It was, however, found that LH release induced by the injection of GnRH was reduced and delayed by a low level of nutrition.

Boland and O'Callaghan (1999), found low levels of feed intake to delay oestrus and decrease LH and FSH secretion rates. In a subsequent study, Gauthier and Mauleon (1983) used a GnRH challenge to study the effects of weight loss on ovarian activity. Cows that lost weight during the first 45 days post partum, had a greater response to a GnRH challenge, when compared to cows that maintained their body weight during the same period. Rutter and Randel (1984) also treated post partum cows with GnRH, but found no effect of the level of nutrition on serum LH response. However, when considering the ovarian response according to body condition score of the cow, it was found that thinner cows exhibited a smaller response. When considering oestrous response, body condition or fat reserves are apparently more important than the level of nutrition.

2.2.3 Effect of supplementing ionophores on the ovarian activity

Monensin is an ionophore that changes the rumen fermentation patterns so that, during volatile fatty acid (VFA) production, a higher proportion of propionate is favoured. The supplementation of monensin has proven to be a useful tool in the studying of effects of energy metabolism on reproduction and associated hormonal secretion patterns (Short & Adams, 1988). Feeding monensin decreased the age at puberty in cows (Ortuno & Carson, 1985), enhanced the LH response to both E_2 (Randel, Rutter & Rhodes, 1982) and GnRH (Randel & Rhodes, 1980) and increased the ovarian response to superovulation treatment (Bushmich, Randel, McCartor & Carroll, 1980; Ortuno & Carson, 1985). The effects of monensin can be mimicked by infusing propionate into the abomasum (Rutter, Randel, Schelling & Forrest, 1983).

2.3 EFFECT OF BODY CONDITION SCORE ON OVARIAN ACTIVITY

Body condition score in cattle is positively correlated to the level of body reserves at any given time in the production cycle (Richards, Spitzer & Warner, 1986), and is shown to be highly correlated to body fat (Dunn, Riley, Murdock & Field, 1983). It can therefore be stated that body condition score is closely related to nutritional status, physiological condition and the general well-being of the animal. Zeitoun, Rodriguez and Randel (1996) also recorded a strong relationship between body condition and fertility in cattle. This is in agreement with pioneering work done in South Africa by Meaker, Coetsee, Smith and Lishman (1980) and Lishman, Snyman and Moolman (1984). Ginther Kastelic and Knopf (1989) recorded thin cows to have a weak oestrous response to superstimulation. Although limited information on the effect of body condition score on the superstimulation response is available in cattle, several studies have been executed to determine the influence of body condition score on the subsequent reproductive performance in non-stimulated animals (Ginther, Kastelic & Knopf, 1989).

Bielanski and Yadav (1990), in investigating the effect of superstimulation in obese dairy cows (body condition score 4.5 to 5) regarding embryo recovery rate of viable embryos, found the correlation between embryo production and subcutaneous fat deposition to be low. Thus indicating random sources of variation to embryo production, although animals with more backfat tended to produce fewer viable embryos. To the contrary, no detrimental effect on the number of viable embryos produced in obese dual-purpose heifers was recorded by Broadbent, Tregaskes, Dolman and Smith (1996). Extreme fat accumulation in the regions of the ovarian bursar and oviducts could interfere with or prevent normal ovum transport into or through the oviducts, although fat accumulation *per se* does not interfere with the superstimulation response, which is on par with that of thinner animals (Bielanski & Yadav, 1990).

2.4 THE EFFECT OF TEMPERATURE AND SEASON ON REPRODUCTION

Seasonal depression of reproductive performance is one of the most serious problems encountered in the cattle (as in bison) industry in tropical and subtropical regions (Rhodes, De'ath & Entwistle, 1995). Reports from field and laboratory trials agree that fertility is negatively related to environmental temperature and humidity (Ingraham, Gillette & Wagner, 1974; Page, Jordan & Johnson, 1985). Conception rates to artificial insemination may range from 55% during months of low temperature and humidity to only 10% during months of high temperature and humidity (Ingraham *et al.*, 1974). Increased heat loads at oestrus or following insemination increase body temperature, which may affect the conception rate (Zeitoun *et al.*, 1996).

Reproductive efficiency was suppressed in cows maintained under environmental conditions of high ambient temperature and humidity (Putney, Mullins, Thatcher, Drost & Gross, 1989; Wadié & Mufeed, 1995). The same applies for the influence of season on reproductive performance. Various reproductive processes are also adversely effected by unfavourable ambient temperatures (Baginda, Collier, Thatcher & Wilcox, 1985). During heat stress, cattle are unable to reproduce due to environmental heat and are hypothermic with an increased body temperature. Thus resulting in an increase in the temperature of the embryo micro-climate, which may adversely effect the embryo's viability. Maternal hypothermia also suppresses embryonic development as well as increasing the incidence of embryonic mortality in mammalian species (Putney, Drost & Thatcher, 1988). The period of embryonic development most sensitive to heat stress appears to be between the initial stages of cleavage and the blastocyst stage (between day 1 and 7 following fertilisation). A high ambient temperature (32°C) for 72 hours immediately following insemination, depressed pregnancy rates by up to 50% in beef heifers (Putney *et al.*, 1988). Exposure of dairy cows to summer heat-stress in tropical regions like Saudi Arabia and Florida (USA), during the first 7 days of pregnancy, resulted in a decreased quality and viability of embryos recovered from cows and an increased incidence of abnormal embryos from heifers (Gordon, Boland, McGovern & Lynn, 1987; Putney *et al.*, 1988).

The physiological response is similar in cattle (and possibly the bison) exposed to heat-stress under natural environmental conditions, with similar fertilisation rates. Results in Holstein cows suggest fertilisation to have occurred prior to exposure to high ambient temperatures (Wadie & Mufeed, 1995).

The rate of embryonic development appears to be retarded in heat-stress heifers (Wadie & Mufeed, 1995). Similar effects of heat-stress on embryonic development have been observed in mice (Massey & Oden, 1984). Bovine embryos seem to remain sensitive to high temperatures, beyond the early stages of cleavage and blastocyst formation. In this regard, Looney (1986) postulated exposure of pregnant beef heifers to thermal stress (37°C) between days 8 and 16 following insemination, to reduce the conceptus weights - compared to heifers maintained at 22°C. Indicating that thermal stress may lead to retarded embryonic growth. However, the effect of temperature and season after day 7 of AI does not appear to be as drastic on embryonic development as the initial 7 day gestation period.

Environmental modification such as the provision of shade, air conditioning and evaporative cooling has been suggested as reproductive management strategies to optimize superstimulation and superovulation response during excessively hot seasons. However, cost effectiveness of a specific system should always be considered. Consequently it is important to consider the effect of environment and temperature on the reproductive performance of embryo donor and recipient animals during any MOET program. Strategies to consider may include practices such as the superstimulation and transfer to donor cattle (or in this case, bison) during mild weather conditions.

2.5 THE ROLE OF THE DOMINANT FOLLICLE IN THE SUPERSTIMULATION PROGRAMME

It has been suggested that superovulation in cattle is impaired in the presence of a dominant follicle, but results to test this hypothesis have been contradictory (Stock, Ellington & Fortune, 1996). Ovulatory size follicles do not develop at random in mammalian ovaries. In a number of species, groups or cohorts of follicles are recruited synchronously and a specie-specific number of follicles are selected from each cohort for further differentiation to the ovulatory size. The factors that regulate the emergence of cohorts of follicles are not well understood. Even less is known about the mechanisms that allow selection of the appropriate number of dominant follicles for further growth and development. Two hypotheses have been put forward to explain this dominance. Firstly, the dominant follicle(s) secretes a circulating factor that directly suppresses the growth of subordinate follicles on both ovaries. Secondly, dominance is exerted via the secretion by the dominant follicle of factors like estradiol and inhibin that would suppress plasma FSH to levels too low to support growth of these subordinate follicles (Fortune, 1994).

The bovine oestrous cycle, with its 2 or 3 successive waves of follicular recruitment, provides a particularly useful model for studying mechanisms associated with follicular dominance in heifers and cows (Savio, Deenan, Boland & Roche, 1988; Knopf, Kastelic, Schallenberger & Gunther, 1989; Ginther, Wiltbank, Fricke, Gibbons & Kot, 1996). One follicle is selected from each wave of cohorts for further development while the other follicles gradually regress. The dominant follicle of any wave can ovulate if luteolysis occurs spontaneously or is induced prematurely by the injection of PGF2α during its period of dominance (Kastelic, Knopf & Gunther, 1990; Draincourt, Thatcher, Terqui & Andrieu, 1991; Kastelic & Gunther, 1991).

Superovulatory treatments can overcome or subvert the normal mechanisms of follicular dominance in humans and domestic animals. Therefore, if follicular dominance results primarily from the direct actions of the dominant follicle on subordinates, superovulatory regimes could be expected to be most successful when initiated at the beginning of a follicular wave (i.e. in the absence of a dominant follicle). It has been empirically determined that the optimal time to start a superovulatory treatment in cattle (and possibly the bison), is day 8 to 12 of the oestrous cycle. This period coincides with the beginning of the second follicular wave (Armstrong, 1993). Results from experiments to determine how the presence of the dominant follicle affects the superovulatory response have been contradictory. Some researchers indicate that the dominant follicle may affect the outcome of the superovulation response (Grasso, Guilbault, Roy & Lussier, 1989; Guilbault, Grasso, Lussier, Rouiller & Matton, 1991; Bungartz & Niemann, 1994;

Kohram, Bousquet, Durocher & Guilbault, 1995; Lussier, Lamothe & Pacholek, 1995). No effect however, was reported in cattle by Wilson, Jones and Miller (1990), Gray, Cartee, Stringfellow, Riddell, Riddell and Wright (1992) and Rajamahendran and Calder (1993).

2.6 ELIMINATION OF DOMINANT FOLLICLE DURING SUPEROVULATION

Both hormonal and physical methods have been attempted to remove the dominant follicle or otherwise subvert its suppressive effects during superstimulation. Hormonal methods include administration of oestrogen to induce dominant follicle regression, gonadotropin releasing hormone (GnRH) to down regulate its gonadotropin receptors and human chorionic gonadotropin (hCG) to luteinize the dominant follicle. Physical methods include manual rapture of follicle, trans vaginal aspiration (puncture of dominant follicle) removal by electrocautery and surgical removal of the ovary containing the dominant follicle (Rajamahendran & Calder, 1993).

a) Oestrogen treatment

Bo, Pierson and Mapletoft (1991) administered estradiol valerate to cows either at the time of administration of progestagen (Syncrho-Mate-B, SMB) implants to replace or supplement the *corpus luteum* prior to superovulation, or 7 days later and recorded the follicular dynamics and superovulatory response. Although superovulatory results were not conclusive in this case, daily ultra-sonographic examinations revealed that oestrogen treatment at the time of SMB implantation caused a reduction in the mean size of the two largest follicles over a 5 day period - followed by an increase in size of the two largest remaining follicles, presumably from the next follicular wave.

In a subsequent study on heifers of Bo, Adams, Nasser, Pierson and Mapletoft (1992) administration of oestradiol treatment at the onset (day 1) or middle (day 3) but not at the end (day 6) of the growing phase of the first follicular wave suppressed the growth of the dominant follicle. Emergence of the subsequent wave was accelerated as a result of oestrogen treatment on day 1 and delayed by treatment on days 3 or 6. These results indicate that the effects of oestrogen treatment on follicle dynamics are complex, but suggest that modulation of follicle dynamics by exogenous estrogen treatment may prove useful in synchronization of follicular waves in cattle, leading to improved responses to superovulatory treatments.

b) hCG treatment

Rajamahendran and Sianangama (1992) reported the ability of hCG administration to induce ovulation and/or luteinization of the dominant follicle present on ovaries at day 7 of the cycle. Postulating that this treatment would remove the suppressive effect of the dominant follicle on the superovulatory response, Calder and Rajamahendran (1992) treated cows with hCG on day 7, followed by a superovulatory regimen of FSH beginning on day 9. Although not significantly different from the controls, a tendency toward more transferable embryos was noted in the cows who received the hCG treatment.

c) GnRH and GnRH agonist treatments

Gonadotropin-releasing hormone agonists (GnRHa) are currently used widely in ovarian stimulation regimens for oocyte aspiration in *in vitro* fertilization (IVF) protocols in women. The mechanism of action is assumed to be via the down-regulation of pituitary gonadotrophins, resulting in follicular regression through removal of gonadotropic (presumably FSH) support. Exogenous FSH is then highly effective in stimulating uniform follicle growth due to the absence of dominant follicles. Similar effects of GnRH agonist treatment have been demonstrated in laboratory rodents and sheep, resulting in enhanced responses to FSH treatment (Hamilton & Armstrong, 1990; Picton, Tsonis & McNeilly, 1990).

Savio, Bongers, Drost, Lucy and Thatcher (1991) examined the superovulatory response of Holstein cows treated with a potent GnRH analogue (Buserelin) on day 3, at the time of insertion of intravaginal progesterone-releasing devices. Although the results may have been confounded by the presence of a progesterone-releasing device, this treatment with GnRHa decreased rather than increased the ovulation rate in response to FSH. Pituitary down-regulation with GnRH agonists may influence ovarian response of cows to gonadotrophic stimulation and thus the possible utility of such treatments in bovine and bison superovulation protocols.

d) Physical removal of dominant follicle

The surgical removal of the dominant follicles from bovine ovaries, either by unilateral ovariectomy (Staigmiller & England, 1982), or by electrocautery (Ko, Kastelic, Del Campo & Ginther, 1991), is followed by the immediate resumption of follicular growth, probably acting through systemic endocrine channels (Ginther *et al.*, 1989). Recent advances in efficient methods for follicle aspiration (Ovum Pickup – OVP) for ooctye collection may provide a more practical method for ablation of the dominant follicle - which may enhance the ovarian response to superovulatory treatments in cattle.

2.7 MANAGING THE DONOR COW TO MAXIMIZE THE EFFICIENCY OF EMBRYO TRANSFER

Ovarian superstimulation in conjunction with embryo transfer to expedite the propagation of animals with superior genetic merit for desirable traits, still remains a feasible procedure. However, the large variation in ovulation rate, production and recovery of viable embryos following a superstimulation procedure limits the extensive use of embryo transfer technology (Roberts, Grizzle & Echternkamp, 1994). Several studies aimed at establishing suitable superstimulation protocols in cattle that would standardise and give a more consistent yield of viable embryos have been investigated (Dieleman, Bevers, Wurth, Gielen & Willemse, 1989; Lucy, McMillan, Thatcher, Drost & Tan, 1990; Calder & Rajamahendran, 1992; Walsh, Mantovani, Duby, Overstrom, Dobrineky, Enright, Roche & Boland, 1993; Roberts *et al.*, 1994).

The most effective procedure currently used for superstimulation in cattle, is the twice daily administration of gonadotrophin (FSH) during the mid-luteal phase of the cycle (days 9 to 13; where day 0 is the day on which the cow exhibits oestrus). This is followed by a prostaglandin (PGF2α) injection 48 hours after the last FSH treatment on day 15, to induce luteal regression, followed by oestrus and ovulation (Elsden, Nelson & Seidel, 1978; Monniaux, Chupin & Saumande, 1983; Moore, Kruip & Green, 1984; Goulding, Martin, Williams, Ireland, Roche & Boland, 1991). However, the variability with regard to the quality and quantity of bovine embryos produced with the above protocol, remains one of the limiting factors in embryo transfer technology (Goulding *et al.*, 1991). Variability in embryo response may be caused by both extrinsic (FSH preparation and mode of administration), as well as intrinsic (ovarian status) factors. Many studies have been conducted to increase follicular development and ovulation rate in individual cows and to reduce the variability between cows. Thusfar, no major breakthrough has been achieved (Goulding *et al.*, 1991; Roberts *et al.*, 1994; Draincourt, 2000).

2.8 EFFECT OF STAGE OF THE OESTROUS CYCLE ON SUPEROVULATORY RESPONSE

The stage of the oestrous cycle is one of the most obvious sources of variability in ovarian response to gonadotrophic stimulation. Lindsell, Murphy and Mapletoft (1986) reported significantly higher ovulation rates and embryo yields in cows administered follicle stimulating hormone (FSH), beginning on day 9 of the oestrous cycle, compared to those in which treatments were initiated on days 3 or 6 of the cycle. Goulding, Williams, Duffy, Boland and Roche (1990) observed lower superovulatory responses to FSH administration when initiated on day 2 than on day 10 of the cycle in beef cattle. When examining the mid-cycle period more closely, Hasler, McCauley, Schermerhorn and Foote (1983) and Donaldson (1984) failed to record significant differences in ovulation rate in cows where gonadotrophin treatment was initiated at day 9 to 13 of the oestrous cycle.

