STRATEGIES TO ENHANCE FERTILITY IN DAIRY CATTLE DURING SUMMER INCLUDING USE OF CRYOPRESERVATION OF IN VITRO PRODUCED EMBRYOS

By

C. MOISÈS FRANCO

A THESIS PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

UNIVERSITY OF FLORIDA

2006

This professional achievement reflects the sacrifice and guidance of my family especially that of my mother, Mercedes Y. Vaca El-Hage, who laid the foundations with strong pillars in my life.

This dissertation is dedicated to my beloved son Talyn Izaak Franco Benton and astonishing father Antonio Vicente Franco Monasterio (†) for their endless love, support and most important, inspiration.

"EL HOMBRE SE AUTORREALIZA EN LA MISMA MEDIDA EN QUE SE COMPROMETE AL CUMPLIMIENTO DEL SENTIDO DE SU VIDA"

Victor Frankl
(1905-1997)

ACKNOWLEDGMENTS

This thesis would not have been possible without the enthusiasm, knowledge, guidance, tenacity, and, perhaps most importantly faith that I received from my academic advisor, Peter J. Hansen. From the very first interview to the last queries on research accomplishments and career plans, he was always eager to entertain my ideas in hope that I fulfilled my dream(s) and become successful. I was not sure I could handle an undertaking of such a magnitude, but was able to thanks to his consistent effort and true desire to keep me on track.

I would like to extend my sincere appreciation to my committee member Dr. Karen Moore, for her insight and willingness to help me academically without fail and regards to time. Despite having other major responsibilities, Dr. Carlos Risco was willing to help whenever asked. I thank him for his assistance and especially for the desire to help me learn to palpate. Thanks are also extended to Dr. Alvin Warnick for his advice and suggestions for improving my research projects and academic training. I would also like to thank Dr. Joel Yelich for his teaching, support, and enthusiasm while providing me with ideas that can help me achieve my goals.

Special thanks are extended to my family for encouraging me to seek for myself a demanding and meaningful education. This thesis could not have taken place without that precious gift.

Most sincere appreciation is also due to to my colleague and friend Dr. Rocío M. Rivera, whose willingness to assist me in my early stages as a master's student helped to

kindle my interest in this exploration. I would not have gotten this far if it was not for her unique and excellent training doing IVF. Dr. Zvi Roth was an inspirational friend whose passion for science was transmitted to me. He also expressed his kindness and love towards my son. I also thank Dr. Joel Hernandez for his support, friendship, and guidance.

Thanks are given to Maria B. Padua for her assistance with the completion of this manuscript and Luis Augusto Castro e Paula. Their unconditional friendship and help at any given time is sincerely appreciated. I am grateful to Dean Jousan for making the time to proofread my writings throughout the years and for his assistance in various research experiments. Special thanks go to Amber Brad for her personality and joy that helped the lab be united. Best of all has been my colleague and friend Jeremy Block for his patience, expertise and engaging conversations that helped develop in me new dreams. In addition, he always remained motivated throughout my transfer experiments.

I also would like to thank Central Packing Co. management and personnel at Center Hill, FL, for providing the ovaries used for various experiments and William Rembert for his assistance in collecting these ovaries. Special thanks go to Mary Russell and Elise Griffin, for their assistance at the University of Florida Dairy Research Unit. I thank Luther White and Mark Saulter of Hilltop Dairy, R.D. Skelton and Mathew Steed of Levy County Dairy, and Mauricio Franco and Faby Grisel of Sausalito Dairy for cooperation and assistance with the projects. And last but not least, I would like to thank Todd Bilby, Osiloam Gomez, Reinaldo Cooke, Patrick Thompson, Saban Tekin, and Paolette Soto.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Abstract of Thesis Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Master of Science

STRATEGIES TO ENHANCE FERTILITY IN DAIRY CATTLE DURING SUMMER INCLUDING USE OF CRYOPRESERVATION OF IN VITRO PRODUCED EMBRYOS

By

C. Moisés Franco Vaca

May 2006

Chair: Peter J. Hansen
Major Department: Animal Sciences

There has been a precipitous decline in fertility of lactating dairy cows. In addition, heat stress can further compromise fertility. The goals of this thesis were to 1) evaluate strategies for enhancing fertility after artificial insemination using mid-cycle GnRH treatment and 2) further develop embryo transfer using in vitro produced embryos as a tool for increasing fertility. For the second objective, experiments tested whether pregnancy rate could be improved by transfer of twin embryos and whether the developmental competence of embryos after cryopreservation could be improved by hyaluronic acid or cytochalasin B treatment.

A series of six experiments were conducted to test the efficacy of GnRH for increasing fertility. Except for one experiment, in which GnRH administration at day 14 after insemination increased pregnancy rate, GnRH was without effect whether given at

day 11, 14 or 15 after insemination or at day 11 after anticipated ovulation in embryo transfer recipients..

Neither unilateral transfer of two embryos nor administration of GnRH at Day 11 after anticipated ovulation improved pregnancy rates of dairy cattle exposed to heat stress. Cytochalasin B treatment before freezing improved cryosurvival of bovine embryos produced in vitro. In contrast, culture with hyaluronic acid was of minimal benefit.

Taken together, GnRH treatment did not consistently increase pregnancy rates when administered at Day 11-15 after insemination and is not recommended as a fertility-enhancing treatment. Similarly, transfer of two embryos to the uterine horn ipsilateral to the CL was not an effective method for increasing pregnancy rates in recipients. Transfer of cryopreserved embryos may be enhanced by treatment of embryos with cytochalasin B since this molecule increased in vitro survival, and it remains to be tested whether survival of IVP embryos after vitrification can be improved by cytochalasin B treatment.

CHAPTER 1
REVIEW OF LITERATURE

Infertility in Modern Dairy Cattle

Fertility is defined as the ability of a cyclic animal to establish pregnancy and is an important economic trait that affects herd productivity in dairy cattle (Pecsok et al., 1994; Plaizier et al., 1998). Unfortunately, there has been a decline in fertility in dairy cows over the last 10-40 years. Fertility, whether traditionally measured as conception rate (number of pregnant animals divided by the number of inseminated animals) or herd pregnancy rate (number of pregnant animals divided by the number of animals eligible to be bred), has declined in North America (Butler, 1998), Ireland (Roche, 2000), Spain (López-Gatius, 2003), and the United Kingdom (Royal et al., 2000). Other important reproductive measurements have changed during this time as well, including increases in days to first service, days to conception, and calving interval (de Vries and Risco, 2005). The magnitude of these changes in reproductive function over time is illustrated for data from herds in the United States (Figure 1-1) and northeastern Spain (Figure 1-2).

The incidence of infertility of dairy cows has been correlated with changes in dairy cattle physiology and improvements in genetic progress, nutrition, and management practices. This literature review will seek to identify physiological causes for this decrease in fertility and describe efforts to improve fertility.

Causes for the Decline in Fertility in Dairy Cattle

Milk Yield

The Animal Improvement Programs Laboratory of the United States Department of Agriculture (USDA) has estimated the genetic trend for milk yield with an average of 37 kg/yr during the 1960s, 79 kg/yr during the 1970s, 102 kg/yr during the 1980s, and 116 kg/yr for the period from 1990 to 1996 (http://aipl.arsusda.gov; Hansen, 2000). It has long been known that fertility is reduced in lactating cows as compared to non-lactating heifers (Ron et al., 1984; Nebel and McGilliard, 1993). Given that milk yield has increased over time as fertility has declined, the possibility must be considered that the increase in milk yield is one reason that has contributed to the decreased fertility in dairy cattle.

