Superfetation in beef cattle

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SUPERFETATION IN BEEF CATTLE

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

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by

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ABSTRACT

Although superfetation has been reported in cattle and other species, there is considerable skepticism as to its existence due to the lack of clear evidence in these reports. Other explanations such as embryonic diapause or differential growth of twins have been suggested as more accurate descriptions of the cases reported as superfetation. The hypothesis of this study is that if a viable pregnancy can ensure maternal recognition in cattle, an asynchronous embryo can develop in a more chronologically advanced uterine environment. The objective was to produce superfetation by (1) ovulation induction and artificial insemination (AI) of pregnant cows and (2) transfer of 7-day embryos to cows with more advanced pregnancies. An attempt to produce superfetation by induction of ovulation in mated cattle with human chorionic gonadotropin (hCG) followed by AI was unsuccessful. Ovulation was induced in pregnant cows on day 7 but could not be induced >23 days of pregnancy. Subsequently, it was shown that uterine stage bovine embryos (7-day) could develop when transferred to recipients on day 14 of pregnancy, but not on day 28, day 60, or day 90 of pregnancy. Twins (7 days asynchronous) were produced in a recipient that received two 7-day embryos 14-days post-estrus. Although the younger twin was a heifer and her co-twin was a bull, the heifer was not a freemartin. FSH and hCG treatment 7 days prior to embryo transfer (ET) did not increase the rate of superfetation in 14-day or 25-day mated recipients. The nonsurgical ET technique may have caused pregnancy loss in recipients receiving embryos >25 days post-estrus. Two sets of asynchronous twins were produced by transfer of 7-day embryos to 14-day pregnant recipients. An additional experiment was undertaken to determine if asynchronous
embryos could develop following maternal recognition in pregnant cows yet prior to invasion of the contralateral uterine horn by the primary conceptus. Asynchronous twins were produced following transfer of 7-day embryos to a 13-day pregnant recipient but not in 19-day pregnant recipients. Asynchronous twin pregnancies (superfetation) were therefore consistently produced in this series of experiments by transfer of embryos to recipients up to 16 days of pregnancy.
INTRODUCTION

Superfetation is a reproductive phenomenon which is said to occur when an animal shows signs of estrus and is mated during pregnancy so that a secondary pregnancy occurs in addition to the previous one (Long, 2001). Therefore, at least two fetuses resulting from different ovulation cycles and conception times would be present in the uterus at the same time (Hurnik et al., 1995). There are numerous reports in several animals, such as the mouse (Littleford and Gysin, 1944; Barnett and Munro, 1970), the rat (Slonaker, 1934), the rabbit (Pickard, 1928), the cat (Jepson, 1883; Hoogeweg and Folker, 1970), the sheep (Smith, 1927; Scanlon, 1960), the pig (Smith, 1927; Larivée, 1972), the buffalo (Rao et al., 1987), the burro (Short, 1964), the horse (Mumford, cited by Leroy and Pechdo, 1950), the monkey (Leakey, 1969) and man (Scrimgeour and Baker, 1974). Superfetation in cattle has been described in cows with calving intervals of 64 to 92 days, or following abortion of twins of apparently different ages (Dalrymple and Jenkins, 1951; Simmons, 1960; Gee, 1971; Hall, 1987).

Reports of superfetation have been met with a great amount of skepticism. Other explanations have been offered, such as embryonic diapause (Vandeplaschche, 1969) and differentiated growth of twin fetuses (Kuntz, 1920). However, although embryonic diapause has been reported in over 70 eutherian and 30 marsupial species (Renfree and Shaw, 2000), only one ungulate species exhibits this phenomenon: the Roe deer (Capreolus capreolus), first observed in 1651 by William Harvey (cited by Eckstein, 1959).

Growth differences between twins for various reasons could explain some of the superfetation cases reported. With the advent of ultrasound technology differentiated
growth of twin fetuses has been well documented in humans (Kol et al., 1993; Weissman et al., 1993). In most cases, growth difference between twins is usually associated with congenital malformations of the smaller twin, which seldom survives until term (Kol et al., 1993).

In order for superfetation to occur in nature, the female must show signs of estrus and be mated during pregnancy. This phenomenon has been widely reported in cattle (Bullard, 1934; Donald, 1943; Branton, 1949; Erb and Morrison, 1958). Williams (1921) estimated that 1 to 2% of all pregnant cows show signs of estrus during pregnancy.

Ovulation, along with other conditions necessary for sperm and ovum transport, would also have to occur in pregnant animals for superfetation to be possible. Follicular waves have been found to continue throughout pregnancy in cattle (Ginther et al., 1989; Bergfelt et al., 1991; Taylor and Rajamahendran, 1991; Ginther et al., 1996), although no cases of ovulation during pregnancy in cattle were found in the scientific literature.

However, follicles present during pregnancy in cattle can be induced to ovulate at different stages of pregnancy using various ovulation inducing hormones, such as human chorionic gonadotrophin (hCG) or luteinizing hormone (LH). Rajamahendran and Sianangama (1992) found hCG to be very effective in causing ovulation in dairy heifers when given on day 7 of pregnancy. Lulai et al. (1994) induced ovulation using exogenous LH after day 35 of pregnancy in cows in which the CL of pregnancy had been regressed and pregnancy was being maintained with norgestomet implants. In a similar study, Bridges et al. (2000) regressed the CL of pregnancy and maintained pregnancy with exogenous progestins in a group of cows prior to day 36 of pregnancy, then treated
the cows with hCG alone or in conjunction with an FSH pretreatment. Ovulation occurred in 59% of these cows.

Bovine oocytes from follicles present during pregnancy have the capability of being fertilized and becoming viable embryos. Embryo development in vitro from oocytes recovered from pregnant cows is similar to that of oocytes from nonpregnant cows (Behboodi et al., 1992; Ryan et al., 1993). Meintjes et al. (1995a) used oocytes recovered from FSH-stimulated pregnant, live cows using an ultrasound-guided technique to produce embryos in vitro at LSU that resulted in 2 pregnancies, resulting in the birth of a normal, healthy calf.

A major obstacle to the concept of superfetation is that close synchrony between embryonic development and uterine environment has been shown to be important to the establishment of pregnancy from the first experiments with embryo transfer by Sir Walter Heape (1890). This concept holds true for all species studied, such as the rat (Nicholas, 1933), the rabbit (Chang, 1950), the sheep (Moore and Shelton, 1964; Rowson and Moor, 1966) and cattle (Rowson et al., 1972). In all of these species, pregnancy rates declined as embryo-uterus synchrony decreased.

The reason for this need for synchrony is now known to be due to a process called maternal recognition. In cattle, embryo-maternal synchrony is important to ensure a complex series of events that prevents lysis of the CL from occurring with subsequent termination of pregnancy. The bovine embryo produces a protein known as bovine interferon-τ (bIFN-τ) around day 16 of the estrous cycle to prevent luteolysis and subsequent termination of pregnancy (Thatcher et al., 1986). If bIFN-τ production is
temporally altered, as in the case of asynchrony between uterine and embryonic
development, luteolysis occurs followed by embryonic death.

Some degree of chronological asynchrony is acceptable for the establishment of
pregnancy. Rowson et al. (1972) reported that embryo transfer to recipients that were in
estrus 72 hours after the donors resulted in a 20% pregnancy rate. Ashworth and Bazer
(1989) showed that day 6 ovine embryos placed in recipients on day 4 of their cycle
caused a shift in the secretory protein profile of the uterus to that of a more advanced
uterine environment, presumably allowing maternal recognition to occur.

Embryonic development is also affected by the degree of asynchrony between
embryo and uterine environment. Lawson et al. (1983) showed that ovine embryos
transferred to less chronologically advanced uterine environments showed retarded
growth, while embryos transferred to more advanced uterine environments showed
accelerated growth. This phenomenon also occurs in cattle (Albihn et al., 1991). This
would imply that an embryo resulting from ovulation and fertilization during pregnancy
would be exposed to a more chronologically advanced uterine environment and would
theoretically accelerate development in an attempt to reach the same stage as the uterus
and would eventually degenerate.

Exposure to elevated progesterone levels can cause acceleration of embryonic
development. Garrett et al. (1988) found that supplemental progesterone on days 1 to 4
of pregnancy in cows resulted in accelerated embryo development compared with
controls. This would pose a threat to the successful embryo development after breeding
during pregnancy, when progesterone levels are elevated. However, pregnancy rates in
that study were similar at 40 days of gestation (Garrett et al., 1988).
Although maternal recognition appears to be localized to the uterine horn ipsilateral to the CL, as indicated by increased blood flow to the ipsilateral uterine horn on days 16 to 17 of the estrous cycle (Ford et al., 1979), bovine embryos can develop in the uterine horn contralateral to the CL when a viable fetus is present in the uterine horn ipsilateral to the CL. Scanlon (1972) reported two cases in cows with dizygotic twin pregnancies in opposite horns although both ovulations occurred on the same ovary and one case in which monozygotic twins developed in opposite horns.

The production of bIFN-τ begins with the process of embryonic elongation (Geisert et al., 1992) and the developing bovine embryo may occupy two thirds of the uterine horn ipsilateral to the CL by days 17 to 18 post-estrus. Further growth into the contralateral uterine horn occurs by days 20 to 24 (Chang, 1952), so it would be difficult for an embryo developing in the contralateral horn to expand sufficiently to signal the CL without the aid of an ipsilateral pregnancy. It must be noted that these studies were undertaken with embryos developing in the uterine horn ipsilateral to the CL, so the rate of development of embryos in the contralateral uterine horn alone may not be the same.

Embryo transfer has also been used extensively to induce twins in opposite horns. Sreenan and Diskin (1989) found no difference between 50-day pregnancy rates, twin pregnancies, or sets of twins born between unilateral or bilateral transfer of 2 embryos by surgical or nonsurgical means. Transfer of embryos into the uterine horn contralateral to the CL of mated recipients has also been used to produce twins in cattle (Testart et al., 1975; Sreenan and McDonagh, 1979; Renard et al., 1979; Heyman et al., 1980; Sreenan et al., 1981; Sreenan and Diskin, 1988, 1989; McEvoy et al., 1995).
Superfetation has been induced artificially in a few cases. Hafez and Pincus (1956) used asynchronous embryo transfer in rabbits to produce superfetation by transferring 3.5-day embryos to the left horn of recipient does at different intervals following mating. The left oviduct of each recipient was removed surgically and decidual formation was induced by mechanically stimulating the left uterine horn with a sterile probe. Embryos implanted successfully when transferred up to 6 days following mating (2.5 days asynchronous). Parturition occurred at the same time for synchronous and asynchronous young in recipient does allowed to complete the pregnancy.

Johansson and Venge (1951) induced superfetation in the mink by mating different males to females at intervals of 8 to 19 days. This was possible because mink are induced ovulators and exhibit delayed implantation (Hansson, 1947; Mead 1993). Gestation length in the mink normally varies according to the time of mating, so blastocysts resulting from earlier matings implanted at the same time as blastocysts resulting from the latter matings.

Superfetation may have resulted following ovulation induction in a woman (Bsat and Seoud, 1987). The patient was treated with clomiphene citrate (CC) for induction of ovulation after an initial CC treatment had apparently failed. Ultrasonography at 25 weeks after the first CC treatment showed twins, one 25 weeks-of-age and the other appeared to be 22 weeks-of-age, corresponding to the second round of treatment. The twins were born at 38 weeks from the first round of CC treatment. One twin was normal, while the other showed signs of prematurity and had characteristics indicative of less than 36 weeks intrauterine gestation.
Wislocki and Snyder (1931) used anterior pituitary lobe extract to induce ovulation in a pregnant rabbit doe 44 hours following natural mating. Four hours later, they artificially inseminated the doe with fresh spermatozoa. The doe was sacrificed 29 hours later and normal developing blastocysts were recovered from the uterus, as well as 3- to 5-cell embryos from the oviducts. Similarly, Edwards and Fowler (1958) used eCG for follicular stimulation and hCG for ovulation induction 2.5 to 3.5 days apart in mice. Females necropsied 18 hours following the second ovulation had both morulae and/or blastocysts and recently ovulated ova. One female in which pregnancy was maintained delivered pups from both matings.

In summary, we believe that superfetation in cattle is possible for the following reasons: (1) pregnant cows show signs of estrus and may be mated during pregnancy, (2) follicles continue to develop throughout pregnancy (3) and oocytes from these follicles have been shown to produce embryos *in vitro* after IVF. Obstacles to this concept are (1) embryos placed in asynchronous uterine environments develop at retarded or advanced rates, eventually degenerating, (2) high progesterone concentrations during pregnancy may block ovulation and/or affect gamete transport in the female reproductive tract (3) and physical barriers resulting from growth of the more advanced fetal-placental unit.

Therefore, the following model for superfetation in beef cattle is proposed: (1) at some point after a successful pregnancy has been established, (2) progesterone levels decrease to a level which allows a dominant follicle to grow on the ovary contralateral to the ongoing pregnancy, increasing estradiol levels enough to (a) allow for behavioral estrus and subsequent mating to ensue and (b) induce a luteinizing hormone (LH) peak and subsequent ovulation of the dominant follicle, (3) then progesterone levels remain
near pregnancy baseline levels for a short period of time, allowing for successful gamete transport and fertilization to occur and finally, (4) implantation occurs in the uterine horn contralateral to the fetus.

In a series of experiments, portions of this model for superfetation will be addressed. The hypothesis is that once an ongoing viable bovine embryo has attained maternal recognition, ensuring maintenance of the CL, a second, asynchronous embryo can establish a secondary pregnancy that will develop concomitantly with the primary conceptus. The objective of this experiment is to attempt to induce superfetation by (1) inducing ovulation and mating in pregnant cattle and (2) placing 7-day bovine embryos in the uterine horn contralateral to the ongoing pregnancy of mated recipient cows at more advanced stages of pregnancy.
CHAPTER I
LITERATURE REVIEW

SUPERFETATION

Superfetation may occur when a pregnant female is mated during pregnancy so that two or more embryos resulting from different ovulation cycles and conception times are present in the uterus at the same time (Hurnik, 1995; Long, 2001). The concept of superfetation has been around for a very long time. Aristotle (384 to 322 BC) spoke at length on the topic (de Generatione Animalium, translation by Arthur Platte, 1910), specifically mentioning hares and humans as examples of species in which superfetation was believed to occur. Reports on superfetation are frequently viewed by many with skepticism due to the rarity of the phenomenon and the difficulty in ascertaining that the presumed case is actually a true case of superfetation that could not be explained by other physiological phenomena.

Superfetation is different from twinning, in which two or more offspring of the same or different genotypes produced during a single ovulation cycle are present in the uterus during a same gestation. Fraternal twinning occurs when a monotococcus female naturally ovulates two or more ova at estrus and is mated by one male so that two or more embryos resulting from the same male are present in the reproductive tract. Identical twins occur following the division of a single embryo after fertilization resulting in two embryos with identical genotypes. Superfetation must also not be confused with superfecundation, which occurs when a female ovulates two or more ova during the course of the same estrous period, with the ova being fertilized by sperm from two or more males (Long, 2001).
The difficulty of validating reports on superfetation begins with the methods by which superfetation is often measured, such as consecutive parturitions occurring at intervals considered shorter than normal, or recovery of fetuses of apparently different ages from the same female (Edward and Fowler, 1958). Due to the nature of some of the reports, it is difficult to determine which cases are well founded and which are simply erroneous observations. For example, Simmons (1960) reports that several cases of superfetation in dairy cows were recorded in England’s National Milk Recording organization, but present no actual cases. Another report by Brough (1964) claims that a heifer calved in January 1963, again in September 1963 (8 month calving interval) and then in March 1964 (6 month calving interval). Nonetheless, some reports seem acceptable as true cases of superfetation.

**Superfetation in Mice**

Superfetation has been reported in mice. Sumner (1916) published several cases in mice with abnormal intervals between subsequent litters. At least one case could possibly be due to superfetation, as the interval between births of litters was 13 days (normal gestation in a mouse is 21 days).

Watt (1931) reported a case in which a mouse with dystocia was observed to have a copulatory plug that was blocking parturition. Nine young were recovered, of which only two survived. Also, 11 unfertilized ova were recovered from the oviducts and freshly formed corpora lutea (CL) were present on the ovaries.

Littleford and Gysin (1944) describe five possible cases of superfetation, all with shorter than normal parturition intervals between litters (7, 12, 13, 16 and 16 days). In three of the cases, the mice raised pups from both litters to weaning. In the other two
cases, only pups from the second litters survived to weaning. In one case, a mouse mated 15 days into pregnancy and produced two separate litters 7 days apart, the first gestation lasting 27 days and the second gestation lasting 21 days, with a 12 day overlap between groups of embryos.

While studying *in vitro* culture of fertilized ova, Rollhauser (1949) euthanized a mouse 24 hours after apparent mating. At necropsy, five embryos were discovered in the oviducts along with seven implantation sites in the uterine horns. Seven distinct CL and four fresh ovulation points were present on the ovaries. The interval between the successive ovulations was estimated to be 7 days.

Barnett and Munro (1970) describe several cases in their breeding colony in which parturition intervals between litters were less than 16 days. Recording errors were initially blamed for these abnormal gestations and care was taken to ensure more rigorous mating of the mice and proper recording of data. However, six cases of parturition intervals of less than 16 days between litters still occurred. Interestingly, some births occurred ~21 days following a previous parturition in which no male had been present with the females since just before the birth of the previous litter. Mating therefore seemed to have occurred just prior to parturition.

Ullman (1976) studied this same set of experimental mice and confirmed the findings of Barnett and Munro (1970). Males were removed from females as soon as the copulatory plug was detected, indicating mating and therefore ovulation and 22 of 304 (7\%) and 30 of 1,149 (3\%) litters with abnormal intervals were produced in outbred and inbred mice, respectively. Parturition intervals between the first and second (“fatherless”) litters ranged from 16 to 34 days. The oviducts of over 200 females from
days 10 to 15 of the first gestation were examined and no free-floating blastocysts were found, as would be expected with a lactational delay of implantation. However, sperm were found in plentiful numbers *in utero* in two mice 8 and 15 days following copulation and male removal in a nonpregnant and pregnant mouse, respectively, suggesting some type of delayed fertilization could be responsible for this phenomenon.

Leonard and Linden (1972) reported similar cases in mice in which males had been removed at least 5 days prior to parturition and second litters were produced 21 to 29 days following parturition. Previous recorded matings were 45 to 58 days prior to the second parturition. It was noted that the phenomenon could be due to delayed implantation of blastocysts resulting from the previous mating, due to the low number of pups in the secondary litters (one to six pups). Interestingly, one female showing this phenomenon of “fatherless” litters was an offspring of one of the others (three mice reported showing four cases of anomalous litters).

**Superfetation in Rats**

Slonaker (1934) presents two cases of possible superfetation in rats. In the first case, a female rat gave birth to five young and killed them within 3 days of parturition. The rat gave birth to a second litter of six pups 15 days after the first parturition (normal gestation length is 22 days) which survived. The second case occurred in a rat that gave birth to nine young, with five pups surviving. Approximately 14 days after the first parturition, a second litter of three pups was born which did not survive.

**Superfetation in Rabbits and Hares**

Superfetation may occur frequently in rabbits and hares. Aristotle cites hares as a species in which superfetation occurs (*de Generatione Animalium*, translation by A.)
Platte, 1910; and Historia Animalium, translation by D.W. Thompson, 1910). Bloch et al. (cited in Stavy and Terkel, 1992) observed two 4-cell embryos in the oviducts and a fully developed fetus in the uterus of a pregnant hare. Pickard (1928) reported a case in which a rabbit doe was seen mating soon after arriving at a new owner’s home. However, 8 days after mating, the doe gave birth to a litter of six young. More importantly, the doe produced a second litter of six kits 23 days after the first litter and 31 days after mating (gestation length is 32 days). Both litters were normal at birth.

Mayer and Klein (1946) were able to induce mating on 12 days post-coitus in rabbits by administering estrogen. Successful implantation of the embryos from the induced mating had occurred 9 days later; however, upon examination, the fetuses from the first gestation had died and were in the process of resorption.

Parturition intervals are commonly shorter than normal in the hare (Lepus europaeus) and mating can occur up to 10 days prior to parturition, though it occurs more commonly 2 to 5 days prior to parturition (Caillol and Martinet, 1976). However, no cases of shorter than normal gestation lengths were reported in this study. In two hares studied, progesterone was still elevated at the time of mating, although progesterone levels fell before the first parturition. Basal progesterone levels at estrus were below 5 ng/ml, yet in the same two does, the progesterone levels at mating (just prior to the first parturition) were 15 ng/ml and 65 ng/ml. However, estrus just prior to parturition seemed to coincide with the beginning of the drop in progesterone levels.

Martinet (1980) observed mating in hares throughout gestation, but more commonly 3 to 4 days prior to parturition. Induction of ovulation by mating in five does at 35 and 36 days of gestation (but not on days 14, 23, or 26) was reported,
although no information on subsequent gestation lengths was presented. However, in one doe induced to ovulate by mating on day 35, parturition occurred on day 38. It is possible, then, that mating prior to parturition may hasten parturition, or may be a signal that parturition is about to occur. Caillol et al. (1991) recorded several cases of mating in hares prior to parturition, with a subsequent interval between parturitions shorter than the normal gestation length of the rabbits. Mating just prior to parturition caused an LH peak, but only when mating occurred after day 34 of the first gestation.

Martinet and Raynaud (1975) cite two cases in which males were isolated from females after copulation. Vasectomized males were introduced 5 days prior to parturition and mating was observed 3 days prior to parturition. Laparotomy performed following parturition revealed cleaved ova resulting from recent ovulations. Therefore, unless the vasectomized males still had viable sperm, it is plausible that sperm was stored from the first series of copulations and fertilized the ova following ovulation induced by mating with the vasectomized males. Interestingly, sperm was found to be present in the uterine glands at the utero-tubal junction at 48 hours, 10 days and 17 days following artificial insemination (AI). Sperm was also present in the uterus at 48 hours, but not at 10 and 17 days following AI. Although sperm fertilizing ability was not, sperm storage seems to be a reality in hares and could explain some cases of apparent superfetation. However, as the previous cases report, mating just prior to parturition is the most probable cause of superfetation in hares and rabbits.

Superfetation in Cats

Superfetation has also been found in other animals, such as cats. As in other species, the reports are usually cases observed after the fact, so no information is
available on mating intervals. Jepson (1883) reported a case in which a queen produced normal kittens along with a live, immature fetus. Harman (1917) describes a case encountered during a routine dissection, in which four fetuses were recovered from a queen. Three were larger, measuring 90 mm in length and showed the characteristics of 6- to 7-week-old fetuses. The other fetus was smaller (10 mm), with the physical characteristics of a 3-week-old fetus. Hunt (1919) describes a case in which a cat induced to abort with chlorinated gas produced two fetuses, one measuring 105 mm and the other measuring 14 mm in length. The larger fetus appeared to be normal and almost full term, while the other fetus was estimated at 2 weeks of gestational age.

Markee and Hinsey (1935) reported the birth of four kittens, with the birth of two in a first litter and with two more being born 13 days later. Upon examination of the uterus, it was determined that the left horn was more enlarged, with two implantation sites, while the right horn seemed to be involuting, so it appeared that the two sets had developed in opposite uterine horns. Kawata and Tiba (1961) reported assisting in the birth of two kittens, one normal and one dead, as well as two normal, yet immature fetuses measuring 15 mm in length. Hoogeweg and Folkers (1970) reported a case in which two normal kittens and one much smaller (115 mm vs. 33 mm in length) fetus were removed by Caesarean section from a cat. The smaller fetus had characteristics of a 5-week-old fetus, with no apparent signs of deformity or degeneration. The smaller fetus was also alive at the time of delivery.

**Superfetation in Sheep**

Smith (1927) reports several cases of superfetation in sheep, with two being more credible than the others. In one case, a ewe gave birth to two lambs of
different breeds 6 weeks apart. The second case involved a ewe, which was exposed to sires of different breeds at different times. She subsequently gave birth to twins with markings of the first sire and 38 days later, to triplets with markings of the second sire. Scanlon (1960) describes a case in which a Suffolk ewe (white with a dark face and limbs) had been placed first with a Suffolk ram and 14 days later, a Finnish Landrace ram (white-faced) was placed with the group after the first ram had been removed. She gave birth to twin lambs with Suffolk markings and 15 days later, to twin lambs with white faces with black markings, seemingly sired by the Finnish Landrace ram. Matter (1965) reported the birth of two normal lambs to a same ewe at an interval of 36 days. The ewe had been mated three times by the same ram over a period of 32 days.

**Superfetation in Goats**

There is one report of superfetation in a goat (Kroon, quoted by De Bruin in Williams, 1909). A doe was mated twice, with an interval of 52 days between matings. The doe gave birth to triplets 5 months after the first breeding, two live and one dead. The next day, three less developed fetuses were expelled.

**Superfetation in Pigs**

There are several instances of superfetation recorded in pigs. Tapken (quoted by DeBruin, in Williams, 1909) described a case that occurred in 1890 in which a sow was mated twice 17 days apart. The sow gave birth to seven piglets 120 days after the first mating and 14 days later, the sow gave birth to 12 more piglets (nine live and three dead). Smith (1927) reports two cases of superfetation. In the first case, a sow gave birth to two litters 6 weeks apart, with six pigs in the first litter and five piglets in the second litter. Only one mating had been observed, but a boar was close by and could
have mated the sow unobserved. In a second case, a sow mated twice to the same boar at an interval of 21 to 25 days, gave birth to two litters of five and seven piglets, respectively, 24 days apart. Swinehart (1939) reported a case of superfetation in a Chester White sow that was mated twice 25 days apart. A litter consisting of eight piglets was born 114 days after the first mating and a second litter consisting of nine piglets was born 114 days after the second mating.

Schütze (1940) reports a case of superfetation with an interval between matings of 50 days and of birth of 51 days, with nine pigs in the first litter and seven pigs in the second litter. Only three piglets survived from the first litter due to lack of milk and four piglets from the second litter survived in addition to the three from the first litter. Grabherr (1948) describes a case of probable superfetation where two litters were produced 37 days apart, with 14 piglets born in the first litter and eight piglets born in the second litter to the same sow. In this case, the interval between the matings was 35 days.

In another report of superfetation in pigs, Klaas (1950) describes a case with an interval between matings of 58 days and between litters of 68 days, with four piglets in the first litter and 14 piglets in the second litter. Gestation lengths were 104 and 114 days, respectively. The piglets from the first litter were weaned and bottle-fed due to insufficient lactation by the sow. Interestingly, the same sow showed signs of estrus and was mated 7 days prior to her second parturition. Larivée (1972) reports a case where a sow was mated twice at an interval of 16 days to two boars of different breeds, giving birth to two litters 14 days apart. Piglets in the different litters showed breed characteristics corresponding to the respective sire breeds.
Superfetation in Monkeys

There has been one report of an apparent superfetation case in the monkey. Leakey (1969) describes a case where a female Patas monkey (*Erythrocebus patas*) gave birth to stillborn infants 3 months apart. In this report, both infants seemed to be full term. No information was available as to matings, but the female was in the presence of a male for several months.

Superfetation in Humans

Aristotle cites humans as a species in which superfetation occurs (*de Generatione Animalium*, translation by A. Platte, 1910) and reports two cases (*Historia Animalium*, translation by D.W. Thompson, 1910). In the first case, a woman gave birth to normal, full-term twins, as well as a single 5-month-old fetus. In another case, a woman gave birth to full-term twins along with a 7-month-old premature baby, who subsequently died.

More recently, several cases have also been reported. Some are difficult to believe, while others present convincing data. In the first category, Murless and McLaughlin (1937) report the case of a woman with a history of stillbirths (two singletons and three sets of twins) and miscarriages. She apparently finally gave birth although there was some doubt as to the actual occurrence of this birth as only her husband was present at the time of the supposed birth and lactation did not occur. The woman then gave birth 6 months later to a healthy baby girl. At this time, lactation occurred normally.

More believably, Scrimgeour and Baker (1974) described a woman hospitalized with prolonged vaginal bleeding. The woman aborted twins of different sizes. Age of
the fetuses was estimated as 40 days and 57 days for twins according to developmental characteristics. They had completely separate placentae, with one amniotic sac being twice as large as the other (4 cm vs. 8 cm in length). Delayed implantation was also considered as a possible cause of this phenomenon.

Bsat and Seoul (1987) report an infertility case involving repeated treatment with clomiphene citrate (CC). The patient was treated with CC a second time 30 days after the first treatment had apparently failed to produce a pregnancy. Ultrasonography at 25 weeks from the first cycle indicated twins of different sizes: one apparently 25-weeks old and one with the characteristics of a 22-week pregnancy. Delivery of twin boys occurred at 38 weeks from the first CC treatment. Twin 1 was normal, weighing 3 kg, while twin 2 weighed 1 kg. Twin 2 presented several characteristics of prematurity commonly observed when delivery occurs at less than 36 weeks, such as neonatal depression, heart failure, hypoglycemia and hypocalcinemia, as well as anemia. It was noted that growth retardation may have occurred in twin 2 due to the twin pregnancy, but great care was taken to prove otherwise.

Krenn et al. (1995) present a case involving gamete intrafallopian transfer (GIFT). A patient with a history of infertility received three mature oocytes and 300,000 sperm laparoscopically in the left fallopian tube. A twin pregnancy was confirmed 18 days later by ultrasound examination. A third pregnancy was detectable 2 weeks later. At 15 weeks of pregnancy, the woman aborted two well-developed fetuses and a third remnant of a fetus (damaged during delivery) with the developmental age estimated as 98 days of gestation. Additionally, two less developed fetuses were produced, with a developmental age estimated to be 41 days. Ovulation apparently
occurred naturally during the GIFT gestation, with fertilization occurring after normal sexual intercourse.

**Superfetation in Buffalo**

There has also been a report of superfetation in buffalo (Rao et al., 1987). A nuliparous buffalo heifer was observed mating naturally. Soon after, the heifer was diagnosed and treated for hoof and mouth disease. Mating was again observed 30 days after the initial mating. The buffalo heifer gave birth to two heifer calves 36 days apart. Both heifer calves weighed 12 kg at birth.

**Superfetation in Equids**

Twins are very rare in equids, so it stands to reason that superfetation would also be extremely rare. Aristotle (*Historia Animalium*, translation by D.W. Thompson, 1910) cites mares as an example of an animal that mates normally during the course of pregnancy, although no cases of superfetation had been reported at the time.