It has been established from previous observations, follicle marking techniques, hormone assays and more recently, ultrasonographic examination of the ovaries, that follicle growth occurs in waves throughout the oestrous cycle. In cattle there may be 2, 3 or sometimes 4 waves during each oestrous cycle (Pierson & Ginther, 1987; Sirois & Fortune, 1988). During each of these waves, several follicles begin to grow with one "dominant" follicle outstripping and suppressing the further growth of the other (subordinate) follicles in its cohort. In the presence of a functional *corpus luteum* the dominant follicle, along with the subordinate follicles in the cohort, undergoes regression (atresia), and a new follicular wave begins. Only in the last wave of the cycle in absence of an active CL, does the dominant follicle normally ovulate (Armstrong, 1993).

Recent studies of hormone levels in heifers during the luteal phase combined with ultrasonographic examination of the ovaries, have addressed the role of FSH in the etiology of follicle waves in cattle (Adams, Matteri, Kastelie, Ko &

Ginther, 1992). Irrespective of whether heifers were of the "two wave" or "three wave" (per cycle) type, a significant surge of FSH was found to consistently precede the emergence of the next wave by 2 days. When the rise in FSH was prevented by administration of a steroid-free extract of bovine follicular fluid as a crude source of inhibin, the emergence of the next follicular wave was prevented for the duration of treatment, and occurred consistently 2 days after the first detectable increase in FSH levels after cessation of inhibin treatment (Turzillo & Fortune, 1990; Adams *et al.*, 1992). These findings offer strong evidence for the major role of FSH in the initiation and emergence of follicular waves in cattle.

2.9 EFFECT OF FREQUENCY OF GONADOTROPHIN ADMINISTRATION ON THE SUPEROVULATORY RESPONSE

It is well documented that PMSG has a long half-life. Therefore a single injection of this gonadotrophin has a long and variable effect on superstimulation of the ovaries in cattle (Mikel-Jensen, Greve, Madej & Edqvist, 1982). FSH, on the other hand, which is a glyco-protein hormone with a short half-life, requires administration twice daily for 3 to 4 days, to induce satisfactory superstimulation in cattle. To the contrary Walsh *et al.* (1993) considered the twice daily injections of FSH to be time consuming and more costly, compared to a single daily injection of FSH.

It has been reported that a single subcutaneous injection of FSH behind the shoulder, induces a superovulatory response comparable to that of a multiple intramuscular injection regime for superovulation in Holstein and Buffalo cows (Demoustier, Beckers, Van der Zwalman, Classet, Gillard & Ectors, 1988; Kasiraj, Mutha Rao, Rangareddi & Misra, 1992; Misra, Chaubal, Krishna Kishore, Rajeshwaran, Joshi & Jaswal., 1992; Yamamoto, Suzuki, Ove, Takage & Kaneaguchi, 1992).

The plasma FSH concentrations increased immediately after the intramuscular treatment of FSH and reached a maximum level after 3 hours. The FSH concentration then gradually decreased and no FSH could be detected 12 hours after administration (Demoustier, *et al.*, 1988). The observed slower release of FSH into the blood when injected by the subcutaneous route, compared to the intramuscular route, suggested that this route of administration could be more effective in ensuring adequate concentrations of FSH in the blood over a given period of time. Walsh *et al.* (1993) indicated that a once-daily subcutaneous FSH injection does not ensure adequate plasma FSH concentrations over a 24 hour period. Hence it necessitates a more frequent administration schedule. There thus appears to be a major difference between single daily injections and the once-off administration of FSH, on the release rate of the product. It was also found that a single subcutaneous daily injection of FSH for 4 consecutive days resulted in a significant decrease in ovulatory response and viable embryo production rate, compared to a twice daily injection of FSH in superstimulated heifers (Yamamoto *et al.*, 1992).

2.10 EFFECT OF GONADOTROPHIN PRIMING PRIOR TO SUPERSTIMULATION

It has been established that elevated blood levels of FSH during proestrus and/or oestrus are important in determining the number of follicles available for ovulation in the next cycle of rodents (Schwartz, 1974). Cows also have both pre- and post-ovulatory peaks of circulating FSH. This has led to several attempts to experimentally modify the post ovulatory FSH levels, in the hope of modifying the response to superovulation in the next cycle. Rajamahendran, Canseco and Denbow (1987), Ware, Northey and First (1987) and Touati, Beckers and Ectors (1991) reported significant increased superovulation responses in cows administrated priming doses of FSH at the onset of the oestrous cycle. In other studies, similar priming treatments either had no significant effect on the superovulatory response (Gray *et al.*, (1992; Rieger, Desaulnier & Goff, 1988), or resulted in a significant reduction in the ovulation and embryo recovery rate (Grasso *et al.*, 1989; Lussier & Carruthers, 1989).

Ultrasonographic analyses of the ovaries have provided insights into some of the differences observed in the dynamics of the ovaries, although not into their underlying causes. In a study of Gray *et al.* (1992), no effect of FSH priming was observed and the dominant follicle in the FSH-primed animals reached a maximum diameter 1.5 days earlier than in the non-primed controls. It would seem as if the dominant follicle may have lost its dominating capabilities by the time of superovulatory treatment. In contrast, Grasso *et al.* (1989) demonstrated that FSH priming decreased the superovulatory response as the development of the dominant follicle was delayed by 2 days following FSH priming. Although the reason for these divergent results is not clear, the delayed regression of the dominant follicle in the latter study probably resulted in the suppression of the subordinate follicles present when the superovulatory treatment begun was administered - resulting in fewer follicles responding to the superovulatory treatment.

The failure of FSH priming to increase ovarian response in a subsequent superovulatory treatment, as observed in the above mentioned studies, may have been caused by inappropriate time intervals between the priming and superovulatory treatments. In histological studies of the ovaries of PMSG-treated cows, increased numbers of follicles of the pre-antral type (<150 μm in diameter) and decreased atresia of early antral follicles (approximately 200 μm in diameter), were observed 6 days after PMSG treatment (Monniaux *et al.*, 1984). Based on estimates of follicle growth rates from measurements of the mitotic tempo (Lussier, Matton & Dufour, 1987), preantral follicles would require a period equivalent to about two oestrous cycles for growth to the preovulatory stage. Thus a beneficial effect of FSH priming on ovulation rate through stimulation of follicles at these early follicular stages would require a considerably longer period of time than the approximately one-half cycle used in these specific studies.

2.11 FERTILIZATION AND EMBRYO DEVELOPMENT IN DONOR COWS

Low fertilization rates or complete absence of fertilization, continue to be a major limiting factor in current superovulation procedures for embryo transfer in cattle and the bison.

Fertilization is indicative of the success of sperm transport, while a lack of fertilization can be ascribed to a failure in sperm transport and/or survival (Hawk, 1988). In a normal, unstimulated domestic cow, there is evidence of a sperm reservoir in the utero-tubal junction and the mutual portion of the isthmus, approximately 5 hours after insemination (Hunter, 1988; Hyttel, Callesen, Greve & Schmidt, 1991). However, in superstimulated animals, a bilateral reservoir of spermatozoa is only found in a quarter of the animals (Hyttel *et al*., 1991). Hunter (1988) ascribes the high incidence of unfertilized embryos in superovulated animals due to a lack of spermatozoa at the site of fertilization. Accessory sperm reserves for up to 3 days after insemination, increasing in time for repeat breeding animals, have been reported (Hawk & Tanabe, 1986). Sperm transport in superstimulated cows following gonadotrophin treatment, is reported as having a moderate inhibiting effect on sperm transport (Hawk, 1988). In repeat breeders, lower conception rates have also been obtained. A fertilization rate of 72% was recorded in fertile cows, compared to only 35% in lower fertile cows (Evans, Adams & Rawlings, 1994). Similarly Greve, Callesen and Hyttel (1983) obtained fertilization rates of 82% in fertile cows and 41% in repeat breeders. These results are in accordance with those of Hasler *et al.* (1983) where a lower number of viable embryos were found in infertile superovulated cows, compared to normal fertile superstimulated cows.

The site of semen deposition also seems to be a limiting factor in determining the fertilization rate. The site of semen deposition within the bounds of the anterior cervix uterine body or posterior part of the uterine horns, has generally been reported to have little effect on the fertility obtained (Mapletoft, Martinez, Adams, Kastelic & Burnley, 1999). However, Moller, MacMillan and Shannon (1972) found that a reduced volume of semen gave better fertilization results if deposited in various locations throughout the female genital tract. No difference in the site of semen deposition was found in terms of fertilization rate. Hawk and Tanabe (1986) found that spermatozoa moved to both oviducts, regardless of the site of semen deposition in the uterus. However, in superstimulated cows the site of semen deposition in the uterine horn has been shown to have a marked effect in fertilization rate. With superstimulation the efficiency with which spermatozoa are transported from one horn to the other is greatly retarded (Pallares, Zavos & Hemken, 1986).

Ovum transport is generally accelerated in superstimulated cows, especially those stimulated by PMSG and normally very few ova are recovered from the uterus 2 to 4 days following insemination (Dowling, 1994). At least half of the embryos reach and enter the uterus by day 4 following ovulation, while the remainder continue entering the uterus until day 7 (Hafez, Sugie & Gordon, 1963; El-Banna & Hafez, 1970; Moore, 1975; Newcomb, Rowson & Trounson, 1980; Hawk, 1988). A few ova remain in the oviducts indefinitely and cannot be recovered non-surgically. Newcomb *et al.* (1980) found no difference in the transport of fertilized and unfertilized ova the oviducts, due to the higher serum progesterone concentration recorded at oestrus in superovulated cattle (Donaldson, Ward & Glenn, 1986). The rapid rise in oestrogen levels due to superstimulation contributes to this rapid movement of the embryo (Booth, Newcomb, Strange, Rowson & Sacher, 1975; Murphy, Mapletoft, Manns & Humphrey, 1984).

Hyttel *et al.* (1991) ascribed the large variation in embryo development partly due to the initiation of premature maturation in a substantial proportion of oocytes, collected prior to the LH peak. This premature triggering of embryos were at the metaphase I stage (Murphy *et al.*, 1984; Goff, Greve, Bousquet & King, 1986; Callesen, Greve & Hyttel, 1987). Thus this deviant rate of maturation represents a series of obstacles in the more efficient use of superstimulation treatments. The number of normal embryos that reached the metaphase II stage (48%) are associated with low oestradiol and high progesterone concentrations in the follicular fluid, similar to these in control animals (Dieleman, Kruip, Fontijne, De Jong & Van der Weiden, 1983). Various factors thus contribute to the successful fertilization rate in ova. These include sperm transport, sperm reservoir, site of sperm deposition, fertility of the animals, number of follicles ovulating, time of ovulation and ovum transport.

2.12 DIFFERENT AGENTS USED FOR SUPERSTIMULATION IN COWS/BISON

Maximum response to superstimulation or superovulation depends on the administration of gonadotrophins at pre-determined stages of the oestrous cycle (Table 2.3). The gonadotrophins most commonly used in embryo transfer programs in cattle are PMSG and FSH, while limited use of human menopausal gonadotrophin (hMG) is also practiced (Goulding, Williams, Roche & Boland, 1996).

Table 2.3 Ovarian observations and plasma progesterone levels during the oestrous cycle in the bison

Day of cycle	Ovarian structure	Plasma progesterone (ng/ml)
0	Graafian follicle Thin-walled translucent bulge	<0.02
1 to 2	Ovulation point Raised, light red spot	0 to 0.6
2 to 4	*Corpus luteum* (early) Red, elevated	0.5 to 0.7
4 to 8	*Corpus luteum* (developing) Dark red protrusion with blood vessels	0.8 to 2.4
8 to 16	*Corpus luteum* (mature) Red to orange protrusion	1.2 to 2.3
16 - 21	*Corpus luteum* (regressing) Shrunken yellow to white protrusion	<0.02

PMSG is a glycoprotein molecule, which defines both FSH and LH-like activity (Goulding *et al.*, 1996). It is well established that PMSG is composed of an $\propto$ and a β sub-unit. The β sub-unit of PMSG is responsible for both FSH and

LH activity of the hormone, although it must be bound to the ∝ sub-unit to achieve it's full biological activity. The high acetyl-neuraminic acid content of the PMSG molecule is responsible for a longer half-life, which greatly exceeds that of LH or FSH. The amino acid sequence of PMSG is more closely related to LH than to FSH. There is variability in both FSH and LH activity of PMSG, not only among pregnant mares, but also between blood samples in the same mare, taken at different times during gestation (Mapletoft, Gonzalez & Lussier, 1988).

FSH and LH, like PMSG, are glycoprotein hormones composed of structurally similar ∝ sub-units and biologically specific β sub-units. In addition, each molecule has a characteristic carbohydrate component consisting of neutral sugars, hexosamines and a highly variable sialic acid content, which determines the half-life of the molecule. The very low level of sialic acid in LH and the somewhat higher content of FSH is reflected in the half-lives of 30 minutes and 110 minutes for LH and FSH respectively (Murphy *et al*., 1984). Large variation in half-life activity has been reported between batches for both FSH and LH in commercial FSH preparations (Murphy *et al*., 1984; Mapletoft, Pawlyshyn, Garcia, Bo, Willmott, Saunders & Schmutz, 1990).

Considerable data has been accumulated over the years on PMSG and FSH as superovulation or superstimulation agents in cattle. In early work on twinning in cattle, purified FSH gave a more predictable ovulation response than PMSG (Bellows & Short, 1972). Goulding *et al*. (1996) confirmed that PMSG induced a greater number of large follicles, compared to FSH treatment. Extensive studies with both gonadotrophins (PMSG and FSH) however, led to the conclusion that the results of superstimulation using any of these hormones are both variable and unreliable. It is now generally recognized that a higher ovulation rate may be obtained using a FSH preparation (Goulding *et al.*, 1991). This increased ovulation response as a result of FSH administration, is followed by a higher number of quality embryos (Goulding *et al.*, 1990) and in some instances an increase in the number of pregnancies (Elsden *et al.*, 1978). However, several studies confirm that due to the availability and cost factor of PMSG, it still remains an important agent for the superstimulation of cattle (Goulding *et al.*, 1990). Injection of either gonadotrophin, appears to be effective in reducing atresia of the smaller follicles. However, it is postulated that if too much LH is present, either premature ovulation or luteinization of FSH-stimulated follicles can occur. Both FSH and PMSG administration may cause premature ovulation, which may be responsible for the failure of prostaglandin $F_{2\alpha}$ to induce complete luteolysis. This may again be associated with insufficient oestradiol and consequently, insufficient LH surges (Callesen, Greve & Hyttel, 1986; Callesen *et al.*, 1987). Foote and Ellington (1988) suggested that the frequency of premature oocyte activation can be reduced with purer FSH preparations. This is supported by Murphy *et al.* (1984) and Donaldson and Ward (1985). The use of recombinant FSH (Lerner, Thayne, Baker, Henschen, Meredith, Inskeep, Dailey, Lewis & Butcher, 1986) could ensure control over the quality of the gonadotrophin used (Boland *et al.*, 1991). However, it must be remembered that between-animal variation in response will always exist (Armstrong, 1993).

A highly purified standardized biological exogenous gonadotrophin (hMG) with low LH activity, induced similar responses to PMSG and FSH (Lauria, Genazzari, Olivia, Inandi, Crensonesi & Moniltola, 1982). Although a non-significant difference in the number of *corpura lutea* was observed, oocytes or embryos recovered, however, the number of pregnancies resulting from embryos originating from FSH treatment were higher than those obtained from hMG (Ben Jabara, Carreire & Price, 1994).

It is thus clear that many variables play a role in the gonadotrophic agents used for superstimulation of cattle and possibly bison. Although it is possible to control the oestrous cycle effectively using progesterone or prostaglandin $F_{2\alpha}$, large variation exists in the production of viable embryos in donors with purified FSH preparations. Further research is justified and required to apply this existing knowledge with regard to intra-ovarian control of growth and development of follicles, following superstimulation with different agents in cattle and the potential application in bison.

CHAPTER 3

MATERIALS AND METHODS

3.1 OESTROUS SYNCHRONIZATION

3.1.1 Introduction

Bison exhibit breeding phenomena such as a) silent oestruses (heats); b) low conception rates; c) seasonality in breeding; d) a higher than optimal age at first calving and e) repeat breeding. Controlled breeding such as synchronization has been effectively accomplished in the bison with commercially accepted bovine protocols using either $PGF_{2\alpha}$ (Lutalyse, 25 mg) or progestagen (Syncro-Mate-B, SMB) treatments. Bison are seasonally polyestrous, with a breeding season extending from late summer to late autumn/fall, with synchronization of oestrus being most effective during the breeding season. However, oestrus can also be induced, outside the natural breeding season with the aid of progestagens (e.g. Synchro-Mate-B).

For effective use of $PGF2_{\alpha}$ (Lutalyse) or one of the synthetic prostaglandin analogues, such as cloprostenol to induce oestrous in the bison, it is essential that the animal is in a luteal phase of her oestrous cycle. A single injection of $PGF2_{\alpha}$ given to a female at an unknown phase of her cycle will induce oestrus in approximately 50% of the total females within 2 to 5 days following treatment. A second injection of $PGF_{2\alpha}$, 11 days after the first injection will effectively synchronize 90% of the females (Matsuda–Motomura, 1994). The use of progestagens, such as Syncro-Mate-B implants (SMB), in oestrous synchronization programs of bison, yield acceptable results, with a higher degree of follicular development (100% vs 90%) and tighter synchrony (2 to 4 days vs 2 to 5 days), but with a lower ovulation rate (33% vs 76%), when compared to $PGF_{2\alpha}$ (Lutalyse) treatments (Matsuda-Motomura, 1994).