There are indications that the genetic correlation between female fertility and milk production is antagonistic (Kadarmideen et al., 2000; Royal et al., 2002). In contrast, Mahanna et al. (1979) suggested that there was no negative genetic correlation between milk yield and reproduction because there was no difference in fertility among heifers with different genetic abilities for milk yield. There may be an environmental effect of milk yield on fertility, however. As described by Lucy (2001), the increase in milk yield over the period from 1970 has been associated with a corresponding decrease in fertility as measured by increased services per conception and calving interval (Figure 1-1). According to Nebel and McGilliard (1993) there was little or no association of increased milk yield compromising fertility prior to the 1970s (Gaines, 1927; Boyd et al., 1954; Currie, 1956; Smith and Legates, 1962) but adverse effects of milk yield have been correlated with reduced fertility in studies conducted since 1975 (Spalding et al., 1975;

Laben et al., 1982; Fonseca et al., 1983; Stevenson et al., 1983; Hillers et al., 1984; Wiggans et al., 1987; Faust et al., 1988).

Using a data set of Holstein, Jersey, and Guernsey cows, it was found that 0.014 more services per conception were required for each additional 100 kg of 120-d milk for Holsteins and 0.028 services per conception for Jersey and Guernsey cows (Olds et al., 1979). Similarly, cows with the highest milk yield had the lowest first service conception rate (Faust et al., 1988) or 90-d non-return rate (Al-Katanani et al., 1999) and highest number of services (Faust et al., 1988). Days to first insemination and days open also increased linearly as milk yield increased in Jersey dairy cattle (Fonseca et al., 1983).

Expression of estrus at first postpartum ovulation is less likely in cows with higher milk production (Westwood et al., 2002). Some studies (Nielen et al., 1989; Kinsel et al., 1998), but not others (Deluyker et al., 1991), correlate the incidence of twins to milk yield. Amount of milk yield, however, was not correlated to increased incidence of multiple ovulations (López-Gatius et al., 2005b), yet the incidence of double ovulations and twinning rate has increased in modern dairy cattle (Wiltbank et al., 2000). Taken together, the associations of milk yield with reduced duration of estrus, increased days to first insemination, increased number of inseminations per conception, reduced first service conception rates, and reduced progesterone levels post-ovulation compromise herd fertility.

Milk yield and energy balance

One way in which milk yield could affect fertility is through effects on energy balance. A critical phase exists in the period following calving when dry matter intake does not meet the increased metabolic demands of lactation, and as a result, the animal

enters a state classified as “negative energy balance” (NEB). During the period of NEB, body reserves of fat and protein are mobilized (Bauman and Currie, 1980; Butler and Smith, 1989). An animal under NEB tends to have low body condition score (BCS), and both NEB and low BCS are associated with low fertility (O’Callaghan, 1999; Butler, 2000; Pryce et al., 2001; Pushpakumara et al., 2003).

Energy deficiency reduces or impairs gonadotropin secretion, and as an animal reaches this state around parturition, gonadotropin secretion to support follicular development and ovulation is compromised and reproductive problems (i.e., cystic ovaries) associated with onset of ovarian activity become prevalent (Zulu et al., 2002ab). Growth hormone stimulates insulin-like growth factor 1 (IGF-1) production by the liver (Jones and Clemmons, 1995), but during NEB growth hormone receptors are downregulated in a process referred to as “Growth Hormone Resistance” (Donaghy and Baxter, 1996). As milk production increases during early lactation and the cow is under NEB, the liver becomes refractory to growth hormone because growth hormone receptors are decreased (Vicini et al., 1991), and this result in reduced plasma concentration of IGF-1 (Pell et al., 1993).

Follicular growth is stimulated by IGF-1 (Webb et al., 2004) and reduced plasma concentrations of this growth factor are observed in cows with high milk yield (Rose et al., 2004) and together are highly correlated to delayed return to ovarian cyclicity (Taylor et al., 2004). After calving, cows with IGF-I concentrations greater than 50 ng/ml at first service were 5 times more likely to conceive than those with lower concentrations (Taylor et al., 2004).

The fact that high-producing cows have greater energetic demands for lactation does not necessarily mean that these cows have greater NEB or low BCS. Staples et al. (1990) found that low-producing cows had lower dry matter intake and were at a greater risk for failure to conceive due to anestrus and infertility than high-producing cows. It was observed that the low-producing group, classified as non-responders, sustained milk production from 28% of body tissue reserve vs 15.9 and 16.7% in the early responder and late responder groups. This interaction was confirmed when low-producing cows had lost the most body weight during the first 2 weeks of lactation and were in the greatest energy deficit (Staples et al., 1990).

Milk yield and endocrine milieu

Cows displaying greater milk production often have higher dry matter intakes (Staples et al., 1990; Hommeida et al., 2004), which has been demonstrated to decrease circulating progesterone concentrations in lactating (Hommeida et al., 2004) and non-lactating cows (Rabiee et al., 2001). Acute feeding reduced circulating progesterone by 25% in pregnant cows (Vasconcelos et al., 2003). Lucy and co-workers (1998) found that circulating progesterone was lower in cattle genetically selected for high milk production.

Sangsritavong et al. (2002) demonstrated that lactating cows have a much greater steroid metabolism than non-lactating cows. As a result, lactating cows may have larger luteal tissue volume on the ovary (Sartori et al., 2002; Sartori et al., 2004) yet experience lower circulating progesterone and estradiol concentrations than heifers and dry cows (De la Sota et al., 1993; Wolfenson et al., 2004). There is evidence that low progesterone

secretion can compromise fertility in dairy cattle (Mann and Lamming, 1999) and an increase in progesterone secretion may facilitate embryonic development.

Progesterone provides nourishment for the conceptus via induction of secretion of proteins and other molecules from the endometrium (Garrett et al., 1988a). Low peripheral concentrations of progesterone are also associated with increased luteinizing hormone (LH) pulses (Ireland and Roche, 1982) that can stimulate luteolytic signals in favor of pregnancy failure. Skarzynski and Okuda (1999) reported that blocking the progesterone receptor with a progesterone antagonist (onapristone) increased prostaglandin F2α ($PGF_{2\alpha}$) production by bovine luteal cells harvested from mid-cycle corpora lutea (CL) (Days 8–12). In addition, it was revealed that the bovine corpus luteum (CL) does not undergo apoptosis until progesterone production has declined (Juengel et al., 1993; Rueda et al., 1995).

Milk yield and heat stress

One reason why milk yield might decrease fertility of lactating cows is because it increases their susceptibility to heat stress. Infertility is a particular problem during heat stress (Ingraham et al., 1974; Putney et al., 1989b; Al-Katanani et al., 1999) and air temperatures as low as 27^{o}C can induce hyperthemia in lactating dairy cows (Berman et al., 1985). Cows exposed to elevated temperatures to induce heat stress experienced reduced pregnancy rates (Dunlap and Vincent, 1971) and increased embryonic mortality (Putney et al., 1988ab; Ealy et al., 1993). On the other hand, provision of cooling in the summer increased pregnancy rates as compared to non-cooled cows (Stott et al., 1972; Roman-Ponce et al., 1981; Ealy et al., 1994).

The ability to regulate body temperature during heat stress is exacerbated by lactation because of the excess heat production. The increase in body temperature in response to heat stress is greater for lactating cows than heifers (Cole and Hansen, 1993) and greater for high-producing cows than low-producing cows (Berman et al., 1985). Data collected on fertility at first service from 8124 Holstein cows located in South Georgia as well as North and South Florida support the idea that a high level of milk production reduces fertility of lactating cows. When cows were grouped according to mature equivalent milk yield, there was a milk yield class x month of breeding interaction that resulted from the fact that the duration and magnitude of summer infertility increased as milk yield increased (Al-Katanani et al., 1999).

Heat stress before, shortly after, and on the day of breeding is associated with reduced fertility. Heat stress can compromise fertility throughout various reproductive processes such as oocyte developmental competence (Picton et al., 1998; McNatty et al., 1999) since the oocyte becomes sensitive to damage throughout the various stages of follicular growth (Badinga et al., 1993). Indeed, follicular steroidogenesis, follicular dynamics and altered concentrations of FSH and inhibin become altered in response to heat stress (Badinga et al., 1994; Wolfenson et al., 1997; Roth et al., 2000). During heat stress sperm can be damaged after insemination due to the generation of reactive oxygen species (Ishii et al., 2005) and embryonic development can be compromised directly (Monty et al., 1987). Not surprisingly the heat stress problem is multifactorial (Hansen et al., 2001).