Mares remain in estrus for an extended period, which averages around 5 days, yet can range from 1 to 24 days (Hughes *et al.*, 1972). Double ovulations can occur, with intervals between ovulations ranging from 0 to 5 days. Ovulation may also occur during the diestrous period, ranging from 2 to 15 days of the luteal phase (Hughes *et al.*, 1972). Leroy and Pechdo (1950) describe a case that could be explained as superfecundation following double ovulation during the same period of estrus as a draft mare was mated by a jack and a stallion at an interval of 4 days. After a 12 month gestation, the draft mare gave birth to a normal mule and also a normal yet smaller than expected horse foal.
Mares also ovulate during diestrus (Cole et al., 1931). Short et al. (1964) describes a case that could fit into this category. A jenny was mated twice 11 days apart and gave birth to a live, healthy, full-term foal, weighing 16 kg, and also a dead foal soon after weighing 8 kg and showing signs of prematurity. However, estrus in mares may range from 1 to 24 days (Hughes et al., 1972), so this could also be another occurrence of superfecundation.

More rarely, mares may show signs of estrus followed by mating during pregnancy. Bournay and Robin (cited by Leroy and Pechdo, 1950), reported a case in which a mare was mated 3 times by a jack during a same period of estrus. The same mare was mated 14 days later by the jack, and 30 days following this mating by a stallion. The mare gave birth to a mule foal and also a horse foal 11 months following the last breeding.

Mumford (cited by Leroy and Pechdo, 1950) reported three cases of superfetation in mares in which mating dates were recorded and foals were easily distinguishable by sire. In the first case, a mare was mated by a stallion and 30 days later by a jack. The mare later delivered a live foal and also a dead mule foal. In a second case, a mare was mated to a stallion and a jack with an interval of 3 months. The mare aborted a foal that was close to term and also an incompletely developed mule foal 8 months after the first recorded mating. In the third case reported, a mare was first observed mating with a stallion, then 30 days later the same mare was observed mating with a jack. The same mare then mated again with a stallion 14 days after mating with the jack. The mare gave birth to a mule foal as well as a horse foal 11 months following the final mating. Both foals were normal and viable.
Superfetation in Cattle

Dalrymple and Jenkins (1951) reported the case of a dairy cow that was mated naturally and confirmed pregnant via rectal palpation 65 days later. On day 83 of pregnancy, the cow was observed in estrus and was mated to the same bull, being diagnosed pregnant 68 days following the second breeding via rectal palpation by the same veterinarian. The cow aborted a developmentally premature heifer fetus 256 days following the first recorded mating (173 days following the second mating) that died within ten minutes of birth. The cow later gave birth to a live, normal heifer calf 281 days after the second mating (109 days following the first calf).

Similarly, Wewer (1952) reported the case of a dairy cow that was observed in estrus 3 months after a previous mating. The cow was again mated at this time. However, 9 months after the first mating, the cow gave birth to a normal bull calf, along with a fetus that appeared to be 6 months of developmental age.

Simmons (1960) describes a case in which a cow was mated by AI over three consecutive estrous cycles, after which the cow showed no signs of estrus and was presumed pregnant. A recently calved heifer calf was found suckling on this cow 2 months prior to the expected calving date, as determined from the date of the last AI. The cow then gave birth to a normal, live bull calf 2 months after being found with the heifer calf. Blood typing was performed on the calves, with both calves confirmed as having been produced by the cow. However, only the second calf was confirmed as having been sired by the bull used for AI. It was concluded that the first calf born had resulted from an unobserved natural mating.
More recently, Hall (1987) reported a case of superfetation involving embryo transfer (ET). A Simmental embryo was transferred to a crossbred recipient. The recipient was diagnosed pregnant at 38 days of gestation and was subsequently turned out to pasture with a group of pregnant cows and a bull. The recipient gave birth to a live Simmental calf resulting from the embryo transfer 12 days prior to the expected calving date. Following the birth of the first calf, a pair of legs was seen protruding from the recipient’s vulva and a fetus estimated to be 6.5 months of developmental age was subsequently delivered, presumably resulting from mating during the ET pregnancy.

Hunsley (1998) reported another apparent case of superfetation involving ET. An embryo collected from a Shorthorn donor cow was transferred to a crossbred recipient. The recipient gave birth to a 28 kg crossbred heifer calf and soon after, a 37 kg phenotypically purebred Shorthorn bull calf. The Shorthorn calf was subjected to blood typing to verify parentage and was found negative for the embryo donor and sire. However, the calf tested positive for the crossbred recipient and a non-Shorthorn bull with which the recipient was placed following embryo transfer. Hair follicle samples were extracted from the bull calf and used to provide DNA for a polymerase chain reaction (PCR) test. The bull calf tested positive for the embryo donor and sire. It was concluded that placental exchange of blood stem cells between the crossbred heifer born co-twin to the Shorthorn bull calf was responsible for the difference in blood type and hair follicle DNA of the bull calf. Twinning was not a possibility as the recipient was placed with the bull following transfer of the embryo and had not been exposed to a bull
at the time of estrus detection for the embryo transfer cycle (Hunsley, personal communication).

Some reports of superfetation provide little evidence to support the claims. These cases are usually reported by veterinarians with information obtained from the owners as to breeding and calving dates of the animals in question. Bell (1964) reported two cases of apparent superfetation. In one case, a Shorthorn cow calved twice with an interval of 1 month between calvings. In another case, a Jersey cow produced two calves in a 3-month interval, with mating dates recorded for both calves. Vandeplassche (1969) reported two cases of cows in which only one mating was recorded, yet at parturition, produced live, normal, calves and also less developed fetuses with the physical characteristics of 6-month old fetuses. Gee (1971) reported a case of superfetation in which a cow gave birth to a healthy calf, as determined by the appearance of the calf and the presence of fetal membranes on the perineal region of the cow. Then 82 to 87 days later, the cow was found suckling a second, heifer, calf, once again giving appearance of having calved recently, as could be determined by rectal palpation. Nottle (1976) reported a beef cow giving birth to a bull calf and then was found with a second calf 15 days later. At the time of the second presumed calving, the cow showed signs of having recently calved, such as an enlarged vulva and a blood-stained perineum.

**Experimental Production of Superfetation**

Edwards and Fowler (1958) induced superfetation experimentally in mice using gonadotropin treatments during the reproductive cycle. Equine chorionic gonadotropin (eCG) was used to induce follicular growth and human chorionic gonadotropin (hCG)
was used 40 hours later to induce ovulation. A second treatment of eCG (higher dosage) was administered within 24 to 48 hours of the first hCG treatment, also followed by hCG to induce ovulation. Natural mating or AI using males with marker genes were used for first and/or second treatments. Superfetation occurred in females naturally mated following the first treatment with eCG and hCG and artificially inseminated following the second treatment with eCG and hCG. Four of six females necropsied 30 hours after the second hCG treatment in this group had recently ovulated ova and also morula or blastocysts from the first ovulation period. Two pregnant females necropsied 21 days after the first hCG treatment (18 days after the second hCG treatment) had fetuses resulting from both ovulation periods. One female allowed to go to term gave birth to pups resulting from both ovulation periods.

Wislocki and Snyder (1931) successfully induced superfetation experimentally in a rabbit doe by administering anterior pituitary lobe extract 4 days after the doe was mated. The doe was artificially mated 4 hours after treatment and then euthanized 30 hours after insemination. At necropsy, five normally developing blastocysts from the first mating were discovered in the uterus and six 3- to 5-cell embryos from the second mating were found in the oviducts of the female.

Hafez and Pincus (1956) also successfully induced superfetation in rabbits. The left oviducts were surgically removed from several does. The does were mated 10 days following the surgery and then subjected to surgical ET at varying times following mating. At the time of transfer, 3.5-day-old blastocysts were placed in the left uterine horn of the does at 4, 5, 6, 7, 8, 10, 15 and 20 days of pregnancy and the horn was traumatized with a sterilized probe to induce the formation of deciduoma. Pregnancies
resulted in does that were 4, 5 and 6 post-mating, so that the longest interval in age of embryos was 2.5 days.

Johansson and Venge (1951) produced superfetation in the mink by mating males of different coat patterns to females at intervals of up to 19 days. Approximately 16% of young in litters born resulted from the first mating when matings were 8 to 19 days apart. It is important to point out that mink exhibit facultative delayed implantation (Hansson, 1947; Mead, 1993). Gestation length varies according to time of mating so that if mating occurs early in the mating season, delayed implantation occurs, but not if mating occurs late in the season. Therefore, Johansson and Venge (1951) did observe superfetation in that the blastocysts resulting from the first mating were in embryonic diapause at the time of the second mating, allowing for young from both matings to develop together and be born at the same time.

Not all attempts to produce asynchronous twins have been successful. Stavy and Terkel (1992) treated hares at various stages of pregnancy with hCG and then immediately artificially inseminated the females by means of a glass pipette with freshly collected epididymal sperm, but no superfetation resulted. In females subjected to hCG and/or AI after day 34 of gestation, parturition was hastened by 3.5 days when compared to non treated females. Lawson and Cahill (cited in Wilmut and Sales, 1981) transferred synchronous ovine embryos with more chronologically advanced or retarded embryos, but no asynchronous embryos developed successfully. Wilmut and Sales (1981) also transferred asynchronous ovine embryos (±3 days) along with synchronous embryos to recipient ewes. However, no lambs resulted from the asynchronous embryos. Camillo et al. (1997) transferred 7-day-old horse embryos nonsurgically to
recipient mares that had ovulated 2 to 7 days prior to the donor mares (9 to 14 days pregnant). No twins resulted in these mares and one mare lost her primary pregnancy following ET.

Other Explanations for Apparent Cases of Superfetation

Other explanations have been offered for reported cases of superfetation. Kuntz (1920) proposed that many superfetation reports are better explained by differential growth of fetuses and presented three cases to support this theory. In the first report, a queen that had been euthanized was found to be pregnant with two nearly term fetuses and also two fetuses that measured 10 and 9 mm in length apiece. The fetuses were distributed evenly between the two uterine horns, with one large and one small fetus per horn. Although blood supply seemed adequate for each fetus, proportionate to the size of the fetus, the smaller fetuses presented necrotic tissues. The ovaries had two CL each, but they were determined to be of the same age. In another case, two 70 mm fetuses and two smaller fetuses in which developmental arrest had occurred at different stages were recovered from a queen at necropsy. All fetuses appeared to have an adequate supply of blood, proportional to the size of the fetuses. In both cases, microscopic evaluation was needed to determine the necrotic changes present.

In a third case described by Kuntz (1920), a bitch was found to be pregnant during a routine surgery, with three fetuses in each uterine horn. The embryonic vesicles appeared to be of the same size. However, 1 month later, the bitch gave birth to four full term fetuses, two live and two dead, as well as one fetus that was not fully developed. The full term fetuses averaged 102 g and the smaller fetus weighed 40 g. Gross observation of the smaller fetus did not reveal obvious abnormalities in
development. However, when examined microscopically, the fetus appeared to be in a state of necrosis. Since the embryonic vesicles were the same size at the time of the operation, it was determined that the fetus had died some time afterwards.

Vandeplassche (1969), in an extensive review, offered a different explanation for what was termed “double-parturition” rather than superfetation, in the pig. Embryonic diapause rather than superfetation was proposed as a more plausible explanation for most cases of double parturition in pigs. Twelve clinical case reports of double parturition following a single mating, with intervals between litters ranging from 4 to 98 days were presented. In 2 of the 12 cases, a boar was present on-site with the sows, so there was a possibility that a second unobserved mating could have occurred.

Two of the sows were examined laparoscopically following the second parturition (35 and 39 days apart, respectively) (Vandeplassche, 1969). In these cases, the ampullar (cranial) regions of the uterine horns showed formations consistent with the number of piglets born to the second litter. The caudal portions of the uterine horns were tubular in shape, an indication that involution had occurred in these portions of the uterine horns, suggesting that the two litters developed in the caudal portions of each uterine horn, and not in opposite uterine horns.

Vandeplassche (1969) theorized that embryonic diapause could have occurred due to the large number of piglets generated (average of 22 piglets per litter, ranging from 11 to 38). Instead of degenerating, the embryos may have entered diapause until uterine capacity became sufficient to allow for development.

However, embryonic diapause has not been confirmed in the pig and may not be a plausible explanation for these cases of unusual parturition intervals. Although
embryonic diapause has been found in over 70 eutherian and 30 marsupial species (Renfree and Shaw, 2000), the only ungulate in which the phenomenon has been confirmed is the Roe deer \( (\text{Capreolus capreolus}) \), first observed in 1651 by William Harvey (cited by Eckstein \textit{et al.}, 1959).

**REPRODUCTIVE DELAY PATTERNS**

Reproductive developmental delay patterns have frequently been offered as alternative explanations for the superfetation phenomenon. The most commonly occurring patterns in certain mammals are (1) delayed fertilization, or sperm storage in the female reproductive tract, (2) delayed implantation, which occurs following fertilization, but prior to implantation and (3) delayed development, which occurs following fertilization and implantation. Delayed implantation and delayed development are generally grouped together and are referred to as embryonic diapause (Renfree and Calaby, 1981). However, these two distinct phenomena will be discussed separately.

**Delayed Fertilization**

Bats are of particular interest, as delayed fertilization, delayed implantation and/or delayed development can be found among different species (Burns, 1981). Delayed fertilization occurs in some species of the mammalian families, Vespertilionidae and Rhinolophidae (Hamlett, 1935). Sperm can be stored by the male, in the epididymis, or by the female, in various locations in the reproductive tract (Racey, 1979).

Spermatogenesis in Rhinolophids and Vespertilionids typically occurs during the summer (Racey, 1979), after which the testes regress and unejaculated sperm are
stored in the cauda epididymides until the following summer. Although mating usually occurs in the fall, ovulation only occurs in the spring (Hamlett, 1935; Racey, 1969), so sperm that will father the young in the following summer are normally released early, with most of the storage time occurring in the female reproductive tract. For example, copulation may occur up to 5 months prior to ovulation and subsequent fertilization in the Big Brown bat (*Eptesicus fuscus*) and Little Brown Myotis bat (*Myotis lucifugus*) (Wimsatt, 1944). Therefore, sperm can be stored extensively in the female reproductive tract.

Ovulation appears to be triggered by emergence from hibernation in the spring (Racey, 1969). Racey (1973) isolated female bats from males following mating and determined that sperm could be stored up to 151 days in pipistrelle bats and 198 days in noctule bats prior to emergence from hibernation and subsequent ovulation.

Sperm can be stored in various places in the female, although it is believed that only sperm stored in the uterus or oviduct are responsible for delayed fertilization (Racey, 1979). In stored sperm, the heads are typically in close contact with the microvilli on the surface of the epithelium in that portion of the reproductive tract (Racey, 1979; Krutzsch *et al*., 1982). This arrangement has been implicated in sperm destruction (Austin, 1959). However, Krutzsch *et al*. (1982) found this function to be true following emergence from hibernation in Little Brown Myotis bat and the Cave Myotis bat (*Myotis velifer*), but not during periods of sperm storage. In fact, sperm clearance accelerates greatly following emergence from hibernation, similar to the phenomenon that takes place following copulation in most mammals that do not present delayed fertilization (Krutzsch *et al*., 1982).
In some species of snakes and turtles, spermatozoa are still capable of fertilization 4 years following mating (for review see Howarth, 1974). However, other than in some species of bats, sperm is only viable for a few hours to a few weeks following copulation in most mammals.

Sperm was found in the reproductive tract of two mice 8 and 15 days following mating, respectively, by Ullman (1976) although at a very low incidence (over 500 females were studied). In Brown Marsupial mice (*Antechinus stuartii*), in which males typically die at the end of the mating period and ovulation is spontaneous, sperm storage is also important (Selwood and McCallum, 1987). Sperm was found to maintain fertilizability after up to 16 days of storage in the female reproductive tract.

According to Chang (1965), ferret sperm is viable in the female ferret reproductive tract following copulation for up to 5 days. Doak *et al.* (1967) found motile sperm up to 11 days following mating in a bitch. Gould *et al.* (1984) reported that sperm recovered from humans following AI could still penetrate human ovum zona pellucidae up to 80 hours following recovery.

In horses, sperm can survive up to 6 days and maintain fertilizing ability following AI (Day, 1942) or natural service (Burkhardt, 1949). However, other than in the horse, sperm has a much shorter fertilizable life in other domestic species. Boar sperm can survive up to 72 hours at the utero-tubal junction (UTJ) and isthmus of the oviduct but not in other parts of the reproductive tract (Hunter, 1975). Ram sperm maintain fertilizability in the female tract up to 48 hours (Hafez, 1993). Duration of sperm storage in cattle is also short. Thibault *et al.* (1975) found living sperm in the UTJ folds up to 72 hours following insemination. However, Laing (1945) found that
inseminations <16 hours before the end of estrus did not result in pregnancies, and concluded that sperm retained their fertilizing ability up to 30 hours following insemination, based on an average interval of 10 hours from the end of estrus to ovulation.

More recently, Hafez (1993) stated that cattle sperm appear to maintain fertilizing ability 30 to 48 hours following insemination. In superovulated cattle, where ovulation appears to occur over a period of hours, a single unit of high quality semen inseminated at 12 to 24 hours following onset of estrus is sufficient to ensure fertilization and high embryo quality (Critser et al., 1980; Schiewe et al., 1987; Lopez-Gatius et al., 1988). Schiewe et al. (1987) found that a single insemination at 36 or 48 hours following onset of estrus resulted in progressively increasing rates of embryo degeneration, which implies aging of ova or adverse hormonal environments. Therefore, sperm storage and delayed fertilization do not appear to be factors in the reported cases of superfetation in cattle, as reported superfetation asynchronous pregnancies tend to have age differences in weeks and months rather than hours and days.

**Embryonic Diapause**

Embryonic diapause is the temporary retardation of embryonic development at any stage of embryogenesis (Mead, 1993) and can be divided into delayed implantation, where development is arrested or retarded at a free-floating blastocyst stage and delayed development, where normal development is slowed following implantation (Renfree and Calaby, 1981). Embryonic diapause occurs in both the animal and plant kingdoms. Typical examples are plant seeds that must wait until favorable conditions occur to
germinate and bird eggs in which females lay several eggs over several days, but
development only increases after all eggs are laid and the female begins to incubate
them (Mead, 1993).

**Mechanisms of Delayed Implantation**

In mammals, some form of embryonic diapause has been confirmed in more
than 70 eutherian species and 30 marsupial species (Renfree and Shaw, 2000). Delayed
implantation can be divided into two categories: facultative, where implantation of
blastocysts is delayed by lactation or seasonality and obligate, when implantation of
blastocysts is delayed regardless of any external stimulus (Mead, 1993). Lactational
delayed implantation was first observed in rats by Lataste (1891). Species that have
facultative delayed implantation generally have a postpartum estrus soon after
parturition, with developing blastocysts implanting when nursing ceases (Vogel, 1981).
Weitlauf and Greenwald (1965) consistently induced delayed implantation in mice for 9
days by allowing standard litters of 10 pups to suckle on females that had recently given
birth and had been mated by fertile males. Implantation was induced on day 9 by
removing the young on day 8 of gestation.

Estrogen can induce implantation (Weichert, 1942) in rats exhibiting lactational
delayed implantation. Raud (1974) induced implantation in rats with lactational
delayed implantation by administration of FSH, but only if large follicles were present
on the ovaries. Suckling decreases FSH levels in blood, so when suckling decreases,
FSH levels start to rise, affecting follicular size and subsequently estrogen levels so that
implantation can take place (Raud, 1974).
Delayed implantation is also facultative in most marsupials and can be lactational or seasonal. In the Red kangaroo (*Megaleia rufa*), for example, mating occurs ~2 days after parturition. If the joey remains in the pouch (~236 days) and is suckling, the blastocyst remains in diapause (Sharman, 1963). When the joey exits the pouch, the embryo accelerates development and is born ~31 days later, after which postpartum estrus again occurs. In other words, the kangaroo female can have an out-of-pouch joey (suckling), an in-pouch joey (also suckling) and a blastocyst awaiting implantation.

In marsupials, CL development is also retarded. Resumption of CL development and progesterone secretion apparently induces resumed development of the blastocyst and subsequently implantation, as exogenous administration of progesterone can cause resumption of development (Clark, 1968). Removal of the suckling in-pouch Red kangaroo joey causes resumed CL development and consequently, embryo development (Sharman, 1963).

Delayed implantation in marsupials can also be seasonal: in the Tamar wallaby (*Macropus eugenii*), removing the young from the pouch during the long day photoperiod does not cause resumption of luteal function (Sharman, 1963). Reactivation of the CL occurs following a decline in prolactin, which is, in turn, regulated by melatonin secretion in response to photoperiod (Renfree and Shaw, 2000). Exposure to short day photoperiods causes resumption of CL and embryonic development (Sadleier and Tyndale-Briscoe, 1977). Interestingly, tamars give birth during the summer, mating only a few hours later. Lactation maintains the embryo in diapause until the winter months, after which photoperiod mediates the maintenance of diapause until
reactivation after the summer solstice (Renfree and Shaw, 2000). Tamars then give birth 1 month later, so that gestation lasts 364 days, with the blastocyst remaining in diapause for 11 months. Since the Tamar wallaby enters puberty at 10 months and mates soon after, she is only nonpregnant for the first 10 months of her life and thereafter only on the day she gives birth (Renfree and Shaw, 2000).

Mink present obligate delayed implantation, although length of delay is variable. In the mink, gestation length varies according to time of mating (Hansson, 1947). Mink mating early in the season have a longer period of delayed implantation of blastocysts, while mink mated late in the season have a shorter period of delayed implantation (Dukelow, 1966). Therefore, litters within a given population are generally born close together. Delayed implantation in mink appears to be a function of photoperiod, as Hansson (1947) was able to shorten gestation length in mink by daily exposure to ultraviolet light for 45 or 90 minutes. This was confirmed by Martinet et al. (1981) who reported that increasing light vs. dark periods significantly decreased gestation lengths. Progesterone secretion by the CL precedes implantation by 7 to 10 days (Moller, 1973) and prolactin is apparently luteotrophic in the mink (Martinet et al., 1981). Plasma prolactin levels also appear to increase as daylight increases, so prolactin may be the trigger for implantation in spring-implanting species such as the mink (Martinet et al., 1981). The Striped skunk (Mephitis mephitis) appears to show a pattern of delayed implantation similar to that of the mink (Wade-Smith et al., 1980).

Several other species present obligate forms of delayed implantation, where delayed implantation occurs apparently without regard to stimuli such as lactation or seasonal effects. Examples of other mammals showing some form of delayed

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implantation are armadillos (Patterson, 1913), some species of bats (Burns, 1981; Bernard and Cumming, 1997), black bears (Wimsatt, 1963), seals (Daniel, 1981) and many other mammals (for reviews see Mead, 1993; Renfree and Shaw, 2000).

Various conditions are necessary to induce delayed implantation, as are conditions necessary to induce resumption of embryonic development. Even members of a same genus may or may not present delayed implantation (Mead, 1993; Bernard and Cumming, 1997). However, the only ungulate known to exhibit delayed implantation is the Roe deer (*Capreolus capreolus*). William Harvey (cited by Eckstein *et al.*, 1959) first observed this phenomenon in Roe deer in Great Britain in 1651, reporting that although mating occurred in September, fetuses could only be detected in November. Blastocysts enter the uterus soon after hatching from their zonae pellucidae (fall season) and become quiescent for 5 months, until calving occurs in the spring (Aitken, 1974). Prolactin does not appear to influence resumption of embryonic development in Roe deer (Mead, 1993), nor does estrogen (Aitken, 1981). Although Lincoln and Guiness (1972) were able to advance molting in Roe deer by altering photoperiod, delayed implantation appeared unaffected, as the deer gave birth at the normal time.

Interestingly, the Red deer (*Cervus elaphus*), a species similar to the Roe deer and which live in the same region as the Roe deer, does not exhibit delayed implantation (Hamlett, 1935). In Red deer, mating occurs in fall-early winter rather than summer and calving occurs in spring (231-day gestation), with an anestrous period during the summer months (Guiness *et al.*, 1971). Embryonic diapause has also been
suspected in Pere David’s deer (*Elaphurus davidianus*) due to its long gestation (280 days), although this has not been confirmed (Brinklow and Loudon, 1993).

**Delayed Development Following Implantation**

Delayed development following implantation is another reproductive delay phenomenon that occurs in bats (Renfree and Calaby, 1981). In these species, blastocysts implant following mating, fertilization and normal embryonic growth (Bernard and Cumming, 1997). Following implantation, embryonic development either completely stops or is retarded. Certain species, such as the common pipistrelle (*Pipistrellus pipistrellus*), have temperature dependent, or facultative delayed development. Racey (1969) extended gestation length by 14.5 days by placing pregnant bats captured as they emerged from hibernation (and presumably ovulated, allowing delayed fertilization to occur) in a low temperature (11 to 14° C) environment for 13 days in which the bats resumed their state of torpor. Other species, such as the California fruit bat (*Macrotus californicus*), have arrested development for 4 to 5 months regardless of temperature and food availability (Burns *et al.*, 1972).

It is important to note that not all species of bats exhibit reproductive delay phenomena. Also, African bats of the nonmolossid species can be polyestrous in the tropics and monestrous with reproductive delays in temperate climates (Bernard and Cumming, 1997).

**Differentiated Growth of Twins**

Another possible explanation for some of the reported cases of superfetation is differential growth of twins. With the advent of ultrasound technology, this phenomenon has been well documented in humans and is generally believed to be an
initial sign of reduced viability of the smaller twin, or even both (Kol et al., 1993; Weissman et al., 1994). Weissman et al. (1994) studied five cases of twins with discordant growth and in all cases, the smaller twin was found to have congenital malformations. Kol et al. (1993) studied 60 multiple pregnancies and found only two instances in five cases of twins with different growth rates in which a twin with a size differential greater than 60% survived to term.

Echternkamp (1992) induced superovulation in cows prior to insemination and reported one case in a cow in which separate placental units were found in each uterine horn at slaughter at 52 days. One placenta contained three live, viable fetuses that were at the correct developmental stage, while the other contained three dead fetuses, which appeared to be 35 days of developmental age.

**Delayed Delivery of Twins**

To further confound the concept of superfetation, delayed delivery of twins can also occur. In humans, in the first recorded case (Carson, 1880) twins were delivered 44 days apart. More recently, the number of multiple births has increased dramatically due to assisted reproductive technologies (Luke, 1994). Complications normally arise during multiple fetus gestations (resulting in fetal death), so it has become increasingly common to manage pregnancies in order to prevent total pregnancy loss (Kalchbrenner et al., 1998; Song et al., 2000; Farkouh et al., 2000). Delays between deliveries of twins as long as 123 days have been recorded in which the first-born did not survive (Farkouh et al., 2000) and 13 weeks for pregnancies in which both twins survived (Kalchbrenner et al., 1998) have been recorded. Chances of fetal survival are greatly reduced when delivery of the first twin occurs prior to 24 weeks (Farkouh et al., 2000).
After 24 weeks, survival rates ranging between 40% and 60% for the firstborn twin and 78% to 100% for the latter born twin have been recorded (Kalchbrenner et al., 1998; Farkouh et al., 2000).

Delayed delivery of twins has also been reported in cattle. Frost (1918) reported two cases. In the first case, delivery of live twin heifer calves occurred 8 days apart and in the second case, assisted delivery of a stillborn calf occurred 39 days following the birth of a live, healthy calf. Either of these cases could have been presented as cases of superfetation, although superfetation is not mentioned.

ECTOPIC PREGNANCIES

Ectopic Pregnancies in Humans

Embryos can also develop in extra-uterine sites, sometimes as far as full-term, although birth by natural means cannot occur. These pregnancies are known as ectopic pregnancies. This possibly deadly form of pregnancy was first described in humans in the 10th century (Cotlar, 2000). Approximately 99% of ectopic pregnancies in humans are tubal pregnancies (Lehner et al., 2000). Interestingly, Cotlar (2000) reported that underdeveloped countries reported the highest incidence of abdominal pregnancies and that rates in Louisiana and Mississippi were similar to these third world countries.

In recent years, there has been a significant increase in the number of ectopic pregnancies reported, which is due in part to advances in diagnostic technology which allow early detection of ectopic pregnancies that might have gone unnoticed in the past and also possibly to increased promiscuity and consequently venereal diseases (Oelsner et al., 1989). Ankum et al. (1996) analyzed several reports on ectopic pregnancies and reported that previous tubal problems (prior ectopic pregnancy, tubal surgery, other
tubal pathologies) and exposure to diethylstilbestrol (DES) *in utero* strongly increase chances of an ectopic pregnancy. A lower risk occurs following genital infections such as pelvic inflammatory disease, chlamydia and gonorrhoea. There is also a mild increase in risk of ectopic pregnancy in women who have more than one sexual partner during their lifetime. Other risk factors included pelvic or abdominal surgery, vaginal douching, smoking and first sexual intercourse at an early age.

**Ectopic Pregnancies Due to Assisted Reproductive Technologies**

It also appears that assisted reproductive technologies (ART) procedures can increase the chances of ectopic pregnancies. For example, in 1994, 4% (246 of 6,114) of standard *in vitro* fertilization (IVF) pregnancies, 3% (45 of 1,342) of gamete intrafallopian transfer (GIFT) pregnancies and 3% (9 of 278) of pregnancies resulting from zygote intrafallopian transfer (ZIFT) in the U.S. were ectopic [Society for Assisted Reproductive Technology (SART), 1996]. In 1997, these numbers decreased slightly for IVF and GIFT to 2% (220 of 8,975) and 2% (16 of 627), respectively (SART, 2000). The rate of ectopic pregnancies established with ZIFT remained the same. Dimitry *et al.* (1990) attributes the high rate of ectopic pregnancies following ART procedures to the fact that more women with tubal damage are now able to conceive.

Interestingly, with uterine transfer of embryos, only 1 of 226 (0.4%) pregnancies reported was ectopic (SART, 2000). Apparently, manipulation of the fallopian tubes increases the chances of ectopic pregnancies, as might be expected. In fact, the actual placement of the transferred embryo in the fallopian tube itself can increase the rate of ectopic pregnancies. Nazari *et al.* (1993) found that midfundal transfers had a lower
ectopic pregnancy rate (3%) than deep fundal transfers, when embryos were transferred <5 mm from the fundus (12%).

Ectopic pregnancies can also occur following ovarian stimulation and ovulation induction with human menopausal gonadotropin (hMG), CC and/or hCG (Lehner et al., 2000). Oelsner et al. (1989) reported that 1.4% (7 of 484) of pregnancies in Israel established following hMG/hCG treatment were ectopic, compared with 1.0% (349 of 35,746) of normally occurring pregnancies. Oral contraceptives have also been implicated in increased rates of ectopic pregnancies (Larimore and Stanford, 2000).