In cattle, superovulation typically results in ovulation and the production of approximately 10 ova. So for example an average of 6 embryos were recovered in an embryo transfer programme (Seidel & Elsden, 1989). In this current trial, 6 bison donors were incorporated in a superovulation program and 12 bison recipients were prepared to act as recipients of the bison embryos (presuming that all grade 1 embryos would be satisfactorily frozen after recovery).

3.1.2 Oestrous synchronization of bison recipients and donors

In total 12 bison recipients were synchronized. All 12 recipients were synchronized using a Syncro-Mate-B (SMB, Ceva Corp., Overland Park, KS) implant behind the ear. Implants were implanted on day-1 of the donor schedule and removed 9 days later on day 8 of the donor schedule (Table 3.1). Oestrus in donors using 2 protocols (n = 6) was synchronized using a 9 day treatment with Synchro-Mate B implants, in combination with a 25 mg im injection of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (Lutalyse, Upjohn), administered on day 0 of the superovulation schedule. Day 6 initiated FSH treatment. Removal of SMB was performed on day 9. The synchro-Mate B implant of the donors was removed on the morning of the fourth day of FSH treatment (Table 3.1).

Oestrous observations in recipients was monitored daily (8:00) at 12h intervals from day 6, by using ultrasound (ovulation) and Kamar heat detection pads. In all bison donors and recipients in which a functional Corpus Luteum (CL) was palpated (per ultrasound examination) on day 6 of the protocol (1 and 2), the onset of oestrus was presumed to have occurred 60 hours after the PGF-treatment.

Donors were bred, using AI at 12 and 24 hours after oestrous observation. With all types of synchronization implemented in the bison, the biggest problem to overcome was stress, associated with the handling of the animals. In order for these programs to be successfully completed, handling stress was reduced to a minimum by proper management.

3.2 ARTIFICIAL INSEMINATION

3.2.1 Introduction

AI has been successfully used to produce viable embryos in the bison. The most notable drawback in the application of AI programs in the bison is a shortage of available viable semen. Synchronization programs are effective, but the post-thawing semen quality is marginal. The viability of fresh bison semen collected using an electro-ejaculation is however comparable to that in domestic cattle, but bison sperm do not survive the freezing process with common bovine extenders and cryoprotectants as effectively as bovine spermatozoa (Sipko, Abilov, Shukhal & Rott, 1993).

Great variation in semen quality exists between individual bison males regarding their post-thawing sperm survival rate. The sperm lose more than 50% of their initial viability and the surviving sperm cells have a very short life span. The best way to evaluate the quality of frozen/thawed semen in the bison, is after a 2 hour incubation period at 38°C (Sipko *et al.*, 1993).

3.2.2 Semen Collection

Semen for fertilising the donors was collected from live bison bulls (n = 2), using an electro-ejaculator. The semen was collected in a 200 ml sachet and then transferred into smaller 5 cc vials, whereafter semen was drawn up into 0.25 cc straws and placed in a petri dish for microscopic evaluation and viability evaluation.

Following chilling in the 0.25 cc straws the semen was stored at 3°C to 8°C, for a period of no longer than 24 hours prior to insemination - if not used immediately after collection to inseminate donor cows. Donors were inseminated with one (0.25 cc) straw of fresh bison semen, after rewarming semen to 38°C, 12 and 24 hours after first observed oestrous signs.

3.3 SUPEROVULATION AND EMBRYO COLLECTION

3.3.1 Introduction

Flushing of embryos entails a series of events that must be carried out meticulously, in order to retrieve the maximal proportion of viable embryos. It is evident that superstimulation experiments are subjected to tremendous individual variation regarding the number and quality of the embryos recovered. Before the incorporation of the donor animals into the different trials, all females are to be subjected to a gaeno examination in order to ascertain if the animals are anatomically and structurally sound i.e. both ovaries active, no cystic ovaries or adhesions, while the uterine horns are also rectally palpated for tone, size and symmetry. A precise superovulation regime with the aid of gonadotrophins is to be adhered to, in conjunction with accurate record keeping (Taylor, 1997).

Superovulation in the bison has been achieved using both pituitary FSH (follicle stimulating hormone) and eCG (PMSG, Equitech). Superovulation has also been attempted in bison females using different dosages of gonadotropin, different synchronization programs, treatments at various times of the year and in different donor age groups. The tremendous variation experienced in treatment protocols, the limited number of bison per treatment group and numerous management and environmental differences make a direct comparison between superovulation treatments very difficult (Dorn, Foxworth, Butler, Olsen, Wolfe, Davis, Simpson & Kraemer, 1990).

One of the first controlled trials on superovulation in the bison evaluated the use of a total dose of 34 mg FSH-P in 18 SMB-implanted females. SMB implants were left in place for 9 days and FSH-P was administered in a decreasing dose schedule over a period of 4 days. A mean (± SEM) ovulation rate of 4.2 ± 1.0 was attained, with a recovery rate of 3.1 ± 0.9 ova/embryos per donor. However, only 0.7 ± 0.3 of the bison embryos per donor were of transferable quality. The low number of fertilized ova obtained was however ascribed to the poor semen quality used in the AI of the donors (Dorn *et al*., 1990).

3.3.2 Superovulation and AI protocols of donor bison cows

Three different protocols were used to superovulate 6 mature bison females, 2 per group. Each group consisted of cows ranging in age between 24 and 40 months of age.

Protocol 1: Bison cows (n = 2), were treated once daily with a total of 18 mg FSH-P in decreasing doses, over a 4 day period, to superovulate the animals. Oestrus was synchronized during superovulation treatment using a SMB (mg) implant over a 9 day period, the implants were removed on day 4 of the FSH-P treatment (Table 3.1).

Protocol 2: Bison cows (n = 2), were treated twice daily with a total of 18 mg FSH-P in decreasing dosages over a 4-day period to superovulate the animals. Oestrus was synchronized during superovulation using a SMB-implant over a 9 day period. The implants were removed on day 4 of the FSH-P treatment (Table 3.2).

Table 3.1 Synchronization and superovulation treatment regimes – Protocol 1

Donors		Recipients	
Day –1		Day –1	SMB implant + injection of PGF (5ml) im
Day 0	SMB implant + injection of PGF (5ml) im	Day 0	
Day 6	6ml of FSH/am	Day 6	
Day 7	5ml of FSH/am	Day 7	
Day 8	4ml of FSH/am	Day 8	Remove SMB implant
Day 9	3ml of FSH/am Also remove implant + inject PGF (5ml) i.m.	Day 9	
Day 10	Oestrus AI 12 & 24 hours after first observed standing oestrus	Day 10	Oestrus
Day 17	Collect embryos	Day 17	Transfer of embryos

Protocol 3: Bison cows (n = 2) were treated with a single dose of 3000 IU eCG (PMSG) i.m. on day 10 of the induced oestrous cycle. The oestrous cycle was induced by 2 injections prostaglandin (Estrumate) 11 days apart. Due to the long half-life of PMSG, a single intramuscular injection of PMSG. This was again followed by 2 (500 ug) intramuscular prostaglandin injections, 48 and 60 hours after PMSG administration (day 12). Animals

were observed for overt signs of oestrus, starting 24 hours after the last prostaglandin (PGF) injection.

Embryo flushing and collection commenced 6 - 7 days after insemination.

Table 3.2 Synchronization and superovulation - Protocol 2

Donors		Recipients	
Day –1		Day –1	SMB implant + injection of PGF (5ml) i.m.
Day 0	SMB implant + injection of PGF (5ml) im	Day 0	
Day 6	3ml of FSH/am + 3ml of FSH/pm	Day 6	
Day 7	2.5ml of FSH/am + 2.5ml of FSH/pm	Day 7	
Day 8	2ml of FSH/am + 2ml of FSH/pm	Day 8	Remove SMB implant
Day 9	1.5ml of FSH/am + 1.5ml of FSH/pm Also remove implant + inject PGF (5ml)	Day 9	
Day 10	Oestrus AI 12 & 24 hours after first observed standing oestrus	Day 10	Oestrus
Day 17	Collect embryos	Day 17	Transfer of embryos

3.3.3 Preparation of the donor cows for embryo flushing

Before the commencement of the embryo recovery technique, all donor animals were rectally palpated to monitor the extent of superstimulation ovarian response. Following the confirmation of a superstimulation response, the tail head of the cow was clipped, washed and disinfected with 70% alcohol or an iodine solution. A lower epidural anesthesia was accomplished by administering 6 to 7ml of 2% procaine hydrochloric solution

(Lidocaine, Centaur) into the spinal canal between two lumbrae vertebrae at the tail attachment. The tail was then secured to facilitate access to the vulva. Once a complete epidural blockage was accomplished (rectal straining and contractions had been completely arrested), the rectum was emptied of faeces. During this procedure of removing the faeces, air was frequently aspirated into the ampulla recti, causing ballooning. This ballooning resulted in an undesirable effect, as it hindered proper palpation and guidance of the embryo collection catheter. The air was removed by grasping a fold of the rectal mucus membrane and moving the arm back and forth. Although this ballooning could be a problem, a good epidural anathesia is a prerequisite for complete relaxation of the rectum and for proper placement of the flushing catheter in the uterus.

Table 3.3 Donor program for cows superstimulated with PMSG – Protocol 3

Day	A.M.	P.M.
-14	Prostaglandin (500 μg Estrumate)	
-3	Prostaglandin (500 μg Estrumate)	
0	Donor in oestrus	
10	PMSG (3000 IU)	
12	Prostaglandin (500 μg Estrumate)	Prostaglandin (500 μg Estrumate)
14		Donor in oestrus
15	AI of donor	AI of donor
22	Collect embryos	Transfer of embryos

Prior to insertion of the catheter into the vagina, the peri-anal region and vulva were washed thoroughly with soap and finally disinfected with a 70% alcohol solution.

Although the epidural has a relaxing effect on the bison, it was not adequate in some cases. Keeping in mind that bison are wild animals, 1-2ml Xilazine - 10% (Rompun), was administered intramuscularly to ensure sedation of the animal.

3.3.4 Positioning of the catheter and inflation of the balloon

Following cleansing, the vulva lips were parted and a Foley catheter (Plate 3.1) introduced into the vagina. By rectal manipulation it was guided through the cervical canal. Difficulties in manipulation the catheter through the cervical canal were seldom encountered, as most of the donor animals had previously calved. The placing of the catheter through the cervical canal is often a problem associated with heifers, requiring the use of a cervical expander before the insertion of the catheter (Elsden *et al.*, 1976). Once the cervix had been successfully surpassed, the catheter was guided towards the uterine horn, ipsilateral to the ovary containing the highest number of ovulation points (*corpora lutea*). As the uterine body is normally very short, the uterus also had to be manually manipulated, as soon as the catheter reached the internal os of the cervix. With great care the catheter was then positioned in the uterine horn. Large individual variation was experienced in cows as to where, and how for the catheter could be advanced and placed in the uterine horn. In animals where the uterus was situated in the pelvic area, proper positioning presented a minor problem. In older cows, with long uterine horns, the uterus was difficult to delineate and maintain in the pelvis. Thus a more posterior localization of the catheter generally occurred. In the anterior region of the inter carnal ligament of the uterine horn, the tip of the catheter would generally lodge in the endometrial folds of the greater curvature. To introduce the catheter further up in the uterine horn, without causing damage to the endometrial epithelium, the stylet was partly removed so that the tip of the catheter was flexible prior to attempting to advance further up the uterine horn. In some cases increased uterine tone tended to cause the horns to curl up. This, plus the nervousness of some cows made further advancement of the catheter difficult and a fair amount of uterine manipulation was required. Once the catheter was placed in the desired position (top third of the horn), a syringe filled with 20ml flushing medium was connected to the air valve of the balloon and 15 to 20ml fluid used to inflate the balloon slowly. The positioning of the balloon was regularly checked throughout the flushing procedure. This procedure was complete with the withdrawal of the stylet, which occurred immediately after inflation of the balloon.

3.3.5 Embryo flushing procedures

Embryos were collected non-surgically 6 to 7 days after the first insemination. Each donor was restrained in a chute especially designed for bison and more specifically, embryo flushing. The ovaries were rectally palpated and the superovulatory response (ovarian activity), recorded. Progressive, ovarian activity or development (ovarian volume) was monitored daily (8:00) in both the PMSG and FSH-treated groups by rectal palpation. These observations were performed from 2 days prior to the superstimulation program, until 2 days after the animals had shown the induced post-superstimulation oestrus (oestrus following the superstimulation program). A subjective method to estimate ovarian volume was used. This entailed rectal palpation and estimating the length, width and height (cm) of the ovary, with the aid of a caliper held in the free hand. These measurements of the ovaries were taken on day 7 (day of normal embryo flushing), after the induced and superstimulated oestrus. The number of ovulation points (*corpora lutea*) were also recorded by rectal palpation for each ovary on day 7. Lower epidural anasthesia, as described previously, was achieved by injecting 6 to 7ml of 2% procaine hydrochloride solution. The cervix dilator was used for subsequent ease in catheterization. A sterile, two-way (18 to 20 French gauge) Foley catheter was introduced into the uterine horn with the aid of a sterile stylet. Each uterine horn was flushed with an average of 500ml of Dulbecco's phosphate buffered saline (DPBS, Sigma, USA, Cat no D6650), supplemented with 9.1% bovine serum albumin (BSA, Fraction V).

A routine bovine embryo recovery procedure (intermittent gravity flow method) was used. The effluent was recovered in a 75µm embryo filter (Em Con, Immuno systems Inc. Biddeford ME). The final 40 to 50ml effluent was decanted into a 100 X 50mm flat bottom grid dish and the filter thoroughly rinsed (fluid) using a 24 French gauge needle and syringe.

The embryos were identified using a stereomicroscope (X25). The recovered embryos were transferred to a small petri dish (35 x 10mm) containing embryo holding medium. The holding medium was similar to flushing medium, except that the concentration of BSA was modified to 0.4%. Embryos were kept in the holding medium for 4 to 9 hours. The holding medium was replenished every 2 hours.

3.4 EMBRYO CLASSIFICATION AND GRADING

The final evaluation or assessment of embryo quality was made under a stereo microscope at high magnification (X75). Based on the morphological appearance, the embryos were graded and allocated to specific categories.

Plate 3.1 **Two way Foley catheter (18 French gauge), an intermittent gravity flow method was used for embryo flushing**

Plate 3.2 Grading and evaluation of bison embryos recovered post flushing, with the aid of a stereomicroscope (X75)

Plate 3.3 Positioning of the catheter in the uterine horn of the bison during embryo flushing

Physical characteristics used in the evaluation of embryos:

1. "Age" of the embryo in relation to the morphological appearance
2. Shape of the embryo
3. Colour of the cytoplasma
4. Scalloping
5. Broken membranes (integrity of the zona pellucida)
6. Presence of vesicles in cytoplasma
7. Extruded cells
8. Abnormalities of the peri-viteline space (PV space)

The following general guidelines for embryo grading as stipulated by the International Embryo Transfer Association were followed (Stringfellow & Seidel, 1990):

a) Grade 1, 2 and 3 embryos

These embryos were classified as embryos at the expected stage of development (day 7), that showed signs of little or no degeneration, protruding cells or vacuolization of the blastomeres. Embryos at day 7 post oestrus, demonstrated the following developmental stages:

i) Compact morula

ii) Early blastocyst

iii) Expanded blastocyst

All these embryos were morphologically normal, fell into these grades, and were designated as viable embryos. All Grade I embryos were thawed while Grade II and Grade III embryos were transferred, fresh, on the day of collection.

b) **Grades 4 and 5 embryos**

All these embryos were retarded or at an earlier developmental stage (8 and 32 cell stage), were regarded as non-viable embryos, and not processed further.

c) **Unfertilized oocytes**

Ova falling into this category, were discarded.

d) **Standardized coding systems**

Standardized numeric coding systems for use in describing the stage of embryonic development and quality are shown in Figure 3.1 (Stringfellow & Seidel, 1990).

Number 1 identifies an unfertilized oocyte or a one cell embryo. Number 2 identifies embryos with 2 to 16 cells (approximately day 2 to day 5 of development). Number 3 identifies an early morula, and number 4 through 9 identifies post-compaction stage embryos as illustrated in Figure 3.1. In commercial embryo transfer, embryos are usually collected on days 6 to 8 of the oestrous cycle (morula to blastocyst stage).