Heat stress of superovulated cows at day 1 after breeding reduced the proportion of embryos that were blastocysts at day 8 after breeding, but heat stress on day 3, 5 or 7

after breeding did not affect subsequent embryonic development (Ealy et al., 1993). Superovulated heifers experienced a high percentage of retarded embryos recovered on day 7 after insemination after exposure to high temperature and humidity at the onset of estrus for 10 h (Putney et al., 1989a). In another study heat stress was induced in Holstein heifers by submitting them from day 1 to day 7 after estrus to 42^{o}C for 7 h (treatment) or 30^{o}C for 16 h (control) and results obtained revealed more retarded embryos with degenerate blastomeres on the day of recovery (20.7% vs. 51.5%, respectively; Putney et al., 1988a).

One cause for the observed reduction in reproductive performance under heat stress conditions is steroidogenic capacity and its effects on oocyte function (Roth et al., 2001; Al-Katanani et al, 2002b; Roth and Hansen, 2004). Under heat stress, low estradiol concentration in the follicular fluid of dominant follicles involves reduced aromatase activity in the granulosa cells (Badinga et al., 1993) and reduced androstenedione production by theca cells (Wolfenson et al., 1997). Although earlier studies were inconsistent in demonstrating that plasma concentrations of estradiol are reduced under heat stress (no change– Gwazdauskas et al., 1981; increase – Rosenberg et al., 1982; decrease – Gwazdauskas et al., 1981), recent work points toward heat stress resulting in lower estradiol concentrations in the follicular fluid (Badinga et al., 1993; Wolfenson et al., 1995; Roth, 1998; Wilson et al., 1998ab).

Heat stress also has been reported to decrease (Rosenberg et al., 1982, Younas et al., 1993; Howell et al., 1994), increase (Abilay et al., 1975; Roman-Ponce et al., 1981; Trout et al., 1998), or have no effect (Wise et al., 1988; Wolfenson et al., 1995) on peripheral concentrations of progesterone. Elevated temperatures in culture can directly

influence endometrium explants by increasing $PGF_{2\alpha}$ secretion (Putney et al., 1988c; Malayer and Hansen, 1990) and from days 8-16 of pregnancy can reduce the size of the embryo at day 17 (Biggers et al., 1987).

A retrospective survey involving 12,711 lactations from high-yielding dairy herds in northeast Spain demonstrated that milk yield per cow increased from 1991-2000 (López-Gatius, 2003; see Figure 2). For each 1000 kg increase in average milk yield in the warm period, there was a decrease of 6% in pregnancy rate, and 7.6% in cyclicity, and an increase of 8% in the incidence of inactive ovaries. During the cool period, however, there was no change in fertility over time. Thus, the continual increase in milk yield might have reduced fertility in Spain, at least, by exacerbating effects of heat stress.

Milk yield and diseases

Increased incidence of certain diseases has been associated with elevated milk yield. High somatic cell score and clinical mastitis (Schukken et al., 1990; Barkema et al., 1998; Chassagne et al., 1998; Fleischer et al., 2001); lameness (Green et al., 2002); cystic ovarian disease (Fleischer et al., 2001; López-Gatius et al., 2002); milk fever (Fleischer et al., 2001); and acute metritis (Kelton et al., 1998) are all correlated with milk yield.

Compared to non-mastitic herd-mates, high producing cows were at a greater risk of developing clinical mastitis (Gröhn et al., 2004). Number of days to conception, artificial inseminations per conception and number of days to first artificial insemination (AI) were significantly greater for cows with clinical mastitis (Barker et al., 1998), and may affect embryonic survival when occurring after insemination (Soto et al., 2003). According to Jousan et al., (2005) an elevated somatic cell count score among lactating

females influenced mid-to-late fetal loss (represented as occurring after day 70 to 90 of gestation) and mastitis has been reported to affect pregnancy loss during the period of embryonic (Chebel et al., 2004) and fetal development (Risco et al., 1999; Santos et al., 2004a).

High yielding cows had an increased likelihood of becoming lame (Green et al., 2002) and cows that had been treated for lameness had a negative influence on pregnancy to first insemination and numbers of inseminations per service period (Petersson et al., 2005). Similarly, non-lame cows were more likely to conceive at first service than lame cows and lameness within the first 30 days after calving was associated with reduced pregnancy rates at first AI and a higher number of services per conception (Hernandez et al., 2001; Melendez et al., 2003). In a meta-analysis of several published papers, leg problems were associated with an average increase of 12 days to conception (Fourichon et al., 2000).

Cows that develop cysts remain infertile as long as this condition persists and early spontaneous cyst recovery was negatively correlated with milk yield (López-Gatius et al., 2002). Similarly, elevated milk yield increased the risk of cows developing cysts (López-Gatius et al., 2002) and days from metritis occurrence to first AI is also correlated to infertility (Loeffler et al., 1999). Milk yield in the current lactation is also correlated with incidence of milk fever (Fleischer et al., 2001) and this disease reduces fertility (Chebel et al., 2004).

Milk yield, estrus detection, and fertility

Milk yield may affect fertility indirectly by reducing the ability to accurately detect estrus. An antagonistic relationship between increased milk production and days to first

visual estrus has already been reported. According to López et al. (2004), duration, standing events, intensity (determined by the number of standing events per hour), and standing time were reduced for high-producing cows as compared to low producers. Similarly, Harrison et al. (1990) reported that elevated milk yield was correlated to a longer period of estrus suppression. Westwood et al., (2002) indicated that high genetic merit for milk yield influenced significantly the chance a cow showed weak signs of estrus as compared to low milk producing cows.

Cows with elevated milk yield also had reduced circulating estradiol concentrations on the day of estrus expression and shorter duration of estrus despite having larger preovulatory follicle diameters (López et al., 2004).

Changes in Herd Size as a Factor in Reduced Fertility

Increased milk yield is not the only change in dairy farming over the last 50 years and some of these other changes could also contribute to decreased fertility. One major change has been the trend towards large farms. In a review, Lucy et al. (2001) cited data from the USDA National Agricultural Statistics Services that nearly 30% of all dairy farms in the United States have more than 500 cows. In addition, Stahl et al. (1999) reported that the expansion of dairy herds comes in large part through the purchase of first-lactation cows. Thus, as Lucy et al. (2001) pointed out, these more infertile primiparous cows (Stahl et al., 1999) may have represented an increasingly larger percentage of the herd as dairy herds have expanded over the last 10-40 years. The importance of changes in herd size as a cause for infertility have been questioned by de Vries and Risco (2005) who found no clear association with reproductive function.

Nevertheless, as the herd size is increased one would expect that the likelihood that it becomes harder for accurately detecting estrus becomes a challenge because factors

associated with herd size such as the surface (concrete floor) on which the cow stands will reduce the preponderance of cows displaying estrus activity (Britt et al., 1986; O'Connor and Senger, 1997).

Inbreeding

Inbreeding represents increased frequency of identical alleles at a gene locus and the inbreeding percent is a measure for the genes of an individual that are identical by descent (Wright, 1922; Falconer, 1981). It is generally considered that reproductive function declines when inbreeding levels in a population rise above 6.25% (Hansen et al., 2005). Increased degree of inbreeding as the result of use of AI could explain some of the declines in fertility experienced by dairy cattle because inbreeding coefficients have increased in all the major U.S. dairy breeds. Estimates of inbreeding in the U.S. dairy population are near 5% currently (Short et al., 1992; Wiggans et al., 1995; Young et al., 1996; Hansen, 2000; Wall et al., 2005) and increasing at a constant rate of about 0.1% per year for U.S. Holsteins (Hansen et al., 2005). At an average of 5%, it is likely that many dairy cows have inbreeding coefficients above 6.25% (Hansen et al., 2005).

Thompson et al. (2000ab) found calving intervals to increase by 12 and 17 d for Jersey and Holsteins cows, respectively, with levels of inbreeding >10%. Similarly, inbreeding had pronounced negative effects on fertility at higher levels (10%) of inbreeding (Wall et al., 2005). In another study, animals with an inbreeding coefficient >9% had fewer transferable embryos following superovulation than animals with a lower inbreeding coefficient (Alvarez et al., 2005).