**Heterotopic Pregnancies**

Ectopic pregnancies can also occur together with a normal intrauterine gestation in a condition known as a heterotopic pregnancy. In humans, these occur very rarely, or approximately 1 in 30,000 pregnancies (Beck et al., 1990). It does appear that with the increased use of ART procedures, heterotopic pregnancies have become more common (Lehner et al., 2000). Dimitry et al. (1990) reported nine cases of heterotopic pregnancies in 315 clinical pregnancies (3%) following standard IVF procedures.

**Ectopic Pregnancies in Animals**

Although not as prevalent as in humans, ectopic pregnancies have also been reported in various animals. During early attempts to study early development of embryos, mouse embryos were successfully cultured for a time in the anterior chamber of the mouse eye as well as the abdominal cavity of the mouse (Fawcett et al., 1947). However, in these cases, development of the embryos appeared retarded and eventually stopped, although implantation was achieved in some cases. Retarded or abnormal
development of embryos transplanted to various extra-uterine sites was also reported in the guinea pig (Bland and Donovan, 1965).

Gosden and Russell (1981) reported a fully formed abdominal pregnancy during necropsy of a Sprague-Dawley rat. Although the fetus was dead, it appeared normal. Naturally occurring ectopic pregnancies have also been reported in the guinea pig (Araujo, 1964), the hamster (Buckley and Cain, 1979; Peter, 1982), the rabbit (Smith et al., 1989), the dog (Lederer and Fisher, 1960; Peck and Badame, 1967) and the horse (Foster, 1918).

Interestingly, the cat appears to have a high incidence of extrauterine pregnancies, due to the large number of reports in the literature. In attempting to explain a case of an ectopic mummified fetus discovered 18 months following spaying, Carrig et al. (1972) postulated that handling of the reproductive tract during ovariohysterectomy may have resulted in the loss of recently fertilized embryos into the abdominal cavity. Similar cases were reported by Reens (1988) and Nack (2000). However, Nack (2000) postulated that the mummified fetuses could have been present but undetected at the time of the ovariohysterectomy.

In fact, ectopic pregnancies have also been reported in intact queens (Bodle, 1979; McKeating, 1979; de Haan, 1991). Hannon (1981) reported ectopic, mummified fetuses in a queen that had been spayed three years earlier. In this case, palpable masses had been present on the abdomen at the time of removal of stitches following ovariohysterectomy, but were assumed to be adhesions resulting from the procedure.

There are several reports of ectopic pregnancies in monkeys. Abdominal pregnancies have been reported in the Owl monkey (Aotus trivirgatus), the Squirrel
monkey (*Saimiri sciureus*) and the Himalayan macaque (*Macaca assamensis*) (Bunte and Hildebrandt, 1975; McClure and Chang, 1975; Bosu and Barker, 1980, respectively). Jerome and Hendrickx (1982) reported a tubal pregnancy in a Rhesus monkey (*Macaca mulatta*). This case is important as most reported ectopic pregnancies in animals have been abdominal, while tubal pregnancies are the more common form of ectopic pregnancy in humans (Lehner *et al.*, 2000).

Extrauterine pregnancies have also been reported in cattle. Botcherby (1980) reported a full-term ectopic pregnancy in a cow following rupture of the uterine wall. The apparent exit site was six inches long, although the calf was fully formed (36 kg), indicating that rupture had occurred earlier in the pregnancy. There were almost no fetal membranes or fluid present. Vallée and Gamelin (1997) reported a similar case of uterine rupture causing an ectopic pregnancy in a heifer, although in this case the fetus was abnormal. In one interesting case, Hedge (1989) reported a full term abdominal pregnancy in a cow in which the placenta had attached to the viscera and the mesentery. No evidence of uterine rupture was found and there was no evidence of fetal membranes in the uterus.

**FACTORS AFFECTING SUPERFETATION**

**Estrus During Pregnancy**

Superfetation can only occur in nature if the pregnant female in question shows signs of estrus and is mated by a male. Estrus during the course of pregnancy has been reported in many animals, including cows. Aristotle (*Historia Animalium*, translation by D.W. Thompson, 1910) cites humans and horses as species in which mating occurs during the course of pregnancy.
Mating during pregnancy has been reported in the mouse by Watt (1931) and Littleford and Gysin (1944). Long and Evans (1922) reported two cases in which pregnant rats mated during gestation. Nelson (1929) reported a case in which a pregnant rat showed regular signs of estrus and was mated at 4-day intervals (beginning on day 5 and ending on day 21) during the course of pregnancy, although no superfetation occurred. Wild hares (*Lepus europaeus*) commonly mate during the course of gestation, though mating usually occurs just prior to parturition (Martinet and Raynaud, 1975; Caillol and Martinet, 1976; Martinet, 1980; Caillol et al., 1991).

Williams et al. (1956) slaughtered 50 ewes 20 to 90 days following mating; 11 of these (22%) had shown at least one estrus prior to slaughter and all were found to be pregnant to the initial mating. However, no ovulations were detected from the secondary heats.

Williams (1921) estimated the number of cows that show signs of estrus during the course of pregnancy to be 1 to 2%, with estrus occurring at variable intervals up to 7 or 8 months, but usually ceasing after 4 months of gestation. One case is cited in which a pregnant cow that was showing signs of estrus was palpated *per rectum*. The CL appeared to have atrophied and a large follicle was present on the ovary contralateral to the pregnancy. No information is offered as to the whether pregnancy was maintained in this cow. Bullard (1934) presented data on 10 cows that showed at least one estrus during pregnancy, some of which resulted in mating, although no superfetation cases resulted from the matings during pregnancy. Time from the initial mated estrus to second and subsequent estruses (during pregnancy) ranged from 24 to 235 days of gestation.
There are several reports of cattle being artificially inseminated while pregnant, indicating that these pregnant cows showed signs of estrus. Donald (1943) reported 36 cases in which cows were mated during the course of pregnancy, with times from initial mating to the secondary estrus ranging from 15 to 232 days. A few cows were mated following up to three estrous periods during the course of an ongoing pregnancy. In a report on 1,855 natural matings over a period of years in Louisiana, Branton (1949) reported that 64 matings (3%) occurred in cows that were already pregnant. Erb and Morrison (1958) studied data compiled on a sample group of dairy cows between 1920 and 1950 and reported that 378 of 6,751 (6%) successful pregnancies in the records showed that the cows were mated at least once during the course of the pregnancy.

Williamson et al. (1972) observed estrus in 7% of pregnant cows during a study on estrous behavior in dairy cows. Perez Garcia et al. (1984) reported 24 of 388 (6%) cases of estrus during pregnancy in a study with dairy cows, which were pregnant and subsequently calved. Estrus was confirmed by the cow being mated by an intact bull as well as having milk progesterone levels lower than 2 ng/ml. Six pregnant cows (2%) showed signs of estrus on two occasions and one (0.2%) showed signs of estrus on three occasions during the course of her pregnancy. Estrus was observed throughout pregnancy (10 days to >200 days).

Thomas and Dobson (1989) studied 43 cases of estrus in 35 pregnant cows. Although estrus was observed in all stages of pregnancy, most cases occurred between 121 and 240 days of gestation. Seven of nine cows presented to a bull at the time of estrus were mounted and mated successfully. Follicles between 10 and 15 mm were detected in 5 of 12 pregnant cows palpated at estrus, but no ovulations were apparent as
determined by palpation and confirmed by ultrasonography 2 and 14 days following estrus. No significant differences were found between levels of plasma estradiol and progesterone at estrus and 14 days later.

However, it must be noted that signs of estrus are not always accompanied by ovulation. Estrus without subsequent ovulation and CL formation has also been observed in nonpubertal heifers (Nelsen et al., 1985; Rutter and Randel, 1986).

**Follicular Waves**

Estrus during pregnancy alone would not be sufficient for superfetation to occur. Ovulation of viable oocytes would also have to occur during pregnancy. Follicles have been shown to develop in groups, or cohorts, with one or more cohorts growing in a wave-like pattern during the estrous cycle in many species. Follicular waves have been described in horses (Ginther, 1993), goats (Ginther and Kot, 1994), sheep (Ravindra et al., 1994; Ginther et al., 1995; Bartlewski et al., 1998, 1999), llamas (Adams et al., 1990), camels (Skidmore et al., 1995) and musk oxen (Hoare et al., 1997; Adams, 1999). Wave patterns may be classified as major (clear follicular dominance), occurring at the beginning and end of the estrous cycle and minor (no clear dominant follicle), occurring during diestrus (Adams, 1999).

Cattle appear to have two to four waves of follicular growth during the estrous cycle (Ireland and Roche, 1983; Ireland, 1987; Savio et al., 1988; Sirois and Fortune, 1988). The waves are generally characterized by a group of follicles emerging over a period of days, with one follicle eventually becoming dominant, so that it continues to grow while the other follicles in the cohort become atretic and regress. Each wave is preceded by a surge of FSH, which decreases as one follicle appears to become
Dominant (Adams et al., 1992). Dominant follicles that grow in the presence of an active CL eventually become atretic and regress, while the dominant follicle that is growing at the time of CL regression becomes the ovulatory follicle (Savio et al., 1988; Sirois and Fortune, 1988; Ginther et al., 1989). If the animal becomes pregnant, the CL is maintained and the otherwise ovulatory follicle also becomes atretic and regresses (Ginther et al., 1989).

**Follicular Waves During Pregnancy**

Follicular waves continue throughout pregnancy in cattle (Ginther et al., 1989; Bergfelt et al., 1991; Taylor and Rajamahendran, 1991; Ginther et al., 1996). Ginther et al. (1989) studied follicular waves in heifers through day 70 of pregnancy and found that the interval between the emergence of waves was similar, ranging between 9 and 10 days. The dominant follicle of wave 1 was the largest, while waves 2 and 3 had the smallest dominant follicles. The dominant follicles for waves 4 to 6 had intermediate diameters. Taylor and Rajamahendran (1991) studied follicular waves in cows through day 60 of pregnancy and also found that follicular waves continued to emerge. However, no differences were found in dominant follicle diameters among waves until day 56.

Ginther et al. (1996) studied follicular waves in pregnant cattle between 90 days of pregnancy and the first follicular wave following parturition. Follicular waves continued throughout pregnancy in most heifers up to 3 weeks before parturition. Intervals between waves decreased after the fourth month of pregnancy. Surges of FSH were also correlated with the emergence of follicular waves, except in the last 3 weeks prior to parturition, when rhythmical surges of FSH were not always followed by the
emergence of follicles. The diameter of the largest follicle also decreased between the fourth month and the remaining months of pregnancy.

Bergfelt et al. (1991) compared follicular waves between progesterone-treated, nonbred heifers and pregnant heifers through day 100 of pregnancy. In this study, the dominant follicle for wave 1 was larger than the dominant follicles of waves 2 to 10. The dominant follicle occurred more often on the ovary contralateral to the CL of pregnancy in waves 3 to 9. More importantly, heifers in the nonbred progesterone-treated group also had continued anovulatory follicular wave cycles even though the CL regressed normally, suggesting an effect of progesterone on suppression of ovulation of dominant follicles.

Ovulation During Pregnancy

Although estrus during pregnancy is not always an indication that ovulation will also take place, (Williams et al., 1956), ovulation has been found to occur during pregnancy in some species. Likewise, ovulation during pregnancy may not always be accompanied by signs of estrus.

Watt (1931) examined a mouse post-mortem that was discovered to have a copulatory plug during pregnancy and found recent ovulations on the ovaries and 11 ova were recovered from both oviducts. Rollhauser (1949) found freshly formed CL on the ovaries of an 8-day pregnant mouse and also apparently fertilized ova.

Swezy and Evans (1930) studied 76 pregnant rats post-mortem and found what appeared to be distinct periods of follicular growth every 4 or 5 days during pregnancy, with large follicles present at the end of each growth period, followed by the appearance
of newly formed CL. No signs of estrus or copulation were found in 23 rats placed with males during the course of pregnancy.

Patterson (1913) found evidence of ovulation during pregnancy in armadillos as determined by the presence of unfertilized ova in the oviducts of a few pregnant armadillos. Ovulation was also found to occur following mating during pregnancy in hares maintained in captivity (Martinet, 1980). Although mating occurred throughout pregnancy, ovulation only occurred in does mated up to 5 days prior to parturition.

Ovulations occur quite commonly during mid-cycle and pregnancy in the mare with no obvious signs of estrus (Cole et al., 1931; Day, 1940; Amoroso et al., 1948; Rowlands, 1949; Allen, 1971). Recently ovulated ova have also been recovered from mares during various stages of pregnancy (Rowlands, 1949). Allen (1971) described several cases of ovulation during pregnancy in mares ranging from 22 to 87 days of gestation. One of the mares also showed estrous behavior when teased, although she would not allow the stallion to mount at those times.

No reports of ovulation during the natural course of pregnancy could be found for cattle. Progesterone levels in cattle rise following ovulation and remain elevated throughout pregnancy (Randel and Erb, 1971). High progesterone levels during the luteal phase block the pre-ovulatory surge of FSH and LH (Kesner et al., 1982). Therefore, it is possible that ovulation of follicles during pregnancy in cattle may only occur if specific hormonal conditions are met.

**Ovulation Induction During Pregnancy**

However, ovulation can be induced in mid-cycle and in pregnant animals of different species using various ovulatory agents. Various agents have been used
successfully to induce ovulation in induced ovulators such as mice (Burdick et al., 1943; Burdick and Ciampa, 1944), rats (Brown-Grant, 1969) and rabbits (Wislocki and Snyder, 1931, 1933; Boyarski et al., 1947; Murphree et al.; 1947; Austin, 1949). Edwards and Fowler (1958) successfully induced ovulation in pregnant mice treated with pregnant mare serum 12 hours following mating and hCG up to 4 days apart. Fertilization occurred in mice in which ovulations occurred up to 3 days apart, with some mice carrying young from each set of ovulations to term.

Ovulations can also be induced during pregnancy in humans. A woman with a history of anovulatory cycles received two subsequent treatments of CC for ovulation induction after the first had presumably failed to establish a pregnancy (Bsat and Seoud, 1987). She eventually delivered twins of apparently different ages that were determined to have resulted from ovulations induced 6 weeks apart.

As ovulation occurs normally during pregnancy in the mare, it is no surprise that hCG has been used to induce ovulation in pregnant mares. Amoroso and others (1948) induced ovulation in a pregnant mare with hCG, although fertilization did not occur following AI. However, fertilization did occur following hCG treatment to induce ovulation in a mare with a persistent CL (Hughes and Stabenfeldt, 1977). The mare was artificially inseminated 24 hours following administration, resulting in the birth of a live filly. Progesterone levels remained elevated at the time of hCG administration and breeding, indicating that the CL was active.

Ovulation can be induced in cattle at different stages of the estrous cycle and pregnancy. Schomberg et al. (1967) induced secondary ovulations in dairy cows using hCG or LH injections on day 15 of their cycles, but ovulation did not occur with
treatments on day 5 or 10 of the same cycle. Price and Webb (1989) found hCG to be very effective in causing ovulation of large mid-cycle follicles in crossbred heifers when given on days 4 to 7 (83%) and 14 to 16 (66%) of the estrous cycle, but less effective when given during days 8 to 13 (35%). It was noted that ovulation induction by hCG during the luteal phase required the presence of large follicles. Walton et al. (1990) induced ovulations and supranumary CL in nonpregnant and pregnant dairy cows by treatment with 1,500 IU hCG on day 5 post-breeding. Fricke et al. (1993) successfully induced ovulations in 100% of cows treated with 1,500 IU of hCG on day 6 of the estrous cycle. Sianangama and Rajamahendran (1996) were successful as well with hCG treatment on day 7 of the estrous cycle in nonpregnant cows. Rajamahendran and Sianangama (1992) found hCG to be very effective in causing ovulation in pregnant cows when given on day 7 of pregnancy (7 of 9 treated cows ovulated) but less effective on day 14 of pregnancy (4 of 9 treated cows ovulated). It was proposed that the dominant follicle present at day 7 of the estrous cycle was less likely to be atretic and has more LH receptors present and was therefore more likely to be responsive to ovulation induction by hCG.

Lulai et al. (1994) induced ovulations with exogenous LH after day 35 in 10 of 10 pregnant beef cows in which pregnancy was maintained with norgestomet implants following induced luteal regression. Interestingly, cows in which the new CL formed on the same side as the previous CL maintained their pregnancy following removal of the norgestomet implants (4 of 4 remained pregnant), while cows with CL forming on the contralateral ovary lost their pregnancies (1 of 6 remained pregnant). Bridges et al. (2000) also induced ovulation in pregnant cows in which the CL of pregnancy had been
removed early in pregnancy and pregnancy was maintained with exogenous progesterone. In this study, hCG treatment alone or in conjunction with an FSH pretreatment caused ovulation in 59% (30 of 51) cows when hCG was administered before day 36 of pregnancy. However, 96% (22 of 23) cows ovulated following treatment with FSH/hCG prior to day 36 of pregnancy followed by another treatment after day 36.

**Quality of Oocytes Recovered from Pregnant Females**

Oocytes originating from follicles growing during pregnancy have been shown to be capable of being fertilized and developing into normal embryos in several species. Lefebvre *et al.* (1990) documented a case in which oocytes were recovered from a woman with a 14-day undiagnosed pregnancy. Five oocytes were transferred to the patient’s Fallopian tube with sperm and nine were subjected to IVF procedures. No pregnancies resulted from the GIFT, but five embryos resulted from IVF and were frozen for later use at the 4-cell stage. In another interesting case, Serafini *et al.* (1985) reported aspirating follicles from a woman with an ectopic (tubal) pregnancy. Five oocytes were recovered and subjected to IVF procedures, with the two resulting embryos being frozen at the 3-cell stage for later use.

Oocytes recovered from transvaginal ultrasound-guided aspiration of follicles in live pregnant mares have produced morulla and blastocyst stage embryos after *in vitro* (Li *et al.*, 1995) or *in vivo* (Meintjes *et al.*, 1995b) maturation. In addition, Cochran *et al.* (1998) produced two foals at Louisiana State University (LSU) following intracytoplasmic sperm injection (ICSI) of oocytes recovered from live pregnant mares.
Behboodi et al. (1992) found no differences in embryo development from oocytes retrieved from ovaries of pregnant or nonpregnant cows. Ryan et al. (1993) at LSU produced embryos from oocytes recovered from stimulated ovaries aspirated surgically from pregnant cows. Meintjes et al. (1995a) produced a live calf at LSU from oocytes recovered from FSH-stimulated pregnant, live cows using an ultrasound-guided technique. The oocytes were matured, fertilized and cultured *in vitro* until they could be transferred into suitable recipients. Two pregnancies resulted, with one going to term, resulting in the birth of a normal, healthy calf.

**Effect of Stage of Cycle on Gamete Transport**

Fertilization and subsequent embryonic development is a result of careful synchronization and timing of various events within the reproductive tract. Cilia within the oviduct are responsible for ovum transport through the oviduct in cattle (Ellington, 1991). Bovine oviductal cilia beat in a downward current, toward the uterus (Gaddum-Rosse and Blandau, 1976). Transport towards the uterus is slower in the fimbria and ampulla, but more rapid in the isthmus of the oviduct. Interestingly, there did not appear to be an effect of estrous phase on the current strength in any of the oviductal sections. However, when progesterone was administered to cows 24 hours following the end of estrus, ovum transport rate through the oviducts was almost doubled (Crisman et al., 1980). No effect was found when estradiol benzoate was administered in a similar manner.

Sperm transport in the reproductive tract is a function of sperm motility and also uterine and oviductal movement of sperm towards the ovum. Muscular activity in the uterus is very low during the luteal phase (Ruckebusch and Bayard, 1975). Activity
increases as estrus approaches as evidenced by electrical activity increasing in amplitude and frequency just prior to estrus, followed by strong, prolonged bursts during estrus and shorter, more frequent bursts after estrus.

At this point, cervical secretions also become more viscous. Oviductal muscular contractions and flow of fluids has been shown to move towards the ovary against the ciliary current in humans (Stone and Hamner, 1975), facilitating sperm movement towards the ovum. Bennet et al. (1988) found that oviductal muscular activity increased 3 to 5 days prior to estrus and decreased 3 to 5 days following estrus. During the luteal phase of the estrous cycle, muscular activity was very low. Muscular contractility was also consistently higher in the oviduct ipsilateral to the ovary containing the ovulatory follicle.

**Maternal Recognition**

It has long been recognized that close synchrony between the uterine environment and embryonic development was necessary to maintain pregnancy (Nicholas, 1933). In fact, pregnancies following transfer of embryos to cattle decrease dramatically as asynchrony between donor and recipient estrus increases (Rowson et al., 1972). In a normal bovine non-mated cycle, CL regression occurs following increases in PGF₂α late in the estrous cycle. The increase in PGF₂α requires a 10-day period of progesterone stimulation (Geisert et al., 1992) and is a result of oxytocin (OT) from the CL binding to OT receptors (induced by estrogen resulting from mid-cycle large follicles) in the uterine endometrium (McCracken et al., 1984), which in turn affect the production of arachidonic acid (AA) via secondary messenger systems.
Arachidonic acid is then converted to PGF$_{2\alpha}$ with help from the enzyme prostaglandin synthase (PGS).

Maternal recognition is the term used to describe the process by which the embryo signals the uterus so that luteolysis does not occur (Short, cited in Thatcher et al., 1984). In cattle, it appears that the signal responsible for inhibiting luteolysis and maintaining pregnancy is provided by interferon-τ (IFN-τ). IFN-τ is secreted by the trophectoderm of the developing conceptus and secretion increases as maternal recognition approaches (Roberts et al., 1992).

Presently, there are two pathways by which it is believed that IFN-τ may regulate PGF$_{2\alpha}$ production. First, it may inhibit PGF$_{2\alpha}$ production by inhibiting transcription of genes for molecules involved in the PGF$_{2\alpha}$ production pathway (Thatcher et al., 1997) and thus decreasing levels of PGF$_{2\alpha}$. IFN-τ may also stimulate production of molecules inhibitory to synthesis of PGF$_{2\alpha}$ (Thatcher et al., 1997).

**Asynchronous Embryo Transfer**

Consequently, embryos transferred asynchronously to recipients do not appear to signal the recipient uterine endometrium in a timely fashion in order to establish pregnancy. Rowson et al. (1972) recommended no more than a 24-hour differential between donor and recipient estrus in order to achieve successful pregnancies with bovine embryo transfer. Early research showed that embryos placed in earlier stage uteri (i.e., day-9 embryos on day-6 uteri) tended to retard their growth (rat: Dickman and Noyes, 1960; mouse: Doyle et al., 1963). Lawson et al. (1983) found that sheep embryos placed in less chronologically advanced uteri showed retarded growth and embryos placed in more chronologically advanced uteri showed accelerated growth up...
to day 12 of pregnancy, after which the embryos degenerated and were resorbed. Albihn et al. (1991) reported that when 7-day bovine embryos were transferred to recipients on day 4 (-3 day asynchrony), development 8 days following transfer was severely retarded when compared with controls, while development of embryos transferred to day 10 recipients (+3 day asynchrony) was significantly advanced when compared with development of controls.

These phenomena could be interpreted as attempts by the embryo to reach the same stage as the uterus in which it was placed. Ashworth and Bazer (1989) found that ovine embryos placed in less chronologically advanced uteri caused a shift in the secretory protein profile of the uterus to a more advanced stage, apparently in an attempt to catch up with the embryo. Therefore, it appears that when asynchronous transfer occurs, there is a concerted effort between the uterine environment and the embryo to become synchronized, although this process is not always successful.

**Effect of Progesterone on Embryo Development**

Timing of exposure of the reproductive tract to progesterone appears to be important in survival of the developing embryo. Progesterone levels are highly correlated to IFN-τ production by conceptuses (Kerbler et al., 1997). Progesterone levels have been found to be elevated in pregnant vs. nonpregnant cows as early as day 10 of the estrous cycle (Łukaszewska and Hansel, 1980; Lamming et al., 1989).

However, progesterone given to inseminated cows on days 1 to 5 of their cycle tended to accelerate embryonic development (Garret et al., 1988). This did not appear to affect maternal recognition, as pregnancy rates were similar to controls at 40 days of gestation. In sheep, progesterone given to ewes on days 1 to 3 of an inseminated cycle
caused reduced pregnancy rates and greater fetal weights on day 74 of pregnancy (Kleemann et al., 1994).

Conversely, it appears that progesterone priming can affect the uterine environment and make the uterus more accepting of later stage embryos. Although progesterone treatment early in the estrous cycle tended to accelerate development of embryos in mated cycles, priming recipients with progesterone early in a nonmated cycle appears to prepare the uterine environment to accept later-stage embryos. Lawson and Cahill (1983) administered progesterone during days 0 to 3 of the estrous cycle of sheep and achieved acceptable pregnancy rates when they placed day-10 embryos in day-6 progesterone-treated recipient ewes. Pool et al. (1987) treated recipient mares with a synthetic progestin beginning the day that they ovulated and found that the highest pregnancy rates (50%) were achieved in mares that had been treated for 2 to 5 days when they received day-6 or day-7 embryos. Geisert et al. (1991) treated bovine recipients with progesterone on days 1 to 5 of their estrous cycles and found no difference in pregnancy rates when 8-day embryos were transferred to day-5 progesterone treated recipients compared with 8-day embryos into day-8 nontreated controls.

However, the effect of progesterone exposure on the embryo is still not fully understood. Thatcher et al. (1989) produced asynchronous pregnancies by using buserelin, a GnRH agonist, on recipients. It was hypothesized that mid-cycle follicles were necessary for luteal regression (providing estrogen which increases OT receptors) and therefore proper maternal recognition. Disrupting follicular dynamics would therefore prolong the CL lifespan and allow the embryo to catch up with the CL.
Recipients were administered buserelin on day 12 following estrus and treatment was repeated every 3 days until 12 after embryo transfer. Embryos were placed in day-9.5 to -10.5 recipients, day-11 to -12.5 recipients and day-13 to -15 recipients. In the first group, 3 of 3 recipients became pregnant (60 to 84 hours asynchronous). In the second group, 3 of 7 recipients became pregnant (96 to 132 hours asynchronous) and in the third group, 1 of 8 recipients became pregnant. This recipient was in estrus 14.5 days prior to embryo transfer (180 hours asynchronous).

**Physical Barriers to Superfetation**

Another barrier to the concept of superfetation in cattle is purely physical. The bovine conceptus completely occupies the gravid horn by 18 to 20 days of pregnancy and by 24 days has partially occupied the contralateral horn (Chang, 1952; King *et al.*, 1982; Thatcher *et al.*, 1986). By 27 days of pregnancy, most of the uterine epithelium is in contact with the placenta (Leiser, 1975, cited in King *et al.*, 1982). Caruncular and intercaruncular regions are in close contact with the choriallantoic mucosa until term (King and Atkinson, 1987). However, although placentomes are present in the contralateral horn, attachment seems to be very loose (King and Atkinson, 1987). King and Atkinson (1987) found no evidence of attachment at the utero-tubal junction of the contralateral horn.

Allantoic fluid increases dramatically between days 27 and 33 (4 ml to 45 ml), forcing contact between the conceptus and the uterine endometrium in preparation for eventual implantation (Eley *et al.*, 1979). Two problems are immediately obvious for successful fertilization and embryonic development to occur in a pregnant cow: the ability of sperm (regardless of hormonal milieu) to reach the site of fertilization and
subsequent implantation and continued development of a newly formed pregnancy, if fertilization and early embryonic development were successful.

**PRODUCTION OF TWINS BY EMBRYO TRANSFER**

*Embryo Transfer to Mated Recipients*

There are many reports of embryo transfer following artificial insemination or natural breeding of recipients to produce twins. Results have been variable. Testart *et al.* (1975) transferred embryos using a nonsurgical transvaginal embryo transfer technique to 17 cows that had been previously inseminated and 11 (65%) became pregnant, with 3 of 11 (27%) producing twins at birth. Renard *et al.* (1979) achieved 44% twinning rates at calving by nonsurgical transcervical transfer of day-10 embryos to the horn contralateral to the CL of day-10 to -12 inseminated recipients. Heyman *et al.* (1980) reported 50% twinning rates at calving using similar procedures.

Sreenan and McDonagh (1979) transferred bovine embryos to the contralateral horn of inseminated recipients and slaughtered the recipients at 30 to 42 days of pregnancy to recover the fetuses. A 60% twinning rate was reported at this stage of pregnancy (9 of 15 females), with 2 of 6 singleton pregnancies (33%) resulting from the contralateral transfer.

In an interesting study, Holy (1980) surgically transferred embryos to the ipsilateral horn in previously inseminated cows and achieved 8 of 19 (42%) twinning at calving, similar to other reports. However, of the remaining single pregnancies, eight calves resulted from the transferred embryo. In this same report, embryos were transferred to the horn contralateral to the CL in inseminated cows and a twinning rate
of 80% (4 of 5) at calving was achieved. The only singleton calf born also resulted from a transferred embryo.

Sreenan et al. (1981) used an AI gun to nonsurgically transfer embryos to the base of the horn contralateral to the CL in cows that had been inseminated at 48 and 72 hours following removal of a progesterone-impregnated sponge (in place for 9 days). Pregnancy rates were 58%, with 1.4 calves produced per calving recipient. In recipients in whom it was possible to distinguish AI-produced or ET-produced calves, 7% of singleton calves resulted from the embryo (transferred to the horn opposite to the CL). Sreenan and Diskin (1989) transferred day 7 embryos surgically to the horn ipsilateral or contralateral to the CL in inseminated heifers and found no difference in twinning rate at 53 days of pregnancy or at calving (55% vs. 60% and 50% vs. 52%, respectively). There was also no difference in pregnancy rates for transfer of embryos nonsurgically into the same or opposite uterine horn as the ongoing pregnancy in inseminated cows (33% vs. 38%). The apparent difference in surgical vs. nonsurgical transfers or heifers vs. cows cannot be compared, since these results were from different experiments. McEvoy et al. (1995) transferred in vitro-produced embryos to the contralateral horn of previously inseminated recipients and reported 35% twinning at calving.