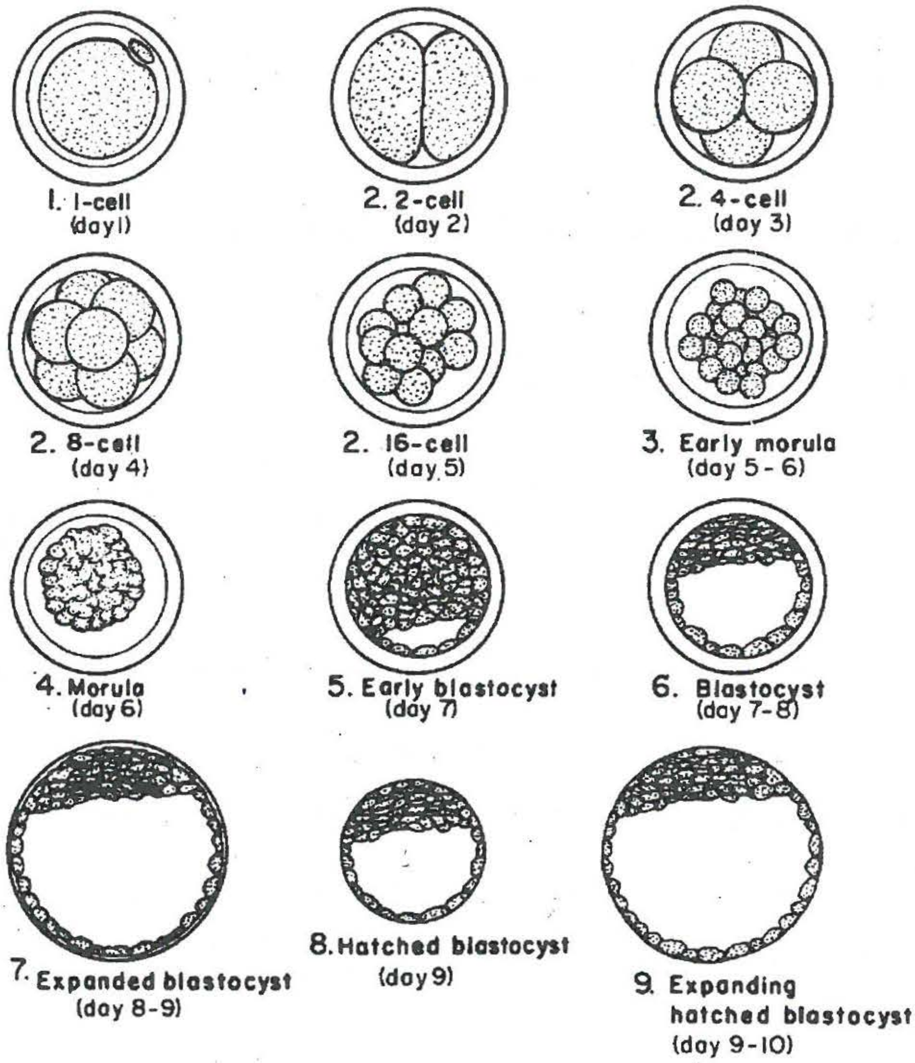

Figure 3.1 Normal bovine embryos recovered at various stages of development (Stringfellow & Seidel, 1990)

3.5 EMBRYO CRYO-PRESERVATION AND STORAGE

3.5.1 Introduction

The procedure of controlled freezing and thawing rates require the artificial induction of seeding at a temperature of approximately -6°C to avoid excessive crystallization of the embryos. The temperature alteration due to release of heat of fusion and change in osmolality requires a equilibrium period of 5 to 10 minutes, but not more than 10 minutes, to equilibrate temperature and cell volume. Manual seeding is performed by touching the medium with the embryo within the straw at the opposite end to the embryo (Saha, 1996). According to Saha, Otoi, Takagi, Boediono, Sumatri and Suzuki (1996), early cryobiological studies with bovine embryos were aimed at obtaining complete dehydration of the embryo.

3.5.2 Preparation of freezing media

Conventional (slow) freezing media

Commercial ViGro ™ Holdingplus Media (AB Technology. Inc. Pullman. Wa. USA. 509 335-4047) was prepared for temporary storage of embryos for *in vitro* culture, before being transferred to 1.5 M ViGro ™ Ethylene glycol Freezeplus (AB Technology. Inc. Pullman. WA – USA). Prior to transfer, ViGro™ EG Freezeplus medium was warmed to 24°C (ViGro ™ EG Freezeplus is a DMPBS–based solution containing 1.5M EG, 0.4% BSA and 0.1M sucrose).

3.5.3 Conventional slow freezing of embryos

i) Day 7, 8 and 9 early blastocyst, blastocyst and expanded blastocyst embryos were removed from IVC (39°C) and randomly placed in holding media (ViGro ™ Holdingplus) at room temperature (24°C). Embryos were then transferred from ViGro ™ Freezeplus medium to 1.5M ViGro ™ EG Freezeplus at room temperature (24°C) and immediately loaded into 0.25ml plastic straws and sealed. In preparation for the freezing of embryos in straws, they were loaded and labeled as follows (Figure 3.3):

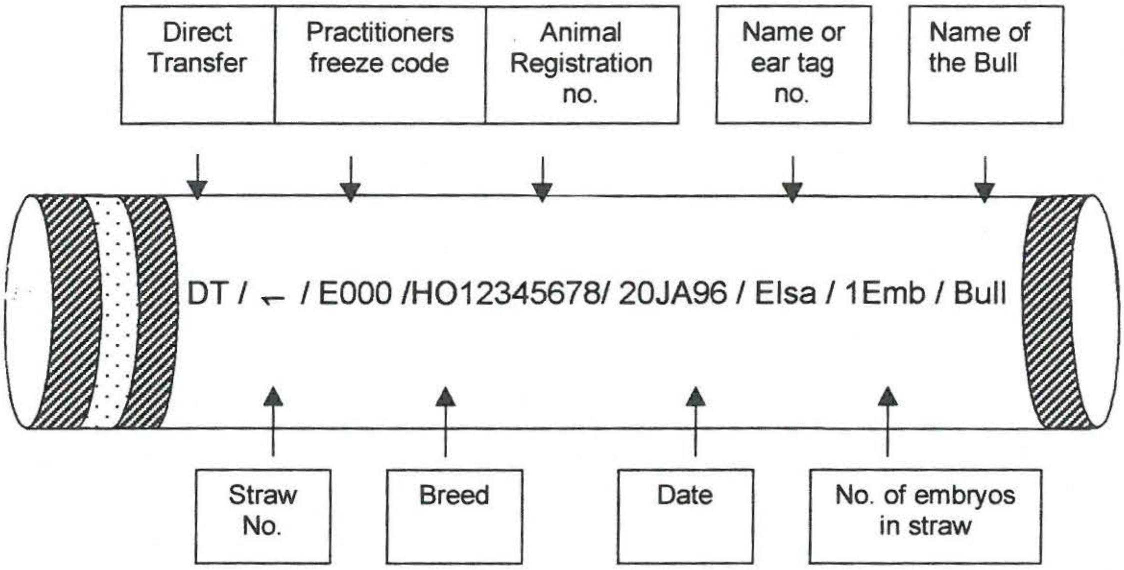

Figure 3.2 Label detail of embryo cryo-preservation

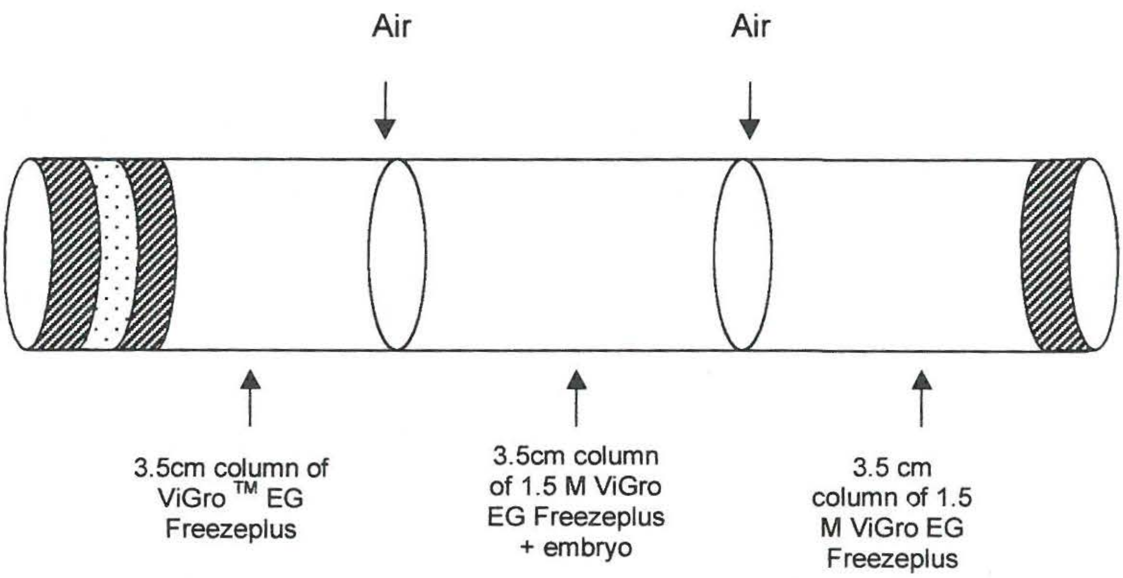

Figure 3.3 Method of embryo placement in cryo-preservation

ii) After sealing, embryos were immediately placed into a CL 863 cryochamber system and the temperature maintained at -6°C. Straws were then seeded after 5 minutes of equilibration, by touching the wall of the straws with a cold forceps at the end of the straw opposite to the embryo. Embryos were cooled at a rate of 0.3°C/minute from -6°C to -30°C. From -30°C to -33°C, the rate changed to 0.1°C/minute. After the target temperature of -33°C was reached, embryos were transferred directly into a container with liquid nitrogen (-196°C) and straws were stored vertically in a liquid nitrogen tank.

3.5.4 Thawing procedures

Regarding thawing, various reports have dealt with procedures for the exposure of the frozen straw to air and water. A low incidence of embryo damage when straws are warmed in air for 10 seconds prior to transfer into water has been recorded in mammals (Rall & Meyer, 1989).

Thawing of the embryos in the straw was carried out in a water bath (35°C) at room temperature (24°C). Each straw was air thawed for 10 seconds and then placed in the water bath (35°C) for 30 seconds. The straw was then dried, cut and the contents expelled into ViGro TM Holdingplus medium. Once the embryos were recovered from the straws, they were washed twice in fresh ViGro TM Holdingplus medium and immediately examined morphologically for survival/re-expanded stage of the blastocyst under a stereo microscope (X 75).

- All thawing procedures were performed in close proximity of the bison recipients in a warm room (± 25°C).

- The reproductive tract of the recipients was rectally palpated to check for abnormalities and the ovaries palpated for *corpora lutea* (CL'S). The ovary on which a CL was present in each recipient was noted.

- A water bath was prepared (35ºC) as it is important that this temperature is accurately maintained when using ethylene glycol as a cryoprotectant.

- Recipients were administered 3 ml of a local anaesthetic (2% Lidocaine) for an epidural block into the medullar colon between the lumber vertebrae at the tail attachment. The vulva was cleaned and disinfected as described previously.

- After the thawed embryo was evaluated and examined, a clean 0.25cc straw was washed with ViGro ™ Holdingplus. The embryo was drawn into the straw in the appropriate columns as shown in Figure 3.3.

3.6 EMBRYO TRANSFER PROCEDURE

3.6.1 Introduction

The first embryo transfer in bison was performed in 1993. A total of 5 fresh bison embryos were transferred non-surgically into bison recipients, resulting in 3 confirmed pregnancies. One of the pregnancies was lost after 40 days of gestation and a second pregnancy produced a normal healthy bison calf. This trial confirmed the possibility to non-surgically recover bison embryos and to successfully transfer them into bison recipients (Jainudeen & Hafez, 2000).

Commercial bison producers in America are more interested in the option of transferring bison embryos into domestic cattle recipients as this would allow a faster multiplication of bison and open up new doors to the industry. Numerous studies and commercial ventures have demonstrated the successful hybridization of bison and cattle to produce the beefalo (Bergfelt, Mapletoft, Adams & Pierson, 1994). In these hybridization programs, bison bulls are mated to domestic cows to produce a F1 hybrid. However, success has been poor, presumably due to the high incidence of placental hydrops. Placental hydrops is an excessive build-up of amniotic and allantois fluid due to an immune response in the uterus. In 75% bovine genotypes, the occurrence is lethal to the developing foetus, causing second and third trimester abortions and in some cases also being fatal to the pregnant cow. The incidence of hydrops can be as high as 50 to 60% following hybridization. The high incidence of hydrops and the associated abortion rates warrant research prior to the success of inter specie embryo transfer between the bison and domestic cattle (Hanna & Scott, 1989).

Few attempts have been made to transfer bison embryos directly to domestic cattle, but no successes have been reported. The first attempt was performed in 1989, when 4 frozen-thawed bison embryos were transferred to 4 Beefmaster cows, resulting in 2 confirmed pregnancies as determined by ultrasound at day 45 of gestation. Both pregnant recipients aborted between

90 and 120 days of gestation. Therefore bison recipients were used in the transfer of bison embryos in this study to overcome the incompatibility between donors and recipients.

3.6.2 Preparation of recipients for embryo transfer

An epidural anathesia was accomplished by administering 5ml of a 2% Lidocaine (Centaur) into the medullar canal between the lumbar vertebrae at the tail attachment. The tail was then secured to facilitate access to the vulva. The pericanal region and the vulva area were washed with an iodine scrub soap, dried and rinsed with a 70% alcohol solution for disinfection. Thereafter excess faeces was removed from the rectum. In removing the faeces, air was frequently aspirated into the ampulla recti, causing ballooning. This ballooning resulted in an undesirable effect, as it hindered proper palpation and guidance at the embryo transfer gun into the cervix and uterine horns.

3.6.3 Transfer of embryos into recipients

Embryos were transferred non-surgically through the cervix to the uterine horn ipsilateral to the active CL of the synchronized recipients, using a miniaturized embryo transfer gun (IMV, L'angle, France). Where oestrus could not be observed in the recipient and a functional CL was rectally identified at the time of transfer, onset of oestrus was presumed to have occurred at 60h after SMB/PGF treatment.

Embryos were temporary stored in holding medium plus 0.95M glycerol prior to being transferred. Straws (as indicated in Figure 3.3) were loaded in a specific manner to ensure that the embryo would not be lost or remain in the straw following transfer. The air column created, prevents the embryo from drifting to the ends of the straw.

CHAPTER 4

RESULTS

4.1 SUPEROVULATORY RESPONSE AND NUMBER OF EMBRYOS RECOVERED

The superovulatory response, in terms of number of *corpora lutea* palpated, anovulatory follicles, the total number of embryos recovered and the number of transferable embryos produced in the 3 protocols, is set out in Table 4.1 and Figure 4.1.

Table 4.1 Ovarian response and embryo recovery rate following superovulation and flushing in the bison

Protocol	No of donors	No of CL (Mean ± SE)		Follicles > 10mm (mean ± SE)		Embryos/ova recovered (mean ± SE)		Embryos transferable (mean ± SE)	
I	2	5	(2.5 ± 0.7)	1	(0.5 ± 0)	4	(2 ± 0)	1	(0.5 ± 0)
II	2	18	(9 ± 1.4)	7	(3.5 ± 0.7)	14	(7.0 ± 0.0)	7	(3.5 ± 1.0)
III	2	7	(3.5 ± 0.7)	2	(1.0 ± 0)	5	(2.5 ± 0)	1	(0.5 ± 0)
TOTAL	**6**	**30**	-	**10**	-	**23**	-	**9**	

No ovulation response was detected in one of the bison females (Protocol I), where FSH-P was administered once daily in decreasing doses. In total only 5 bison cows responded to the superovulation treatment (Figure 4.1). The overall mean response rate recorded for all treatment groups in this study was

5.0 ± 7.7 embryos/ova per donor, with a total of 7 follicles (mean 1.4 ± 1.4) failing to ovulate.