Strategies to Improve Fertility in Lactating Dairy Cattle

Four general approaches to improve reproductive function in dairy cattle have been developed. The first is to regulate the timing of ovulation using gonadotropin

releasing hormone (GnRH) and PGF2□ utilized in timed AI (TAI) programs. The advantage of this approach is that this program maximizes the number of animals inseminated and allows inseminations to be made at some pre-planned time to eliminate the need for estrus detection. Pioneering studies (Thatcher et al., 1989; Twagiramungu et al., 1992; Wolfenson et al., 1994) were able to synchronize estrus effectively, however, subsequent studies at the University of Florida (Schmitt et al., 1996a) and University of Wisconsin (Pursley et al., 1995) led to the development of the Ovsynch TAI program and the demonstration that good pregnancy rates can be achieved (Thatcher et al., 2001; Thatcher et al., 2002). Although this approach is an effective one and is widely used in dairy herds, it involves regulation of events occurring before conception and is beyond the scope of the present review. The second approach is to use information regarding the hormonal basis for establishment of pregnancy and signaling between the maternal and embryonic units during early pregnancy as the basis for pharmacological treatments to improve embryonic survival. Failure of essential biochemical dialogue between the conceptus and the maternal unit undoubtedly contributes to embryonic mortality and termination of pregnancy (Spencer et al., 1996; Spencer and Bazer, 2002). The third approach has been to regulate the nutrition of the dairy cow to improve energy balance or to provide specific nutrients that favor establishment and maintenance of pregnancy. Finally, recent work has focused on use of embryo transfer to bypass early embryonic death and perhaps coupled with crossbreeding may become an important alternative since Holsteins have become more inbred (Hansen et al., 2005).

Treatment with Bovine Somatotropin (bST) to Enhance Fertility

Circulating concentrations of IGF-I, glucose, and cholesterol are reduced in lactating animals (de la Sota et al., 1993; Beam and Butler 1997). Circulating

concentrations of IGF-I is influenced by nutrition (Adam et al., 1997) and closely related to energy balance of the cow (Ginger et al., 1997; Beam and Butler, 1998; 1999). Present in serum and in various tissues, IGF-I is produced mainly by the liver but other organs as well (Murphy et al., 1987; Thissen et al., 1994). IGF-I regulates ovarian function in dairy cattle (Breukink et al., 1998; Chase et al., 1998), is necessary for proper follicular development in which a fully competent oocyte capable of inducing ovulation develops (Lucy et al., 1992a), and is required for normal CL formation and function (Leeuwenberg et al., 1996; Chase et al., 1998). Dairy cows that initiated estrous cyclicity during the postpartum period had higher plasma IGF-I than anestrous cows (Thatcher et al., 1996), cystic and inactive ovary or persistent CL cows (Zulu et al., 2002a).

Bovine somatotropin (bST) increases plasma concentrations of insulin, IGF-I, and growth hormone (Bilby et al., 2004), perhaps by stimulating ovarian function especially after IGF-1 plasma levels are reduced in lactating animals (de la Sota et al., 1993). In addition, injection of bST stimulates conceptus growth by day 17 of pregnancy (Bilby et al., 2004). Additional studies provided evidence that bST can improve pregnancy rates in lactating cows (Moreira et al., 2000b; Morales-Roura et al., 2001; Santos et al., 2004b). Superovulated donor cows that received bST treatment experienced reduced number of unfertilized oocytes, increased number of embryos that developed to the blastocyst stage, and increased number of transferable embryos (Moreira et al., 2002). Collectively, these studies indicate that critical thresholds of GH and IGF-I concentrations are needed to stimulate reproductive performance (Bilby et al., 2004).

Treatment with GnRH to Delay Luteolysis

The estrous cycle is characterized by 2, 3, and sometimes 4 waves of follicular growth (Sirois and Fortune, 1988; Ginther et al., 1996). During the second half of the

luteal phase, development of an estrogenic follicle facilitates the luteolytic process via secretion of estradiol. Non-pregnant cows have higher peripheral concentrations of estradiol on days 16 and 18 after breeding compared to pregnant animals (Ahmad et al., 1997). Thatcher et al. (1991) examined the largest and second largest follicles present on day 17 after estrus in pregnant and cyclic dairy cows. In the cyclic cows, the largest follicle had greater aromatase activity and contained more estradiol and less progesterone in the follicular fluid than the second largest follicle. These relationships were reversed in pregnant animals, which indicated an earlier recruitment of the third wave of follicular development in the pregnant animal associated with delayed luteolysis and higher pregnancy rates. That these follicles play an important role in luteolysis was shown by Villa-Godey et al. (1985), who reported that electrocautery to destroy large follicles was associated with an extension of the estrous cycle.

Estradiol is now known to be one of three hormones that control uterine secretion of $PGF_{2\alpha}$, with progesterone and oxytocin also being involved. Pulsatile release of $PGF_{2\alpha}$ from the luminal epithelium of the endometrium is stimulated via oxytocin (Roberts and McCracken, 1976; Silvia and Taylor, 1989; Milvae and Hansel, 1980). Progesterone and estradiol regulate this process because estradiol induces formation of oxytocin receptors (Silvia and Taylor, 1989; Zingg et al., 1995; Robinson et al., 2001) after progesterone exposure (Ginther, 1970; Garrett et al., 1988b; Lafrance and Goff, 1988). While progesterone initially suppresses $PGF_{2\alpha}$ secretion by blocking oxytocin receptors during the early and mid-luteal phase of the estrous cycle, the endometrium becomes responsive to oxytocin and progesterone receptors become down regulated as the estrous cycle progresses (Lafrance and Goff, 1988; Spencer and Bazer, 1995).

Delaying luteolysis might improve pregnancy rate by allowing embryos more time to produce sufficient quantities of interferon-τ (IFN- τ). Eliminating or decreasing estradiol production from the dominant follicle during the critical period of early pregnancy could be one strategy to improve pregnancy establishment (Thatcher et al., 2000; Binelli et al., 2001). One approach for doing this is to use GnRH to regulate follicular function.

Gonadotropin releasing hormone is a decapeptide that plays a central role in regulating reproductive processes. Release of GnRH from the hypothalamus occurs in a pulsatile fashion and can be regulated by various internal and external signals. Hypothalamic GnRH is synthesized in cell bodies of neurosecretory neurons, and is transported to and released from the median eminence into the hypothalamic-hypophyseal portal system (Loucopoulos and Ferin, 1984). GnRH has its primary effects at the pituitary gonadotrope and stimulates the pulsatile release of the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) into the peripheral circulation (Chenault et al., 1990). Two potential gonadotropin responsive tissues within the ovary are the CL and the follicle. LH release induces ovulation or luteinization of large ovarian follicles present at the time of treatment (Thatcher and Chenault, 1976).

One strategy tested for increasing pregnancy rate is to inject GnRH or GnRH analogues at day 11-14 after estrus to increase progesterone secretion (Willard et al., 2003) and delay luteolysis (Macmillan and Thatcher, 1991), thereby increasing the chance for an embryo to initiate its own antiluteolytic mechanism. Injection of GnRH at this time can lead to decreased estrogen secretion (Rettmer et al., 1992a; Mann and

Lamming, 1995a) in an action that likely involves luteinization of the dominant follicle (Thatcher et al., 1989; Rettmer et al., 1992a; Ryan et al., 1994).

Improvement of fertility has been seen by administration of GnRH or its analogues at day 11-14 in nulliparous beef heifers (Rettmer et al., 1992b) and lactating dairy cows (Macmillan et al., 1986; Lajili et al., 1991; Sheldon and Dobson, 1993; Drew and Peters, 1994; Willard et al., 2003; López-Gatius et al., 2005a). In contrast to these positive results, there was no favorable effect of similar treatments of GnRH or GnRH analogues on pregnancy rates in other studies (Jubb et al., 1990; Stevenson et al., 1993; Ryan et al., 1994; Bartolome et al., 2005). In a meta-analysis of published results, Peters et al. (2000) concluded that the overall effect of GnRH administration between day 11 and 14 after anticipated ovulation was positive but that results were not consistent between studies.