Transfer of Two Embryos

Twins were first induced successfully with embryo transfer in cattle by Rowson et al. (1969a,b). In a later study, Rowson et al. (1971) reported 73% and 45% twinning rates for cows receiving two embryos in either different or same uterine horns. This seems to confirm the observations of Gordon et al. (1962), who reported in a study
in which eCG was administered to cows prior to estrus that twins resulting from ovulations on opposite ovaries had a greater chance of survival (62% vs. 29%). Newcomb et al. (1980) transferred embryos to different combinations of location (tip or base of horn) in opposite horns and found that the highest embryo survival rates (35 days of pregnancy) occurred when an embryo was transferred to the tip of the horn ipsilateral to the CL. Embryo transfer site on the contralateral horn had no effect on pregnancy rate or twinning rate. There are many reports confirming early studies when transferring two embryos to the same (Lu et al., 1989) or opposite horns (Sreenan and Beehan, 1974; Sreenan et al., 1975; Anderson et al., 1978, 1980).

Lu et al. (1989) reported acceptable twinning rates at calving (45%) for unilateral transfers of two in vitro-produced (IVP) embryos. Reichenbach et al. (1992) found no differences when they transferred IVP embryos either bilaterally or unilaterally (42% and 33%) but reported that even though twinning rates were similar, fetal loss was greater when two embryos were transferred to the same horn (22% vs. 6%). McEvoy et al. (1995) transferred two IVP embryos to the uterine horns ipsilateral or contralateral to the CL of recipients and reported 36% and 29% twinning rates at calving, respectively. The different methods were utilized in subsequent years. Takada et al. (1991) reported a similar twinning rate (29%) at calving for IVP embryos transferred bilaterally to the uterine horns. Sakakibara et al. (1996) reported similar rates of fetal loss following the transfer of two frozen-thawed embryos to the same side in Holstein and Japanese Black cattle (29% and 32%, respectively). On an interesting note, it was found that twinning rate at calving was higher for Holstein recipients than
Japanese Black recipients. This difference was attributed to differences in uterine size and capacity.

**Calving Difficulty**

Gestation lengths for twins are generally shorter than single pregnancies for both *in vivo*-produced (Anderson *et al.*, 1978; Davis *et al.*, 1989; Reichenbach *et al.*, 1992) or IVP twins (Vasques *et al.*, 1995). Twin pregnancies may also cause an increase in retained placentae (Anderson *et al.*, 1978; Renard *et al.*, 1979; McEvoy *et al.*, 1995). Although there are some reports of an increase in calving difficulty with twinning, others report no such effect (for review see Anderson, 1978). McEvoy *et al.* (1995) found that calving difficulty varied over the three years studied between single and twin pregnancies with IVP embryos. However, calving difficulty was positively correlated to birthweight for both single and twin pregnancies. Interestingly, there was a decrease in large birthweights and calving difficulty over the 3 years studied and it was noted that this may have been due to improvements in the production of embryos *in vitro*. Anderson (1978) suggested that intense management of twin calvings could decrease loss of calves due to calving difficulties.

**Freemartinism**

One of the problems associated with twinning is freemartinism. A freemartin is a sterile female born co-twin to a male. This syndrome has been reported in sheep (Saba *et al.*, 1977; Wilkes *et al.*, 1978), goats (Smith and Dunn, 1981) and pigs (Cukierski, 1989), although occurrences in these species are rare. It is more common in cattle, occurring in 92% of heterosexual twin pairs (Long, 1990).
The condition is characterized by changes in female reproductive organs such as differentiation of ovaries into testis or even possession of reproductive organs of both sexes (Khan and Foley, 1994). Freemartins in cattle may lack a uterus or cervix (Kennedy and Miller, 1993). It was previously thought that these changes occurred due to exchange of male hormones between the twins via anastomoses of placental vessels of the twins (Lillie, 1917), as male differentiation occurs earlier than female differentiation (Jost et al., 1972). It does appear that the degree of freemartinism depends on when and if anastomoses develop between the placentae of the male and female twins (Long, 1990). However, although androgens can cause masculinization, they do not inhibit the ovaries or affect the differentiation of the Mullerian ducts (Kennedy and Miller, 1993). It has been reported that anti-Mullerian hormone (or Mullerian inhibiting substance – MIS) produced by the testis of the male fetus is present in peripheral blood of the female fetus, thus, preventing development of the Mullerian ducts into oviducts, uterus, cervix and vagina in the female fetus (Vigier et al., 1984).

Hematopoietic cells and other substances are also exchanged via the shared placental vessels. Herschler et al. (1966) showed that freemartins (both male and female) were chimeric, containing both XX and XY leukocytes in peripheral blood, due to cross-colonization of hematopoietic cells from each twin. The expression of the testis determining factor (TDF) by the Y chromosomes present in the female is thought to be responsible for the sterilization of the freemartin (Kennedy and Miller, 1993). Interestingly, in one study 93% of heifers born co-twin to bulls that were fertile contained no evidence of XX/XY chimerism in cultured leukocytes (Eldridge and Blazak, 1970).
Laster et al. (1971) also found that the degree of masculinization varied depending on female:male ratios in superovulated females allowed to go to term. Freemartins resulting from multiple fetus pregnancies with a higher female to male ratio had a higher ratio of XX to XY chromosomes following chromosomal analysis of leukocytes than those which had an equal or lower female:male ratio.

It must be noted that early tests for chimerism were not 100% effective as at least 100 cells were needed to be 98% accurate (Dunn and Johnson, 1981) and that early tests for Y chromosome may not have been sensitive enough to detect chimerism in all freemartins. The advent of the polymerase chain reaction (PCR) method has made it easier to detect chimerism even when only a few cells were available. Fujishiro et al. (1995) used PCR to successfully detect chimerism in an animal with less than 0.1% XY cells. Kadokawa et al. (1995) used PCR to positively identify a heifer as a freemartin that had been born as a singleton following transfer of two embryos to a recipient. No twin was detected by ultrasound at 34 days of gestation, so a twin must have been present prior to this date, resorbing prior to detection by ultrasound. Anastomoses must have formed prior to this date, allowing exchange of XY cells. Although these cells were not detected using a simple chromosomal analysis technique, PCR allowed for detection of the chimerism. The heifer showed no obvious signs of freemartinism, as all reproductive organs appeared normal. However, following histological examination of the ovaries, structures resembling seminiferous tubules were detected alongside apparently normal Graafian follicles.
OBJECTIVES

Superfetation has been reported in many species, including cattle, although these reports are viewed with skepticism. Other phenomena have been used to attempt to explain at least part of these reports, such as embryonic diapause and differentiated growth of twins. The hypothesis of the experiment is that once a viable fetus has assured maternal recognition and the continuation of pregnancy, additional embryos can develop in a more advanced uterine environment. The objective of this study is experimentally induce superfetation by (1) induction of ovulation followed by mating in pregnant cows and (2) asynchronous transfer of uterine-stage embryos to cows in an advanced stage of pregnancy.
CHAPTER II

OVULATION INDUCTION FOLLOWED BY ARTIFICIAL INSEMINATION DURING PREGNANCY IN BEEF CATTLE: AN ATTEMPT TO PRODUCE SUPERFETATION

INTRODUCTION

Superfetation is believed to occur when a pregnant animal comes into estrus and is mated again, with the ensuing pregnancy occurring in addition to the previous pregnancy (Long, 2001). Therefore, the fetuses would be from different ovulation cycles and conception times (Hurnik et al., 1995). This has been reported in several animals, such as the mouse (Littleford and Gysin, 1944; Barnett and Munro, 1970), the rat (Slonaker, 1934), the rabbit (Pickard, 1928), the cat (Jepson, 1883; Hoogeweg and Folker, 1970), the sheep (Smith, 1927; Scanlon, 1960), the pig (Smith, 1927; Larivée, 1972), the buffalo (Rao et al., 1987), the burro (Short, 1964), the horse (Mumford, cited by Leroy and Pechdo, 1950), the monkey (Leakey, 1969) and man (Scrimgeour and Baker, 1974).

There have been reports of superfetation in cattle where cows calved at intervals of 64 to 92 days or aborted twins of different ages (Dalrymple and Jenkins, 1951; Simmons, 1960; Gee, 1971; Hall, 1987). These reportedly occurred following successive natural matings (Dalrymple and Jenkins, 1951; Gee, 1971), natural mating followed by artificial insemination (AI) (Simmons, 1960) and embryo transfer followed by natural mating (Hall, 1987; Hunsley, 1998).

Several alternative explanations for apparent cases of superfetation have been offered. Vandeplassche (1969) reviewed several cases of apparent superfetation in pigs and concluded that the parturition intervals, which ranged from 4 to 98 days, could be
explained as cases of embryonic diapause. However, embryonic diapause has been found in over 70 eutherian and 30 marsupial species (Renfree and Shaw, 2000) but the only known ungulate to exhibit this phenomenon is the Roe deer (*Capreolus capreolus*), first observed in 1651 by William Harvey (cited by Eckstein *et al*., 1959).

Kuntz (1920) reported two cases involving cats and one involving a dog where fetuses of different sizes were recovered at necropsy or surgery and credited the size difference to death of some of the fetuses without resorption. In humans, differentiated growth of twins has been well documented with the advent of ultrasound technology (Kol *et al*., 1993; Weissman *et al*., 1993). In most cases, growth difference between twins is usually associated with congenital malformations of the smaller twin, which seldom survives until term (Kol *et al*., 1993). Growth differences between twins for various reasons could therefore explain some of the superfetation cases reported.

There are other obstacles to the concept of superfetation. Sir Walter Heape (1890) produced the first successful embryo transfer by transferring embryos from an Angora rabbit doe (collected 32 hours following mating) to a Belgian doe that had been mated 3 hours prior to the transfer. Thus, although there was a 29-hour differential in age of the embryos implanting in the Belgian doe, they were still relatively synchronous. This concept was has been shown to hold true for all species studied, such as sheep (Moore and Shelton, 1964; Rowson and Moor, 1966) and cattle (Rowson *et al*., 1972). In these species, pregnancy rates declined as embryo-uterus synchrony decreased.

Researchers have found that embryos placed in earlier stage uteri (i.e., day-9 embryos on day-6 uteri) tended to retard their growth (rat: Dickman and Noyes, 1960; mouse: Doyle *et al*., 1963; sheep: Lawson *et al*., 1983; cattle: Albihn *et al*., 1991). The
reverse was also true, as younger embryos placed on older stage uteri accelerated their growth (sheep: Lawson et al., 1983; cattle: Albihn et al., 1991). These phenomena could be interpreted as attempts by the embryo to reach the same stage as the uterus in which it was placed to ensure maternal recognition and subsequent survival.

Bovine embryos can develop successfully in nature in the uterine horn contralateral to the CL if a fetus is developing in the ipsilateral uterine horn, such as in the case of twins (Scanlon, 1972). This has been shown by experiments with the transfer of embryos to opposite uterine horns or to the contralateral uterine horn of previously inseminated recipients (Rowson et al., 1971; Anderson et al., 1978; Sreenan and McDonagh, 1979; Renard et al., 1979; Heyman et al., 1980; Sreenan and Diskin, 1989; Takada et al., 1991; McEvoy et al., 1995). Therefore, it seems possible that superfetation could occur in nature if maternal recognition were accomplished by the first fetus, allowing the secondary conceptus to develop.

In nature, it would be necessary for the female to come in to estrus during pregnancy for superfetation to be possible. There are many reports of pregnant cattle standing to be mounted during the normal course of a successful pregnancy. Williams (1921) estimated that 1 to 2% of all pregnant cows exhibit signs of estrus during pregnancy. Bullard (1934) presented data on 10 cows exhibiting various signs of estrus during pregnancy, some of which stood to be mounted by a bull. Donald (1943) studied mating records on 1,072 dairy cows in Great Britain and found 36 cows (3.4%) that were mated at least once during an ongoing pregnancy. Branton (1949) reviewed data on 1,855 natural services in cattle belonging to the Louisiana State University dairy herd over a period of years and found 64 cases (3.4%) of natural mating occurring in cows.
already pregnant. Erb and Morrison (1958) studied records on dairy cows belonging to the Carnation Milk Farm from 1920 to 1950 and found 378 of 6,751 (5.6%) cases of pregnant cows being mated during an ongoing pregnancy. In this report, 42.5% of all cases occurred within 25 days of mating, though intervals of 2 days to over 200 days were noted.

Estrus during pregnancy has also been observed on four separate occasions at this laboratory. During the course of an experiment, one pregnant cow stood to be mounted twice, once at 56 days and again at 80 days of a successful gestation (personal observation). Another female presented signs of estrus, including standing to be mounted by other cows, at 289 days of gestation and calved the next day (personal observation). A third cow observed in estrus at 42 days of a gestation resulting from embryo transfer was still pregnant at 49 days when this recipient was slated for a second embryo transfer attempt (personal observation).

Estrus during pregnancy would have to be accompanied by a successful ovulation, along with other conditions necessary for sperm and ovum transport, for superfetation to be possible. In cattle, follicles grow in cohorts in a wave-like manner during the estrous cycle, with one dominant follicle per wave and two to four waves per cycle (Ireland and Roche, 1983; Ireland, 1987; Savio et al., 1988; Sirois and Fortune, 1988). Dominant follicles that grow during the luteal phase become atretic and regress, while the dominant follicle that is growing at the time of CL regression becomes the ovulatory follicle (Savio et al., 1988; Sirois and Fortune, 1988; Ginther et al., 1989).

Follicular waves continue throughout pregnancy in cattle (Ginther et al., 1989; Bergfelt et al., 1991; Taylor and Rajamahendran, 1991; Ginther et al., 1996). However,
no cases of ovulation during pregnancy in cattle were found in the scientific literature. The apparent lack of ovulation during pregnancy in cattle could be due to the fact that progesterone levels in cattle rise following ovulation and remain elevated throughout pregnancy (Randel and Erb, 1971). Elevated progesterone levels block the pre-ovulatory surge of FSH and LH (Kesner et al., 1982) and, therefore, could prevent ovulation in pregnant cows.

However, superfetation has been induced experimentally in some animals by using different agents to induce ovulation in pregnant females. Wislocki and Snyder (1931) induced ovulation in a pregnant rabbit doe by injecting anterior pituitary lobe extract 44 hours following natural mating. After 4 hours, the doe was artificially inseminated with fresh spermatozoa. The doe was euthanized 29 hours later and normal developing blastocysts were recovered from the uterus and 3-cell to 5-cell embryos were recovered from the oviducts. Edwards and Fowler (1958) induced superfetation in mice using equine chorionic gonadotropin (eCG) for stimulation and human chorionic gonadotropin (hCG) for ovulation 2.5 to 3.5 days apart. Males used for mating were from marker strains so that offspring would be easily recognizable. Four females necropsied 18 hours following the second ovulation had morulae and/or blastocysts along with recently ovulated pronucleate ova. Two females necropsied 18 days after insemination (21 days after initial mating) were found to be carrying offspring from both matings. One female delivered pups from both matings at parturition.

Johansson and Venge (1951) successfully induced superfetation in the mink by mating different males to females at intervals of 8 to 19 days. Mink are induced ovulators and exhibit delayed implantation (Hansson, 1947; Mead 1993). Gestation
length normally varies according to the time of mating, so blastocysts resulting from earlier matings apparently implanted at the same time as blastocysts resulting from the latter matings.

In humans, superfetation was induced unintentionally in a woman with consecutive clomiphene citrate (CC) treatments for ovulation induction (Bsat and Seoud, 1987). A patient that had been treated with CC for induction of ovulation underwent CC treatment a second time after the first treatment had apparently failed to produce a pregnancy. Ultrasonography at 25 weeks from the first CC treatment revealed twins, one showing appropriate characteristics for a 25-week gestation and the other presenting the characteristics of a 22-week fetus, corresponding to the second CC treatment. The twins were born at 38 weeks from the first CC treatment; one was normal, while the other showed signs of prematurity and had physical characteristics that indicated less than 36 weeks intrauterine gestation.

Ovulation induction has also been used successfully to produce a pregnancy in a mare with a persistent CL (Hughes and Stabenfeldt, 1977). The mare displayed classical signs associated with persistent CL, such as lack of estrus and elevated levels of progesterone, similar to levels normally associated with diestrus. Attempts to lyse the CL with prostaglandin were not successful, so the mare was treated with hCG and artificially inseminated, resulting in a pregnancy. Interestingly, progesterone levels remained elevated throughout treatment.

Although there are no reports of attempts to produce superfetation in cattle by inducing ovulation during pregnancy, secondary ovulations can be induced in cattle at different stages of pregnancy using various ovulatory inducing hormones such as hCG or
LH. Rajamahendran and Sianangama (1992) found 1,000 IU of hCG to be very effective in causing ovulation when given on day 7 of pregnancy (7 of 9 treated dairy heifers ovulated), but less effective on day 14 of pregnancy (4 of 9 treated dairy heifers ovulated). It was noted that the results could be attributed to the fact that the dominant follicle present at day 7 of the estrous cycle was less likely to be atretic and has more LH receptors present and was more likely to be responsive to ovulation induction by hCG.

Lulai et al. (1994) successfully induced ovulation using exogenous LH treatment in pregnant beef cows after day 35 of pregnancy. In this case, the cows received norgestomet implants and luteal regression was induced 2 days later with prostaglandin (cloprostenol). LH was administered 3 days following the cloprostenol injection (following the confirmation of CL regression), inducing ovulation and secondary CL formation in all of the treated cows. Norgestomet implants were removed after 20 days. Pregnancy was maintained in all cows in which the new CL formed ipsilaterally to the previous CL following removal of the norgestomet implants. However, cows in which the induced CL formed on the contralateral ovary lost their pregnancies (only 1 of 6 remained pregnant).

In a similar study, Bridges et al. (2000) regressed the CL of pregnancy and maintained pregnancy with exogenous progestins in a group of beef cows prior to day 36 of pregnancy and then treated them with 2,500 IU of hCG treatment alone or in conjunction with 10 mg of FSH 60 hours prior to hCG treatment. Ovulation occurred in 58.8% of the treated cows. Pregnancy was maintained in 78.9% of cows in which the induced CL formed ipsilaterally to the regressed CL of pregnancy following cessation of
exogenous progesterone treatment. However, all cows in which the secondary CL formed contralaterally to the regressed CL of pregnancy lost their pregnancies.

Oocytes from follicles present during pregnancy have the capability of being fertilized and becoming viable embryos. Embryo development in vitro from oocytes recovered from pregnant cows is similar to that of oocytes from nonpregnant cows (Behboodi et al., 1992; Ryan et al., 1993). Meintjes et al. (1995a) used oocytes recovered from FSH-stimulated pregnant cows using an ultrasound-guided technique to produce embryos in vitro at LSU that resulted in the birth of a normal, healthy calf.

The hypothesis of this experiment is that ovulation induction in mated bovine recipients followed by AI will produce superfetation. The first objective of this experiment is to induce ovulation with hCG treatment on day 7 and day 25 of pregnancy. The second and primary objective is to produce secondary pregnancies (superfetation) using timed AI following ovulation induced by hCG treatment.

MATERIALS AND METHODS

Experimental Animals

Cattle used in this experiment were part of the Louisiana State University, St. Gabriel Experiment Station reproductive physiology research herd. This herd consists of crossbred beef cattle with varying degrees of Bos indicus (Brahman) and Bos taurus (Angus, Hereford, Gelbvieh and/or Charolais) breeds. Only parous cows between 3 and 10 years of age with body condition scores (BCS) of 5 through 7 were used in this study.

Experimental Design

Cows were synchronized with one prostaglandin treatment at two different times so that all groups would receive treatment at the same time to reduce seasonal effects.
Cows (n = 33) that would be treated on day 25 following AI (Groups 2 and 3) were treated with prostaglandin to induce estrus prior to the cows (n = 19) that would be treated on day 7 following AI (Group 1).

Following estrus synchronization, cows that showed signs of estrus in the herd were mated to bulls of similar coat color patterns and further randomly assigned to groups as follows: (Group 1) cows to be artificially inseminated following administration of 1,000 IU of hCG 7 days after being mated at estrus (controls); (Group 2) cows to be inseminated on day 24 of pregnancy following administration of 1,000 IU of hCG; and (Group 3) cows to be inseminated on day 24 of pregnancy following administration of 3,000 IU of hCG (Figure 2.1).

Pregnancy was determined by ultrasound evaluation prior to treatment for cows in Groups 2 and 3 and nonpregnant cows were removed from the study. Since pregnancy could not be determined prior to treatment in Group 1, cows initially synchronized for AI were removed from this group prior to AI for ovarian abnormalities such as follicular cysts (Table 2.1).

**Experimental Procedures**

Estrus synchronization was accomplished with 25 mg of prostaglandin F$_{2\alpha}$ (Lutalyse®, Pharmacia & Upjohn, Kalamazoo, MI) in a single treatment. All cows in the herd exhibiting signs of estrus at the appropriate time were inseminated regardless of whether estrus resulted from synchronization.

Frozen thawed semen from bulls (n = 3) of different breeds with similar coat color patterns to each female was used to AI each female so that offspring from this initial mating would have the same coat color at birth as its dam. Black cows were mated to a
Figure 2.1. Cows in Groups 2 and 3 were initially mated 14 days prior to cows in Group 1 so that treatment with human chorionic gonadotropin (hCG) followed by the second artificial insemination (AI) 16 hours post-hCG would occur at the same time for all groups. The hCG treatment followed detection of at least 1 follicle ≥10 mm on either ovary in each female.
Table 2.1. Cow assignment to the treatment groups

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>1st mating (n)</th>
<th>Females removed (n)</th>
<th>hCG + 2nd mating (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>20</td>
<td>4†</td>
<td>16</td>
</tr>
<tr>
<td>Group 2</td>
<td>9</td>
<td></td>
<td>9</td>
</tr>
<tr>
<td>Group 3</td>
<td>26‡‡</td>
<td>13‡‡</td>
<td>4</td>
</tr>
</tbody>
</table>

†Females were removed from Group 1 for cystic ovarian syndrome or failure to develop a follicle ≥10 mm during the treatment period.
‡‡Females to be assigned to Groups 2 and 3 were synchronized together for the initial mating, and randomly assigned to either group following ultrasonic detection of pregnancy.
‡‡‡Females were removed from Groups 2 and 3 for failure to establish a pregnancy following the initial mating.
Holstein sire and cows with other color patterns were mated to a Gelbvieh bull or a Polled Hereford bull. All bulls used in this study were of proven fertility.

Follicular status following estrus synchronization was monitored by transrectal ultrasonography using an ultrasound equipped with a 5 MHz linear rectal probe (Aloka 500-V, Corometrics, Wallingford, CT). hCG was administered i.v. when the largest follicle on either ovary reached a minimum of 10 mm in diameter. Monitoring of the ovaries for follicular status and appearance of secondary CL began on day 5 of the estrous cycle for Group 1 and on day 23 of pregnancy for Groups 2 and 3. Ovulation was determined to have occurred if ultrasound scanning initially revealed the disappearance of the follicle followed by of the appearance of accessory CL. One cow in the group synchronized for Groups 2 and 3 did not develop a follicle ≥10 mm during the course of ultrasound evaluation and was removed from the experiment.

Sirois (1994) reported the interval between hCG administration and ovulation in cattle to be 24 to 28 hours so the cows were artificially inseminated the second time 16 hours following hCG administration. For this second mating, black cows were mated to a Gelbvieh bull or a Polled Hereford bull and cows with all other color patterns were mated to a Holstein sire so that offspring resulting from the first mating would be distinguishable from offspring resulting from the second mating.

Circulating progesterone levels were used to support ultrasound pregnancy data and to determine the effect of hCG treatment on circulating progesterone levels. Blood samples were obtained by venipuncture of the jugular vein for subsequent hormone analysis. The samples were centrifuged at 300 x g for 10 minutes and the resulting plasma was poured off into plastic tubes and frozen for analysis at a later time. Plasma
samples were analyzed following extraction with acetone for circulating progesterone levels using a commercially available progesterone assay kit (Diagnostic Systems Laboratory, Webster, TX).

**Statistical Analysis**

Plasma progesterone levels were analyzed across treatment groups using repeated measures analysis of variance (ANOVA). Only pregnant cows for each group were used for comparison of plasma progesterone levels, as only pregnant cows were available for analysis in Groups 2 and 3. Pregnancy rates and ovulation rates were compared using Fisher’s Exact Test.

**RESULTS**

All of the cows in Group 1 (16 of 16) receiving 1,000 IU of hCG 5 to 8 days after inseminated estrus ovulated and developed accessory CL regardless of pregnancy status. However, none of the pregnant cows (n = 9) receiving 1,000 IU of hCG on 23 to 29 days of pregnancy (Group 2) ovulated. For pregnant cows in Group 3 that received 3,000 IU of hCG on days 23 to 29 of pregnancy (n = 4), only one ovulated and developed an accessory CL (25%) on the ovary ipsilateral to the CL of pregnancy following hCG administration on day 23 of pregnancy. This cow eventually lost her pregnancy.

Plasma progesterone levels increased within 2 days after hCG treatment for cows in Group 1 and Group 2 (P<0.05) (Figure 2.2). However, plasma progesterone levels did not increase following treatment with 3,000 IU of hCG for Group 3. Although ovulation occurred in 100% of the pregnant cows in Group 1 treated with 1,000 IU of hCG 5 to 8 days following estrus, superfetation did not result in this or any of the other groups.
Figure 2.2. Plasma progesterone (P₄) levels (±SEM) for mated recipients on the day of human chorionic gonadotropin (hCG) treatment and 2 days following hCG treatment (hCG + 2 d). (*) Denotes a significant difference within groups (P<0.05).
The initial pregnancy rate on day 28 post-estrus was 62.5% (10 of 16) for Group 1, 60% (9 of 15) for Group 2 and 42.9% (3 of 7) for Group 3 (Figure 2.3). By day 60 of pregnancy, one cow in Group 1 had lost her pregnancy, resulting in a pregnancy rate of 56.2% (9 of 16). However, pregnancy rates dropped dramatically to 20% (3 of 15) for Group 2 and 14.3% (1 of 7) for Group 3 by day 60.

In summary, for Group 1, only one cow receiving 1,000 IU of hCG and mated on day 5 to 8 post-estrus lost her pregnancy between day 28 and day 60 of gestation. In contrast, 6 of 9 pregnant cows receiving 1,000 IU of hCG and artificially inseminated at 23 to 29 days of pregnancy (67%) lost their pregnancies by day 60 post-estrus. In fact, the pregnancy rate on day 28 was significantly different than the pregnancy rate on day 60 for Group 2 (P<0.05). In Group 3, 2 of 3 (67%) pregnant cows receiving 3,000 IU of hCG and artificially inseminated on days 23 to 29 of pregnancy lost their pregnancies.

**DISCUSSION**

In this experiment, all pregnant and nonpregnant cows (n = 16) receiving 1,000 IU of hCG between days 5 and 8 following mating ovulated. Similar results have been published with nonpregnant (Price and Webb, 1989; Sianangama and Rajamahendran, 1996) and pregnant cows (Rajamahendran and Sianangama, 1992). Price and Webb (1989) found different responses to hCG during the estrous cycle, with good success on days 4 to 7 (83% ovulation) and 14 to 16 (66% ovulation) of the cycle but poor success on days 8 to 13 of the cycle (35% ovulation). Furthermore, Rajamahendran and Sianangama (1992) reported that pregnant cows administered hCG on day 14 of their estrous cycles did not ovulate as frequently as those that received hCG on day 7 (44% vs. 78%). In both studies, the differences were believed to have been caused by the
Figure 2.3. Pregnancy rate on day 28 and day 60 of gestation for cows in Group 1 (n = 16) receiving 1,000 IU of human chorionic gonadotropin (hCG) on day 7, cows in Group 2 (n = 15) receiving 1,000 IU of hCG on day 25 and cows in Group 3 (n = 7) receiving 3,000 IU of hCG on day 25 of gestation. (*) Denotes a significant difference within groups (P<0.05).
presence or lack of growing dominant follicles responsive to hCG at the time of treatment.

In the present experiment, cows were only treated with hCG after the appearance of a new follicle $\geq 10$ mm on one of the ovaries so that a responsive growing dominant follicle would be present at the time of hCG treatment. Even using this criterion, only one cow receiving 3,000 IU of hCG on day 23 of pregnancy (Group 3) ovulated and formed a secondary CL, while none of the cows administered 1,000 IU of hCG (Group 2) on or after day 23 of pregnancy ovulated.

Other researchers have successfully induced ovulation later in pregnancy. Lulai et al. (1994) reported induction of ovulation following two LH treatments on days 33 and 34 of pregnancy in 10 of 10 cows in which pregnancy was maintained with norgestomet implants following induced luteal regression 5 days prior to LH treatment. Bridges et al. (2000) achieved 50% ovulation following a single treatment with 2,500 IU of hCG in cows on days 28 to 31 of pregnancy. Once again, CL had been removed or regressed during early pregnancy in these cows and pregnancy was maintained with exogenous progesterone treatment. It is likely that new follicular waves with less atretic, more responsive follicles were present at the time of ovulation induction following CL removal in these experiments.

Initial pregnancy rates were similar for all groups. However, pregnancy rates were markedly lower on day 60 post-estrus for Groups 2 and 3, but not for Group 1. Pregnancy loss occurred between 37 and 47 days of pregnancy, around the time of implantation, as evidenced by ultrasound scanning and decreasing levels of plasma progesterone.
Treatment with hCG was implicated, but not confirmed, as a cause of early CL regression in at least one cow in a study by Schomberg et al. (1967). Furthermore, Allen (1974, 1975) reported that treatment with hCG caused pregnancy loss in pony mares following repeated injections of 2,000 IU of hCG between day 27 and day 38 of pregnancy and by a single injection of 2,000 IU of hCG in one mare on day 24 of pregnancy. Pregnancy loss did not occur in mares in which treatment was initiated after day 38 of pregnancy (Allen, 1975). It had been previously reported that hCG treatment during diestrus in the mare caused a temporary decrease in the concentration of progesterone in peripheral blood (Allen, 1973, cited in Allen, 1975), so it was proposed that the decrease in circulating progesterone levels may have caused pregnancy loss in the pony mares. It was suggested that this failure for hCG to induce pregnancy loss after day 38 in mares might be due to the presence of eCG by this time (Allen, 1975).

However, Breuel et al. (1989) reported elevated progesterone levels within 3 days of hCG treatment on day 4 or 7 of the estrous cycle, although not when hCG was administered on day 10 of the estrous cycle. Similarly, Fricke et al. (1993) reported an increase in progesterone within 2 days in nonpregnant cows treated with hCG on day 6 when compared with nontreated cows. In the present experiment, plasma progesterone levels increased within 2 days following hCG treatment for pregnant and nonpregnant cows treated with hCG between 5 and 8 days post-estrus (P<0.05). Furthermore, hCG administered early in pregnancy in the present study did not seem to affect pregnancy loss, as the pregnancy rate on day 30 for Group 1 were similar to pregnancy rates for Groups 2 and 3.
Although the pregnancy rate at day 60 was lower than the pregnancy rate at day 28 for cows in Group 2, which received one treatment of 1,000 IU of hCG on days 23 to 29 post-estrus, plasma progesterone levels actually increased for cows in this group (P<0.05) within 2 days of treatment. Progesterone levels were not affected by hCG treatment for cows in Group 3.