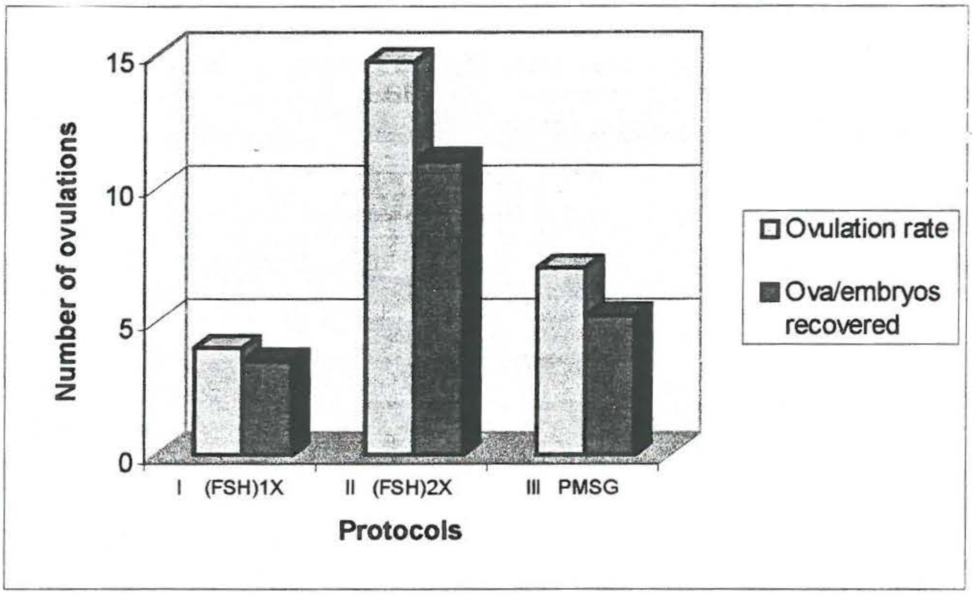

Figure 4.1 **Ovarian response following protocols of a daily (1X), twice daily (2X) FSH or PMSG treatment in adult and heifer bison**

The maximum ovulation rate (9.0 ± 1.4 CL/donor) was observed in Protocol 2, compared to a minimum ovulation rate (2.0 ± 0.7) recorded in Protocol 1 (Table 4.1). The most embryos recovered (n = 14) were from the 2 donors (7 embryos/donor), when implementing Protocol 2. The least number of transferable embryos per donor was 1, for animals treated with Protocol 3. Overall one or more embryos were recovered from 5 out of the 6 bison cows. A total of 23 embryos were recovered from the 5 donors with the mean number of embryos recovered per donor being 3.8 ± 4.2 embryos/donor. Of the 23 embryos and ova recovered, 3 were at the 16-cell stage (retarded), and 11 were unfertilized oocytes and could not be processed further. The remaining 9 viable embryos were further classified as Grade 1 (n = 2), Grade 2 (n = 3) and Grade 3 (n = 4) embryos. All Grade 1 and Grade 2 (n = 5) embryos were frozen. Grade 3 (n = 4) embryos were transferred as fresh embryos. The FSH (2X) produced 60.9% of the total number of embryos/ova, Protocol 1 FSH (1X) produced 17.4% of the total number of embryos, while in Protocol 3 the PMSG treated animals produced 21.4%. The one FSH (1X) bison which did not respond, was not flushed. The percentage of embryos recovered (Table 4.1) and the number of viable embryos produced per donor protocol was higher in the FSH (2X)-treated group (60.8%), compared to the FSH (1X)-treated group (17%) and the PMSG-treated group (22%).

In the FSH (2X)-treated group, the number of unfertilized oocytes could positively be related to the poor motility of the frozen/thawed semen used. The percentage unfertilized ova recorded in the three treatment groups, was 25%, 42% and 40% respectively for Protocol 1, 2 and 3 (Figure 4.2).

The estimated ovarian volume in all donors (indicator of ovarian activity) for the different treatment protocols when rectally palpated, felt considerably different, with the PMSG-treated cows having much larger ovaries, compared to the other FSH-treated animals. The status of ovarian growth and development and progressive increase in size of the ovaries during the course of the superstimulation program using PMSG, FSH (2X) and FSH (1X) are set out in Table 4.2 and Table 4.3, respectively. A graphic presentation of the ovarian volumes, by rectal palpation, is illustrated in Figure 4.4.

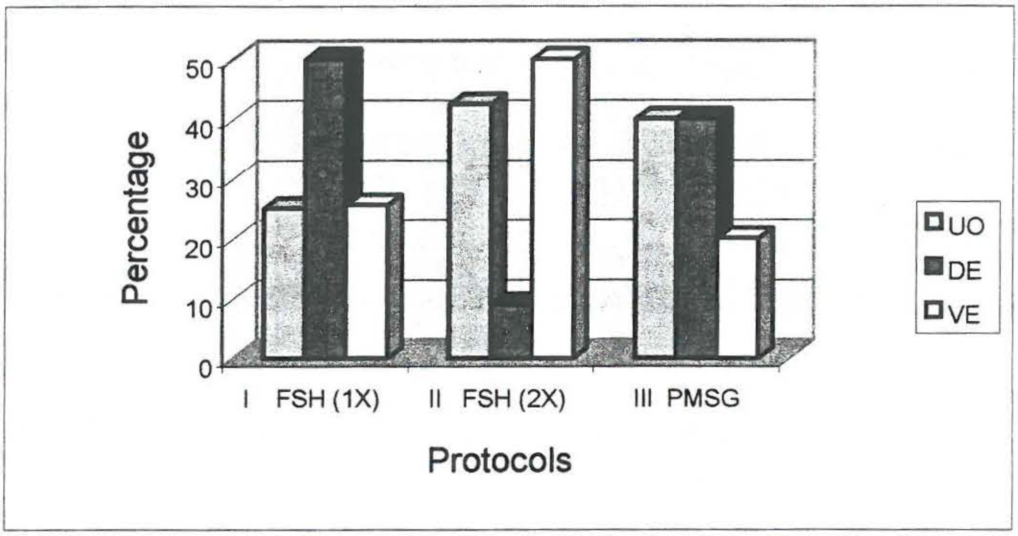

Figure 4.2 **The percentage viable embryos (VE), retarded (degenerated) embryos (DE) and unfertilized oocytes (UO) in FSH (1X)-treated, FSH (2X)-treated and PMSG-treated bison cows**

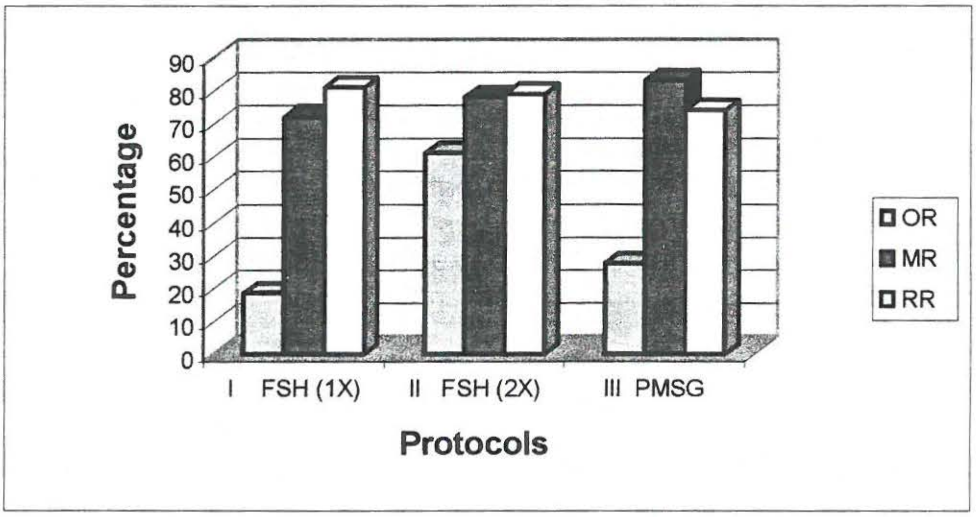

Figure 4.3 **The oestrous response (OR), the percentage flushing medium recovered (MR) and the embryo recovery rate (RR) in the FSH (1X)-treated, FSH (2X)-treated and PMSG-treated animals following superstimulation**

Table 4.2 The estimated ovarian volume of bison cows prior to superstimulation with FSH (1X), FSH (2X) or PMSG

	FSH (1X)		FSH (2X)		PMSG	
	Mean	± SE	Mean	± SE	Mean	± SE
Volume prior to superstimulation:						
Left ovary (cm^3)	20.4	0.3	18.4	0.2	19.1	0.2
Right ovary (cm^3)	18.3	0.2	16.1	0.2	17.5	0.3
Total volume (cm^3)	19.3	0.3	17.3	0.3	18.3	0.4

A definite increase in ovarian volume was recorded in all three treatment protocols, following the induced superstimulated oestrus. The increase in ovarian volume was prominent soon after the administration of the gonadotrophic hormones, relative to prostaglandin administration. The progressive increase in ovarian volume continued until shortly after the onset of the induced oestrus. This ovarian expansion later slowed down and even decreased during the post oestrous period (Figure 4.3). Spongy small *corpora haemorhagica* that had not yet formed luteal tissue, were palpated during this period. This decrease in size of the ovary was again rapidly followed by an increase in size, coinciding with the development functional *corpora lutea* and/or follicles. The ovaries continued to grow progressively and reached their maximum size between days 6 and 9 following oestrus – indicating only a slight increase in ovarian size after the day of collection (day 7).

Luteal regression and follicular atresia prior to and in conjunction with the first post superstimulation oestrus, resulted in a pronounced decrease in ovarian volume. Although still somewhat enlarged, the dimensions of the palpated ovaries were similar to dimensions observed prior to the start of the superstimulation program.

Table 4.3 The estimated ovarian volume on the day of embryo collection (day 7), following superstimulation with FSH (1X), FSH (2X) and PMSG in bison cows

		FSH (1X) (n = 1)		FSH (2X) (n = 2)		PMSG (n = 2)	
		Mean	± SE	Mean	± SE	Mean	± SE
Length:	Left ovary (cm)	4.9	0.3	5.1	0.2	6.2	0.3
	Right ovary (cm)	5.5	0.3	5.8	0.2	7.0	0.3
Width:	Left ovary (cm)	4.2	0.2	3.8	0.2	4.3	0.2
	Right ovary (cm)	4.0	0.2	4.1	0.2	5.1	0.2
Height:	Left ovary (cm)	3.0	0.2	3.2	0.3	4.1	0.2
	Right ovary (cm)	3.4	0.2	3.5	0.2	4.8	0.2
Volume:	Left ovary (cm^3)	61.7	7.4	62.2	8.4	109.3	12.1
	Right ovary (cm^3)	74.8	13.2	83.2	15.0	171.4	17.3
Total volume (cm^3)		68.3	17.8	72.8	18.2	140.4	24.1
Increase in volume (%):							
	Left ovary	302.6		338.1		571.7	
	Right ovary	409.8		517.0		980.3	
	Total volume	353.2		421.8		767.0	

The mean volume of left ovaries from the PMSG group increased from an average 19.1cm^3 to 109.3cm^3, resulting in a 571.7% increase. Concurrently the right ovaries increased in volume from 17.48cm^3 to 171.36cm^3, resulting in an increase of 980.3%.

The ovaries of the FSH (1X) treated group showed an average increase in ovarian volume of 302.6% for the left ovary and 409.8% for the right ovary, respectively. Similarly the ovaries of the FSH (2X) treated group showed a similar increase in average ovarian volume, from 18.4cm^3 to 62.2cm^3 (338.1%) in volume for the left ovary and 16.10cm^3 to 83.23cm^3 (517%) for the right ovary, respectively.

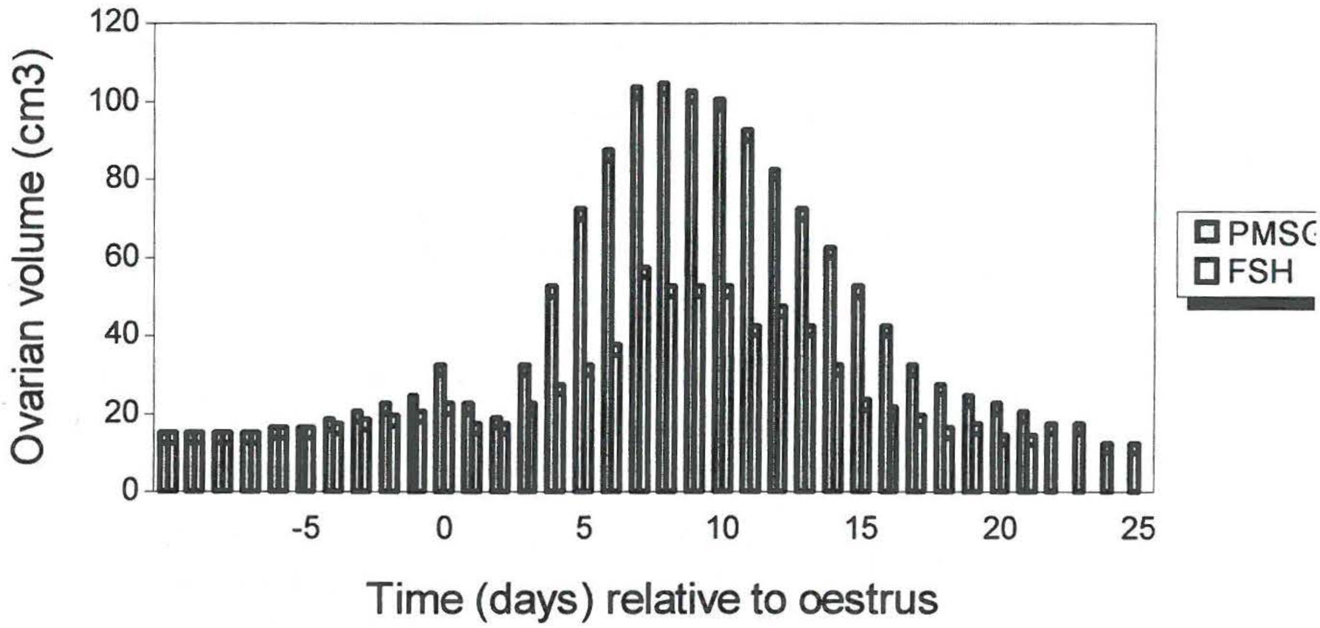

Figure 4.4 **Changes in ovarian volume following PMSG and pooled FSH superstimulation in bison cows**

4.2 SYNCHRONIZATION RESPONSE OF THE RECIPIENTS

All 12 of the recipient animals exhibited definite signs of oestrus as reflected by the uterine tone, cervical dilation and vaginal mucous secretion observed following rectal palpation.

The use of progestagens (SMB) in the synchronization of recipient cows (n = 12) yielded effective synchronization results (Figure 4.5), with a high degree of ovarian (oestrus) response (100%) and a compact synchrony (2 to 4 days) for the SMB treatment.

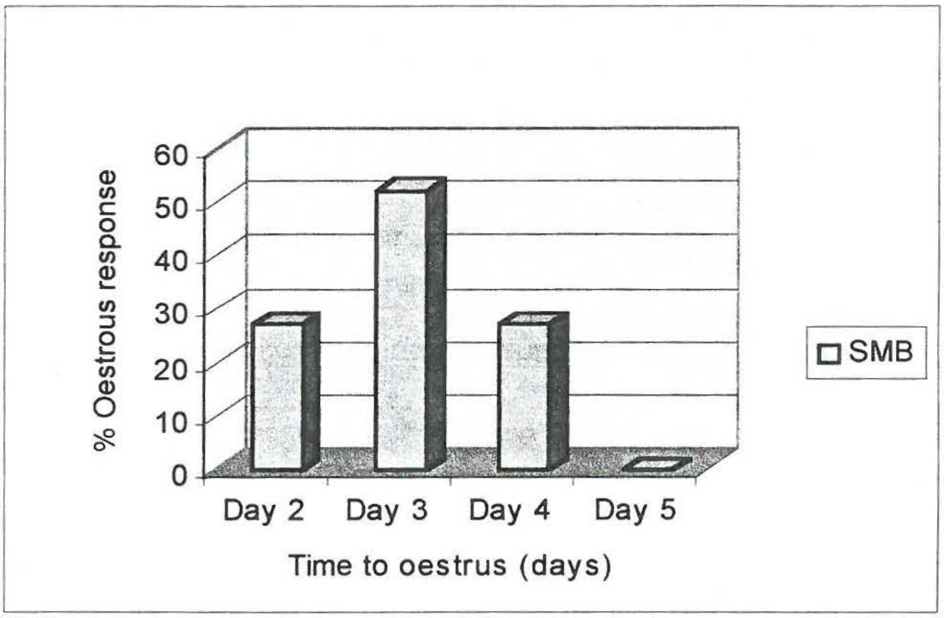

Figure 4.5 **Oestrous response following SMB synchronization treatment in recipient bison cows**

4.3 THAWING OF FROZEN BISON EMBRYOS

Five bison embryos were thawed prior to transfer. One embryo was lost during the thawing process and 3 out of the 4 remaining embryos were found to be of transferable quality following microscopic evaluation (Plate 4.1) and were transferred to 3 bison recipients (two embryos and one embryo per recipient cow, respectively). Two pregnancies were diagnosed by rectal palpation 50 days after embryo transfer (in the recipients). One of the cows aborted at 60 days of gestation and the second pregnancy produced a normal healthy bison calf.