Increase in the Size of the Preovulatory Follicle to Generate a Larger Corpus Luteum

As mentioned earlier, high-yielding dairy cows are more likely to have lower circulating concentrations of progesterone throughout the estrous cycle than cows with lower milk yields because of increased rate of progesterone catabolism (Lucy et al., 1998; Vasconcelos et al., 1999). Given the importance of progesterone concentration for embryonic survival (Man and Lamming, 2001), efforts have been made to increase progesterone secretion in cows. One possible effect of mid-cycle treatment with GnRH is to increase progesterone secretion (Schmitt et al., 1996b; Willard et al., 2003). Another approach for increasing progesterone concentrations has been to regulate the size of the preovulatory follicle to affect subsequent CL function.

Optimum differentiation and growth rate of the CL varies according to the duration and amplitude of the ovulatory LH surge such that inhibition of LH release preceding the

preovulatory surge of LH resulted in development of a smaller CL in diameter (Quintal-Franco et al., 1999). Induced ovulation of small follicles resulted in a smaller CL and reduced secretion of progesterone than when a larger follicle ovulated (Vasconcelos et al., 2001). In another study (Perry et al, 2005), regression analysis indicated that pregnancy rate for cows with induced ovulation with an ovulating follicle of 14.5 mm was higher than for cows ovulating follicles <10.3 mm in diameter. It was further revealed that 39% of cows that lost their pregnancy had ovulatory follicles ≤11 mm in diameter. Among cows that ovulated spontaneously, however, pregnancy rates at day 27 and 68 were independent of ovulatory follicle size (Perry et al., 2005). In contrast to this result, Vasconcelos et al (1999) found that the group of cows ovulating larger follicles had lower pregnancy rates on day 28 and 98 after AI and higher pregnancy loss between these times.

Administration of GnRH just prior to or at the time of the LH surge causes an amplified preovulatory surge of LH (Lucy and Stevenson, 1986; Yoshioka et al., 2001). Injection of GnRH at or near the time of estrus increased the proportion of large luteal cells in the CL on day 10 of the estrous cycle (Mee et al., 1993), peripheral progesterone concentrations during the first 7 days of the estrous cycle (Lucy and Stevenson, 1986), and increased pregnancy rates in repeat breeding cows (Stevenson et al., 1990; Mee et al., 1993).

Ullah et al. (1996) observed that GnRH treatment at estrus in dairy cows improved pregnancy rates and increased peripheral progesterone concentration. Conversely, GnRH administered to lactating dairy cows at the time of AI did not affect pregnancy rates (Ryan et al., 1994). Similarly, Mee et al. (1990) concluded that GnRH treatment at 1 h or

12 to 16 h after first detected estrus did not improve pregnancy rates at first service. Mee et al. (1990) mentioned that 16 studies in the literature suggest an overall advantage in pregnancy rate of 6 percentage points (53 vs. 59%) or an 11% improvement for cows receiving GnRH treatment at the time of AI or up to 6 h preceding AI.

Induction of an Accessory Corpus Luteum

Progesterone concentrations following ovulation have been positively correlated to volume of uterine secretions (Garrett et al., 1988a), conceptus development (Garrett et al., 1988a; Mann et al., 1996), the embryos ability to secrete IFN-τ (Kerbler et al., 1997; Mann et al., 1998), embryo viability for subsequent survival (Stronge et al., 2005), and perhaps most importantly conception rates (Hansel, 1981; Fonseca et al., 1983; Shilton et al., 1990; Larson et al., 1997). One possible approach to increasing progesterone secretion has been to induce formation of an accessory CL by administering GnRH or hCG, LH or their analogues at a time when the first wave dominant follicle is present after ovulation (metestrus) (Rajamahendran and Sianangama, 1992; Schmitt et al., 1996b; Santos et al., 2001). Santos et al. (2001) reported that hCG treatment on d 5 of a synchronized estrous cycle induced an accessory CL in 86.2% of treated cows, increased plasma progesterone by 5 ng/ml, and increased conception rates on day 28 from 38.7% to 45.8% and on day 90 of pregnancy from 31.9% to 38.4%. Lactating dairy cows treated with GnRH on d 5 (Willard et al., 2003) and hCG on day 7 (Rajamahendran and Sianangama, 1992) or day 4 in heifers (Breuel et al., 1989) reported successful accessory CL formation and an increase in conception rates and pregnancy rate.

Besides stimulating luteal tissue formation, treatment of cows to induce ovulation of the first wave dominant follicle with GnRH or GnRH analogues also reprograms follicular growth to increase the proportion of estrous cycles composed of three follicular

waves as compared to two waves (Diaz et al., 1998). Such an effect could reduce the probability that a large, highly estrogenic follicle is present during the critical period of pregnancy recognition. Compared to animals with two-wave cycles, Holstein cows (Townson et al., 2002) and beef cows (Ahmad et al., 1997) with a three-wave cycle had higher conception rates and a longer luteal phase (Ginther et al., 1989).

Progesterone Supplementation

The ability of the conceptus to secrete IFN-τ is related to its developmental progress and progesterone concentration of the pregnant female (Mann et al., 1999). Low progesterone concentration in plasma as early as day 6 after insemination has been implicated as a contributing factor for cows failing to conceive (Bulman and Lamming, 1978; Lukaszewska and Hansel, 1980; Kimura et al., 1987; Lamming and Darwash, 1995; Inskeep, 1995; Mann and Lamming, 1999; Hommeida et al., 2004). Enhanced luteolytic signals also result from suboptimal progesterone concentrations after insemination (Mann and Lamming, 1995b). Another approach to increase fertility of lactating dairy cows has been to directly supplement cows with progesterone. A meta-analysis of 17 studies revealed that progesterone supplementation after insemination produced an overall improvement in conception rate of 5% and that the timing of progesterone supplementation was a critical factor (Mann and Lamming, 1999). One study revealed depressed conception rates when controlled internal drug releasing (CIDR) devices containing progesterone were inserted in heifers on day 1 or day 2 following estrus (Van Cleef et al., 1989). In contrast, injection of progesterone (100 mg) on day 1, 2, 3, and 4 of pregnancy advanced development of conceptuses to 14 days of gestation in beef cows (Garrett et al., 1988a). These conceptuses had increased length and secreted a greater array of proteins into medium following a 24 hour culture. When

progesterone supplementation was initiated beginning at day 10 of pregnancy, Macmillan et al. (1991) found a slight decrease in pregnancy rate (-2.7%), Sreenan and Diskin, (1983) obtained a small increase (4.3%), and Robinson et al. (1989) obtained a large increase (29.3%) in pregnancy rate. Villarroel et al. (2004) found that first and second lactation repeat-breeder Holstein cows were 3.26 times more likely to become pregnant when cows received progesterone releasing intravaginal device (PRID®, 1.55g of progesterone) on day 5 through 19 post-AI.

Inhibition of Luteolysis

The maintenance of a functional CL depends directly upon the intensity of embryonic signals that attenuates endometrial secretion of $PGF_{2\alpha}$. Pregnancy fails if an embryo does not produce sufficient amounts of IFN-τ or if production is delayed until after the critical time-period between days 14 and 17 when the luteolysis would otherwise occur.

Intrauterine infusions of recombinant bovine IFN-τ from days 14 to 24 of the estrous cycle increased lifespan of the CL and duration of the estrous cycle (Meyer et al., 1995). Further studies with a large number of cows needs to test whether this treatment increases pregnancy rates. Co-transfer of embryonic vesicles to increase trophoblastic signals has been reported to increase pregnancy rates in embryo transfer recipients (Heyman et al., 1987). Administration of IFN-α by intramuscular injection, which can also block luteolysis, decreased pregnancy rates in heifers (Barros et al., 1992) because IFN-α has several adverse actions such as causing hyperthermia (Newton et al., 1990).