An increase in progesterone levels following hCG treatment could be due to increased production of progesterone by the primary CL (Donaldson and Hansel, 1965; Veenhuizen et al., 1972; Rajamahendran and Sianangama, 1992). De novo production of progesterone by the secondary CL has also been implicated as a cause of increased progesterone following hCG treatment in dairy cattle (Rajamahendran and Sianangama, 1992). Morris et al. (1976) reported elevated progesterone levels in beef heifers within 5 days following treatment with hCG on day 4 following AI. However, Rajamahendran and Sianangama (1992) reported that although secondary CL were present on the ovaries of dairy heifers following hCG treatment on day 7 post-of the estrous cycle, progesterone levels only became significantly elevated 18 days post-AI.

hCG treatment later in pregnancy also does not appear to cause pregnancy loss (Bridges et al. (2000). In that study, the primary CL of pregnancy was removed and pregnancy was maintained with exogenous progestin treatment. Secondary CL were induced with hCG at 36 days of pregnancy. The secondary CL successfully maintained pregnancy in 17 of 25 pregnant cows (68%) following cessation of progestin treatment. Therefore, it is not likely that hCG treatment caused loss of pregnancy in the present experiment, although it cannot be ruled out as a possible cause.
Another possible cause of loss of pregnancies in Groups 2 and 3 is the AI procedure itself. The recto-cervical technique for AI in cattle involves grasping the cervix *per rectum* and passing an insemination gun or pipette through the cervix, subsequently depositing the semen in the body of the uterus. During pregnancy in cattle, the conceptus elongates quickly and by day 24 of pregnancy, has partially occupied the contralateral horn (Chang, 1952; King *et al.*, 1982; Thatcher *et al.*, 1986). Therefore, pregnancy loss could have been caused by disruption of fetal membranes, since there did not appear to be pregnancy loss from the treatment for cows in Group 1, which were artificially inseminated ~7 days following estrus. At this point, the conceptus has not yet begun to elongate.

Vandemark *et al.* (1952) compared mid-cervical insemination of pregnant cows with uterine body deposition of semen and found that uterine deposition of semen with antibiotics caused loss of pregnancy in 33% of inseminated cows, while mid-cervical insemination did not result in loss of pregnancy. Weaver *et al.* (1989) also found that reinsemination into the uterine body of pregnant cows with semen processed by current standards resulted in a markedly lower pregnancy rate at 35 to 45 days after the initial mating than cows that did not undergo reinsemination (4% vs. 40.6%).

Introduction of different types of bacteria at insemination has also been implicated in loss of pregnancy following insemination during pregnancy (Tanabe *et al.*, 1955). However, current standards for semen processing have greatly reduced the risk of bacteria transmission during AI.

Induction of ovulation during pregnancy followed by AI did not result in superfetation in any of the groups. It is likely that for superfetation to occur in cattle in
nature hormonal conditions similar to those at normal estrus must occur such as lowered progesterone and elevated estrogen levels, so that fertilization and appropriate embryo development may occur.

Progesterone and estrogen levels have been reported to influence gamete transport so that sperm and the ovum have the best chance for syngamy. In cattle, cilia in the oviduct pulsate towards the uterus (Gaddum-Rosse and Blandau, 1976), facilitating ovum transport. Sperm transport towards the ovum is facilitated by oviductal muscular contraction toward the ovary in humans (Stone and Hamner, 1975). Oviduct muscular activity increases 3 to 5 days prior to estrus in cattle and decreases 3 to 5 days following estrus (Bennet et al., 1988). Muscular activity in the uterus is also low during the luteal phase in cattle (Ruckebusch and Bayard, 1975). Therefore, during the luteal phase (elevated progesterone) of the estrous cycle, oviduct and uterine muscular activity would be very low.

Crisman (1980) reported that progesterone administered 24 hours following the end of estrus caused an increase in the ovum transport rate through the oviducts so that the ovum would reach the uterus sooner than in nontreated cows. Likewise, oocytes ovulated during pregnancy (elevated progesterone) may be pushed too quickly through the oviducts, while sperm transport is impaired by decreased muscular activity of the uterus. Also, even if fertilization would occur normally, it is possible that the embryo would reach the uterus too quickly and then degenerate.

In the present experiment, superfetation did not occur after AI of mated cows following hCG treatment in any of the groups, even though hCG induced ovulation in all cows treated during early pregnancy (5 to 8 days post-estrus). hCG at varying doses did
not consistently cause ovulation induction in cattle when administered after 23 days of pregnancy. It is possible that gamete transport may have been affected by hormonal conditions at the time of AI. Therefore, it is likely that for superfetation to be induced during pregnancy, a hormonal environment similar to that of estrus must be mimicked somehow, so that the highly synchronized process of fertilization and embryo development may have a better chance of occurring.
CHAPTER III
SUPERFETATION PRODUCED BY ASYNCHRONOUS EMBRYO TRANSFER

INTRODUCTION

Superfetation occurs when a pregnant female shows signs of estrus, is mated and subsequently carries two fetuses of different ages during the same pregnancy period (Long, 2001). There are several reports of supposed superfetation in many species, including cattle (Dalrymple and Jenkins, 1951; Simmons, 1960; Gee, 1971; Hall, 1987; Hunsley, 1998). However, there is considerable controversy about the actual existence of such a phenomenon.

One of the primary concerns with the concept of superfetation is that for a successful pregnancy to occur in most species, several highly synchronized events must take place. Early experiments with embryo transfer (ET) showed the importance of synchronization of the stage of reproductive cycle between embryo donors and embryo recipients in species such as sheep (Moore and Shelton, 1964; Rowson and Moor, 1966) and cattle (Rowson et al., 1972).

It later became clear that this was due to a process called maternal recognition. In cattle, embryo-maternal synchrony is important to ensure a complex series of events that prevents lysis of the corpus luteum (CL) from occurring with subsequent termination of pregnancy. It has been determined that the bovine conceptus produces a protein known as bovine interferon τ (bIFN-τ) around day 16 of the estrous cycle to prevent luteolysis (Thatcher et al., 1986). In short, it is currently believed that luteolysis occurs in the following manner: estrogen produced from follicles on the ovary stimulates the production of oxytocin receptors on the uterine endometrium as progesterone declines at
the end of the luteal phase of the estrous cycle (McCracken et al., 1984). Oxytocin from the CL binding to these receptors initiates a cascade of events leading to the synthesis of PGF$_{2\alpha}$ from arachidonic acid in the uterine endometrial epithelial cell (Thatcher et al., 1997). The increased secretion of PGF$_2$ ultimately culminates in the lysis of the CL (McCracken, 1984).

However, in the pregnant cow, bIFN-τ produced by the embryo acts by altering gene expression in the endometrial epithelial cell to affect expression of molecules that in turn affect the synthesis of PGF$_{2\alpha}$ (Thatcher et al., 1997). bIFN-τ may also act by producing an endometrial prostaglandin synthetase inhibitor (EPSI) that blocks prostaglandin production by the endometrium (Thatcher et al., 1989). If bIFN-τ production is affected in any way so that it does not occur at the appropriate time, as in the case of asynchronous embryonic development, luteolysis occurs followed by embryonic death.

Some degree of asynchrony is acceptable for the establishment of pregnancy. Although Rowson et al. (1972) reported an optimal differential of ±24 hours between bovine donors and recipients for best results (67% pregnancy rate), 35% of recipients ±48 hours of the donors became pregnant in that study and 20% of the recipients that were in estrus ±72 hours of the donors became pregnant following ET.

In sheep, Rowson and Moor (1966) found that ovine blastocysts collected on day 7 after estrus and transferred to recipients that were in estrus ±48 hours from the donors became pregnant in 63% of the transfers. Ashworth and Bazer (1989) showed that day-6 ovine embryos placed in recipients on day 4 of their cycle caused a shift in the secretory
protein profile of the uterus to one more similar to a more advanced uterine environment, presumably allowing maternal recognition to occur.

Embryonic development is also affected by the degree of asynchrony between embryo and uterine environment. Lawson et al. (1983) reported that sheep embryos placed in less advanced uterine environments (day-4 embryos in day-1 or -2 uterine horn) exhibited retarded growth, while embryos placed in more advanced uterine environments (day-4 embryo in day-6 or -7 uterine horn) exhibited accelerated growth up to day 12 of pregnancy, after which they degenerated and were resorbed. Similarly, Albihn et al. (1991) transferred day-7 bovine embryos to either a day-4 or a day-10 recipient and found that the embryos degenerated, showing either retarded or advanced development, respectively.

In cattle, it appears that this retardation or acceleration of embryonic development is affected by timing and/or length of uterine exposure to progesterone. This could pose a threat to the successful development of an embryo resulting from breeding during pregnancy, when progesterone levels are naturally high. Garrett et al. (1988) administered progesterone on days 1 to 4 of pregnancy in cows and found that embryos recovered from a sample group of cows were in an advanced stage of development compared with controls although pregnancy rates were similar at 40 days of gestation. However, Ashworth and Bazer (1989) administered progesterone to ewes beginning on day 4 of the inseminated estrous cycle, but found no apparent change in the secretory protein profile. This could simply be a species difference, or could possibly be due to the difference in the timing of initiation of progesterone treatment, as Garrett et al. (1988)
began treatment on day 1, while Ashworth and Bazer (1989) began treatment on day 4 of the estrous cycle.

It appears that the uterus must be exposed to progesterone for a minimum length of time for pregnancy to become possible. Lawson and Cahill (1983) administered progesterone to ewes beginning on day 0 (estrus) of the estrous cycle and were able to achieve acceptable pregnancy rates after placing day-10 embryos in day-6 progesterone-treated recipient ewes. Similarly, Geisert et al. (1991) administered progesterone on days 1 to 4 of the estrous cycle of cows and achieved acceptable pregnancy rates after placing day-8 embryos into day-5 progesterone-treated recipients.

However, it is not clear if there is an upper limit for length of progesterone exposure after which the uterus could no longer become pregnant. Thatcher et al. (1989) disrupted mid-cycle follicular dynamics and extended CL life by administering buserelin, a GnRH agonist, on day 12, 15, 18, 21 and 24 of the estrous cycle to potential recipients. Embryos (7 and 8 days-of-age) were transferred into recipients at 9.5 to 15 days post-estrus and several asynchronous pregnancies resulted. In one extreme case, a buserelin-treated recipient became pregnant after receiving a day-7 embryo on day 14.5 post-estrus (7.5 days asynchronous).

Maternal recognition appears to be localized in the uterine horn ipsilateral to the CL, as indicated by increased blood flow to the ipsilateral uterine horn on days 16 to 17 of the estrous cycle (Ford et al., 1979). In fact, Scanlon (1972) found no instances of pregnancies in cattle developing in the uterine horn contralateral to the CL in 643 cases of singleton pregnancies studied.
Production of bIFN-τ has been shown to begin with the process of embryonic elongation (Geisert et al., 1992). Although the developing bovine embryo begins elongating at ~12 days post-estrus and may fill two thirds of the uterine horn ipsilateral to the CL by day 17 to 18, it only begins to occupy the contralateral uterine horn by days 20 to 24 (Chang, 1952), so it would be difficult for an embryo developing in the contralateral horn to expand sufficiently to signal the CL.

However, bovine embryos can develop in the horn contralateral to the CL when a viable fetus is present in the uterine horn ipsilateral to the CL. Scanlon (1972) reported two cases in cows with dizygotic twin pregnancies in opposite horns although both ovulations occurred on the same ovary and one case in which monozygotic twins developed in opposite horns.

ET has also been used to produce twins in opposite horns. The first reported use of ET in cows to achieve twins was by Rowson et al. (1969a,b). Rowson et al. (1971) compared transfers of two bovine embryos in the same (unilateral) or opposite (bilateral) uterine horns. A sample group of the pregnant cows was slaughtered at day 90 of gestation and the remaining animals were allowed to calve. Pregnancy rates were similar (71% vs. 73%), but twinning rates were different. In the group that received embryos in both horns, 5 of 7 had viable sets of twins at 90 days of gestation and 3 of 4 heifers left to carry the pregnancies calved twins. At necropsy, it was noted that for heifers that received two embryos in the same horn, only 1 of 7 had viable twins in the same horn. Another set occurred, but one embryo had migrated to the opposite horn. In contrast to these findings, Sreenan and Diskin (1989) found no difference between 50-day pregnancy rates (64% vs. 72%), twin pregnancies (55% vs. 60%), or sets of twins born (33% vs.
38%) between unilateral or bilateral transfer of two embryos by surgical or nonsurgical means.

There are several reports of the use of the transfer of embryos into the uterine horn contralateral to the CL of mated bovine recipients to produce twins (Testart et al., 1975; Sreenan and McDonagh, 1979; Renard et al., 1979; Heyman et al., 1980; Sreenan et al., 1981; Sreenan and Diskin, 1988, 1989; McEvoy et al., 1995). In a few cases, fetuses developed in the contralateral uterine horn without the presence of a fetus in the opposite uterine horn, although it could not be determined if a fetus had been present in that horn at the time of maternal recognition (Sreenan and McDonagh, 1979; Renard et al., 1979; Heyman et al., 1980; Sreenan et al., 1981; Sreenan and Diskin, 1989). Nevertheless, the uterine horn contralateral to the CL appears to be able to maintain a pregnancy as long as the embryo in the horn ipsilateral to the CL is viable and capable of achieving maternal recognition.

Hafez and Pincus (1956) successfully used asynchronous ET in rabbits to produce superfetation. Day-3.5 embryos were transferred to the left horn of recipient does at different intervals following mating. To ensure that pregnancies found in the left horn resulted from ET, the left oviduct was removed surgically. A sterile probe was used to mechanically stimulate the left uterine horn to induce deciduoma formation. Implantation of transferred embryos occurred successfully when embryos were transferred up to 6 days following mating (2.5 days asynchronous). Parturition occurred at the same time for synchronous and asynchronous young in recipient does allowed to complete the pregnancy.
The hypothesis addressed by this experiment is that as long as an ongoing viable embryo signals pregnancy recognition and ensures CL longevity, a second, asynchronous, embryo can establish a secondary pregnancy. Therefore, a uterus at an advanced stage of pregnancy would be receptive to an embryo at an earlier stage of development as long as maternal recognition has already been ensured by the ongoing pregnancy. The objective of this experiment is to produce superfetation by placing 7-day bovine embryos in the uterine horn contralateral to the ongoing pregnancy of mated recipient cows at more advanced stages of pregnancy.

**MATERIALS AND METHODS**

**Experimental Animals**

The animals used in this experiment were crossbred, parous, nonpregnant beef cows with varying degrees of *Bos indicus* (Brahman) breeding, typical of cattle in the southeastern United States. The cows were obtained from the St. Gabriel Experiment Station of the Louisiana State University. Body condition scores were recorded prior to synchronization and only cattle with body condition scores between 5 and 8 were used in this experiment.

**Experimental Design**

Prostaglandin F$_{2\alpha}$ treatment was used to synchronize cows for mating in the herd at different times so that the embryos would be transferred on the same day (Figure 3.1). Cows were then artificially inseminated or mated naturally to provide pregnant recipients for ET. Cows to be used for the 90-day group (ET-90) were mated first (n = 14). Cows for the 60-day group (ET-60) were mated 30 days later (n = 19); 32 days following that, cows (n = 14) were mated for the 28-day group (ET-28); 14 days later, cows (n = 15)
Figure 3.1. Prospective recipients were artificially inseminated (AI) so that mated recipients at different stages of pregnancy [90, 60, 28, 14 and 7 days (Ctrl = Control) post-estrus] would receive the transfer of embryos (ET) on the same treatment day.
were mated for the 14-day group (ET-14) and then, 7 days later, cows (n = 20) were mated for the 7-day control group (ET-Control).

Black cows were inseminated with semen from black Angus bulls of known fertility to ensure that offspring resulting from these matings would be of similar coat color as the dams. Likewise, red/other colors were naturally mated or inseminated with semen from Red Angus or yellow Gelbvieh bulls of known fertility. Embryos to be transferred were produced so calves could be easily distinguished from calves resulting from artificial insemination (AI) or natural breeding. These embryos were either frozen-thawed, in vivo-produced embryos collected from cows mated to bulls of similar coat color, or in vitro-produced (IVP) embryos resulting from slaughterhouse oocytes inseminated with semen from a Red Brahman bull. Brahman semen was chosen because the distinctive ear forms of Brahman cattle would make calves resulting from IVP clearly distinguishable from the Bos taurus-sired calves.

Experimental Procedures

Prospective recipients were synchronized at different times with one dose of 25 mg of prostaglandin F\(_2\alpha\) (Lutalyse®, Pharmacia & Upjohn, Kalamazoo, MI) so that embryos would be transferred at the same time for all groups. Cows that exhibited signs of estrus were then either placed with a bull at estrus following synchronization or artificially inseminated 12 hours after onset of estrus. Bulls used for each cow were of similar coat color so that calves resulting from these matings would have similar coat colors at birth.

Embryos to be transferred were obtained using two methods. First, embryos were collected from superstimulated cows (n = 18) residing at the LSU physiology herd at St.
Gabriel Experiment Station. Prospective donor cows were synchronized with one dose of 25 mg of prostaglandin F2\alpha prior to superstimulation. Donor cows that exhibited estrus began the superstimulation treatment 7 to 13 days following estrus. Donor cows that did not exhibit estrus received a norgestomet implant (SyncroMate-B, Rhone Merieux, Athens, GA) 3 to 6 days prior to the beginning of the superstimulation treatment.

A dominant follicle removal (DFR) via ultrasound-guided follicular aspiration of all follicles >5 mm was performed 48 hours prior to the beginning of the superstimulation treatment in all donor cows. Cows were superstimulated with 20 to 32 units of porcine FSH (pFSH; Sioux Biochemical, Sioux Center, IA) depending on parity and breed given in a decreasing dosage twice daily over 4 days. Donor cows without a norgestomet implant were induced into estrus during superstimulation with two treatments of 25 mg of prostaglandin F2\alpha at the same time as the fifth and sixth FSH treatments. Donor cows with norgestomet implants were induced into estrus during superstimulation with 25 mg of prostaglandin F2\alpha with the fifth FSH treatment followed by removal of the implant at the same time as the sixth FSH treatment. Donor cows were either artificially inseminated with one straw of semen from bulls of similar coat color at 12 hours and again at 24 hours post-estrus or naturally mated at estrus to bulls of similar color pattern.

Embryos were collected from the donors 7 days after onset of estrus. Donor cows were immobilized in a hydraulic squeeze chute and given 5 ml of 2% lidocaine into the first intercocygeal space as a caudal epidural block to reduce rectal contractions. The perineal region was thoroughly washed and a 2-way 18 French silicon catheter with a 30-ml cuff (PETS, Canton, TX) was introduced through the cervix into the right uterine horn. The cuff was inflated with 6 to 10 ml of phosphate-buffered saline (PBS) to keep the
catheter in place and avoid leakage of fluid. PBS supplemented with 1% calf serum and antibiotics was introduced into the uterine horn via gravity flow and then allowed to return via plastic tubing into an embryo filter (SureFlush, PETS, Canton, TX). The distal portions of the uterine horn were lightly manipulated to dislodge the embryos.

The process was repeated several times, with ~500 ml of PBS being rinsed through the horn. The catheter was then removed, placed in the left uterine horn and the procedure was repeated. The filter with ~50 ml of recovered PBS was then rinsed into a square gridded petri dish and the recovered PBS was searched for embryos. A total of 86 good quality embryos (4.8 transferable embryos per collection) resulted from the embryo collections and were frozen for transfer at a later date.

Embryos to be frozen were placed in 1.5M ethylene glycol (EG) or 1.4M glycerol solutions, loaded into 0.25 ml straws and held at room temperature for 5 minutes. The straws containing the embryos were placed in a methanol bath at -7°C. The embryos were held in the methanol bath for 2 minutes, seeded by touching supercooled forceps to the uppermost column of solution and then held for an additional 8 minutes at -7°C. The embryos were subsequently cooled to -35°C at -0.5°C per minute. At this point, the straws were plunged into liquid nitrogen. Groups of straws containing embryos from individual donors were placed in labeled goblets, which were then placed on aluminum canes and placed in dewar tanks containing liquid nitrogen for storage until needed.

Secondly, IVP embryos were purchased from a commercial company (Bomed, Madison, WI). These embryos were produced from slaughterhouse oocytes using semen from a Red Brahman bull for easy recognition at birth. Embryos were produced in Wisconsin and shipped overnight in a portable incubator in culture medium consisting of
TL-Hepes plus 10% fetal calf serum at 39°C to Louisiana as day-6 and day-7 embryos to be transferred into the recipients.

At the time of transfer, frozen embryos were thawed in air for 10 seconds and then in a 30°C water bath for an additional 10 seconds. Embryos that had been frozen in 1.4M glycerol were rehydrated using a standard 3-step rehydration protocol, which consists of placing the embryos in a solution of 6% glycerol + 0.3M sucrose in PBS for 5 minutes, then into a solution of 3% glycerol +0.3M sucrose for 5 minutes, and finally in a solution of 0.3M sucrose for 5 minutes, after which the embryos are placed in PBS supplemented with 10% bovine serum awaiting transfer. Embryos that had been frozen in 1.5M EG were rehydrated by placing them directly in PBS plus 10% serum following thawing, awaiting transfer.

Following thawing and rehydration, one thawed embryo was loaded together with one IVP embryo into a 0.25 ml straw or a tomcat catheter for nonsurgical or surgical transfer, respectively, so that each recipient would receive two embryos. Embryos were transferred surgically to the 90-day and 60-day recipients and nonsurgically to the 28-day, 14-day and 7-day recipients.

Recipients in the ET-90 and ET-60 groups received embryos surgically due to the difficulty with placing embryos nonsurgically into cows with a late gestation as well as concern over terminating the pregnancy. Surgery was performed with the cows in a standing position. The left flank region of the recipients was surgically prepared and a line block was performed using 2% lidocaine, after which an incision was made in the left paralumbar fossa. The horn contralateral to the CL and pregnancy was exteriorized, although with some difficulty. No attachment of the placenta at the uterotubal junction
could be found in any stage of pregnancy. Therefore, the contralateral uterine horn was stretched up in an attempt to avoid puncturing fetal membranes. A puncture was made at the tip of the horn with a sterile nail and the embryos were introduced into the uterine horn using a tomcat catheter.

Nonsurgical ETs were performed on the ET-28, ET-14 and ET-Control groups. Briefly, recipients were placed in a hydraulic squeeze chute for immobilization and palpated for uterine and ovarian normality as well as for the presence of a CL. Acceptable recipients were administered 5 ml of 2% lidocaine into the first intercocygeal space as a caudal epidural block. The perineal region was thoroughly washed. Straws containing the embryos were loaded into an ET gun (IMV, France) and a side delivery sheath (IMV, France) was placed over the gun. A plastic chemise (IMV, France) was placed over the transfer gun prior to transfer to avoid contamination. The transfer gun with the chemise was inserted into the vagina up to the cervix and then pushed through the chemise prior to entering the external os cervix. The transfer gun was then manipulated through the cervix and gently passed deep into the uterine horn contralateral to the CL. The embryos were then quickly deposited at the distal portion of the uterine horn.

Recipients were checked for signs of twin pregnancies via transrectal ultrasonography at ~30 days following ET and every 14 days following the initial check. Each uterine horn was scanned carefully beginning at the uterine body and scanning towards the distal portion of the uterine horns. Pregnancies were considered established by day 90 and a rectal palpation was performed at ~180 days of gestation to check for conceptus loss.
**Statistical Analysis**

Pregnancy rates and twinning rates were compared among groups using Fisher’s Exact Test. Gestation lengths and calf birth weights were analyzed across sire breeds using analysis of variance (ANOVA).

**RESULTS**

Following the initial synchronization, 14 cows were mated for the ET-90 group. Of the 14 cows mated, eight were pregnant (57.1%) at 60 days post-mating. Of the eight cows, two did not receive embryos, as one lost her pregnancy prior to ET at 90 days of gestation. Another cow exhibited signs of estrus, including standing to be mounted, at 56 days of gestation and again at 80 days of gestation and did not receive an embryo. Therefore, six cows received embryos at ~90 days of gestation (n = 6).

Although ultrasound scanning was used to attempt to verify if superfetation pregnancies were developing, no asynchronous pregnancies could be detected due to the advanced stage of the primary pregnancies resulting from AI and the large amount of fluid present at this stage. Therefore, it was necessary to await birth to determine successful twinning. Unfortunately, this does not take into account embryonic development and loss prior to calving. One cow aborted between 5 and 6 months of gestation, but no fetuses were recovered. The remaining five cows calved single calves, all resulting from AI, with no signs of additional fetuses (Table 3.1).

As noted previously, one cow prepared for this group was in standing estrus at 56 and 80 days of gestation (prior to the projected ET date). The cow was inseminated 12 hours following observation of estrus signs at 80 days of gestation. Transrectal ultrasonography revealed no follicle ≥10 mm at the time of insemination. Superfetation
Table 3.1. Breeding, embryo transfer, palpation and calving results†

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Mated (n)</th>
<th>Received embryos (n)</th>
<th>45 day pregnancy rate (%)</th>
<th>Twin sets detected</th>
<th>Twin sets born</th>
<th>AI + IVP twins (n)</th>
<th>ET + IVP twins (n)</th>
<th>Single AI calves (n)</th>
<th>Single IVP calves (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ET-Control</td>
<td>20</td>
<td>18</td>
<td>11 (61%)</td>
<td>7 (64%)</td>
<td>3 (27%)</td>
<td>2 (18%)</td>
<td>1 (9%)</td>
<td>3 (27%)</td>
<td>1 (9%)</td>
</tr>
<tr>
<td>ET-14</td>
<td>15</td>
<td>13</td>
<td>7 (54%)</td>
<td>1 (14%)</td>
<td>1 (14%)</td>
<td>1 (14%)</td>
<td>0 (0%)</td>
<td>6 (86%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>ET-28</td>
<td>14</td>
<td>7</td>
<td>7 (50%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>3 (43%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>ET-60</td>
<td>n/a††</td>
<td>7</td>
<td>7 (n/a)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>7 (100%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>ET-90</td>
<td>14</td>
<td>6</td>
<td>6 (43%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>5 (83%)</td>
<td>0 (0%)</td>
</tr>
</tbody>
</table>

†AI = artificial insemination, ET = embryo transfer, and IVP = *in vitro*-produced calves.
††Recipients from the ET-60 group were part of another project and pregnancy rates are not applicable.
did not occur following AI. The cow did not lose her ongoing pregnancy following AI and calved normally at 274 days of gestation.

Pregnant cows for the ET-60 group were obtained from a concurrent project involving deep uterine placement of low numbers of semen from a Holstein bull using an ultrasound-guided transvaginal AI technique (Hylan et al., unpublished data). Cows that became pregnant using this method were used in the ET-60 group, receiving embryos at ~60 days of gestation (n = 7).

As in the ET-90 group, it was not possible to determine the presence of twinning following ET by palpation or ultrasonography due to the advanced stage of the primary pregnancy. All seven cows calved single calves at term resulting from AI, with no additional fetuses found to be present in the placental tissue, or by transrectal palpation of the uterus (Table 3.1).

For the ET-28 group, 14 cows were artificially inseminated 32 days after recipients in the ET-60 group to bulls of similar coat color pattern following estrus synchronization. These cows were checked for pregnancy prior to receiving embryos via transrectal ultrasonography using an ultrasound equipped with a 5 MHz linear rectal probe (Aloka 500-V, Corometrics, Wallingford, CT). Of the 14 cows mated, 7 (50%) were pregnant at 24 to 28 days of gestation and received embryos at that time (n = 7). Following ET, four cows (57.1%) were still pregnant at ~60 days of gestation and one cow aborted between 90 and 120 days of gestation. No twins were determined by palpation or ultrasonography at any stage. The remaining three cows calved single, normal calves in this group resulting from AI. No additional fetuses were found in the placental tissue, or by palpation of the recipients (Table 3.1).
Fourteen days after recipients in the ET-28 group, 15 cows were artificially inseminated to bulls of similar coat color for the ET-14 group 12 hours after onset of estrus. As it is not possible to confirm pregnancy ultrasonically at this stage, these cows were checked for uterine and ovarian abnormalities via transrectal ultrasonography prior to ET.

Of the 15 cows inseminated for this group, 13 were deemed acceptable and received embryos by nonsurgical ET (Table 3.1). At 45 days of gestation, an ultrasound examination was performed and seven (53.8%) were found to be pregnant (n = 7). Twins were detected in opposite horns in one recipient and two more were suspected, but not confirmed, to have twins. The remaining four pregnancies were determined to be single pregnancies.

At 60 days of gestation, only the cow with confirmed twins at 45 days was still found to be carrying twins, for a twinning rate of 14.3%. The remaining six cows, including the two cows in which twins had been suspected, had normal single calves at birth, all resulting from AI (Table 3.1). The recipient with twins, a black Brangus crossbred cow, calved at 285 days of gestation (Figure 3.2). One calf was produced by AI (Angus sire) and the other calf was IVP (Red Brahman x Holstein). The Angus-sired calf, a male, was born first and the IVP calf, a female, was born within 15 minutes of the first, weighing 30.3 kg and 28.4 kg, respectively (Table 3.2).

Blood typing performed by an independent contractor (Immgen, College Station, TX) confirmed that the bull calf was a product of the sire to which the recipient had been mated and the recipient herself. More importantly, the heifer was confirmed to be a product of the Red Brahman sire used for in vitro fertilization, but not of the recipient
Figure 3.2. Superfetation: 7-day asynchronous twins. The calf in the foreground was produced by artificial insemination of the recipient 12 hours following onset of estrus, and the calf in the background is a result of transfer of a 7-day embryo to the mated recipient 14 days post estrus.
Table 3.2. Calving results for cows diagnosed with twin pregnancies by ultrasound scanning. The twin pregnancy for recipient 4924 resulted from transfer of a 7-day asynchronous embryo at 14 days of gestation (superfetation).