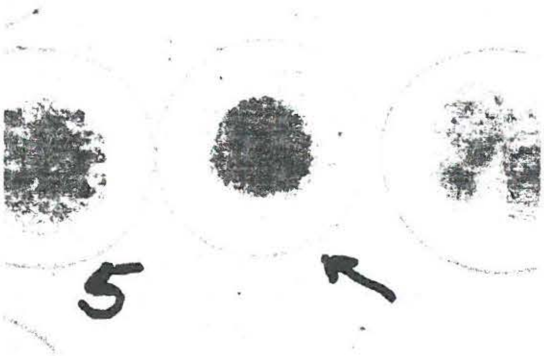

Plate 4.1 **Bison embryo recovered (day 7) Grade 1, retarded**

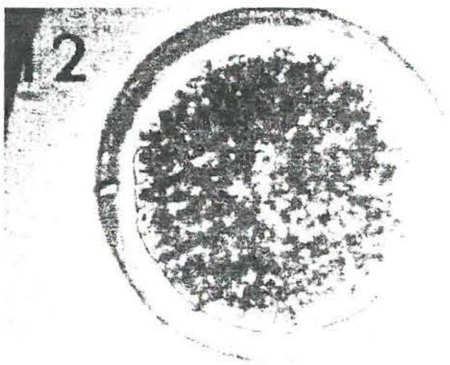

Plate 4.2 **Bison embryo recovered (day 7) Grade 1.5, retarded**

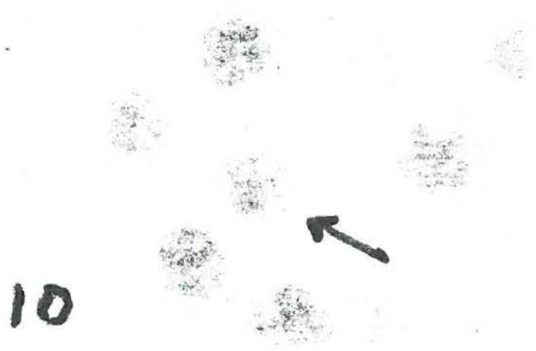

Plate 4.3 **Bison embryo recovered (day 7) Grade 2, retarded**

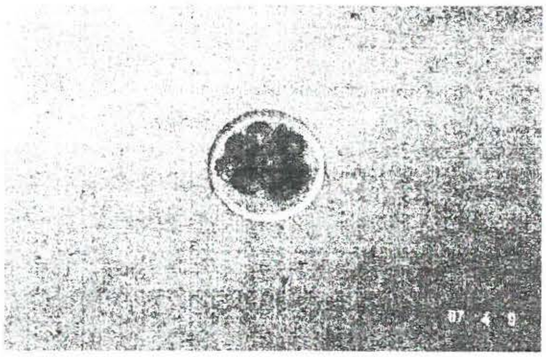

Plate 4.4 **Bison embryo recovered (day 7) Grade 2.5**

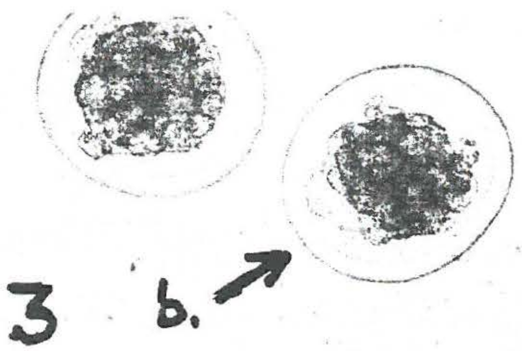

Plate 4.5 Bison embryo recovered (day 7) Grade 3

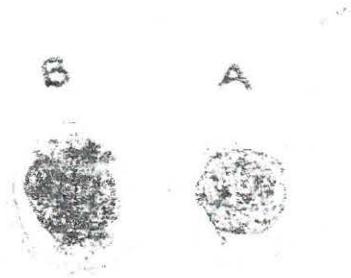

Plate 4.6 Bison embryo recovered (day 7) Grade (B) 3 (A) 4

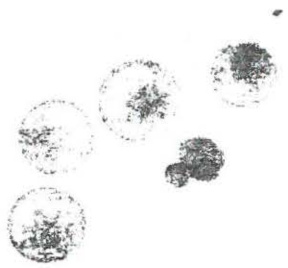

Plate 4.7 Degenerated and unfertilized bison embryos recovered at 6.5 to 7 days

Plate 4.8 Degenerated and unfertilized bison embryos recovered at 6.5 to 7 days

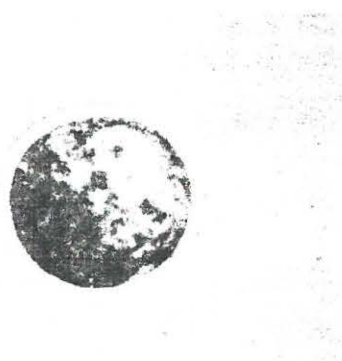

Plate 4.9 Degenerated and unfertilized bison embryos recovered at 6.5 to 7 days

4.4 TRANSFER OF FRESH BISON EMBRYOS

A total of 4 fresh bison embryos were transferred non-surgically to 3 bison recipients and one hybrid recipient (one embryo/recipient). This resulted in 3 confirmed pregnancies, recorded by rectal palpation, 40 days following embryo transfer. One of these cows aborted at 65 days of gestation (hybrid) and a second pregnancy produced a stillborn/dead calf. The third pregnancy produced a normal healthy bison calf.

CHAPTER 5

DISCUSSION

5.1 SUPEROVULATION OF THE DONORS

The superstimulation rate (Table 4.1) achieved in the trial with twice daily treatments of FSH (2X) (9 ± 1.4 CL's/donor) is in line with that quoted by other researchers on cattle (Boland *et al.*, 1991; Bo, Hockley, Nasser & Mapletoft, 1994). The response following PMSG stimulation (3.5 ± 0.7 CL's/donor) is lower when compared to that achieved by the use of the FSH (2X) protocol. This lower ovulation rate obtained with PMSG is in agreement with Dieleman, Bevers and Gielen (1987) and could possibly be related to the long half-life of PMSG.

From the high flushing media recovery rates (average of 75%) obtained during flushing, it can be safely said that the technique could be seen as very efficient. These recovery rates are in line and comparable with those obtained by other researchers in other species (Mapletoft *et al.*, 1988; Mapletoft & Pierson, 1993).

The variability and possible inability of PMSG to induce multi-ovulation in the bison, is demonstrated by the fact that an average ovulation rate of only 3.5 was recorded. The higher production rate of embryos obtained in the FSH-treated groups, compared to the PMSG-group also indicates that stimulation by FSH is more advantageous and effective. The overwhelming stimulation by PMSG could negatively effect the embryo production rate. The relatively high recovery rate of the embryos during flushing as such (66% vs 71% for the FSH and PMSG-stimulated groups respectively) is a reflection of acceptable embryo flushing and stimulation techniques, especially in the FSH-stimulated groups. These embryo recovery rates obtained by the two treatments are in line with other studies (Breuel, Baker, Butcher, Townsend, Inskeep, Dailey & Lerner, 1991). The results obtained also seem to indicate the production of more viable embryos following stimulation with FSH. This once again could possibly be ascribed to the longer half-life and over stimulation of PMSG. The difference in the two superovulation regimes (FSH and PMSG) could also be due to the more even distribution (over a 4 day period) of gonadotrophin (FSH) administration which consequently results in healthier and more normal embryos. In the PMSG-treated group, the single gonadotrophin administration with its longer half-life, results in a higher number of cystic follicles with more fragile and less viable ova being released. Dieleman *et al.* (1983) reported that the administration of exogenous gonadotrophins could disturb the normal maturation process of oocytes. The higher purified and more potent FSH stimulation (administered over a longer period of time) also leads to a more efficient follicular growth, with a higher percentage of follicles ovulating. In the administration of PMSG on the other hand (which is a combination of FSH and LH), the ratio of the gonadotrophic hormones released by the pituitary following stimulation is disturbed which often leads to the formation of cystic follicles, which is the primary cause of follicles not ovulating (Elsden *et al.*, 1978). This treatment with PMSG thus results in a lower ovulation rate and the production of less viable embryos than e.g. when using FSH.

From the trial it is evident that barring the fact that treatment with PMSG produced less embryos, it also produced a greater number of degenerating and abnormal embryos. This phenomenon could be ascribed to the hormonal imbalance (FSH:LH ratio) induced by superovulation using PMSG. These observations confirm findings by Booth *et al.* (1975) and Brynar, Garcia-Winder, Lewis, Inskeep and Butcher (1990). A negative aspect of this trial, was the high percentage of unfertilized oocytes recovered (47%), which reflects on an unsatisfactory fertilization rate. The unfertilized oocytes recorded could be due to the poor quality of bison semen used, and not as such the technique of AI. Suggestions have been made regarding the effect of higher serum progesterone levels, following increased *corpora lutea*, on a decreased fertilization rate at embryo recovery (Greve *et al.*, 1983). The effect of stress on the females and males is an aspect not to be underestimated in the low fertilization rates obtained in this study.

The effect of dose schedules and route of administration of the FSH preparation on fertility rates in cattle has been reported by several groups (Staigmiller, Short, Bellows & Hall, 1994; Tribulo, Bo, Kastelic, Pawlyshyn, Barth & Mapletoft, 1995). The reduction of the number of gonadotrophin administrations under normal circumstances, results in a decreased superovulatory response - as was experienced in this study. Several groups have now shown that, with certain formulations, single injections or administrations can be successfully used rather than the conventional 8-10 doses spread over a period of 4-5 days. Such simplification may be particularly important when dealing with beef cattle and especially the bison, where the restraint of animals for multiple injections constitutes an unwelcome source of stress and inhibition of response (Mapletoft & Pierson, 1993; Rodriguez, Neuendorff, Lewis, Chase Jr. & Randel, 1994).

5.2 OESTROUS SYNCHRONIZATION OF DONOR AND RECIPIENT BISON

Synchromate-B treatment in the synchronization of bison, as implemented in this trial, generally results in a high degree of synchronization, while conception rates have often been recorded as variable. It is known from work done with ovariectomized cattle that Synchromate-B treatment can even induce oestrus, irrespective of the status of the ovaries (McGuire, Larson & Kiracofe, 1990). Further, studies by Larson and Kiracofe (1995) using ovarectomised cows suggest that this induced oestrus is a result of the residual oestradiol from the pre-implant injection. These workers also concluded that in some instances the occurrence of reduced or variable fertility, associated with Synchromate B treatment, may be due to an anovulatory oestrus or an improper timing of insemination relative to the time of ovulation. D'Occhio and Kinder (1995), reported that when exogenous progestagens, both natural and synthetic are employed in the oestrous synchronization of cattle, it often leads to the development of a persistent dominant follicle. It is believed that exogenous progestagens may not always exert the same level of feedback on LH secretion that is imposed by the *corpus luteum*, with the result that circulating levels of LH are higher than those normally associated with the luteal phase of the oestrous cycle. This results in the continued development of the dominant follicle, which would otherwise become atretic due to a lack of gonadotrophin stimulation. The persistent follicle that ovulates upon withdrawal of the exogenous progestagen can then also be associated with decreased fertility. In this trial it was found that the use of SMB was effective in synchronizing the oestrous cycles of bison – similar to that reported in cattle.

Maturation of the bovine (and in this case bison) *corpus luteum* is associated with increased sensitivity to prostaglandin treatment. This may be due to a rapid increase in the concentration and total content of specific $PGF_{2\alpha}$ receptors in the *corpus luteum* early in the oestrous cycle. Several studies reported that regression of the *corpus luteum* in cattle could be induced by twice-daily doses of prostaglandin on days 3 and 4 of the oestrous cycle, suggesting that the effectiveness of a given treatment in cattle may depend on the interaction between the age of the *corpus luteum* and the frequency of prostaglandin administration. It was suggested by Watts and Fuguay (1985) that synchronization programmes using prostaglandin ($PGF_{2\alpha}$) should be modified due to its sub-optimal oestrous synchronization efficiency of prostaglandin recorded in the early di-oestrus phase of the cycle and the resultant decreased conception rate obtained. Laverdiere, Roy, Proulx, Lavoie and Dufour (1995) showed that prostaglandin was more efficient at synchronizing oestrus, when given at 7 rather than at 4 days after oestrous detection. The day 7 programme permitted the insemination of 93% of cows over a 12-day period and resulted in 68% of the animals being pregnant, irrespective of their post partum interval or physiological status. The degree of synchrony obtained in this study by using two injections of PGF, 2X 11 days apart, was similar to that obtained in cattle. Currently this technique is more practical to use in the bison than e.g. SMB implants.

5.3 THAWING OF BISON SEMEN

Various reports have dealt with thawing procedures and the exposure of frozen bovine semen (straw) to air and water. Thundathil, Palomino, Barth, Mapletoft and Barros (2001) for example, used zona fracture as an indicator of an appropriate thawing procedure. It was found that a low incidence of zona damage occurred when straws were exposed to air for 10 seconds prior to being transferred into the water. The short exposure to air allows the straw to warm to about 100°C prior to rapid warming in water (31°C). The quality of semen obtained following thawing depends on the quality semen initially frozen. Unfortunately the number of males from which semen was obtained were limited, while the stress factor was always an aspect to be borne in mine – influencing the quality of semen obtained. With better quality semen better fertilization rates would have been possible in this trial.

5.4 TRANSFER OF BISON EMBRYOS

Bison producers are more interested in the option of transferring bison embryos into domestic cattle recipients. It would be easier and more feasible. Numerous studies and commercial ventures have demonstrated the successful hybridization of bison and cattle to produce the beefalo or cattalo. In hybridization programmes, bison bulls are mated to domestic cows to produce a F1 hybrid. However, success rates have been poor, presumably due to the high incidence of placental hydrops. Placental hydrops is an excessive build up to amniotic and allantoic fluid due to an immune response by the uterus. In most cases, this occurrence is lethal to the developing fetus, causing second and third trimester abortions and in some cases, it can also be fatal to the pregnant cow (Hanna & Scott, 1989). The incidence of hydrops has been reported to be as high as 50 to 60% with hybridization in the bison. The high incidence of hydrops and the associated abortion rate, merit great concern for the success of inter-specie embryo transfer between e.g. bison and hybrid (domestic cattle). In this trial the limited success obtained in terms of successful pregnancies following embryo transfer could be ascribed to several still unknown factors. Much more research is required before definite recommendations and conclusions can be made.

CHAPTER 6

GENERAL CONCLUSIONS

From the trial undertaken to evaluate the development and evaluation of a method for multi-ovulation and embryo transfer (MOET) in the bison and the effect of the different gonadotrophic hormones on progressive ovarian stimulation following superstimulation response, it is evident that ovarian response increases considerably following the administration of the exogenous gonadotrophins, PMSG and FSH.

When assessing the effect of PMSG and FSH on embryo quality and quantity following superstimulation, some of the most pertinent observations noted were that the FSH-treated bison cows had a higher ovulation rate, a higher embryo recovery rate and more viable embryos, compared to that obtained in cows following PMSG-treatment. The technique of flushing as such was also highly satisfactorily, as is indicated by the high flushing medium recovery rate (mean 75%). In all trials it was attempted to minimize variation regarding age, location, body condition, nutrition and handling of these wild animals. However, considerable variation still existed in the superstimulation response (in terms of the number of ovulations and number and quality of embryos recovered).

It can further be deduced from the results obtained, that individual variation and unpredictable superstimulation is the consequence of fundamental physiological hormonal responses, which regulate the number of developing follicles and the subsequent ovulation rate. From the results obtained, it would seem that although more labour intensive, time consuming and more expensive, the use of FSH as a superstimulatory agent, is justified in the superovulation of the bison. More research however, is warranted in the administration program of the superovulatory agent (time, dose, route, etc.) for a better and more reliable ovarian response in the bison.

From responses obtained following the administration of 3000 IU of PMSG in the superstimulation of bison, it can be concluded that the response of bison to PMSG is lower than that for FSH treatment given as a single injection. Certain bison (if not all) could be more susceptible to stress and endocrine imbalances cause by a single PMSG stimulation, due to its long half-life and high LH ratio. However, individual animal variation remains an over-riding factor. Caution should be taken when interpreting these results due to the limited number of animals. The single dose of PMSG and its overwhelming effect on the ovary could also have a negative effect on the ovarian response. Thus PMSG stimulation needs to be further investigated if it is to be used in this type of program.

The bison is unique in its response to reproductive technologies, which are commonplace in domestic cattle. Unlike other exotic wild ruminants, the bison respond to protocols used in domestic cattle, when administered under ideal conditions. The problems associated with the use of these reproductive technologies in bison are not the technologies themselves, but the handling and management of the animals. While bison are reproductively similar to cattle, behaviorally they are very different. Most bison are not amenable to domestication and are managed as wild animals. The bison therefore, responds accordingly and presents a very real and dangerous problem when being handled. The danger is not limited to injury of the person, but also to the animal itself. Bison ranchers are aware of normal handling losses and are therefore reluctant to adopt procedures requiring multiple or time consuming handling of the animals.

The application of new reproductive technologies to increase the number of the North American and European bison, will continue to play a major role in the growth of their associated industries. Conservation and commercialization have brought this specie back from the verge of extinction and currently its impact as an enterprise is growing beyond all expectations. By exploiting the use of reproductive technologies in bison, it can possibly also be applied to other relevant wild species, particularly the African Buffalo (*Syncerus Caffer*) with possible similar results. As the bison industry grows, a new market will be created for embryo transfer technology and its expansion beyond the domestic commercial market.