Administration of a prostanoid synthesis inhibitor could suppress the luteolytic stimulus in early pregnancy. Injection of flunixin meglumine (a prostaglandin synthesis inhibitor) neutralized oxytocin-induced $PGF_{2\alpha}$ release, reduced the frequency of short

cycles, and increased pregnancy rate from 33.3% in oxytocin challenged cows to 80% in oxytocin treated cows that received a flunixin meglumine injection (Lemaster et al., 1999). In another study, effects of flunixin meglumine on pregnancy rate were farm or location dependent (Purcell et al., 2005). Together, these results suggest that certain conceptuses are unable to inhibit uterine $PGF_{2\alpha}$ secretion and that reducing prostaglandin synthesis and stimulating IFN-τ secretion could improve pregnancy rates.

Nutritional Strategies

Dairy cows reach peak production on average within the first 4 to 6 weeks after parturition. Unfortunately, feed and energy intake do not reach maximum levels until approximately 10 – 12 weeks postpartum. The end result is a lactating cow with insufficient nutritional requirements that enters a NEB status.

As mentioned before, energy balance is defined as the difference between energy gain from feed intake minus the energy expenditure associated with maintenance of physiological function, growth, and milk production (Staples et al., 1990). Several studies have reported that negative energy status impaired reproductive performance (Butler and Smith, 1989; Jorritsma et al., 2000). Different nutritional strategies to improve energy balance or alter nutrient delivery to improve reproductive function are described in this section.

Fat feeding to improve energy balance

Fats are glyceride esters of fatty acids that can have a direct effect on the transcription of genes that encode proteins that are essential to reproductive events (Mattos et al. 2000). Dietary fats typically increase concentrations of circulating cholesterol, the precursor of progesterone (Grummer and Carroll, 1991). Ruminants fed

supplemental fat often have a slight increase in blood progesterone concentrations [see Staples et al. (1998) for review]. Hawkins et al. (1995) suggested that the increase seen in circulating progesterone when cows are fed supplemental fat was from a reduced rate of clearance of progesterone rather than an increase in progesterone synthesis. Fat supplementation has also been shown to stimulate programmed growth of a preovulatory follicle (Lucy et al., 1993), total number of follicles (Lucy et al., 1991ab; Wehrman et al., 1991; Thomas and Williams, 1996; Beam and Butler, 1997; Lammoglia, 1997), and size of preovulatory follicles (Lucy et al., 1990, 1991a, 1993; Beam and Butler, 1997; Oldick et al., 1997).

Garcia-Bojalil et al. (1998) reported that accumulated plasma progesterone from 0 to 50 days in milk (DIM) was greater, pregnancy rates improved, and energy status did not change when cows were fed diets of 2.2% calcium salts of fatty acids (CSFA) compared to non fat-supplemented cows. Similarly, Scott et al. (1995) fed CSFA at 0 or 450 g/d from 1 to 180 or 200 DIM and reported a tendency for CSFA to increase the proportion of cows exhibiting standing estrus (71.4% vs. 65.6) and a reduction in the proportion of cows with inactive ovaries.

Other studies have also found a beneficial effect of feeding supplemental fats on fertility of lactating cows (Erickson et al., 1992; Sklan et al., 1994) while some studies have found no beneficial effect. Although fertility results are inconsistent when cows were evaluated after being fed supplemental fat, Staples et al. (1998) suggested that positive effects (17 percentage unit improvement) are more often reported. When first AI service and conception or pregnancy rate data was examined, ten studies (Schneider et al., 1988; Bruckental et al., 1989; Sklan et al., 1989; Armstrong et al., 1990; Ferguson et

al., 1990; Sklan et al., 1991; Garcia-Bojalil, 1993; Scott et al., 1995; Burke et al., 1996; Son et al., 1996) report an improvement ($P < 0.10$) while two studies (Erickson et al., 1992; Sklan et al., 1994) revealed a strong negative influence accompanied by a large increase in milk production. Among studies that reported an improvement (Armstrong et al., 1990; Ferguson et al., 1990; Sklan et al., 1991), a reduced number of services per conception by feeding a fat supplemented diet occurred as well.

Dietary fats could favor reproductive processes through actions related to energy balance or through specific actions of individual fatty acids on tissue function. Mattos et al (2000) has suggested that altered uterine and ovarian function can be mediated through specific fatty acid precursors in the diet to allow increased steroid and/or eicosanoid secretion. There are many examples of effects of feeding diets high in specific fatty acids. Linoleic acid supplemented in the diet prepartum can stimulate arachidonic acid synthesis and lead to higher concentrations of the series 2 prostaglandins (Thatcher et al., 1994). It is speculated that linolenic acid may compete with arachidonic acid for binding sites of a key enzyme, cyclooxygenase 2 (PGHS-2), which is necessary for the synthesis of $PGF_{2\alpha}$ (Mattos et al., 2000; 2004).

Supplementation of the diet with fish meal has been reported to reduce uterine $PGF_{2\alpha}$ secretion of lactating dairy cows (Thatcher et al., 1997). Fish meal contains relatively high concentrations of two polyunsaturated fatty acids of the n-3 family, EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid). Concentrations of EPA and DHA in fish oil have been reported to be 10.8 and 11.1% of total fatty acids (Donovan et al., 2000). EPA and DHA can inhibit secretion of $PGF_{2\alpha}$ in different cell culture systems (Levine and Worth, 1984; Achard et al., 1997) including bovine endometrial cells

(Mattos et al., 2001). Using fish meal to replace soybean meal as a source of protein, Bruckental et al. (1989) and Armstrong et al. (1990) reported higher pregnancy and conception rates. These results suggest that high concentrations of EPA and DHA in the diet can reduce $PGF_{2\alpha}$ endometrial secretion and aid in establishment of pregnancy rates.

Administration of antioxidants

Reactive oxygen species are a possible source of infertility because ovarian steroidogenic tissue (Carlson et al., 1993; Margolin et al., 1992), spermatozoa (Rivlin et al., 2004), and preimplantation embryos (Fujitani et al., 1997) become compromised as a consequence of free radical damage. Vitamin E (i.e., α-tocopherol) and β-carotene are major antioxidants present in plasma membranes of cells (Wang and Quinn, 1999; 2000). Treatment of cows with vitamin E and selenium can increase the rate of uterine involution in cows with metritis (Harrison et al., 1986) and improve fertilization rates in ewes (Segerson and Ganapathy, 1980) and cows (Segerson et al., 1977). In general, however, treatment of lactating cows with vitamin E alone, through feeding or injection, had little or no benefits on postpartum cows (Kappel et al., 1984; Stowe et al., 1988; Aréchiga et al., 1998a; Paula-Lopes et al., 2003).

β-carotene is another cellular antioxidant and is thought to be present at the interior of membranes or lipoproteins (Niki et al., 1995). Cows fed diets deficient in β-carotene had lower amounts of progesterone in the CL (Ahlswede and Lotthammer, 1978). In spite of this, its effect on fertility is controversial. Some authors report benefits of feeding supplemental β-carotene (Ahlswede and Lotthammer, 1978; Rakes et al., 1985; Aréchiga et al., 1998b) whereas others do not (Wang et al., 1982; Akordor et al., 1986). There was no strong relationship between serum concentrations of β-carotene and fertility

in dairy cattle (Gossen et al., 2004; Gossen and Hoedemaker, 2005). Injection of vitamin A, a metabolite of β-carotene, resulted in an increase in the number of recovered blastocysts from superovulated cows (Shaw et al., 1995).

Crossbreeding

Two bulls (Chief and Elevation) make up about 30% of the gene pool of U.S. Holsteins (Hansen et al., 2005). As mentioned previously, inbreeding coefficients are rising in American dairy cattle (Short et al., 1992; Wiggans et al., 1995; Young et al., 1996; Hansen, 2000; Wall et al., 2005) and there is some evidence that this has contributed to the decline in fertility seen in dairy cattle (Thompson et al., 2000ab; Alvarez et al., 2005; Wall et al., 2005). Crossbreeding represents a strategy for preventing effects of inbreeding especially if the milk yield of crossbreds can approach that of Holstein cattle.