<table>
<thead>
<tr>
<th>Dam number</th>
<th>Group</th>
<th>Aborted</th>
<th>Born</th>
<th>Calf number</th>
<th>Gestation</th>
<th>BW, kg</th>
<th>Sex</th>
<th>Origin †</th>
<th>Sire breed ††</th>
</tr>
</thead>
<tbody>
<tr>
<td>1062</td>
<td>ET-Control</td>
<td>&lt;150 d</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1063</td>
<td>ET-Control</td>
<td>No</td>
<td>2</td>
<td>Stillborn</td>
<td>279</td>
<td>43.1</td>
<td>F</td>
<td>IVP</td>
<td>RR</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Died 4 days</td>
<td>279</td>
<td>32.2</td>
<td>M</td>
<td>FT</td>
<td>GV</td>
</tr>
<tr>
<td>4902</td>
<td>ET-Control</td>
<td>&lt;80 d</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4910</td>
<td>ET-Control</td>
<td>No</td>
<td>2</td>
<td>9130</td>
<td>273</td>
<td>29.5</td>
<td>F</td>
<td>AI</td>
<td>AN</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>9129</td>
<td>273</td>
<td>36.3</td>
<td>F</td>
<td>IVP</td>
<td>RR</td>
</tr>
<tr>
<td>4936</td>
<td>ET-Control</td>
<td>No</td>
<td>2</td>
<td>Stillborn</td>
<td>280</td>
<td>31.8</td>
<td>F</td>
<td>AI</td>
<td>AN</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Stillborn</td>
<td>280</td>
<td>40.9</td>
<td>F</td>
<td>IVP</td>
<td>RR</td>
</tr>
<tr>
<td>9006</td>
<td>ET-Control</td>
<td>&lt;180 d</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9031</td>
<td>ET-Control</td>
<td>No</td>
<td>1</td>
<td>Died &lt;1 day</td>
<td>298</td>
<td>63.6</td>
<td>F</td>
<td>IVP</td>
<td>RR</td>
</tr>
<tr>
<td>4924</td>
<td>ET-14</td>
<td>No</td>
<td>2</td>
<td>9138</td>
<td>285</td>
<td>35.0</td>
<td>M</td>
<td>AI</td>
<td>AN</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>9139</td>
<td>285</td>
<td>32.7</td>
<td>F</td>
<td>IVP</td>
<td>RR</td>
</tr>
</tbody>
</table>

†IVP = *in vitro*-produced; FT = frozen-thawed, *in vivo*-produced; AI = artificial insemination.

††RR = Red Brahman; GV = Gelbvieh; AN = Angus.
dam. This heifer calf has also been determined not to be a freemartin, as evidenced by karyotype (no XY present). More importantly, the heifer was found to be fertile, as she has since been mated and has calved successfully (Figure 3.3).

For the ET-Control group, 20 cows were artificially inseminated to bulls of like coat color 12 hours after onset of estrus 7 days prior to the projected ET date. As in the ET-14 group, it was not possible to confirm pregnancy prior to ET. The cows were checked via transrectal ultrasonography for uterine and ovarian abnormalities at the time of transfer and 18 of the original 20 cows received embryos (n = 18).

Of the 18 cows that received additional embryos at 7 days post-AI (ET-Control), 11 (61.1%) were determined to be pregnant at ~40 days of gestation (n = 11). Within 90 days of mating, ultrasonic scanning was performed and 7 of the 11 (63.6%) pregnant recipients were found to be carrying twins (Table 3.1). This is not different from the proportion of 14-day mated recipients found to be carrying twins (1 of 7, or 14.3%). Of the seven cows determined to have twins, three aborted prior to 180 days of gestation (recipients 1062, 4902 and 9006; Table 3.2).

One cow detected with twins during pregnancy resorbed one of the twins at an unknown point in her gestation (recipient 9031). At 298 days of gestation, she produced a single IVP calf (Red Brahman x Holstein) weighing 63.6 kg (Table 3.2). The calf died soon after birth.

One of the remaining three recipients produced twins at 279 days of gestation (recipient 1063; Table 3.2), one IVP calf (Red Brahman x Holstein heifer calf) and one originating from the frozen-thawed (FT) embryo (Gelbvieh x Hereford bull calf). However, it could not be determined if the recipient did not become pregnant by AI or
Figure 3.3. Cow 9138 (born co-twin to a bull calf produced by transfer of an IVP embryo to a mated recipient 14 days following mating) with her first bull calf 2 days after calving.
resorbed the AI-produced fetus at some stage of gestation following maternal recognition. The IVP calf died at birth (very difficult breech birth) and the FT embryo calf died 4 days later.

The remaining two sets of twins in this group consisted of an Angus-sired (AI) calf and an IVP calf (Table 3.2). One recipient lost both twins following a very difficult delivery. The remaining recipient calved normally, but did not accept the IVP calf, which had to be bottle raised and was later successfully grafted onto another cow.

In the control group, Gelbvieh-sired calves (n = 3) were heavier at birth than Angus-sired calves (n = 14) (42.4 ± 3.4 kg vs. 34.3 ± 1.2 kg; P<0.05) but not Holstein-sired calves (n = 7) (42.4 ± 3.4 kg vs. 35.8 ± 1.1 kg) (Table 3.3). Gestation lengths for cows mated to the Gelbvieh sire were also longer than those for cows mated to Angus sires (289.0 ± 0.6 days vs. 282.96 ± 1.0 days; P<0.05) but not to Holstein sires (289.0 ± 0.6 days vs. 283.4 ± 0.8 days) (Table 3.3). There were no differences in gestation lengths or birth weights of males vs. females (284.0 ± 1.2 days vs. 283.4 ± 0.7 days and 36.6 ± 1.4 kg vs. 34.3 ± 1.3 kg, respectively).

Twin gestations were significantly shorter than single gestations (277.3 ± 2.2 days vs. 283.8 ± 0.8 days, respectively; P<0.05) in the control group (Table 3.4). Gestation length for the lone twin pregnancy in the 14-day group was 285 days.

Within twin gestations in the control group, IVP calves (n = 3) were significantly heavier at birth than their co-twins (n = 3) resulting from AI of the recipient or in one case, from a frozen-thawed embryo (40.1 ± 2.0 kg vs. 31.2 ± 0.8 kg, respectively; (P<0.05), although neither was different from singleton calf birth weights (35.8 ± 0.9 kg)
Table 3.3. Mean gestation lengths (±SEM), birth weights and calf survival rate of singleton calves resulting from artificial insemination compared by breed

<table>
<thead>
<tr>
<th>Sire breed</th>
<th>Calves (n)</th>
<th>Gestation length (days)</th>
<th>Birth weight (kg)</th>
<th>Calf survival (1st week)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angus</td>
<td>14</td>
<td>282.9 ± 1.0\textsuperscript{a}</td>
<td>34.3 ± 1.2\textsuperscript{a}</td>
<td>14/14 (100%)</td>
</tr>
<tr>
<td>Holstein</td>
<td>7</td>
<td>283.4 ± 0.8\textsuperscript{a,b}</td>
<td>35.8 ± 1.1\textsuperscript{a,b}</td>
<td>5/7 (71%)</td>
</tr>
<tr>
<td>Gelbvieh</td>
<td>3</td>
<td>289.0 ± 0.6\textsuperscript{b}</td>
<td>42.4 ± 3.4\textsuperscript{b}</td>
<td>3/3 (100%)</td>
</tr>
</tbody>
</table>

\textsuperscript{a,b}Means within columns with different superscripts are different (P<0.05).
Table 3.4. Mean gestation lengths (±SEM), birth weights and calf survival rates for calves compared by method of production [artificial insemination (AI), frozen thawed *in vivo*-produced (FT) or *in vitro*-produced (IVP)] and singleton/twin status

<table>
<thead>
<tr>
<th>Origin (twin or single)</th>
<th>Calves (n)</th>
<th>Gestation length (days)</th>
<th>Birth weight (kg)</th>
<th>Calf survival (1st week)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AI Single</td>
<td>24</td>
<td>283.8 ± 0.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>35.8 ± 0.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20/24 (83%)</td>
</tr>
<tr>
<td>Non-IVP Twin</td>
<td>3</td>
<td>277.3 ± 2.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>31.2 ± 0.8&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>1/3 (33%)</td>
</tr>
<tr>
<td>IVP Twin</td>
<td>3</td>
<td>277.3 ± 2.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>40.1 ± 2.0&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td>1/3 (33%)</td>
</tr>
</tbody>
</table>

<sup>a,b,c</sup> Values within columns with different superscripts are different (P<0.05).
Interestingly, for the 14-day twin calves, in which the IVP embryo was 7 days younger (fetal age) than the AI-produced embryo, the IVP calf was actually born lighter than its AI-sired co-twin: 28.4 kg vs. 30.3 kg. This is the only set of twins in which the IVP calf was born lighter than the calf resulting from AI, which may have been due to the fact that the IVP calf had 7 fewer days of growth during the pregnancy (Table 3.2).

DISCUSSION

In this study, 7 of the 11 cows (63.6%) becoming pregnant in the control (7-day) group were diagnosed with twins by ultrasonography. These numbers are similar to results obtained by others. Using surgical transfer of one embryo per horn in beef heifers, Rowson et al. (1971) produced 73% sets of twins and Sreenan et al. (1975) obtained a 74% twinning rate in closely synchronized recipients. Testart et al. (1975) transferred in vivo-collected embryos to the contralateral uterine horn in previously inseminated recipients using a transvaginal transfer method and 30% were diagnosed with twins. Sreenan and McDonagh (1979) transferred in vivo-collected embryos nonsurgically using the now common transcervical method to the contralateral uterine horn of previously inseminated recipients and reported 60% twinning rates at slaughter (30 to 42 days). Lu et al. (1989) transferred two IVP embryos nonsurgically to the horn ipsilateral to the CL and 73% of the recipients were diagnosed with twins at 60 to 90 days of gestation.

However, only 3 of the 11 cows (27.3%) originally diagnosed as pregnant produced twins and 4 of 7 cows (57.1%) originally diagnosed with twins aborted or
resorbed fetuses during the course of pregnancy. This is similar to results achieved in
cattle by Anderson et al. (1978). By transferring in vivo-collected embryos to opposite
uterine horns, 62% of the pregnant recipients produced twins at calving. However, 28%
of the original pregnancies confirmed between 45 and 60 days during the course of
gestation were lost during the course of that experiment.

The results in this experiment are also similar to observations by Takada et al.
(1991) with nonsurgical transfer of in vitro-derived embryos to each uterine horn who
reported 29% of recipients palpated pregnant at 40 to 60 days had twins at calving,
although 25% of the pregnancies were lost during the course of gestation. McEvoy et al.
(1995) also transferred single IVP embryos to the contralateral horn of inseminated
recipients and obtained a 35% twinning rate at calving.

In the control group, 10 calves were produced from 7 cows calving, or 1.4 calves
per calving recipients, with 3 of the 7 calving twins. This is also consistent with the
literature: using nonsurgical transfer of single in vivo-derived embryos to the contralateral
horn of artificially inseminated recipients, Renard et al. (1979) produced 44% sets of
twins at calving. Using a similar method, Heyman et al. (1980) produced 1.5 calves per
recipient calving, with a twinning rate of 48.5% and Sreenan et al. (1981) produced 1.4
calves per recipient calving. Takada et al. (1991) produced 1.4 calves per calving
recipient with transfer of IVP embryos to each uterine horn, while Lu et al. (1989)
produced 1.4 calves per calving recipient transferring two IVP embryos to the same horn.

ET-Control recipients (day-7 embryos in day-7 pregnant recipients) calving twins
had some of the classical outcomes of twin pregnancies (Table 3.2). Gestation lengths
were shorter than singleton gestations, which is similar to reports by others (Anderson et al., 1980; Sreenan et al., 1981; Lu et al., 1989; Takada et al., 1991). Only one of the three sets of twins in the control group (ET-Control) was born without assistance. Even so, the dam did not accept the second calf born, so this calf had to be grafted onto another cow. Two of the three cows that produced twins in this group had breech births and had to be assisted at calving. All four calves in these two twin sets were either stillborn or were lost shortly after calving.

Takada et al. (1991) lost 43% of twins resulting from bilateral nonsurgical transfer of IVP bovine embryos soon at calving. This may have been related to birth weights of IVP calves. In the present experiment, calves resulting from IVP embryos were heavier at birth than their co-twins resulting from AI or frozen-thawed in vivo produced embryos (40.1 ± 2.0 kg vs. 31.2 ± 0.8 kg, respectively; P<0.05). McEvoy et al. (1995) found a positive correlation between birth weight and calving difficulty in inseminated recipients receiving IVP embryos. Interestingly, the heaviest calves resulted from single pregnancies that had been originally diagnosed with twins. Similarly, in the present experiment, one such calf resulted from a recipient originally diagnosed with twins. This calf weighed 63.6 kg at birth.

The large birth weight of calves resulting from IVP in this experiment may have been a result of the in vitro system of production. Production of embryos in vitro has been associated with increased size at birth of calves and is referred to as “large calf syndrome” (Behboodi et al., 1995; Walker et al., 1996), although current methods of production of embryos in vitro have led to smaller, more normal calf birth weights. McEvoy et al. (1995) transferred IVP embryos over a 3-year period and reported large
calves over the first 2 years, but this syndrome was not evident in the third year of the experiment.

The hypothesis of this experiment was that a viable, synchronous fetus was capable of attaining maternal recognition, preventing luteolysis and therefore allowing an asynchronous, progesterone-stage embryo (day-7) to develop to term. No twins were produced in the 28-day, 60-day, or 90-day groups. However, one set of twins was produced in the 14-day group of seven cows diagnosed as pregnant. In this case, the fetuses were ~6.5 days apart in age, as a 7-day IVP embryo (along with a frozen-thawed in vivo-produced embryo) was transferred to a cow 13.5 days following estrus. The twinning rate at calving was not different for the 14-day group when compared with the twinning rate at calving of the ET-Control group (14.3% vs. 27.3%).

Others have used similar approaches in unsuccessful attempts to produce asynchronous sets of twins (Wilmut and Sales, 1981; Camillo et al., 1997). Lawson and Cahill (cited in Wilmut and Sales, 1981) transferred synchronous embryos with advanced or retarded embryos, but only synchronous embryos developed and produced lambs. Wilmut and Sales (1981) transferred ±3-day asynchronous sheep embryos together with synchronous embryos to recipient ewes, but no pregnancies from asynchronous embryos resulted. Camillo et al. (1997) inseminated recipient mares and transferred a day-7 asynchronous embryo to each on day 9, 12, or 14 post-insemination following confirmation of pregnancy. No additional pregnancies resulted from the transferred embryos.

It appears that there may be a minimum exposure of the uterus to progesterone in order for maternal recognition to occur, as evidenced by data showing that progesterone
treatment early in the cycle allows later stage embryos to establish pregnancies in sheep (Lawson and Cahill, 1983) and cattle (Geisert, 1991). However, it is unclear whether there is a maximum exposure of the uterus to progesterone (at least up to 14.5 days) for a pregnancy to become established Thatcher et al., 1989). Thatcher et al. (1989) noted that mid-cycle follicles are important to the maternal recognition process, as estradiol produced by these follicles are involved in the production of oxytocin receptors which are in turn involved in the production of PGF$_{2\alpha}$ by the uterine endometrium in the luteolysis process (McCracken, 1984). By disrupting the second wave of follicles, the need for maternal-embryonic synchrony would be affected and the timing of IFN-τ production by the embryo would not be as critical. Potential recipients were treated with a GnRH agonist on days 12, 15, 18 and 21 of the estrous cycle and became pregnant following transfer of 7-day embryos as far out as day 14.5 of the cycle, or 7.5 days asynchronous.

The set of twins from the 14-day group (6.5 days asynchronous) did not exhibit any of the classical problems associated with twinning in cattle (Table 3.2). Calving was unassisted, with both calves being expelled within 15 minutes. Gestation length for this asynchronous twin pregnancy was similar to gestation lengths of singleton pregnancies (285.0 and 283.9 ± 0.8 days, respectively), although they cannot be compared statistically. The asynchronous calf, a heifer, resulted from the transfer of an IVP embryo and was lighter than her twin bull calf (28.4 kg vs. 30.3 kg) at birth, possibly due to the fact that she had 7 less days of gestation for growth.

Another problem commonly associated with twin pregnancies is that 92% of heifers born co-twin to bull calves are sterile (Long, 1990). Current freemartin theory is that anastomoses form between placental vessels in twin pregnancies (Lillie, 1917),
allowing for the exchange of hematopoietic cells and subsequent cross-colonization of these cells (Herschler et al., 1966). The presence of the Y chromosome in the female is thought to be responsible for the presence of testis-determining factor (TDF) in the female twin (Kennedy and Miller, 1993), which in turn is thought to be responsible for sterilization of the female. The anastomoses also allow for the exchange of anti-Müllerian hormone (AMH, or Müllerian-inhibiting substance – MIS) due to the male fetus developing more rapidly (Vigier et al., 1984), which prevents the development of tubular parts of the reproductive tract (vagina, cervix, uterus). Degree of freemartinism also appears to be related to timing of anastomosis formation (Long, 1990).

Freemartinism did not occur in the asynchronous, opposite-sexed twin pair, although it must be noted that ~8% of heifers born co-twin to bulls are not freemartins and may be fertile (Long, 1990). Therefore, it does appear that anastomoses did not occur in this case. The heifer showed no evidence of admixing following chromosomal analysis and also has a normal reproductive tract, evident following ultrasound examination of her reproductive tract during her development. More importantly, she has been mated and has calved (Figure 3.3). Whether this occurrence was due to the fact that the twins were asynchronous and would have developed at different rates, affecting the development or not of anastomoses and subsequent exchange of hematopoietic cells and MIS, remains to be determined. More research is needed with more sets of twins produced in a similar manner to more accurately assess this phenomenon.

In the present experiment, a case of superfetation occurred as a result of transferring day-7 embryos to a recipient 13.5 days after estrus (6.5 days asynchronous). However, no twin pregnancies resulted in pregnant recipients receiving 7-day embryos at
28, 60, or 90 days of gestation, where fetal age differences would have been more similar to reported superfetation cases. Although it appears that an asynchronous pregnancy can be established and maintained in the uterine horn contralateral to an ongoing pregnancy, more research is needed to ascertain the reality of superfetation in nature.
CHAPTER IV

USE OF HORMONAL MANIPULATION AND EMBRYO TRANSFER FOR INDUCTION OF SUPERFETATION IN BEEF CATTLE

INTRODUCTION

Superfetation occurs when a pregnant animal shows signs of estrus and is mated again, with the ensuing pregnancy occurring in addition to the previous one (Long, 2001). This has been reported in several animals, including cattle. Apparent cases of superfetation have been reported in cattle following natural breeding (Dalrymple and Jenkins, 1951; Gee, 1971), natural breeding followed by artificial insemination (AI) (Simmons, 1960) and also embryo transfer (ET) followed by natural breeding (Hall, 1987; Hunsley, 1997).

Superfetation cases have generally been viewed with skepticism, primarily because the establishment of pregnancy in animals is a complex phenomenon. Exposure to a specific hormonal milieu and/or follicular wave status may be necessary to ensure embryonic development and subsequent implantation. For example, it has been known since the early days of ET that donor/recipient estrous cycle synchrony was important to produce pregnancies. In cattle, Rowson et al. (1972) reported that successful ET required close donor/recipient synchrony, which should not vary by more than ±1 day.

It later became clear that the need for synchrony was due to maternal recognition of pregnancy. In cattle, viable embryos produce a substance (bovine interferon-τ, or bIFN-τ) around day 16 that prevents luteolysis and ensures the maintenance of pregnancy (Thatcher et al., 1986). Bovine embryos that are too young do not produce bIFN-τ at the appropriate time and will not prevent luteolysis and subsequent termination of the
pregnancy. Similarly, bovine embryos that are too old will not be producing bIFN-τ at the appropriate time, with the same result.

Length of progesterone exposure prior to maternal recognition may also affect embryo survival. Researchers have reported that embryos placed in less chronologically advanced (“younger”) uteri exhibited retarded growth (rat: Dickman and Noyes, 1960; mouse: Doyle et al., 1963; sheep: Lawson et al., 1983; cattle: Albihn et al., 1991), while embryos placed in more chronologically advanced (“older”) uteri exhibited accelerated growth (sheep: Lawson et al., 1983; cattle: Albihn et al., 1991). Progesterone given to ewes on days 1 to 3 of an inseminated cycle caused reduced pregnancy rates, as well as greater fetal weights on day 74 of pregnancy (Kleemann et al., 1994). In cattle, however, although progesterone given to inseminated cows on days 1 to 5 of their cycle appeared to accelerate embryonic development, this did not affect maternal recognition, as pregnancy rates at 40 days of gestation were similar to controls (Garret et al., 1988).

Ashworth and Bazer (1989) noted that ovine embryos placed in “younger” uteri induced a shift in the secretory protein profile of the uterus to that of a more chronologically advanced uterus, apparently in an attempt to reach the same stage as the embryo. Therefore, it seems that when asynchrony between the uterus and embryonic development exists, there is a concerted effort between the uterine environment and the embryo to become synchronized.

It does appear that once maternal recognition in cattle has occurred, the uterus is capable of allowing a second, asynchronous pregnancy to develop and go to term. In a prior experiment (Chapter III), asynchronous twins were produced by transferring 7-day
embryos to the uterine horn contralateral to the corpus luteum (CL) in a recipient that had been inseminated ~14 days prior to ET (Carter et al., 2000).

However, if superfetation does occur in nature, estrus and ovulation must occur in a pregnant animal so that she may be mated and achieve a secondary pregnancy. Although estrus has been well documented in pregnant cows (Bullard, 1934; Donald, 1943; Branton, 1949; Erb and Morrison, 1958; Williamson et al., 1972; Perez Garcia et al., 1984), no resulting ovulations have been reported.

Thomas and Dobson (1989) studied 43 cases of estrus in 35 pregnant cows. Estrus was observed in all stages of pregnancy, with most cases occurring between 121 and 240 days of gestation. Mounting and mating occurred successfully in 7 of 9 cows presented to a bull at the time of estrus. Although follicles between 10 and 15 mm were detected in 5 of 12 pregnant cows palpated at standing estrus, no ovulations were apparent as determined by palpation and ultrasonography 2 and 14 days following estrus. High progesterone levels block the pre-ovulatory surge of FSH and LH (Kesner et al., 1982). Therefore, it is probable that a similar mechanism effectively blocks ovulation during pregnancy in the cow, when circulating progesterone levels are also elevated.

However, ovulation can be induced with various agents during pregnancy in cattle, although success rates may vary according to the stage of pregnancy. Rajamahendran and Sianangama (1992) reported treatment with human chorionic gonadotropin (hCG) to be very effective in causing ovulation in pregnant dairy heifers when given on day 7 of pregnancy, but not on day 14 of pregnancy. This was thought to be due the possibility that the large follicle present at day 7 of the estrous cycle was more likely to be responsive to hCG. Lulai et al. (1994) induced ovulations with exogenous
LH treatment after day 35 in 100% of treated pregnant beef cows. In that experiment, luteal regression was induced and pregnancy was maintained with norgestomet implants following luteal regression. In a similar study, Bridges et al. (2000) used exogenous progesterone to maintain pregnancy following CL removal. hCG treatment alone or in conjunction with an FSH pretreatment caused ovulation in 59% of treated beef cows when hCG was administered before day 36 of pregnancy and 96% of treated cows ovulated following a second treatment after day 36.

In a prior experiment (Chapter II), treatment with 1,500 IU of hCG alone did not result in ovulation in pregnant cows when treatment occurred after day 23. Treatment with 3,000 IU of hCG after day 23 was also not effective, as only 1 of 4 cows treated ovulated. However, Bridges et al. (2000) were highly successful in inducing ovulation in pregnant beef cows by using an FSH pretreatment prior to hCG treatment.

Although follicles continue to develop as pregnancy progresses, dominant follicles become smaller than dominant follicles present during a normal estrous cycle (Ginther et al., 1989). Rajamahendran and Sianangama (1992) suggested that the lower response to hCG at day 14 of the estrous cycle in their experiment was due to the presence of smaller, less responsive follicles, which had a higher chance of being atretic. Pierson and Ginther (1986) reported that pregnant heifers had larger follicles on day 8 than on day 21 of pregnancy (14.4 ± 0.6 mm vs. 12.7 ± 0.5 mm). Guilbault et al. (1986) studied follicles on the CL-bearing ovary in pregnant beef cows and found that there appeared to be a higher turnover of follicles during pregnancy. Also, larger follicles were more likely to be atretic.
AI following hCG-induced ovulation of pregnant beef cows did not result in secondary pregnancies in a prior experiment (Chapter II). This may have been a result of impaired sperm transport and/or increased rate of embryo transport through the oviducts due to elevated progesterone levels during pregnancy. Low levels of oviductal (Bennet et al., 1988) and uterine (Ruckebusch and Bayard, 1975) muscular activity occur during the luteal phase of the estrous cycle (high progesterone) and most likely during pregnancy as well, possibly impairing sperm transport. Also, Crisman (1980) found that ovum transport rate through the oviducts in cows doubled following progesterone treatment so that ova reached the uterus more quickly than normal. However, this possible hormonal influence may be bypassed by transfer of uterine-stage embryos (>5.5 days following estrus) directly to the uterus, as shown by the successful asynchronous twin pregnancy produced with this procedure in a prior experiment (Chapter III).

Therefore, the primary objective of this study is to utilize porcine FSH (pFSH) and hCG treatment during early pregnancy in previously inseminated recipients to simulate periestral ovarian conditions (follicular growth followed by ovulation) and then produce superfetation by placing an embryo in the recipient 7 days following treatment with hCG.

In a previous experiment in which hCG followed by AI were used in an attempt to produce superfetation (Chapter II), there was a high incidence of pregnancy loss following treatment. Due to the specific objectives of the experiment, it was not possible to determine the exact cause of pregnancy loss. hCG treatment and/or the AI procedure itself were suspected as possible causes of the perceived pregnancy loss. Therefore, a
secondary objective of this experiment is to determine if hCG treatment during early pregnancy could cause fetal loss in pregnant cows.

MATERIALS AND METHODS

Experimental Animals

Crossbred, parous, nonpregnant cows with body condition scores greater than 5 and less than 8 were obtained from the St. Gabriel Experiment Station of the Louisiana State University for this experiment. Cows in this herd have varying degrees of Brahman (Bos indicus) breeding, an essential aspect for raising cattle in humid southern Louisiana. Bos taurus breeds represented in the genetic pool of the herd are Hereford, Angus, Gelbvieh and Charolais.

Experimental Design

The cows were initially randomly assigned to two synchronization groups: 28-day and 14-day recipients. One treatment with prostaglandin F2α was used to synchronize estrus in potential recipients. Care was taken to ensure even distribution of breed type, body condition and parity among groups. Cows were initially artificially inseminated ~12 hours after onset of estrus with semen from bulls of proven fertility to provide pregnant recipients for ET. The prospective recipients cows were mated at different times so that they would receive embryos at the same time. In other words, animals in Groups 3, 4 and 5 were synchronized to be artificially inseminated 14 days prior to cows in Groups 1 and 2 (Figure 4.1).

Mated cows were further randomly assigned to either a treatment or control group. Cows were assigned to the following groups: (1) 14-day controls (14-SAL), (2) 14-day FSH/hCG (14-hCG), (3) 28-day controls (28-SAL), (4) 28-day pFSH/hCG
Figure 4.1. Cows in Groups 3, 4, and 5 were artificially inseminated (AI) 14 days prior to cows in Groups 1 and 2 so that embryos could be transferred (ET) on the same day. Mated recipients in Group 5 were treated the same as Group 4 but did not receive an embryo. Day 0 = Estrus.
receiving embryos (28-hCG) and (5) 28-day pFSH/hCG not receiving embryos (28-NO ET).

**Experimental Procedures**

Synchronization of estrus was accomplished with one treatment of 25 mg of prostaglandin F$_{2\alpha}$ (Lutalyse®, Pharmacia & Upjohn, Kalamazoo, MI). Initially, 36 cows were mated following onset of estrus for the 28-day groups (Groups 3, 4 and 5). Cows for the 14-day groups (Groups 1 and 2) were synchronized 14 days later with one treatment of 25 mg of prostaglandin F$_{2\alpha}$ (Lutalyse®) and 39 cows were mated following onset of estrus for Groups 1 and 2.

Prospective recipients were artificially inseminated with bulls of similar coat color patterns to ensure that calves would be distinctive at birth. Bulls of proven fertility from three different sire breeds were used. An Angus bull was used to mate black cows and a Gelbvieh bull and a Charolais bull were used to mate cows with other coat patterns.

Mated recipients were monitored via transrectal ultrasonography using an ultrasound machine (Aloka 500-V, Corometrics, Wallingford, CT) equipped with a 5 MHz linear rectal probe for follicular activity beginning on day 4 for 14-day recipients (Groups 1 and 2) and day 16 for 28-day recipients (Groups 3, 4 and 5). When at least one follicle $\geq$10mm was detected on either ovary for each cow, cows received a bolus injection of 10 units pFSH (Sioux Biochemical, Sioux Center, IA) s.c. in physiological saline (Groups 2, 4 and 5) or an equivalent volume of physiological saline in a similar manner (Groups 1 and 3) (Table 4.1). After 48 hours, cows that had been treated with pFSH received 3,000 IU of hCG i.v. and cows that had received saline were treated with
Table 4.1. Designated treatments for cows in each treatment group

<table>
<thead>
<tr>
<th>Group</th>
<th>Day 0†</th>
<th>Day 0 + 48 h</th>
<th>ET††</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>Saline</td>
<td>Saline</td>
<td>Yes</td>
</tr>
<tr>
<td>Group 2</td>
<td>FSH</td>
<td>hCG</td>
<td>Yes</td>
</tr>
<tr>
<td>Group 3</td>
<td>Saline</td>
<td>Saline</td>
<td>Yes</td>
</tr>
<tr>
<td>Group 4</td>
<td>FSH</td>
<td>hCG</td>
<td>Yes</td>
</tr>
<tr>
<td>Group 5</td>
<td>FSH</td>
<td>hCG</td>
<td>No</td>
</tr>
</tbody>
</table>

†Day 0 = 5 days post-estrus for Group 1 and 2 and Day 0 = 19 days post-estrus for Group 3, 4 and 5.
††ET = embryo transfer.
an equal volume of saline i.v. (Table 4.1). Recipients continued to be monitored by ultrasonography until 60 days of gestation.