To date, the application of reproductive technologies in the bison have produced limited successes in terms of semen collection, semen freezing, AI, pregnancy detection, super ovulation, embryo recovery and transfer. It is time to measure the efficiencies of these techniques on a commercial scale and incorporate them to bison ranching. The amount of published work regarding the bison is limited, but the successes obtained hold promise. Further research in this unique animal is warranted – especially regarding synchronization, MOET and AI techniques and thus the potential use of the bison for animal protein production and its role in satisfying this need.

Developing and evaluating a method for multi-ovulation and embryo transfer (MOET) in the bison (Bison bison)

by

Andrew Stephen van der Walt

Supervisor:	Prof JPC Greyling
Co-Supervisor:	Dr C van der Westhuizen
Department:	Agriculture
Technikon:	Technikon Free State
Degree:	M TECH : Agriculture

ABSTRACT

The aim of the study was to investigate the efficiency of different superovulation regimes for a MOET program in bison. The two main variables evaluated were (i) a protocol for superovulation, oestrous synchronization and embryo recovery for bovine and evaluate it for the American bison – to determine the possibility of use in the bison and (ii) to determine the ovarian response using different gonadotrophins (FSH or PMSG) and administration protocols – as possible

sources of variation in the ovulation rate, quality and quantity of embryos recovered following flushing of the bison.

Three different protocols were used to superovulate mature bison cows: Protocol 1: Bison cows (n = 2) were treated once daily (FSH 1X) in decreasing doses over a 4-day period (6, 5, 4 and 3mg FSH-p i.m. per day) with a total of 18mg FSH-p. Oestrus was synchronized with a SMB-implant over a 9 day period. FSH treatment commenced on day 6 of synchronization. Protocol 2: Bison cows (n = 2), were treated twice daily (FSH 2X) also with a total of 18mg FSH-p in decreasing doses over a 4 day period (6, 5, 4 and 3 mg FSH-p i.m. per day). Oestrus was synchronized with a SMB-implant over a 9-day period. FSH treatment commenced on day 6 of synchronization. Protocol 3: Bison cows (n = 2) were treated with a single injection of 3000 IU eCG (PMSG) i.m. on day 10 of the induced oestrous cycle, followed by 2 (500 ug each) i.m. PGF injections 48 and 60 hours later (day 12).

Ovulation rate and ovarian volume was determined by rectal palpation on day 7 following the cessation of treatment in all animals. Embryo flushing was performed on day 7 and recovered embryos were then graded and acceptable embryos frozen or transferred to recipient bison cows.

The ovarian response recorded (mean number of CL ± SE) was 2.5 ± 0.7; 9.0 ± 1.4 and 3.5 ± 0.7 for Protocol 1, 2 and 3 respectively. With the corresponding mean number of embryos/ova recovered per donor for the respective groups being 2, 7 and 2.5. The total increase in ovarian volume (indicator of ovarian activity) was 353.2% for Protocol 1 (FSH 1X) while Protocol 2 (FSH 2X) induced a 421.8% increase and Protocol 3 (PMSG) a 767% increase. The overall mean embryo recovery rate was 3.8 ± 4.2 per donor. Of the 23 embryos and ova recovered, 3 were retarded and 11 unfertilized. The remaining 9 viable embryos were further classified and 4 were transferred as fresh embryos and 5 embryos frozen. The FSH (2X) group (Protocol 2) produced 60.9% of the total number of embryos produced. The occurrence of unfertilized ova was 25%, 42% and 40% for Protocol 1, 2 and 3 respectively. For the recipients all 12 of the bison cows exhibited oestrus. SMB treatment recorded a high oestrus response and compact synchrony in the recipients. Of the 4 frozen embryos and fresh embryos transferred 2 and 3 confirmed pregnancies were recorded respectively.

No ovulation was detected in one of the donor bison cows in the Protocol 1 (FSH 1X) treatment. When assessing the response in FSH-treated bison on embryo quality and quantity, it was evident that the FSH (1X)-treated group had a lower ovulation rate, a lower embryo recovery rate and fewer viable embryos than the FSH (2X) group. The technique of embryo flushing was seen as satisfactory as reflected by a high flushing medium recovery rate (75%). The larger ovaries recorded following PMSG stimulation could be ascribed to the longer half-life of PMSG and possible over-stimulation, compared to FSH. It would seem as if PMSG is less efficient as a superovulation agent in the bison when compared to a single administration of FSH. Certain bison cows could be more susceptible to stress and endocrine imbalances, caused by especially PMSG treatment were factors to be borne in mind. Caution should be taken when interpreting these results due to the limited number of animals. Individual variation remains an over-riding factor and along with the stress factor complicates the interpretation of the results obtained. Further research is needed to optimize the use of MOET in the bison.

Ontwikkeling en evaluering van 'n metode vir multi-ovulasie en embrio-oorplasing in die bison (Bison bison)

deur

Andrew Stephen van der Walt

Studieleier: Prof JPC Greyling

Mede-studieleier: Dr C van der Westhuizen

Departement: Landbou

Technikon: Technikon Vrystaat

Graad: M TECH : Landbou

OPSOMMING

Die doel van die studie was om die doeltreffendheid van verskillende superovulasiebehandelings in 'n embrio-oorplasingsprogram by die bison te ondersoek. Die twee veranderlikes wat geëvalueer is, was (i) 'n protokol vir superovulasie, oestrus sinkronisasie en embrio herwinning by die bees te evalueer vir die Amerikaanse bison - en dus die moontlikheid van gebruik in die bison te ondersoek en (ii) die ovariale respons, deur gebruik te maak van

verskillende gonadotrofiene (FSH of DMSG) en dosisse – as 'n moontlike bron van variasie in ovulasietempo, kwaliteit en hoeveelheid embrios herwin in die bison na spoeling te ondersoek.

Drie protokoile is gebruik om volwasse skenker bison koeie te superovuleer: Protokol 1: Bison koeie (n = 2) is daagliks eenmalig (FSH 1X) behandel (afnemende dosisse) oor 'n 4 dae periode (6, 5, 4 en 3mg FSH-P) i.m. per dag met 'n totaal van 18 mg FSH-p. Die oestrussiklusse is gesinkroniseer met 'n SMB implantaat vir 'n 9 dae periode. FSH behandeling het 'n aanvang geneem op dag 6 van sinkronisasie. Protokol 2: Bison koeie (n = 2) is twee maal per dag (FSH 2X) behandel ook met 'n totaal van 18 mg FSH-p in afnemende dosisse oor 4 dae (6, 5, 4 en 3 mg FSH i.m. per dag). Oestrussiklusse is gesinkroniseer met SMB-implantate oor 'n 9 dae periode. FSH behandeling het 'n aanvang geneem op dag 6 van sinkronisasie. Protokol 3: Bison koeie (n = 2) is behandel met 'n enkele inspuiting van 3000 IE eCG (DMSG) i.m. op dag 10 van die geinduseerde oestrussiklus, gevolg deur 2 (500 ug elk) i.m. PGF inspuitings 48 en 60 uur later (dag 12). Ovulasietempo en ovariale volume is bepaal deur rektale palpasie en sonografie op dag 7 na die staking van behandeling in alle diere. Embriospoeling is ook uitgevoer op dag 7 en herwinde embrios is geëvalueer en aanvaarbare embrios oorgeplaas na ontvangerkoei of gevries.

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Drie protokoile is gebruik om volwasse skenker bison koeie te superovuleer: Protokol 1: Bison koeie (n = 2) is daagliks eenmalig (FSH 1X) behandel (afnemende dosisse) oor 'n 4 dae periode (6, 5, 4 en 3mg FSH-P) i.m. per dag met 'n totaal van 18 mg FSH-p. Die oestrussiklusse is gesinkroniseer met 'n SMB implantaat vir 'n 9 dae periode. FSH behandeling het 'n aanvang geneem op dag 6 van sinkronisasie. Protokol 2: Bison koeie (n = 2) is twee maal per dag (FSH 2X) behandel ook met 'n totaal van 18 mg FSH-p in afnemende dosisse oor 4 dae (6, 5, 4 en 3 mg FSH i.m. per dag). Oestrussiklusse is gesinkroniseer met SMB-implantate oor 'n 9 dae periode. FSH behandeling het 'n aanvang geneem op dag 6 van sinkronisasie. Protokol 3: Bison koeie (n = 2) is behandel met 'n enkele inspuiting van 3000 IE eCG (DMSG) i.m. op dag 10 van die geinduseerde oestrussiklus, gevolg deur 2 (500 ug elk) i.m. PGF inspuitings 48 en 60 uur later (dag 12). Ovulasietempo en ovariale volume is bepaal deur rektale palpasie en sonografie op dag 7 na die staking van behandeling in alle diere. Embriospoeling is ook uitgevoer op dag 7 en herwinde embrios is geëvalueer en aanvaarbare embrios oorgeplaas na ontvangerkoei of gevries.

Die ovariale respons (gemiddelde aantal CL ± SA) was 2.5 ± 0.7; 9.0 ± 1.4 en 3.5 ± 0.7 vir Protokol 1, 2 en 3 respektiewelik. Die ooreenstemmende gemiddelde aantal embrios/ova herwin per skenker vir die respektiewe groepe was 2, 7 en 2.7. Die totale toename in ovariale volume (indikator van ovariale aktiwiteit) was 353.2% vir Protokol 1 (FSH 1X), terwyl Protokol 2 (FSH 2X) 'n 421.8% toename in volume geinduseer het en Protokol 3 (DMSG) vir 'n 767% toename verantwoordelik was. Die algemene embrio herwinningstempo was 3.8 ± 4.2 per skenker. Van die 23 embrios en ova wat herwin is, was 3 se ontwikkelingstadium vertraag en 11 onbevrug. Die oorblywende 9 lewensvatbare embrios is geklassifiseer en 4 is oorgeplaas as vars embrios terwyl 5 embrios gevries is. Die FSH (X2) groep (Protokol 2) het 60.9% van die totale aantal embrios geproduseer. Die voorkoms van onbevrugte ova was 25%, 42% en 40% vir Protokol 1, 2 en 3 respektiewelik. Vir die ontvanger bison koeie, het al 12 van die diere oestrus getoon. SMB behandeling het 'n hoë oestrus respons en kompakte voorkoms van oestrus in die ontvanger koeie tot gevolg gehad. Van die 4 vars en bevrore embrios wat oorgeplaas is, is 2 en 3 bevestigde dragtighede respektiewelik genoteer.

Geen ovulasie is in een van die skenker bison koeie waargeneem met die Protokol 1 (FSH 1X) behandeling nie. Met die evaluering van die respons in die FSH-behandelde bison aangaande embrio kwaliteit en kwantiteit, is dit ooglopend dat die FSH (1X)-behandelde groep 'n laer ovulasie tempo, 'n laer embrio herwinnings tempo en minder lewensvatbare embrios geproduseer het vergeleke met die FSH (2X)-groep. Die tegniek van embriospoeling was bevredigend as gekyk word na die hoë embrio spoelingsmedium herwinningstempo (70%). Die groter ovaria waargeneem na DMSG behandeling kan toegeskryf word aan die langer half-lewe van DMSG en die moontlike oorstimulasie van die ovaria, vergeleke met FSH-behandeling. Dit wil blyk asof DMSG minder doeltreffend is as 'n superovulasiemiddel in die bison, as FSH. Sekere bison koeie is meer vatbaar vir stres en endokriene abnormaliteite, veroorsaak hoofsaaklik deur DMSG behandeling. Sorg moet geneem word by die ontleding van hierdie resultate as gevolg van die beperkte aantal diere. Individuele variasie bly 'n faktor wat in ag geneem moet word en tesame met die stresfaktor bemoeilik dit ontleding van die resultate. Verdere navorsing is nodig om die gebruik van superovulasie en embrio-oorplasing in die bison te optimaliseer.

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DEVELOPING AND EVALUATING A METHOD FOR MULTI-OVULATION AND EMBRYO TRANSFER (MOET) IN THE BISON (Bison bison)

A.S. van der Walt[1], J.P.C. Greyling[2], C. van der Westhuizen[3]
[1]Taurus Co-op, Private Bag X5, Irene, South Africa
[2]Department of Animal Science, University of the Free State, P.O. Box 339, Bloemfontein, South Africa
[3] Agriculture Department, Free State Technikon, Bloemfontein, South Africa

INTRODUCTION

The number of North American bison (Bison bison) has increased progressively since the turn of the previous century when there were fewer than 2000 head[6]. The commercialization and privatization at bison herds has played a major role in increasing the numbers. With the development of bison ranching enterprises, the application of reproductive technologies currently utilized in domestic cattle operations, are being considered. The similarities between bison and domestic cattle have made it possible to directly utilize conventional bovine reproductive technologies in this wildlife specie.

Reproductively bison are anatomically and physiologically similar to domestic cattle with slight differences. One notable difference is the apparent seasonality in the bison. Bison are seasonally polyestrous, having an oestrous season commencing from late summer and extending into late fall. Many bison breeders consider the season to be shorter, ranging from July through September (late summer to early fall). This shorter time-frame may be due to the fact that more sexual activity is seen during the early part of the season and that cows which do not conceive early in the season, often do not become pregnant because of the stress of winter and the related decrease in available nutrition[1].

A bovine protocol for superovulation, oestrous synchronization and embryo collection was tested in the American bison (Bison bison) to determine its efficiency and feasibility for use in embryo transfer programs.

The two main variables evaluated were (i) a protocol for superovulation, oestrus synchronization, embryo recovery and the evaluation for bovine – to determine the possible use in the bison and (ii) to determine the ovarian response using different gonadotrophins (FSH or PMSG) and dosages – as a possible source of variation in the ovulation rate and quality of embryos following flushing in the bison.

MATERIAL AND METHODS

Three different protocols were used to superovulate mature bison cows: Protocol 1: Bison cows (n = 2), were treated once daily (FSH 1X) in decreasing doses over a 4-day period with a total of 18mg FSH-p (6, 5, 4 and 3mg i.m. per day). Oestrus was synchronized with a SMB-implant over a 9 day period. FSH treatment commenced on day 6 of synchronization (Table 1). Protocol 2: Bison cows (n = 2), were treated twice daily (FSH 2X) with a total of 18mg FSH-p (6, 5, 4 and 3 mg i.m. per day), in decreasing doses over a 4 day period. Oestrus was synchronized with a SMB-implant over a 9-day period. FSH treatment commenced on day 6 of synchronization (Table 2). Protocol 3: Bison cows (n = 2) were treated with a single injection of 3000 IU eCG (PMSG) i.m on day 10 of the induced oestrous cycle (following PGF synchronization), followed by 2 (500 ug each) i.m. PGF injections 48 and 60 hours later (day 12) (Table 3).

Table 1 Synchronization and superovulation of bison cows-Protocol 1

Donors		**Recipients**	
Day –1		Day –1	SMB implant + injection of PG 5ml im
Day 0	SMB implant + injection of PGF (5ml) im	Day 0	
Day 6	6ml of FSH/am	Day 6	
Day 7	5ml of FSH/am	Day 7	
Day 8	4ml of FSH/am	Day 8	Remove SMB implant
Day 9	3ml of FSH/am, also remove implant + inject PGF (5ml i.m.)	Day 9	
Day 10	Oestrus Breed 12 & 24 hours after first observed standing oestrus	Day 10	Oestrus
Day 17	Collect embryos	Day 17	Transfer embryos

PGF = Lutalyse estrumate

Table 2 Synchronization and superovulation of bison cows – Protocol 2

Donors		**Recipients**	
Day –1		Day –1	SMB implant + injection of PGF 5ml im
Day 0	SMB implant + injection of PGF (5ml) im	Day 0	
Day 6	3ml of FSH/am + 3ml of FSH/pm	Day 6	
Day 7	2.5ml of FSH/am + 2.5ml of FSH/pm	Day 7	
Day 8	2ml of FSH/am + 2ml of FSH/pm	Day 8	Remove SMB implant
Day 9	1.5ml of FSH/am + 1.5ml of FSH/pm Also remove implant + inject PGF (5ml i.m.)	Day 9	
Day 10	Oestrus Mate 12 and 24 hours after first observed standing oestrus	Day 10	Oestrus
Day 17	Collect embryos	Day 17	Transfer embryos

PGF = Lutalyse estrumate

In total 12 bison recipients were synchronized. The recipients were synchronized using a Syncro-Mate-B (SMB, Ceva Corp. Overland Par, KS) implant behind the ear. Implants were implanted on day-1 of the donor schedule and removed 9 days later on day 8 of the donor schedule. Oestrus in recipients was detected daily (08:00) at intervals by observation using Ka-mar heat detection pads.

Table 3 Donor program for bison cows superstimulated with PMSG – Protocol 3

Day	A.M.	P.M.
-14	Prostaglandin (500 μg Estrumate)	
-3	Prostaglandin (500 μg Estrumate)	
0	Donor in oestrus	
10	PMSG (3000 IU)	
12	Prostaglandin (500 μg Estrumate)	Prostaglandin (500 μg Estrumate)
14		Donor in oestrus
15	AI of donor	AI of donor
22	Flushing of donor	

Ovulation rate and ovarian volume was determined by rectal palpation on day 7 following the cessation of treatment in all animals. Embryo flushing was also performed on day 7 and recovered embryos were then graded and acceptable embryos frozen or transferred to recipient bison cows.