A study in Canada revealed that some groups of crossbred cattle were equivalent to Holstein controls in lifetime net profit (McAllister et al., 1994). Hansen et al. (2005) conducted a study using seven large dairies in California to compare characteristics of several crossbred animals (Normande-Holstein, Montebeliarde-Holstein, and Scandinavian Red-Holstein) versus Holsteins. Milk production as well as fat and protein production during the first 150 DIM among first lactation cows was not significantly different among breed types. Holsteins produced an average of 29.9 kg, followed by Scandinavian Red-Holstein with 29.7 kg, Montebeliarde-Holstein with 28.8 kg, and Normande-Holstein with 26.5 kg. Calving difficulty and stillbirths were reduced in crossbred animals. Survival rates indicate that purebred animals left these dairies sooner. The first service conception rate was 22% for Holsteins compared to 30 - 35% for crossbreds. There were also significantly fewer days open for crossbred cows. Thus,

crossbreeding offers some promise for enhancing fertility. One unanswered question is the optimal type of mating scheme for the crossbred animals themselves and whether the resultant loss of heterosis in the F2 animals will reduce any advantage over purebred cows.

Embryo Transfer

The concept of using embryo transfer (ET) as a tool to increase pregnancy rates is based on the observation that disruptive events such as anovulation, ovulation of oocytes with low developmental competence, compromised oviductal transport or uterine environment, and insemination errors or damaged spermatozoa all occur before the time when embryos are ordinarily transferred (day 6 - 8 after estrus) (Hansen and Block, 2004). Selection of morula and blastocyst stage embryos for transfer offers the chance to avoid pregnancy failure associated with the early stages of embryonic development (day 0 - 8 after estrus).

It has been proposed that during absence of heat stress, pregnancy rates following embryo transfer as compared to AI in lactating cows are not optimal (Putney et al., 1989b; Drost et al., 1994; Ambrose et al., 1997). However, ET may become a more effective strategy to increase pregnancy rates as compared to AI in lactating cows during periods of heat stress, and the magnitude of the increased temperature does not seem to influence overall success following transfer (Hansen and Aréchiga, 1999). As embryos advance in their development, the effects of elevated temperatures become less significant because embryos become more resistant to the deleterious effects of elevated temperatures (Ealy et al., 1992; Ealy and Hansen, 1994; Ealy et al., 1995; Edwards and Hansen, 1997; Rivera and Hansen, 2001). As a result, pregnancy rates following ET

during heat stress are higher than pregnancy rates to AI (Putney et al., 1989b; Ambrose et al., 1999; Al-Katanani et al., 2002a) although not in the absence of heat stress.

One potential constraint for embryo transfer in lactating cows is the short duration of estrus and lack of intense mounting activity seen in dairy cows (Dransfield et al., 1998). This phenomenon is exacerbated by heat stress (Nebel et al., 1997) and will limit the number of embryos transferred in lactating cows in a program that is dependent upon estrus detection. The first report of a timed embryo transfer (TET) protocol, where ovulation was synchronized using an Ovsynch protocol, was by Ambrose et al. (1999) who evaluated the efficiency of TET using either fresh or frozen-thawed in vitro produced (IVP) embryos and TAI under heat stress conditions. Pregnancy rates in cows that received a fresh IVP embryo were higher compared to cows in the TAI group.

Limitations to Optimal Pregnancy Rates Using IVP - TET

For ET to replace AI on a wide scale in commercial herds ET must become an economical breeding alternative and embryos must be inexpensive to produce (Hansen and Block et al., 2004). Superovulation provides the best source of embryos while the most likely inexpensive source of embryos will be produced from slaughterhouse oocytes by IVP since superovulation is costly and requires intensive management and careful synchronization of the donor cows.

Although embryos produced using IVP systems are relatively inexpensive as compared to embryos produced by superovulation, pregnancy rates achieved following transfer of an IVP embryo are often less than what is obtained following transfer of an embryo produced by superovulation. For example, Hasler (2003) reported a 36.7% pregnancy rate for in vitro derived embryos vs. 54.8% for in vivo embryos. The reason for the poor survival of IVP embryos is not known. However, IVP embryos are different

from in vivo embryo in terms of morphology (Massip et al., 1995; Crosier et al., 2001; Rizos et al., 2002), gene expression (Bertolini et al., 2002a; Lazzari et al., 2002; Lonergan et al., 2003), metabolism (Krisher et al., 1999; Khurana and Niemann, 2000b) and chromosomal abnormalities (Iwasaki et al., 1992; Viuff et al., 2000). One or more of these alterations likely contributes to the poor embryo survival after transfer. Calves born as the result of in vitro production are also more likely to experience developmental defects (Hasler et al., 2003; Farin et al., 2006).

One possible strategy for increasing pregnancy rates is to transfer two embryos into the uterine horn ipsilateral to the CL. This approach is based on the idea that the likelihood is increased that the cow receives at least one embryo competent for sustained development. In addition, the transfer of two embryos into the ipsilateral uterine horn to the CL is likely to increase the amounts of IFN-τ and other embryo-derived signaling molecules in the uterus needed to maintain pregnancy and prevent luteolysis. Co-transfer of embryonic vesicles to increase trophoblastic signals has been reported to increase pregnancy rates in ET recipients (Heyman et al., 1987).

In a recent study, there was a tendency for higher calving rates for recipients that received two embryos in the uterine horn ipsilateral to the CL as compared to recipients that received one embryo (Bertolini et al., 2002a). The requirement for the antiluteolytic signal in cattle to be locally administered (del Campo et al., 1977, 1983) means that one should expect pregnancy rates to be higher in cows that received two embryos in the same uterine horn (unilateral transfer) than for cows that received two embryos distributed in both uterine horns (bilateral transfer). The opposite was true for heifers (Anderson et al., 1979). In other studies, transfer of embryos to create two pregnancies in

the uterine horn ipsilateral to the CL has produced a similar pregnancy rate as bilateral twins and single pregnancies (Sreenan and Diskin, 1989; Reichenbach et al., 1992) or reduced pregnancy rate as compared to bilateral transfer (Rowson et al., 1971).

Cryopreservation of IVP Embryos

An additional limitation to the widespread use of IVP embryos in cattle is their poor survival following cryopreservation. Hasler et al. (1995), Ambrose et al., (1999) and Al-Katanani et al. (2002a) indicated that IVP embryos do not survive freezing as well as embryos produced in vivo based on pregnancy rates following transfer as compared to non-frozen embryos. In vitro survival rates following thawing (Pollard and Leibo, 1993; Enright et al., 2000; Khurana and Niemann, 2000a; Diez et al., 2001; Guyader-Joly et al., 1999) and pregnancy rates following thawing and transfer (Hasler et al., 1995; Agca et al., 1998; Ambrose et al., 1999; Al-Katanani et al., 2002a) are consistently lower for IVP embryos as compared to embryos produced in vivo by superovulation.

Among the metabolic changes associated with IVP embryos linked to poor freezability is an increase in lipid content (Abe et al., 1999; Rizos et al., 2002). Mechanical delipidation (Tominaga et al., 2000; Diez et al., 2001) and addition of inhibitors of fatty acid synthesis (De la Torre-Sanchez et al., 2005) can improve embryo survival following cryopreservation. Hatching rates were higher for delipidated embryos compared to controls when day 7 blastocysts were frozen (Murakami et al., 1998), but pregnancy rates after the transfer of delipidated embryos was 10.5% compared to 22% for control embryos (Diez et al., 2001). Although delipidated embryos can survive freezing conditions when tested in vitro, special consideration must be taken since these embryos do not reflect higher pregnancies and remain less viable than control embryos.

Manipulating the cryopreservation process to minimize damage to the embryo has also been considered. Of most promise are procedures based on vitrification, which is defined as "the solidification of a solution (glass formation) brought about not by crystallization but by extreme elevation in viscosity during cooling" (Fahy et al., 1984). Vitrification depends on rapid cooling and thawing of embryos while using high concentrations of cryoprotectants associated with elevated cooling rates (~2500^{o}C/min, Palasz and Mapletoft, 1996). Although vitrification does not eliminate toxic effects of cryoprotectants and osmotic damage, the rapid cooling has been reported to decrease chilling injury and prevent damage associated with high lipid content (Dobrinsky, 1996; Martino et al., 1996ab). In vitro survival rates following the thawing of vitrified IVP embryos was either equal (Van-Wagtendonk et al., 1995) or superior to embryos frozen conventionally (Dinnyés et al., 1995; Agca et al., 1998; O'Kearney-Flynn et al., 1998).