Embryos for transfer were *in vitro*-produced (IVP) by an independent company (Bomed, Madison, WI) and shipped on day 6 of development at 39°C in TL-Hepes plus 10% fetal calf serum so that they would be at the morula or blastocyst stages at transfer. Semen from a Grey Brahman bull (*Bos indicus*) was used to inseminate oocytes obtained from an abattoir so that calves resulting from the IVP embryos could be distinguishable from the AI-produced calves (Gelbvieh, Charolais or Angus) by ear shape and length. Two IVP embryos were transferred deep into the horn contralateral to the CL in each of the recipients using a standard nonsurgical ET procedure in Groups 1 to 4, 7 days following hCG or second saline treatment. Recipients in Group 5 (28-NO ET) were not subjected to ET (Table 4.1).

Follicular status was monitored via transrectal ultrasonography at 2- to 3-day intervals in cows beginning on day 4 post-estrus for 14-day groups (Groups 1 and 2) and day 16 post-estrus for 28-day groups (Groups 3, 4 and 5). Follicular size was recorded at the time of the first and second treatments for comparison. The disappearance of a dominant follicle and/or appearance of secondary CL was considered to be indicative of ovulation resulting from treatment with hCG or saline. Pregnancies were also monitored via transrectal ultrasonography.

Blood samples were obtained by venipuncture of the jugular vein for hormone analysis. Blood samples were placed on ice immediately following collection and later centrifuged at 300 x g for 10 minutes. The resulting plasma was frozen and stored for analysis at a later time. Plasma samples were analyzed following extraction with acetone.
for progesterone levels using a commercially available progesterone assay kit (Diagnostic Systems Laboratory, Webster, TX).

**Statistical Analysis**

Progesterone values at different time points were compared using two way repeated measures analysis of variance (ANOVA). Ovulation and pregnancy rates were compared using Fisher’s Exact Test. Gestation lengths and calf birth weights were compared using ANOVA.

**RESULTS**

Initially, 39 cows were artificially inseminated in the 14-SAL (Group 1) and 14-hCG (Group 2) groups. Four of these cows did not develop follicles ≥10 mm on either ovary within 4 days of the other recipients and were removed from the experiment. The remaining 35 cows were then further randomly assigned to Group 1 (14-SAL) or Group 2 (14-hCG).

In Group 1 (14-SAL), 17 randomly assigned cows were treated on days 6 to 9 post-estrus (average = 6.6 days) with an equivalent volume of saline, followed 48 hours later with another saline treatment (Table 4.2). In this group, 6 of 17 (35.3%) cows inseminated were pregnant at 45 and 60 days. Follicle size increased in 50% of the cows (n = 6) in this group later determined to be pregnant by ultrasound scanning (Table 4.3) and 5 of 11 (45.4%) cows later determined not to be pregnant. No cows formed secondary CL following treatment with saline (Table 4.3).

In Group 2 (14-hCG), 18 cows were treated with 10 units of pFSH on days 5 to 8 and were then treated with 3,000 IU of hCG 48 hours after FSH treatment (Table 4.2). Average day of hCG treatment was 8.4 days post-estrus. In this group, eight cows
Table 4.2. Number of cows undergoing artificial insemination (AI), receiving the first and second treatments and undergoing embryo transfer (ET) in the different treatment groups

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Females inseminated (n)</th>
<th>Females removed (n)</th>
<th>1st treatment (n)</th>
<th>Females removed (n)</th>
<th>2nd treatment (n)</th>
<th>Females removed (n)</th>
<th>ET (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>17</td>
<td>0</td>
<td>17</td>
<td>0</td>
<td>17</td>
<td>0</td>
<td>17</td>
</tr>
<tr>
<td>Group 2</td>
<td>39†</td>
<td>4††</td>
<td>18</td>
<td>0</td>
<td>18</td>
<td>0</td>
<td>18</td>
</tr>
<tr>
<td>Group 3</td>
<td>12</td>
<td>4</td>
<td>8</td>
<td>2</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 4</td>
<td>36†††</td>
<td>9††††</td>
<td>11</td>
<td>0</td>
<td>11</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>Group 5</td>
<td>4</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>n/a††††</td>
<td></td>
</tr>
</tbody>
</table>

†Cows for Groups 1 and 2 were taken from the same group of estrus-synchronized cows.
††Females removed for failure to develop follicles ≥10 mm on either ovary during the treatment period.
†††Cows for Groups 3, 4 and 5 were taken from a same group of cows in which estrus was synchronized 14 days prior to cows synchronized for groups 1 and 2.
††††Females removed for failure to develop follicles ≥10 mm on either ovary during the treatment period, for showing signs of estrus prior to or during the treatment period and for natural double ovulation (n = 1).
†††††Females assigned to Group 5 were not subjected to embryo transfer (ET).
Table 4.3. Follicle response of pregnant recipients following treatment with saline plus saline (Groups 1 and 3) or FSH plus human chorionic gonadotropin (hCG) (Groups 2, 4, and 5)

<table>
<thead>
<tr>
<th>Group</th>
<th>Pregnant recipients (n)</th>
<th>Treatment</th>
<th>Follicle size increased</th>
<th>Follicles ovulated†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>6</td>
<td>Saline + Saline</td>
<td>3 (50%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Group 2</td>
<td>8</td>
<td>FSH + hCG</td>
<td>5 (62.5%)</td>
<td>4 (50%)</td>
</tr>
<tr>
<td>Group 3</td>
<td>6</td>
<td>Saline + Saline</td>
<td>3 (50%)</td>
<td>1 (16.7%)</td>
</tr>
<tr>
<td>Group 4</td>
<td>8</td>
<td>FSH + hCG</td>
<td>4 (50%)</td>
<td>3 (37.5%)</td>
</tr>
<tr>
<td>Group 5</td>
<td>4</td>
<td>FSH + hCG</td>
<td>0 (0%)</td>
<td>2 (50%)</td>
</tr>
</tbody>
</table>

†Not all follicles that increased in size ovulated. Conversely, some secondary CL were detected following treatment when follicle size decreased or remained the same.
(44.4%) were later determined to be pregnant by ultrasound scanning (n = 8). The largest follicle increased in 62.5% of the pregnant recipients in this group (Table 4.3). Ovulation was detected in 4 of 8 (50%) pregnant cows following treatment with hCG, although three cows in which the largest follicle increased between pFSH and hCG treatments did not ovulate (Table 4.3).

Similarly, 36 cows were inseminated to provide recipients for the 28-SAL, 28-hCG and 28-NO ET groups (Groups 3, 4 and 5) (Table 4.2). Since treatment began ~18 days post-estrus, some cows exhibited various signs of estrus prior to, during, or immediately following treatment with saline or pFSH/hCG and were removed from the experiment. Similarly, cows that did not develop any follicles ≥10 mm between days 17 to 21 post-estrus on either ovary were also removed from the experiment. One cow ovulated two follicles naturally prior to any treatment and was also removed from the experiment.

Also, since pregnancy could be detected by ultrasound scanning on day 25 post-estrus, cows that were not pregnant at the time of ET were also removed from the experiment, even though they may have received treatment, since timing of embryonic mortality could not be determined in these cows. Ovulation data could have been distorted by the fact that these cows may have been in estrus, although estrus signs had not been observed (Table 4.2).

Therefore, 12 cows received the initial saline treatment in Group 3 (28-SAL). However, four of these cows were in estrus prior to the second saline treatment and were removed from the experiment, while two more cows were not pregnant at the time of
transfer and did not receive embryos. Therefore, only six cows (n = 6) received both treatments of saline and received embryos and were used for data analysis (Table 4.2).

A secondary CL was unexpectedly detected in a cow in the control group (28-SAL) during the course of the experiment (1 of 6 or 16.7%) (Table 4.3). This CL could only be detected via ultrasonography and may have been an atretic CL that had not previously been recorded, as no other cases of such an occurrence could be found in the literature.

In Group 4 (28-hCG), 11 cows received pFSH following detection of at least one follicle ≥10 mm on either ovary, followed 48 hours later by hCG. No signs of estrus were detected prior to the transfer date in cows in this group. However, three cows were not pregnant at the time of transfer and did not receive embryos. Therefore, only eight cows received both treatments and received embryos (n = 8) (Table 4.2).

In this group, 3 of 8 cows (37.5%) ovulated and developed secondary CL following treatment with pFSH and hCG. In fact, one cow developed four secondary CL in this group. Follicle sizes had increased between pFSH and hCG treatments in two cows that ovulated, including the cow with multiple ovulations. However, follicle size had decreased following pFSH treatment in the remaining cow that ovulated in this treatment group.

Two cows ovulated follicles ipsilateral to the primary CL and one cow ovulated a follicle contralateral to the primary CL. Although pFSH caused superstimulation in one cow, pFSH was not consistent in increasing follicular size in this group. Follicle size had increased in four cows by the time of hCG treatment (two ovulated), while in the
remaining four cows, follicle size had either decreased or remained the same (one ovulated).

In Group 5 (28-NO ET), four cows were treated with pFSH following detection of a follicle ≥10 mm between days 23 to 26 of pregnancy and were treated with hCG on days 25 to 28 of pregnancy (n = 4) (Table 4.2). No embryos were transferred to females in this group.

Two cows (50%) in Group 5 developed secondary CL, both on the ovary ipsilateral to the primary CL. In each of these cows, the largest follicle at the time of pFSH treatment had decreased in size by the time of hCG treatment (48 hours following pFSH treatment).

Data on ovulation rates in Group 4 and Group 5 were combined to determine effectiveness of pFSH pretreatment followed by hCG treatment for ovulation induction, since both groups were treated similarly. The only difference in these two groups was that ET was performed in cows in Group 4 but not in cows in Group 5. No difference could be detected in ovulation rate in animals receiving pFSH followed by hCG (Groups 4 and 5) when compared with nontreated controls (Group 3).

Two cows in Group 3 (33%) lost their pregnancy between ET and 45 days post-estrus. An additional pregnancy was lost between 45 and 60 days of gestation. Interestingly, no cows lost their pregnancies prior to 35 days in 28-day recipients that had been treated with pFSH and hCG 7 days prior to ET (Group 4). However, four cows (50%) lost their pregnancies between 35 and 60 days in this group. Therefore, 60-day pregnancy rates were the same (50%) for the control and treatment groups (Figure 4.2).
Figure 4.2. Pregnancy loss in cows treated with saline (Group 3) or human chorionic gonadotropin (hCG) (Groups 4 and 5) on day 21 of pregnancy. Cows in Groups 3 and 4 were also subjected to nonsurgical embryo transfer (ET) ~28-days of pregnancy, while cows in Group 5 were not subjected to ET.
The incidence of pregnancy loss did not appear to be affected by the presence of a secondary CL induced by hCG treatment, as 2 of 3 cows ovulating in this group were still pregnant on day 60 of gestation.

More importantly, all four cows in Group 5 (28-NO ET) were still pregnant at 60 days post-estrus. Although pregnancy rates at 60 days tended to be lower for cows receiving embryos nonsurgically after day 25 of pregnancy than for cows not receiving embryos nonsurgically after day 25 of pregnancy, this difference was not significant (Figure 4.2). hCG treatment did not affect pregnancy loss, as pregnancy loss was similar for pregnant cows treated with hCG or saline that received embryos after day 25 of pregnancy.

Twins resulting from asynchronous transfer were detected at 45 days of gestation by ultrasound in a recipient in the 14-day control group (Group 1) that had received two expanded blastocysts (day 7.5) on day 16 post-estrus. Fetal age difference was estimated at ~8.5 days. Two bull calves were born at 287 days of gestation, weighing 36 kg and 29 kg respectively, for the AI produced calf and the IVP calf. The AI calf was Charolais-sired and the IVP calf was Brahman-sired, easily distinguishable by ear length and hair coat color. Parentage for each calf was later confirmed by chromosome analysis by an independent company (ImmGen, College Station, TX). Unfortunately, the recipient had a uterine prolapse and died within 1 week of calving. The calves were successfully grafted onto an available dairy cow, which raised them through weaning. The asynchronous twin was diagnosed with a left ventricular valve defect, but is still alive and well at the time of this writing.
An additional set of asynchronous twins that had not been detected by ultrasound scanning was also born to a recipient in Group 2 (14-hCG). This recipient received pFSH on day 6 following estrus and then was treated with hCG on day 8. She had a follicle that measured 15 mm on the ovary ipsilateral to the CL at the time of pFSH, which subsequently grew to 18 mm at the time of hCG treatment and ovulated following treatment. One IVP expanded blastocyst and one IVP blastocyst were transferred to the uterine horn contralateral to the primary and secondary CL at 14 days post-estrus. Although this recipient was repeatedly palpated following transfer (day 45, day 66 and day 83 of pregnancy), the asynchronous twin was not detected until birth.

The twin bull calves were born at 285 days of gestation, weighing 33 kg and 39 kg, respectively, for the AI-produced and IVP calves. The calves could be distinguished by ear length, as the AI calf was Angus-sired and the IVP calf was Brahman-sired. Parentage was confirmed by chromosome analysis by an independent contractor (ImmGen, College Station, TX). The AI calf died from complications during calving (skin samples were saved for testing), but the recipient successfully raised the IVP calf through weaning. The calf is alive and well at the time of this writing.

Bulls of proven fertility from three different breeds were used for the initial AI: Angus for black recipients and Charolais and Gelbvieh for red and other color recipients. Data from all groups were combined for comparisons of gestation length and birth weights for single and twin calves, since twin calves were only produced in the 14-day groups (Group 1 and 2).

Gestation lengths for single calf gestations were not different among sire breeds (285.2 ± 2.1 days, 285.0 ± 1.4 days and 291.8 ± 2.0 days for Angus, Charolais and
Angus- and Charolais-sired calves had similar birth weights (33.7 ± 1.7 kg vs. 34.5 ± 1.2 kg), but Angus-sired calves weighed less at birth than Gelbvieh-sired calves (33.7 ± 1.7 kg vs. 41.1 ± 1.9 kg; P<0.05) (Table 4.4). No differences were detected between males and females for gestation length or calf birth weight.

Gestation lengths were similar between singleton and the twin gestations (287.3 ± 1.3 days vs. 286.0 ± 1.0 days, respectively). There were also no differences in birth weight for singleton calves resulting from AI and twins calves resulting from AI or IVP (36.4 ± 1.2 kg vs. 34.5 ± 1.8 kg and 33.8 ± 4.8 kg, respectively) (Table 4.5). It must be noted that the twin calves resulting from AI were 7 to 8.5 days older (gestational age) than their IVP co-twins, as the IVP embryos were transferred to recipients on days 14 and 16 post-estrus.

Only mated recipients later palpated pregnant were used in comparing plasma progesterone levels following treatment in all groups. Also, data for Groups 4 and 5 were combined for analysis, as these two groups were treated with pFSH and hCG at similar timepoints; the difference between the two groups was that Group 5 did not receive an embryo 7 days after hCG treatment.

There were no differences in plasma progesterone levels following pFSH treatment ~5 days post-estrus followed within 48 hours with hCG treatment in mated recipients in Group 2 when compared to mated recipients treated with saline at similar time points in Group 1 (Figure 4.3). Likewise, there were no differences in plasma progesterone levels following pFSH treatment 18 to 25 days post-estrus followed within
Table 4.4. Mean gestation lengths (±SEM) and birth weights of singleton calves sired by bulls of different breeds

<table>
<thead>
<tr>
<th>Sire breed</th>
<th>Calves (n)</th>
<th>Gestation length (days)</th>
<th>Birth weight (kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angus</td>
<td>6</td>
<td>285.2 ± 2.1</td>
<td>33.7 ± 1.7(^a)</td>
</tr>
<tr>
<td>Charolais</td>
<td>4</td>
<td>285.0 ± 1.4</td>
<td>34.5 ± 1.2(^{a,b})</td>
</tr>
<tr>
<td>Gelbvieh</td>
<td>5</td>
<td>291.8 ± 2.0</td>
<td>41.1 ± 1.9(^b)</td>
</tr>
</tbody>
</table>

\(^{a,b}\)Values within columns with different superscripts are different (P<0.05).
Table 4.5. Mean gestation lengths (±SEM) and birth weights of singleton calves (both sexes) resulting from artificial insemination (AI) and twin calves (all males) resulting from AI and in vitro-production (IVP)

<table>
<thead>
<tr>
<th>Type of pregnancy</th>
<th>Calves (n)</th>
<th>Gestation length (days)</th>
<th>Birth weight (kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AI Singleton</td>
<td>15</td>
<td>285.1 ± 4.1</td>
<td>36.4 ± 1.3</td>
</tr>
<tr>
<td>AI Twin</td>
<td>2</td>
<td>286.0 ± 1.4</td>
<td>34.5 ± 1.8†</td>
</tr>
<tr>
<td>IVP Twin</td>
<td>2</td>
<td>286.0 ± 1.4</td>
<td>33.8 ± 4.8†</td>
</tr>
</tbody>
</table>

†AI twin fetal age is ~7 days greater than IVP fetal age.
Figure 4.3. Progesterone ($P_4$) levels (±SEM) for Groups 1 and 2 at the time of the 1$^{st}$ treatment (Saline or FSH) on days 5 to 7, 2$^{nd}$ treatment (Saline or hCG) on days 7 to 9, 48 to 72 h following the 2$^{nd}$ treatment and 120 to 144 h following the 2$^{nd}$ treatment for Groups 1 and 2.
48 hours with hCG treatment in mated recipients in Groups 4 and 5 when compared with mated recipients treated with saline at similar time points in Group 3 (Figure 4.4).

On an interesting side note, one cow (9069) in Group 2 had very low plasma progesterone levels (below 1.0 ng/ml) up to 12 days post-estrus (Figure 4.5), after which plasma progesterone levels began to rise. This mated recipient was diagnosed pregnant by ultrasonography at 45 days of gestation, although the pregnancy was lost by 60 days post-estrus.

DISCUSSION

Treatment with pFSH prior to treatment with hCG did not improve ovulation induction in pregnant cattle. In fact, pre-treatment with pFSH prior to hCG may have actually decreased the effectiveness of hCG in inducing ovulation in cows treated early in pregnancy by delaying the day of hCG treatment. In this experiment, only 62.5% (5 of 8) cows treated with hCG early in pregnancy ovulated. However, in a prior experiment (Chapter II), 100% of cattle treated with hCG around day 7 of pregnancy ovulated, which is similar to results obtained by other researchers (Rajamahendran and Sianangama, 1992).

However, hCG was administered 48 hours after pFSH treatment, which occurred when a follicle of at least 10 mm was detected by ultrasound. This was the same criterion used for hCG treatment in the previous experiment, which caused a 2-day shift in the average day of treatment between the separate experiments. In the previous experiment (Chapter II), the average day for hCG treatment was 6.3 days of pregnancy. In the present experiment, the average day of pFSH treatment was 6.4 days of pregnancy, which
Figure 4.4. Progesterone (P₄) levels (±SEM) at 1st treatment (Saline or FSH) on day 18 to 25, 2nd treatment (Saline or hCG) on days 20 to 27, 48 to 72 h following 2nd treatment (day 0) and 120 to 144 h following 2nd treatment for Groups 3, 4, and 5. Data for Groups 4 and 5 were combined since they were treated similarly with respect to FSH and hCG treatments.
Figure 4.5. Plasma progesterone (P₄) values for recipient 9069 (Group 2). Note the low progesterone values through 12 days post-estrus. This cow was pregnant at 45 days but lost the pregnancy by 60 days.
delayed the average day of hCG treatment to 8.4 days-post-estrus, ~48 hours after the average day of treatment in the prior experiment.

Price and Webb (1989) found hCG to be very effective in causing ovulation of large mid-cycle follicles when given on days 4 to 7 (83%) and 14 to 16 (66%), but not when given during days 8 to 13 (35%) of the cycle in nonpregnant cattle. It was noted that this could be due to the absence of large, responsive follicles at the time of treatment. In this experiment, 2 of 5 pregnant cows (40%) in which follicle size increased between pFSH treatment on days 5 to 7 post-estrus and hCG treatment 48 hours later did in fact ovulate (Table 4.3). Follicles either decreased or remained static in 3 of 8 (37.5%) cows in this group (14-hCG), although 15 of these cows (33%) ovulated.

pFSH treatment prior to hCG treatment induced superstimulation in at least one cow in the 28-hCG group. This cow had a 14 mm follicle on the ovary ipsilateral to the CL at the time of pFSH treatment and four follicles ranging from 9 to 15 mm on that ovary on the day of hCG treatment, three of which apparently ovulated, with four CL resulting on the ovary (original CL plus three secondary CL). Two other cows ovulated in this group, so 3 of 8 (37.5%) cows treated with hCG between days 20 and 22 of pregnancy ovulated (28-hCG) (Table 4.3). Also, 2 of 4 cows in Group 5 (28-NO ET) treated with hCG ovulated as well. This is similar to results obtained in a previous experiment at this station (1 of 4 cows treated with 3,000 IU of hCG at approximately the same period of pregnancy ovulated).

However, this is different from results published by Bridges et al. (2000) who pre-treated 29- to 50-day pregnant cows with pFSH prior to treatment with hCG and were successful in 23 of 30 cases (77%). In that report, the primary CL were either manually
removed or regressed with prostaglandin prior to hCG treatment and pregnancy was maintained with exogenous progestin treatments. Lulai et al. (1994) reported similar results using two LH treatments on days 33 and 34 of pregnancy to induce ovulation in 10 of 10 cows following induced luteal regression 5 days prior to LH treatment. Once again, pregnancy was maintained with exogenous progestins (norgestomet implants). It is possible that CL regression prior to hCG/LH treatment induces waves of follicles that may be less likely to be atretic and, therefore, more responsive to induction of ovulation during pregnancy.

Although hCG treatment has been implicated in pregnancy loss in pregnant pony mares (Allen, 1975), no such phenomenon has been reported in cows. In a prior experiment, there was a high incidence of pregnancy loss following hCG treatment and AI, but the exact cause of pregnancy loss could not be determined. In the present experiment, cows receiving hCG but no embryo did not lose any pregnancies between days 25 and 60 of pregnancy (4 of 4, or 100% maintenance of pregnancy. However, 50% of cows treated with hCG or saline at a similar date and submitted to the ET technique after day 25 lost pregnancies (7 of 14) by day 60.

Weaver et al. (1989) found that cows reinseminated in the uterine body during pregnancy had a lower pregnancy rate by day 45 post-breeding. The nonsurgical ET technique is even more invasive than the AI technique. In AI, the cervix is grasped per rectum while an insemination device is passed through the cervix and the semen is deposited into the uterine body. With the ET technique, the transfer gun is passed as far as possible into the uterine horn (normally the ipsilateral horn to the CL, but in this experiment, into the contralateral horn).
In pregnant cows, the bovine conceptus has partially occupied the contralateral horn by day 24 of pregnancy (Chang, 1952; King et al., 1982; Thatcher et al., 1986). Therefore, pregnancy loss could be caused by the transfer procedure itself, possibly by disruption of fetal membranes or by a localized prostaglandin release caused by abrasions on the uterine endometrium. It is encouraging to note that although pregnancy loss may occur following ET to a pregnant recipient, this does not happen in 100% of the cases. ET to a pregnant recipient can occasionally occur in commercial ET when the pregnant recipient shows signs of estrus (personal observation).

Treatment of pregnant recipients with pFSH and hCG prior to ET did not result in a greater twinning rate (1 of 8 or 12.5% vs. 1 of 6 or 16.7%, respectively) in the 14-day treated and the 14-day nontreated groups. No asynchronous twins were produced in the 28-day groups. These results are similar to a prior experiment in which embryos were transferred to pregnant recipients at day 14 post-estrus without hCG treatment (one set of twins resulted from seven pregnant recipients (14.3%) receiving ET embryos) (Chapter III).

Gestation lengths in this experiment were not different for singleton pregnancies vs. asynchronous twin pregnancies (287.3 ± 1.3 days vs. 286.0 ± 1.0 day). This is an interesting finding, as normal twin pregnancies are shorter than singleton pregnancies (Anderson et al., 1978; Davis et al., 1989; Reichenbach et al., 1992; Vasques et al., 1995). In fact, gestation lengths resulting from transfer of synchronous IVP embryos to previously mated recipients (Chapter III) in a prior experiment at this station were shorter for twin pregnancies when compared with singleton pregnancies (277.3 ± 2.2 days vs. 283.8 ± 0.8 days; P<0.05).
Birth weights for AI produced twin calves were not different from their 7-day asynchronous IVP co-twins (34.5 ± 1.8 kg vs. 33.8 ± 4.8 kg, respectively). This is also different from the results obtained in Chapter III with synchronous twin sets with IVP calves being heavier at birth than their co-twins resulting from AI (40.1 ± 2.0 kg vs. 31.2 ± 0.8 kg; P<0.05).

It is generally accepted that close synchrony between the embryo and the uterine environment is necessary for pregnancy to be maintained (Nicholas, 1933; Rowson, 1972). Ovine embryos placed in “younger,” asynchronous uteri have been shown to cause a shift in the uterine secretory protein profile to a more advanced stage (Ashworth and Bazer, 1989). Similarly, day-7 embryos placed in recipients 10 days post-estrus exhibited an advanced stage of development 8 days after transfer when compared with controls (Albihn et al., 1991).

However, it appears from these and previous data that uterine stage embryos can develop successfully in an asynchronous uterine horn once maternal recognition has been achieved by an ongoing synchronous pregnancy. Thatcher et al. (1989) used a different approach to produce pregnancies in day-14 recipients with the transfer of 7-day embryos. The recipients were treated with buserelin beginning on day 12 and treatment was repeated every 3 days until 12 days after ET. Day-7 embryos (morula- or blastocyst-stage) were placed in recipients at 9.5 to 15 days post-estrus. In the recipients receiving embryos on days 9.5 to 12.5 days post-estrus, 6 of 10 (60%) became pregnant. One recipient receiving a day-7 embryo on day 14.5 post-estrus also became pregnant (1 of 8 or 12.5%).
No asynchronous twins were produced in the cows receiving embryos nonsurgically ~ 28 days of pregnancy. In fact, it appears that the ET technique may cause pregnancy loss in pregnant recipients when the transfer occurs around day 28 of pregnancy. If pregnancy loss following ET at this stage is indeed caused by some stimulus caused by the nonsurgical ET technique, surgical transfer may be necessary to achieve asynchronous twins past 24 days of pregnancy.

FSH and hCG treatment 6 to 7 days prior to ET did not improve asynchronous twinning rates in this experiment over non-treated controls, nor did it affect plasma progesterone levels. However, asynchronous twins were once again produced in recipients receiving day-7 embryos at days 14 to 16 of pregnancy, demonstrating that asynchronous embryos can develop in a more chronologically advanced uterine environment.
CHAPTER V

NONSURGICAL ASYNCHRONOUS EMBRYO TRANSFER TO PREVIOUSLY INSEMINATED BEEF RECIPIENTS PRIOR TO AND FOLLOWING MATERNAL RECOGNITION

INTRODUCTION

Superfetation occurs when a pregnant animal shows signs of estrus during the course of pregnancy and is mated so that two or more fetuses resulting from different ovulation cycles and conception times are present in the uterus at the same time (Hurnik et al., 1995; Long, 2001). This phenomenon has been described in rodents such as the mouse (Littleford and Gysin, 1944; Barnett and Munro, 1970), the rat (Slonaker, 1934), the rabbit (Pickard, 1928) and hares (Martinet, 1980; Caillol et al., 1991). Superfetation has also been reported in domestic species such as sheep (Smith, 1927; Scanlon, 1960), pigs (Smith, 1927; Larivée, 1972), buffalo (Rao et al., 1987), horses (Mumford, cited by Leroy and Pechdo, 1950) and a burro (Short, 1964). There have also been credible reports of superfetation in cattle (Dalrymple and Jenkins, 1951; Simmons, 1960; Gee, 1971; Hall, 1987; Hunsley, 1998). These reportedly occurred following natural breeding (Dalrymple and Jenkins, 1951; Gee, 1971), embryo transfer (ET) followed by natural breeding (Hall, 1987; Hunsley, 1998) and natural breeding followed by artificial insemination (AI) (Simmons, 1960).

There is controversy over the actual existence of such a phenomenon, in part due to lack of credible evidence in the case reports. In cattle, for example, superfetation has been suggested in reports of consecutive parturitions at less than normal intervals (Dalrymple and Jenkins, 1951; Simmons, 1960; Bell, 1964; Brough, 1964; Gee, 1971; Nottle, 1976) or instances where a normal calf and a less developed fetus or calf were
expelled at parturition (Wewer, 1952; Vandeplassche, 1969; Hall, 1987; Hunsley, 1998). However, some reports are more credible, as in reports of superfetation in cattle following natural breeding of recipients following ET (Hall, 1987; Hunsley, 1998).

Scientists have attempted to explain superfetation by other reproductive phenomena. For example, Vandeplassche (1969) reviewed several cases of apparent superfetation in pigs and cows and suggested that these reports could be explained by embryonic diapause. However, the only known ungulate to exhibit embryonic diapause is the Roe deer (Renfree and Shaw, 2000), first noted in 1651 by William Harvey (cited by Eckstein et al., 1959).

Other researchers believe that at least some cases of apparent superfetation may be explained by differentiated growth of twins in utero. This phenomenon has been well documented in humans with the advent of ultrasound technology (Kol et al., 1993; Weissman et al., 1993). In humans, growth discordance between twins is generally associated with congenital malformations of the smaller twin, usually resulting in the death of the malformed twin (Kol et al., 1993). Although no reports of growth discordance could be found in animals, Kuntz (1920) reported two cases involving cats and one involving a dog where fetuses of different sizes were recovered at necropsy or surgery. The size difference was attributed to death of some of the fetuses without resorption.

Another important barrier for the occurrence of superfetation for most species is that it has been clear since early experiments with ET that a high degree of synchrony between the embryo and the uterine environment is necessary for a pregnancy to be successful. This concept was shown to hold true for domestic species such as the sheep.
and cow (Moore and Shelton, 1964; Rowson et al., 1972). It was later shown that this was due to a phenomenon termed maternal recognition, where the embryo signals the uterus to prevent luteolysis and permit the pregnancy to continue (Thatcher et al., 1986).

In cattle, the bovine embryo produces a protein known as bovine interferon-τ (bIFN-τ) at approximately day 16 of the estrous cycle to prevent luteolysis (Thatcher et al., 1986). It is presently believed that IFN-τ acts by inducing the production of an endometrial prostaglandin synthetase inhibitor (EPSI) by the uterine endometrial cells that blocks prostaglandin production by the endometrium (Thatcher et al., 1989). bIFN-τ may also act by altering gene expression in the endometrial epithelial cell to affect expression of molecules that in turn affect the synthesis of PGF2α (Thatcher et al., 1997). If IFN-τ production is affected in any way so that it does not occur at the appropriate time, luteolysis occurs, followed by embryonic death.