Embryo flushing and collection commenced 7 days after insemination.

A conventional slow freezing method was used, with the aid of a programmable freezer, to freeze embryos, at a rate of 0.3°C/minute from -6°C to -30°C. From -30°C to -33°C the rate changed to 0.1°C/minute. After the target temperature of -33°C was reached, embryos were transferred directly into a container containing liquid nitrogen (-196°C)[3].

Semen was recovered from live bison bulls (n = 2), using an electro-ejaculator. Semen was collected in 200 ml sachets and then transferred into 5 cc vials, whereafter semen was drawn up in 0.25 cc straws and then placed in a petri dish for microscope evaluation and a viability examination. The collected semen was then stored following chilling, in 0.25 cc straws at 3ºC to 8ºC, for not longer than 24 hours before insemination, if not used immediately to inseminate donor cows. Donors were inseminated with one (0.25 cc) straw of fresh bison semen, following rewarming to 38ºC, 12 and 24 hours after first observed oestrus.

Thawing of embryos within the straw was carried out in a water bath (35°C) at room temperature (24°C). Each straw was air thawed for 10 seconds before being placed in a water bath for 30 seconds[2]. The straw was then dried, cut and the contents expelled into ViGro ™ Holdingplus medium. Once the embryos were recovered, embryos were washed twice in fresh ViGro TM Holdingplus medium and immediately examined morphologically for survivability/re-expanded stage of the blastocyst under a stereo microscope.

Embryos from the donors were collected non-surgically 6 to 6.5 days after the first insemination. Each donor was restrained in a chute especially designed for the bison and specifically for embryo collection. The ovaries were rectally palpated and the superovulatory response (ovarian activity) recorded. Progressive, ovarian activity or development (ovarian volume) was monitored daily (8:00) in both the PMSG and FSH-treated groups by rectal palpation. These observations were performed from 2 days prior to the superstimulation program, until 2 days after the animals had exhibited the induced post-superstimulation oestrus. A subjective method to measure ovarian volume was used. This entailed rectal palpation and estimating the length, width and height (cm) of the ovary, with the aid of a caliper held in the right arm. These measurements of the ovaries were taken on day 7 (day of normal embryo flushing), after the induced or superstimulation oestrus. The number of ovulation points (*corpora lutea*) were also recorded by rectal palpation for each ovary on day 7. Lower epidural anesthesia was achieved by injecting 6 to 7ml of a 2% procaine hydrochloride solution. The cervix dilator was used for subsequent ease in catheterization. A sterile, two-way (18 to 20 French gauge) Foley catheter was introduced into the uterine horn with the aid of a sterile stylet. Each uterine horn was flushed with an average of 500ml of Dulbecco's phosphate buffered saline (DPBS, Sigma, USA, Cat no D6650), supplemented with 9.1% bovine serum albumin (BSA, Fraction V).

The routine bovine embryo recovery procedure (intermittent gravity flow method), was used. The effluent was recovered in a 75μ embryo filter (Em Con, Immuno systems Inc. Biddeford ME). The final 40 to 50ml effluent was decanted into a 100x50mm flat bottom grid dish and the filter thoroughly rinsed (fluid), using a 24 French gauge needle and syringe.

Embryos were located using a stereomicroscope (x 25). The recovered embryos were transferred to a small petri dish (35 x 10mm) containing holding medium. The holding medium was the same as the flushing medium, except that the concentration of BSA was 0.4% in the holding medium. Embryos were kept in the holding medium for 4 to 9 hours. The holding medium was replenished every 2 hours.

The final evaluation or assessment of embryo quality was made under a stereo microscope at high magnification (x 75). Based on the morphological appearance, the embryos were graded and allocated to specific categories[7].

RESULTS

The superovulatory response, in terms of number of *corpora lutea*, anovulatory follicles, the total number of embryos recovered and the number of transferable embryos produced, is set out in Table 4 and Figure 1.

Table 4 The ovarian response and embryo recovery rate following superovulation and flushing in the bison

Protocol	No of donors	No of CL (Mean ± SE)		Follicles > 10mm (mean ± SE)		Embryos/ova recovered (mean ± SE)		Embryos transferable (mean ± SE)	
I	2	5	(2.5 ± 0.7)	1	(0.5 ± 0)	4	(2 ± 0)	1	(0.5 ± 0)
II	2	18	(9 ± 1.4)	7	(3.5 ± 0.7)	14	(7.0 ± 0.0)	7	(3.5 ± 1.0)
III	2	7	(3.5 ± 0.7)	2	(1.0 ± 0)	5	(2.5 ± 0)	1	(0.5 ± 0)
TOTAL	**6**	**30**	**-**	**10**	**-**	**23**	**-**	**9**	

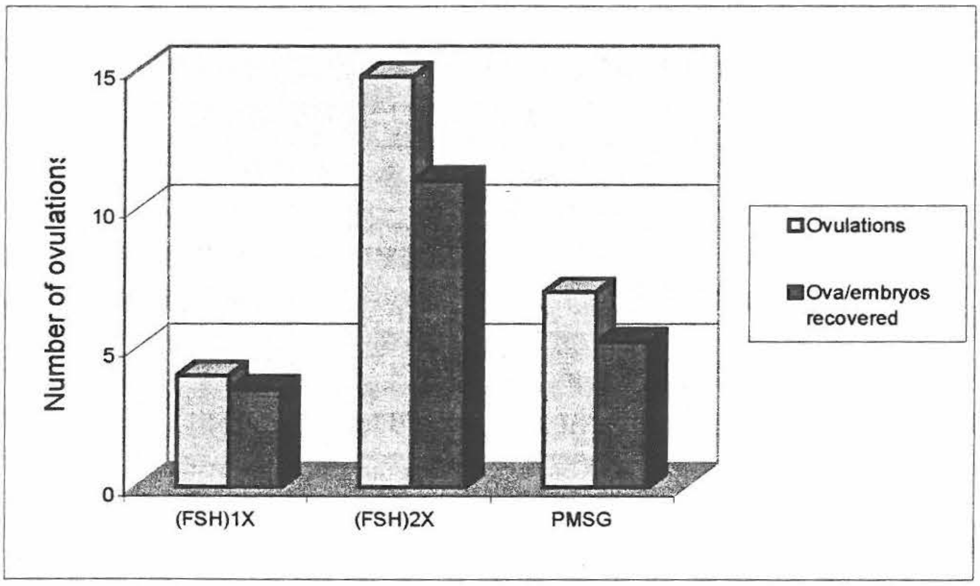

Figure 1 Ovarian response using a daily (1X); twice daily (2X) FSHp or PMSG treatment in bison cows

The mean (± SE) ovarian response was 2.5 ± 0.7; 9.0 ± 1.4 and 3.5 ± 0.7 for Protocol 1, 2 and 3 respectively (Table 4 and Figure 1). With the corresponding mean number of embryos/ova recovered per donor for the respective groups being 2, 7 and 2.5. The total increase in ovarian volume (indicator of increased ovarian activity) was 353.2% for Protocol 1 (FSH 1X), while Protocol 2 (FSH 2X) induced a 421.8% increase and Protocol 3 (PMSG) a 767% increase in ovarian volume (Table 5 and 6). The overall mean embryo recovery rate was 3.8 ± 4.2 per donor. Of the 23 embryos and ova recovered, 3 were retarded and 11 unfertilized. The remaining 9 viable embryos were further processed and 4 were transferred as fresh embryos and 5 embryos frozen. The FSH (2X) group (Protocol 2) produced 61% of the total number of embryos recovered. The occurrence of unfertilized ova was

25%, 42% and 40% for Protocol 1, 2 and 3 respectively (Figure 2). For the recipients, all 12 bison cows exhibited oestrus. SMB treatment recorded a higher oestrus response and more compact synchrony in the recipients compared to the donors. Of the 4 frozen embryos and fresh embryos transferred 2 and 3 confirmed pregnancies were recorded respectively.

Table 5 **The mean ovarian size of bison cows prior to superstimulation with PMSG, FSH (2X) or FSH (1X)**

	FSH (1X)		FSH (2X)		PMSG	
	Mean	**± SE**	**Mean**	**± SE**	**Mean**	**± SE**
Volume prior to superstimulation:						
Left ovary (cm^3)	20.4	0.3	18.4	0.2	19.1	0.2
Right ovary (cm^3)	18.3	0.2	16.1	0.2	17.5	0.3
Total volume (cm^3)	19.3	0.3	17.3	0.3	18.3	0.4

Table 6 **The mean ovarian size on the day of embryo collection (day 7), following superstimulation with PMSG, FSH (2X) and FSH (1X) in bison cows**

		FSH (1X) (n = 1)		FSH (2X) (n = 2)		PMSG (n = 2)	
		Mean	**± SE**	**Mean**	**± SE**	**Mean**	**± SE**
Length:	Left ovary (cm)	4.9	0.3	5.1	0.2	6.2	0.3
	Right ovary (cm)	5.5	0.3	5.8	0.2	7.0	0.3
Width:	Left ovary (cm)	4.2	0.2	3.8	0.2	4.3	0.2
	Right ovary (cm)	4.0	0.2	4.1	0.2	5.1	0.2
Height:	Left ovary (cm)	3.0	0.2	3.2	0.3	4.1	0.2
	Right ovary (cm)	3.4	0.2	3.5	0.2	4.8	0.2
Volume:	Left ovary (cm^3)	61.7	7.4	62.2	8.4	109.3	12.1
	Right ovary (cm^3)	74.8	13.2	83.2	15.0	171.4	17.3
Total volume (cm^3)		68.3	17.8	72.8	18.2	140.4	24.1
Increase in volume (%):							
	Left ovary	302.6		338.1		571.7	
	Right ovary	409.8		517.0		980.3	
	Total volume	353.2		421.8		767.0	

No ovulation was detected in one of the donor bison cows in the Protocol 1 (FSH 1X) treatment. When assessing the response in FSH-treated bison in terms of embryo quality and quantity, it was evident that the FSH (1X)-treated group had a lower ovulation rate, a lower embryo recovery rate and fewer viable embryos than the FSH (2X) group.

DISCUSSION

From the trial undertaken to evaluate the development of a method for multi-ovulation and embryo transfer (MOET) in the bison (Bison bison) and the effect of the different gonadotrophic hormones on progressive ovarian stimulation following superstimulation response, it is evident that ovarian response increases considerably following the administration of the exogenous gonadotrophins, PMSG and FSH.

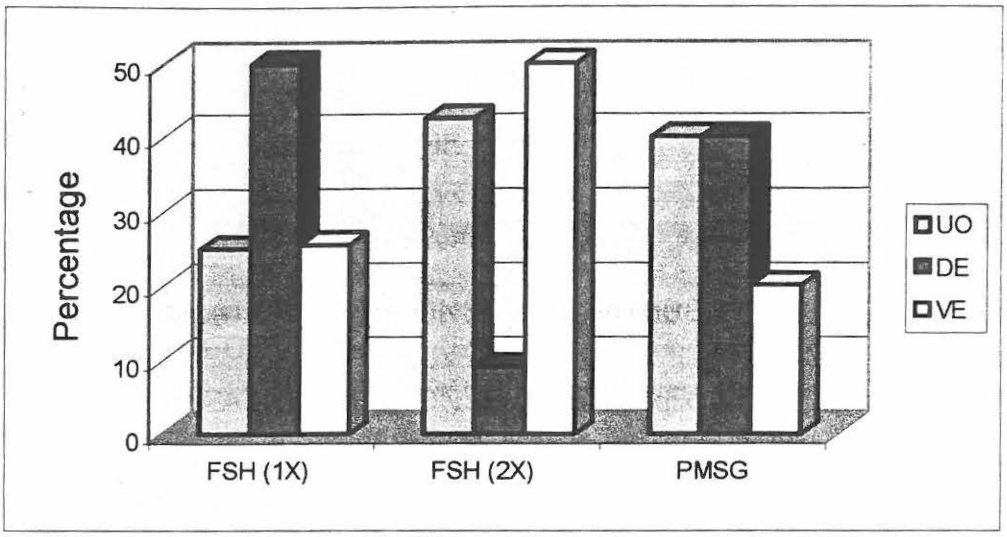

Figure 2 **The percentage viable embryos (VE), retarded (degenerated) embryos (DE) and unfertilized oocytes (UO) in FSH (1X)-treated, FSH (2X)-treated and PMSG-treated bison cows**

When assessing the effect of PMSG and FSH on embryo quality and quantity following superstimulation, some of the pertinent observations were that the FSH-treated bison cows had a higher ovulation rate, a higher embryo recovery rate and more viable embryos, compared to that obtained following PMSG-treatment. The technique of flushing as such was also highly satisfactorily, with a high flushing medium recovery rate (mean 70%) (Figure 3). In all three trials it was attempted to minimize variation regarding age, location, body condition, nutrition and handling of these wild animals. However, considerable variation still existed in the superstimulation response (in terms of the number of ovulations and number and quality of embryos recovered).

It can further be deduced from the results obtained, that individual variation and unpredictable superstimulation is the result of the fundamental physiological hormonal responses, which regulate the number of developing follicles and the subsequent ovulation rate. From the results obtained, it would seem that although more labour intensive, time consuming and more expensive, the use of FSH as a superstimulatory agent, is justified in the bison. More research however, is warranted in the administration program of the superovulatory agent (time, dose, route, etc.) for a better and more reliable ovarian response.

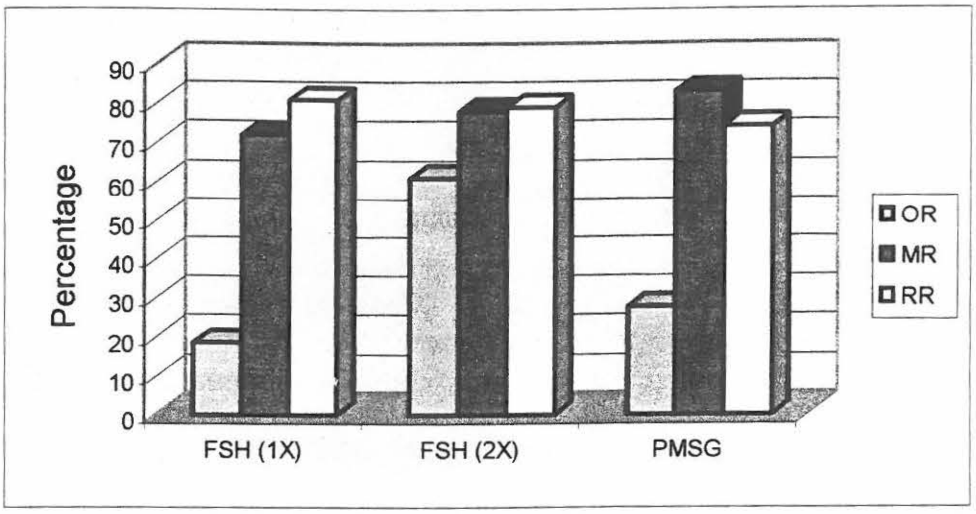

Figure 3 **The oestrous response (OR), the percentage flushing medium recovered (MR) and the embryo recovery rate (RR) in the FSH (1X), FSH (2X) and PMSG-treated animals following superstimulation**

From responses obtained following the use of PMSG in the superstimulation of bison, it can also be assumed that the response of bison to PMSG is less than that for FSH treatment. Certain bison (if not all), could be more susceptible to stress and endocrine imbalances cause by PMSG stimulation, due to its long half-life and high LH ratio. However, individual animal variation remains an over-riding factor. Caution should be taken when interpreting these results due to the limited number of animals. The single dose of PMSG and its overwhelming effect on the ovary could also have a negative effect on the ovarian response[5, 8]. Thus PMSG stimulation needs to be investigated if it is to be used in this type of program. Individual variation remains an over-riding factor and along with the stress factor complicates the interpretation of the results obtained. Further research is needed to optimize the use of MOET in the bison.

The bison is unique in its response to reproductive technologies, which are commonplace in domestic cattle. Unlike other exotic wild ruminants, the bison do respond to protocols used in domestic cattle, when administered under acceptable conditions. The problems associated with the use of these reproductive technologies in bison are not the technologies themselves, but the handling and management of the animals. While bison are reproductively similar to cattle, behaviorally they are very different. Most bison are not amenable to domestication and are managed as wild animals. The bison therefore, responds accordingly, and presents a very real and dangerous problem when being handled. The danger is not limited to injury of the person, but also to the animal itself. Bison ranchers are aware of normal handling losses and are therefore reluctant to adopt procedures requiring multiple or time consuming handling.

The application of new reproductive technologies to the North American bison and the European bison, will continue to play a major role in the growth of their associated industries. Conservation and commercialization have brought this specie back from the verge of extinction and its impact is growing beyond all expectations. By exploiting the use of reproductive technologies in bison, it can possibly also be applied to other wild species, with possible similar results. As the bison industry grows, a new market will be created for embryo transfer technology and its expansion may be even beyond the domestic cow market. The amount of published work with the bison is very limited, but the successes obtained thus far, show promise.

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