Several signs indicate that maternal recognition in cattle is localized in the uterine horn ipsilateral to the CL. First, blood flow to the ipsilateral uterine horn increases on days 16 to 17 of the estrous cycle (Ford et al., 1979). Secondly, at this stage, the embryo has not yet elongated to fill the contralateral uterine horn (Chang, 1952). However, bovine embryos can develop naturally in the horn contralateral to the CL when a viable fetus is present in the uterine horn ipsilateral to the CL (Scanlon, 1972). This has been shown to be true following embryo transfer of embryos to opposite horns in bovine recipients (Rowson et al., 1971). Transfer of embryos into the uterine horn contralateral to the CL of mated bovine recipients has since been used extensively to produce twins (Testart et al., 1975; Sreenan and McDonagh, 1979; Renard et al., 1979; Heyman et al., 1980; Sreenan et al., 1981; Sreenan and Diskin, 1988, 1989; McEvoy et al., 1995). There
have also been a few reports of fetuses developing in the contralateral uterine horn following ET to the contralateral uterine horn of mated recipients without the presence of a fetus in the ipsilateral uterine horn (Sreenan and McDonagh, 1979; Renard et al., 1979; Heyman et al., 1980; Sreenan et al., 1981; Sreenan and Diskin, 1989). However, it could not be determined if a fetus had been present in that horn at the time of maternal recognition, eventually resorbing, yet ensuring the maintenance of the CL.

Hafez and Pincus (1956) successfully produced asynchronous sets of twins in rabbits by transfer of asynchronous embryos to the left uterine horn of mated recipient does which had undergone left ovariectomies. Fetuses were 3.5 days apart in age but were born at the same time. Camillo et al. (1997) transferred day-7 embryos to inseminated recipient mares that ovulated 2 to 4 days after the donor, or 2 to 7 days prior to the donor. They achieved successful twin pregnancies by transferring embryos in mares that had ovulated and were mated up to 3 days after the donor, however, no pregnancies resulted from transferred embryos to recipient mares that ovulated and were mated 2 to 7 days prior to the donor.

Lawson and Cahill (cited by Wilmut and Sales, 1981) transferred synchronous embryos together with asynchronous advanced or retarded embryos, but all resulting lambs were derived from the synchronous embryos. Wilmut and Sales (1981) transferred synchronous embryos with embryos that were ±3 days asynchronous and were also unsuccessful in establishing pregnancies from the asynchronous embryos. It was concluded that a synchronous embryo, although capable of preventing luteolysis and establishing a pregnancy, could not protect an asynchronous embryo from degeneration and/or resorption.
It has been reported that placing embryos in asynchronous uterine environments prior to maternal recognition may alter the rate of development of the embryo. Lawson et al. (1983) reported that sheep embryos placed in less chronologically advanced uterine environments (4-day embryos in recipients 1 to 2 days post-estrus) appeared to decrease the rate of embryonic development when compared to controls, while embryos placed in more advanced uterine environments (day-4 embryo in recipients 6 to 7 days post-estrus) increased their rate of development when compared to controls, up to day 12 of pregnancy, after which they degenerated and were resorbed. A similar phenomenon has been reported in the cow (Albihn et al., 1991).

Ashworth and Bazer (1989) showed that later stage embryos placed in less chronologically advanced recipients caused a shift in the uterine secretory protein profile to one more similar to that of more chronologically advanced recipients. Therefore, it appears that there is a concerted effort by the uterus and the embryo to establish some degree of synchronicity so that maternal recognition can occur.

In fact, transfer of ovine and bovine embryos to recipients ±48 hours of the donor’s stage of the estrous cycle produces acceptable pregnancy rates (Rowson and Moor, 1966; Rowson et al., 1972, respectively). However, although pregnancies were produced with embryos placed in recipients that showed estrus ±72 hours of donor estrus, pregnancy rates were less than usual.

Maternal recognition occurs during the time of trophoblast expansion, ensuring that the signal occurs throughout the uterus (Geisert et al., 1992). The bovine blastocyst begins to elongate at approximately day 12 post-mating and has filled the horn ipsilateral to the CL by day 18 to 20 (Chang, 1952). By day 20 to 24 of pregnancy, the conceptus
has partially occupied the contralateral horn (Chang, 1952; King et al., 1982; Thatcher et al., 1986).

In previous experiments (Chapters III and IV), twin pregnancies have been produced in beef cows from the nonsurgical transfer of day-7 embryos to mated recipients 13 to 16 days post-estrus. No twin pregnancies resulted from day-7 embryos transferred to mated recipients after day 23 of pregnancy. It also appeared that nonsurgical embryo transfer to recipients after day 23 may have caused resorption of ongoing pregnancies.

In cattle, maternal recognition is generally accepted as occurring on day 16 of pregnancy, as shown by experiments where embryos transferred to synchronous recipients up to day 16 resulted in pregnancies, but no pregnancies resulted from synchronous transfer of embryos older than 16 days (Bettermidge et al., 1980). It has also been shown that asynchronous embryos transferred prior to maternal recognition degenerate (Albihn et al., 1991). However, it is possible that once maternal recognition has been achieved, this mechanism may no longer be a factor in degeneration of embryos.

Due in part to the apparent loss of pregnancy following transfer of embryos to pregnant cows >25 days post mating, possibly caused by the embryo transfer procedure, no asynchronous twins have been produced in prior experiments in cows >16 days post-mating (Chapter IV). Therefore, the primary objective of this experiment is to produce asynchronous twin pregnancies by transferring day-7 embryos to the contralateral uterine horn of inseminated recipients ±3 days of maternal recognition, prior to invasion of the contralateral uterine horn by the elongating conceptus.
Plasma progesterone levels have been found to be higher in pregnant cows versus nonpregnant cows as early as day 10 of the mated cycle (Lukaszewska and Hansel, 1980). There is evidence that bovine embryos produce progesterone (Shemesh et al., 1979; Chiappe et al., 2002). Lukaszewska and Hansel (1980) suggested that the embryo might be responsible for at least a portion of the observed elevated progesterone level in pregnant cows. Therefore, a secondary objective of this experiment is to determine if progesterone levels in mated bovine recipients increase following the transfer of embryos to the uterine horn contralateral to an ongoing pregnancy at different stages of pregnancy.

**MATERIALS AND METHODS**

**Experimental Animals**

The cows used in this experiment were part of the St. Gabriel Experiment Station (Louisiana State University) reproductive physiology research herd. Recipients consisted of crossbred, multiparous, nonpregnant beef cows <12 years of age. Body condition scores (BCS) were assessed prior to the experiment and only cows with BCS between 5 and 8 were used in the experiment.

**Experimental Design**

Three groups of cows were synchronized 3 days apart with one treatment of prostaglandin F2α and mated at different times so that the embryos could be transferred on the same day (Figure 5.1). Cows were artificially inseminated ~12 hours after onset of estrus to provide pregnant recipients for embryo transfer.

Cows were assigned to three groups according to estrus dates. Group 1 (n = 12) consisted of mated cows that received 7-day embryos ~7 days post-estrus (controls). Group 2 (n = 10) consisted of mated cows that received 7-day embryos on day 13 of
Figure 5.1. Recipients were synchronized and mated so that embryos would be transferred (ET) on the same date. Artificial insemination (AI) occurred ~12 hours following onset of estrus. Shaded area (__) depicts time period during the mated estrous cycle in which blood was collected for analysis for each group. Day 0 = estrus
pregnancy (3 days prior to maternal recognition). Group 3 (n = 9) consisted of mated cows that received 7-day embryos 19 days following estrus (3 days after maternal recognition).

**Experimental Procedures**

All cows were artificially inseminated with semen from a Charolais bull (to ensure ease of recognition at birth) of proven fertility following estrus synchronization with 25 mg of prostaglandin F$_{2\alpha}$ (Lutalyse®, Pharmacia & Upjohn, Kalamazoo, MI).

Embryos for transfer to mated recipients were produced *in vitro* by a commercial company (Bomed, Madison, WI) and shipped overnight in a portable incubator at 39°C in culture medium consisting of TL-Hepes plus 10% fetal calf serum. The embryos were shipped on day 6 of culture so that they would be day-7 to day-8 embryos on the day of transfer. Oocytes used to produce the embryos were collected from ovaries obtained at a slaughterhouse and Brahman (*Bos indicus*) semen was used for fertilization so calves resulting from these matings would be clearly distinguishable from the Charolais-sired calves (*Bos taurus*).

Two IVP day 7 embryos (one blastocyst plus one blastocyst or morula) were transferred nonsurgically to the uterine horn contralateral to the CL in each recipient cow on day 7, day 13 or day 19 for each appropriate group using a standard nonsurgical ET technique. Recipients were immobilized by means of a hydraulic squeeze chute and palpated *per rectum* for uterine and ovarian normality as well as for the presence of a CL. Ultrasonic scanning of the ovaries and reproductive tract was performed with an ultrasound machine (Aloka 500-V, Corometrics, Wallingford, CT) equipped with a 5 MHz rectal linear probe.
Recipients were checked for pregnancy by palpation per rectum at ~60 days of pregnancy. Ultrasonography was used to confirm pregnancies and determine the presence of twins. Since cows were checked for pregnancy on the same day, cows in Group 1 were first checked for pregnancy on day 54 of pregnancy, cows in Group 2 were checked for pregnancy on day 60 of pregnancy and cows in Group 3 were checked for pregnancy on day 66 of pregnancy.

Blood samples were collected daily for analysis of circulating hormone levels beginning 2 days prior to embryo transfer until 3 days following embryo transfer. Blood samples were obtained from all animals by venipuncture of the jugular vein. The samples were placed on ice immediately after collection and later centrifuged at 300 x g for 10 minutes. The resulting plasma was placed in cryotubes, frozen and stored for analysis at a later time. At a later date, the samples were extracted with acetone and analyzed for progesterone levels using a commercially available progesterone assay kit (Diagnostic Systems Laboratory, Webster, TX).

**Statistical Analysis**

Plasma progesterone levels at different time points were analyzed using repeated measures analysis of variance (ANOVA). Pregnancy rates and twinning rates were compared using Fisher’s Exact Test. Gestation lengths and calf birth weights were recorded at birth and compared using ANOVA.

**RESULTS**

One cow in Group 2 died (struck by lightning) after undergoing ET prior to pregnancy confirmation and was removed from the experiment. Pregnancy rates at ~60
days were similar for Groups 1 and 2 (83.3% or 10 of 12 females and 77.8% or 7 of 9 females, respectively), but lower for Group 3 (33.3% or 3 of 9 females) (P<0.05).

Progesterone concentrations in 3 of the 9 cows in Group 3 were gradually declining at the time of transfer (Figure 5.2). In fact, 2 of these cows were recorded in estrus within 12 hours of ET. Progesterone levels declined dramatically following ET in one cow (2074) in this group (Figure 5.3), although it cannot be ascertained that this was due to the ET procedure, as this occurred on day 19 of the estrous cycle, when levels may have decreased following lack of maternal recognition. The fact that progesterone declined at this stage of the cycle (day 19) is not unusual. However, the dramatic decline from 9.9 ng/ml to 2.0 ng/ml for cow 2074 is unusual. Two additional cows in this group that had elevated progesterone levels up to day 23 and 24 of pregnancy lost their pregnancies prior to 66 days.

Following ultrasonography ~60 days of gestation, 30% (3 of 10) of the pregnant recipients in Group 1 were determined to have twin fetuses in opposite horns. In Group 2, twins were detected in one recipient, with a fetus in each uterine horn (1 of 7 females or 14.3%). This cow received an expanded blastocyst and an early blastocyst on day 13 of her mated cycle, so the estimated age differential for the fetuses was 6 days. Twinning rate was not different between Groups 1 and 2. No twins were could be detected following ultrasound evaluation in Group 3.

In Group 1, 9 of 10 cows pregnant at 54 days of gestation (90%) calved. Six calves were singletons, all resulting from AI. Three recipients calved twin sets consisting of one Charolais-sired (AI) calf and one Brahman-sired (IVP) calf. One of the twins resulting from IVP was born dead.
Figure 5.2. Plasma progesterone ($P_4$) for cows 2047, 5001 and 6003 in Group 3. Note that progesterone values were declining prior to embryo transfer (ET). In fact, recipients 2047 and 5001 were in estrus on the day of ET.
Figure 5.3. Plasma progesterone ($P_4$) for cow 2074 in Group 3. Note the dramatic decline of progesterone values following embryo transfer (ET).
In Group 2, 6 of 7 cows (85.7%) that had been diagnosed pregnant at ~60 days calved, with five singleton calves resulting from AI (Charolais-sired). The remaining cow calved a set of twins consisting of a Charolais-sired bull calf resulting from AI and a Brahman-sired bull calf resulting from transfer of an IVP embryo. Parentage of each calf was later confirmed by chromosome analysis by an independent company (ImmGen, College Station, TX).

In Group 3, 2 of 3 cows (67%) diagnosed pregnant ~60 days post-estrus calved singleton calves. No twins resulted in this group.

Gestation length for singleton pregnancies in all groups was not different from gestation length for synchronous twin pregnancies in Group 1 (285.7 ± 1.6 days vs. 285.0 ± 1.5 days) (Table 5.1). Gestation length for the lone asynchronous twin pregnancy in Group 2 was 283 days.

Birth weights for singleton calves in all groups were not different from birth weights for AI twins or IVP co-twins in the Group 1 (40.1 ± 1.3 kg vs. 37.2 ± 3.2 and 35.3 ± 0.8 kg) (Table 5.1). Birth weights for the AI-produced (Charolais-sired) twins were not different from birth weights for their IVP co-twins (37.2 ± 3.2 kg vs. 35.3 ± 0.8 kg). For the lone twin pregnancy in Group 2, the Charolais-sired bull calf (AI) weighed 40.4 kg at birth, while the IVP co-twin bull calf weighed 26.8 kg at birth.

No calving difficulties or retained placentae were recorded for any of the cows in all groups. One cow in the control group (Group 1) calving twins had a normal live Charolais-sired (AI) calf at birth, while the Brahman-sired (IVP) co-twin was born dead.

Only plasma progesterone values from pregnant females were used for analysis of progesterone levels prior to and following ET. A difference between plasma
Table 5.1. Mean gestation lengths (±SEM) and birth weights of singleton calves (all groups) resulting from artificial insemination (AI) and twin calves (Group 1) resulting from AI and in vitro production (IVP)

<table>
<thead>
<tr>
<th>Type of pregnancy</th>
<th>Calves (n)</th>
<th>Gestation length (days)</th>
<th>Birth weight (kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AI Singleton</td>
<td>13</td>
<td>285.7 ± 1.6</td>
<td>40.1 ± 1.3</td>
</tr>
<tr>
<td>AI Twin</td>
<td>3</td>
<td>285.0 ± 1.5</td>
<td>37.2 ± 3.8</td>
</tr>
<tr>
<td>IVP Twin</td>
<td>3</td>
<td>285.0 ± 1.5</td>
<td>35.3 ± 0.8</td>
</tr>
</tbody>
</table>
progesterone levels on the day of ET and 3 days following ET was detected in Group 1, however, this difference is probably attributable to the normal increases in plasma progesterone levels expected following estrus and ovulation. No differences were detected in plasma progesterone levels following ET in groups 2 or 3 (Figure 5.4).

DISCUSSION

In this study, pregnancy rates at ~60 days were similar for Groups 1 and 2 (83.3% and 77.8%, respectively), but different for Group 3 (33.3%) (P<0.05). Embryo transfers were performed at ~19 days following mating for Group 3, which would be within the normal range for return to estrus for nonpregnant mated cows. Progesterone data showed that 3 of the 9 cows in Group 1 had declining progesterone levels prior to transfer, suggesting that these cows did not become pregnant following mating. In fact, 2 of these cows were in estrus 12 hours after ET.

Progesterone levels for one cow in this group dropped dramatically following embryo transfer on day 19 of her mated cycle (Figure 5.3) compared with a slow decline in progesterone levels for other cows in which progesterone began declining prior to ET (Figure 5.2), so it is possible that the embryo transfer procedure itself may have caused the loss of pregnancy for this cow. The five remaining cows in this group did not show signs of estrus, with progesterone levels remaining elevated at least until day 23 to 24 of their mated cycles (3 days following embryo transfer). However, an additional two cows (40% of cows pregnant at 23 days) lost their pregnancies between day 23 and ultrasonography at ~60 days post-mating.

The bovine conceptus begins elongating ~12 days following mating and fills the uterine horn ipsilateral to the CL of pregnancy within 18 days of gestation, although the
Figure 5.4. Progesterone levels (±SEM) beginning on day of embryo transfer (ET) through 3 days following embryo transfer for the 3 groups. (*) Denotes a significant difference (P<0.05).
conceptus only begins to invade the contralateral uterine horn 20 to 24 days of pregnancy (Chang, 1952; King et al., 1982; Thatcher et al., 1986). In this experiment, an attempt was made to transfer embryos to the contralateral uterine horn prior to this invasion by the elongating conceptus. More specifically, Group 3 cows received embryos ~19 days following mating. However, progesterone and ultrasound results suggest that 40% of cows in this group that were pregnant ~23 days following mating lost their pregnancies following the embryo transfer procedure. This is similar to results from previous experiments (Chapters II, III and IV) where pregnancy rates decreased following AI or embryo transfer of mated recipients after day 23 of gestation.

Similar observations have been made by other researchers following AI of pregnant cows at different stages of pregnancy. The problem was first associated with AI of already pregnant cows that showed signs of estrus (Vandenmark et al., 1952). Mid-cervical deposition of semen was compared to uterine body placement of semen without antibiotics and uterine body placement of semen with antibiotics for their effects on ongoing pregnancies. Cows were artificially inseminated between 64 and 152 days of pregnancy. In 100% of cattle artificially inseminated during pregnancy with semen without antibiotics, the pregnancies were lost and 67% of cattle artificially inseminated during pregnancy with semen with antibiotics also lost their pregnancies. No cattle artificially inseminated with semen mid-cervically lost their pregnancies. More recently, Sturman et al. (2000) found that 19% of reinseminated animals in the Cornell dairy herd had progesterone levels > 10 ng/ml at the time of reinsemination and were pregnant. It was reported that 17% of the reinseminated pregnant cows lost their pregnancies compared with a 7% embryonic loss in cows that were not reinseminated.
In a study similar to the present experiment, Weaver et al. (1989) found that uterine body re-insemination of pregnant cows with commercially prepared semen (with antibiotics) 12 to 24 days following successful breeding was detrimental to pregnancy (4% vs. 41% pregnancy rates at 35 to 40 days). In our experiment, there was no apparent loss of pregnancy following embryo transfer to recipients ~ day 13 (Group 2), as indicated by the high pregnancy rate at 60 days in this group (7 of 9 or 77.8%).

No twins resulted for cows in Group 3, which received additional embryos nonsurgically ~19 days following AI. Pregnancy rates in this group were low (33.3%), as only three cows remained pregnant at ~60 days. Therefore, the lack of asynchronous twinning in this group may have been influenced by the small number of recipients available with viable pregnancies. It is possible that fetal membranes may already have begun to fill the contralateral horn by the day of transfer in this group and the nonsurgical embryo transfer may have caused pregnancy loss.

Pregnancy rates were higher for Group 2 (77.8%), which received additional embryos nonsurgically ~ 13 days of pregnancy. In this group, seven cows had viable fetuses 60 days following AI. One recipient in this group (14.3%) was found to be carrying asynchronous twins following ultrasound evaluation at day 60, subsequently calving twin bull calves at 283 days of gestation. One bull calf was Charolais-sired, resulting from AI and the other bull calf was Brahman-sired, resulting from an IVP embryo. Fetal age difference was ~6 days for the twin calves, as this recipient received an expanded blastocyst and an early blastocyst on day 13 of pregnancy.

Pregnancy rates were also high in the control group (10 of 12, or 83%) which received additional embryos ~7 days following AI. Twins were detected by ultrasound
scanning in 3 of the 10 pregnant cows in this group (30%). Nine of the 10 (90%) cows that were pregnant ~60 days calved, with one cow that had been pregnant with a single fetus losing her pregnancy. All three cows that were carrying twins successfully completed gestation, each calving one Charolais-sired calf resulting from AI and one calf resulting from an IVP embryo. One fully developed IVP twin was dead at birth.

Results in the control group are similar to observations by McEvoy et al. (1995), who transferred single IVP embryos to the contralateral horn of inseminated recipients and obtained a 35% twinning rate at calving. Twinning rate at calving in Group 1 (controls) of this experiment was 33.3% (3 of 9 cows). Using in vivo-derived embryos, Renard et al. (1979) produced 44% sets of twins at calving following nonsurgical transfer of single embryos to the contralateral horn of artificially inseminated recipients. Using similar methods, Heyman et al. (1980) produced 1.5 calves per recipient calving using a similar method, with a twinning rate of 48%. Sreenan et al. (1981) produced 1.4 calves per recipient calving.

No differences were detected in plasma progesterone levels within 3 days of embryo transfer in Groups 2 or 3 (Figure 5.4). However, the number of animals in this experiment may not have been sufficient to detect such an effect. Plasma progesterone levels were higher by day 3 following transfer of two IVP embryos to mated recipients in Group 1 (Figure 5.4), although this effect may be confounded in that these cows were in the early part of gestation (day 6 to 7 of their cycle) at the time of ET and circulating progesterone concentrations may have been increasing naturally during this period.

In this experiment, an attempt was made to produce asynchronous twins by nonsurgical embryo transfer to recipients prior to and following maternal recognition.
Although invasion of the contralateral uterine horn by the fetal-placental unit has been reported to only occur following day 20 to 24 of pregnancy (Chang, 1952; King et al., 1982; Thatcher et al., 1986), it is possible that in at least some cows this phenomenon may occur as early as day 18 of gestation, with loss of pregnancy resulting from the ET procedure at this time.

Pregnancy and twinning rates were similar to prior experiments, with apparent pregnancy loss following nonsurgical ET ~19 days following insemination. Future attempts to produce superfetation must include surgical transfer to the contralateral uterine horn with the hope of bypassing the fetal membranes and maintaining the primary pregnancy, ensuring an adequate number of pregnant recipients for transfer of asynchronous recipients.
CHAPTER VI

SUMMARY AND CONCLUSIONS

Superfetation has been reported in many species, including cattle. Most cases are regarded with skepticism, due in part to the uncertainty with which they are reported. In cattle, for example, there are many physiological barriers to superfetation, such as lack of evidence of ovulation in pregnant cows, maternal recognition, effects of high progesterone levels during pregnancy on embryo development and simply physical barriers caused by the filling of the uterus by the placental-fetal unit. Many scientists have attempted to discredit reports of superfetation by attempting to explain the phenomenon with other physiological phenomena such as embryonic diapause and differentiated growth of twins. Although these are plausible explanations and may be responsible for some of the reports in some species, superfetation is still an intriguing explanation for some of the cases and difficult to disregard.

Estrus throughout pregnancy in cattle has been well documented. There are also reports of natural breeding or artificial insemination (AI) following estrus signs in cattle. Although secondary ovulations are common in some species, such as mares, no reports could be found in the literature of ovulation during pregnancy in cattle.

However, follicular waves continue throughout pregnancy in cows and follicles in these waves can be induced to ovulate with exogenous hCG or LH. Oocytes recovered from pregnant cows have been shown to be viable and capable of producing embryos in vitro. These factors make superfetation a plausible phenomenon in cattle. The hypothesis for this study was that once maternal recognition has been attained by an ongoing pregnancy, additional embryos could develop in a more advanced uterine
Therefore, this series of experiments was designed to experimentally induce superfetation by (1) induction of ovulation followed by mating in pregnant cows and (2) transfer of uterine-stage embryos to cows in an advanced stage of pregnancy.

hCG has been used successfully to induce ovulation and produce a pregnancy in a mare with a persistent CL with high progesterone values. However, an attempt to produce superfetation by induction of ovulation in pregnant cattle with hCG followed by AI was unsuccessful, although ovulation was induced successfully in cattle during early pregnancy (day 7) but not in later pregnancy (day 25). In cattle, ovarian and uterine muscular activity has been shown to be low during diestrus, which is a period of high serum progesterone levels, possibly interfering with sperm transport. Also, progesterone administered beginning on day 1 following estrus has been shown to double the rate of ova transport through the oviducts. The failure to produce superfetation in this study may have been in part due to lack of proper conditions for fertilization and/or rapid transport of ova into the uterus, prior to the embryo attaining uterine-stage status. It appears that certain hormonal conditions would have to be met to produce superfetation following AI or natural mating in pregnant cows, such as lower progesterone levels without loss of the primary pregnancy.

However, uterine-stage embryos would not be dependent on sperm or ovum transport for successful development. Therefore, uterine-stage embryos (morulas and blastocysts) were transferred to recipients at different stages of gestation. Superfetation was produced successfully when uterine-stage embryos (~7 days) were transferred nonsurgically to the uterine horn contralateral to the ongoing pregnancy of day-14 mated recipients. However, although day-7 embryos were also transferred to 28-day
pregnant recipients (nonsurgically), 60-day pregnant recipients (surgically) and 90-day recipients (surgically), no superfetation was produced in these cows.

An attempt was also made to mimic conditions around estrus and also increase the chances of ovulation in pregnant recipients, by treating pregnant recipients with FSH and hCG treatment 7 days prior to embryo transfer. Superfetation was achieved following the transfer of day 7 embryos to day 14 mated recipients, although FSH/hCG treatment did not increase the rate of twinning, since asynchronous twins were produced in both treated and control groups. No superfetation was produced following FSH/hCG treatment 7 days prior to embryo transfer in mated recipients at later stages (~26 days) of pregnancy. However, it appeared that the nonsurgical embryo transfer technique was causing loss of pregnancy in some cases, possibly due to disruption of fetal membranes and/or implantation.

Maternal recognition has been shown to occur ~16 days post-mating in cows. This coincides with conceptus elongation in cattle. The blastocyst begins elongation ~12 days after estrus and mating and has occupied most of the uterine horn ipsilateral to the CL by day 18 post-mating. By days 20 to 23 post-mating, it has been reported that the bovine conceptus has begun to fill the contralateral horn as well. An additional study was undertaken to determine the upper limit of use of nonsurgical embryo transfer to produce superfetation, with embryos being transferred prior to day 19, following maternal recognition in an attempt to avoid disruption of fetal membranes. Once again, asynchronous twins were produced by placing 7-day embryos in day-14 mated recipients. However, pregnancy loss still occurred in the later stage recipients, so it
appears that some yet unknown stimulus caused by the nonsurgical ET technique may already induce loss of pregnancy as early as day 19 post mated estrus.

We have shown with this series of experiments that uterine-stage embryos can develop asynchronously in more chronologically advanced uterine environments as long there is a viable, ongoing pregnancy to signal maternal recognition. However, the upper limit for fetal age interval was not determined, since the nonsurgical embryo transfer technique appeared to be causing pregnancy loss in the recipients, decreasing the number of pregnant recipients available for superfetation. It seems that surgical transfers would be necessary to produce superfetation in more advanced pregnant recipients, at least up to a point where the fetal-placental unit completely fills both horns, restricting the development of an additional, younger pregnancy.

If superfetation does occur in nature, it would be necessary for certain hormonal conditions to be met. For example, high progesterone levels during pregnancy may interfere with gamete transport and embryonic development. Therefore it would be necessary for progesterone levels to drop for at least a short enough period of time to allow for ovulation to occur, but not long enough to cause the loss of the pregnancy.

Prior to beginning this series of experiments, the following model for superfetation in beef cattle was proposed: (1) a cow is mated and the resulting fetus successfully attains maternal recognition, (2) then sometime during pregnancy, progesterone levels decrease for a yet unknown reason to a level which allows estradiol levels from a growing dominant follicle on the ovary contralateral to the CL of pregnancy to increase enough to (a) allow for signs of estrus and subsequent mating to occur and (b) induce a luteinizing hormone (LH) peak and subsequent ovulation, (4)
after which progesterone levels remain near pregnancy baseline levels for a short period, allowing for successful gamete transport and fertilization to occur and (5) implantation occurs in the contralateral uterine horn following embryonic development.

In conclusion, in this series of experiments, a portion of the proposed model was addressed, proving that asynchronous embryos can develop successfully to parturition when placed in a more advanced uterine environment. It also appears that simple induction of ovulation during pregnancy is not sufficient to allow for fertilization to occur. More studies are needed to determine the latest stage of pregnancy in which a secondary pregnancy would be able to develop successfully and also the hormonal profile necessary to ensure ovulation and successful embryonic development without loss of the primary pregnancy.

Furthermore, in one case, a heifer resulting from transfer of a 7-day embryo to a 14-day pregnant mated recipient born co-twin to a bull calf (7 day fetal age difference) was not a freemartin. It is possible that the age differential between fetuses developing in opposite uterine horns may have prevented the formation of anastomoses and exchange of hematopoietic cells and/or Mullerian inhibiting substance. However, fertile heifers do occur in nature in 8% of heifers born co-twin with bulls, so more studies with more sets of superfetation twins are needed to clarify this observation.
LITERATURE CITED


VITA

Joel Andrew Carter was born on September 2, 1965, in Rio de Janeiro, Brazil, to Jimmie Dale and Birdie Sue Carter. Joel has three siblings: Timothy Carter and Rebecca Macedo live in Brazil, while Jonathan Carter, his younger brother, lives in the U.S. Joel grew up in Corrente, Piaui, Brazil, where he attended elementary school at the Instituto Batista Correntino and also completed home schooling courses in English provided by Calvert School of Maryland and the University of Nebraska. He graduated from high school from the Fortaleza Academy in Fortaleza, Ceará, Brazil, in May, 1983. He then attended Texas A&M University in College Station, Texas, where he received his Bachelor of Science degree from the Department of Animal Science in May, 1987. Joel immediately entered graduate school at Texas A&M University under the direction of Dr. Duane C. Kraemer, receiving his Master of Agriculture degree from the Department of Animal Science in December, 1989. He returned to Brazil following graduation, initially working for the Fazenda Estancia das Cascatas in Aragarças, in the state of Goias, as an embryo transfer technician and later started his own company, LoneStar Genetica, in Brasilia, Brazil. In 1995, Joel returned to the United States and enrolled in the doctoral program in the Department of Animal and Dairy Sciences at Louisiana State University, Baton Rouge, Louisiana, under the direction of Dr. Robert A. Godke. During the course of the program, Joel met Renée Brooke Thompson and they were wed on May 13, 2000. He will earn his doctorate in December of 2002.