Sensitivity of in vivo derived embryos to cryopreservation is much less and the complex environment where the embryo develops is key. It has been reported that embryos cultured in the sheep oviduct (26%) compared to synthetic oviductal fluid in culture systems (7%) were better able to tolerate freezing conditions. Embryos cultured in Buffalo rat liver cells or oviductal cells were more resistant to freezing as well as compared to embryos not subjected to co-culture (Massip et al., 1993; Leibo and Loskutoff, 1993; Tervit et al., 1994).

Summary and Objectives of the Thesis

There has been a precipitous decline in fertility of dairy cows over the last 10-40 years and heat stress is associated with infertility in lactating dairy cows. To characterize events associated with infertility is important and the purpose of the present series of experiments described in this thesis was to evaluate strategies that help overcome

reproductive failure. Improving reproductive function in dairy cattle is of major interest and experiments were designed to 1) evaluate strategies for enhancing fertility after AI using GnRH treatment and 2) further develop ET using IVP embryos as a tool for increasing fertility by testing whether pregnancy rate could be improved by transfer of twin embryos and whether the developmental competence of embryos after cryopreservation could be improved.

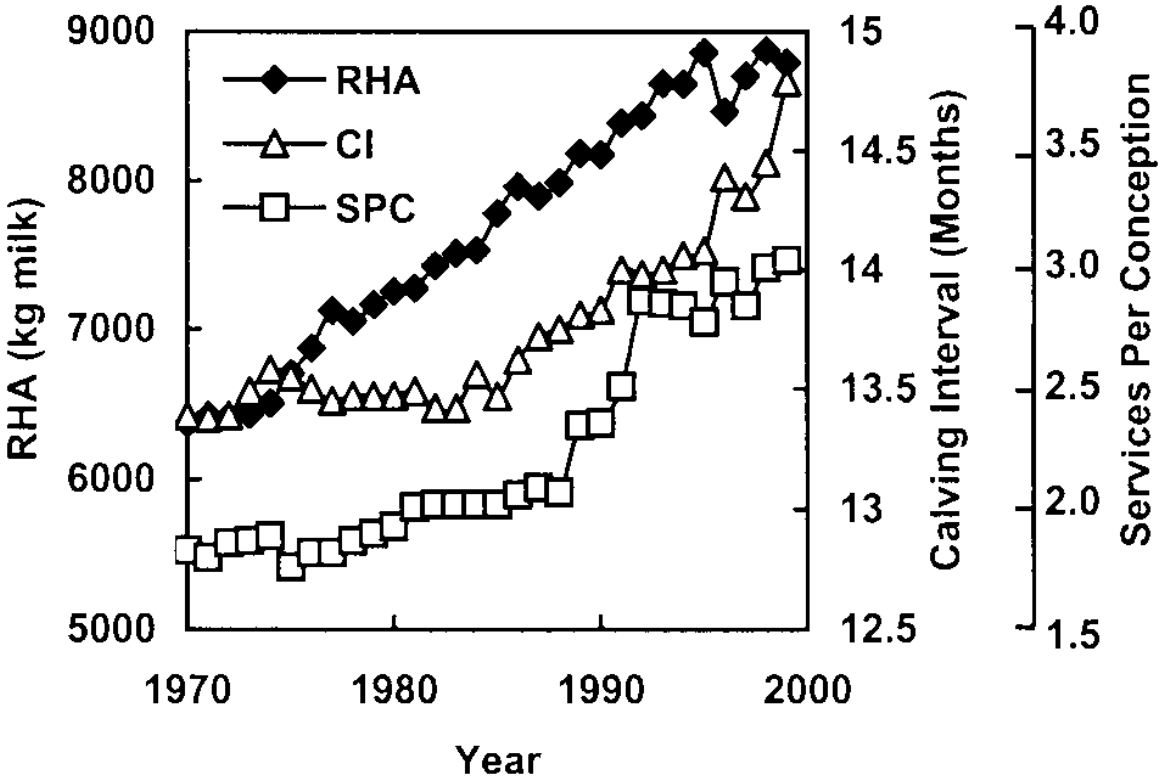

Figure 1-1. Rolling herd average (RHA, kg milk per lactation), calving interval (CI), and services per conception (SPC) for 143 dairy herds continuously enrolled in the Raleigh DHIA record system from 1970 to 1999 (Lucy, 2001).

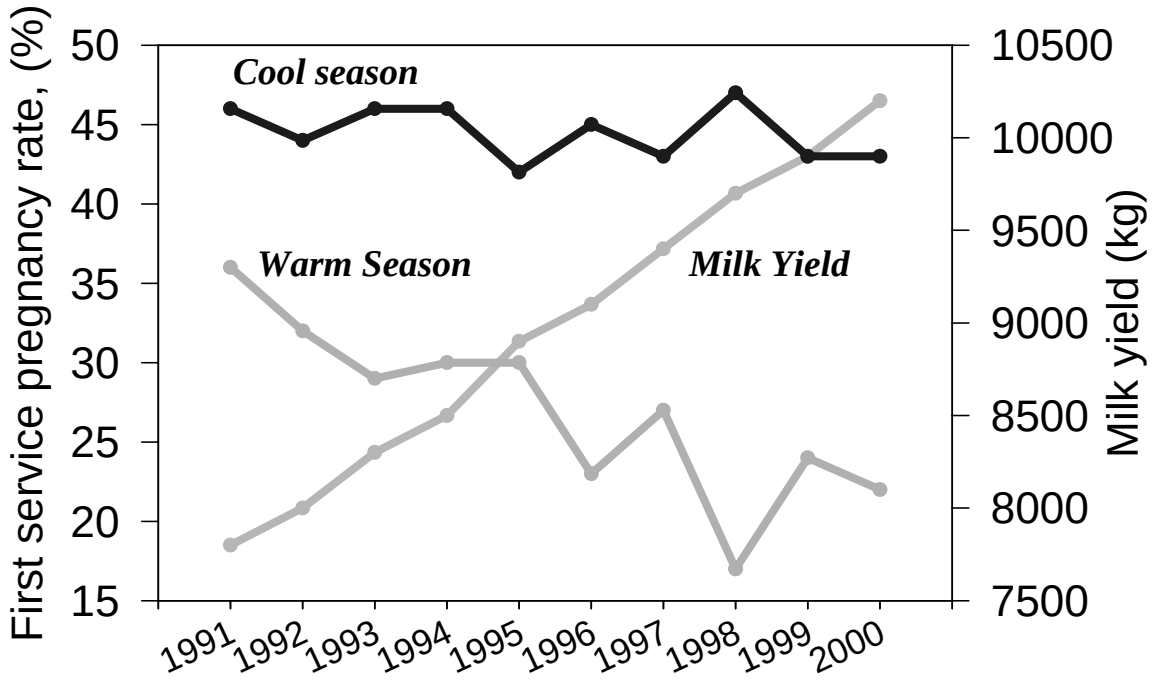

Figure 1-2. Temporal changes in first service pregnancy rate and annual average milk production from high-producing Holstein-Friesian dairy herds in north-eastern Spain. Data for pregnancy rate were recorded in the cool (October - April months) and warm season (May-September months). Data were drawn by P.J. Hansen (unpublished) based on data of Lopez Gatius (2003).

CHAPTER 2
EFFECTIVENESS OF ADMINISTRATION OF GONADOTROPIN RELEASING HORMONE AT DAY 11, 14 OR 15 AFTER ANTICIPATED OVULATION FOR INCREASING FERTILITY OF LACTATING DAIRY COWS AND NON-LACTATING HEIFERS

Introduction

One of the approaches proposed to improve fertility in cattle is administration of GnRH or GnRH analogues at day 11-15 after estrus. Injection of GnRH at this time can lead to decreased estrogen secretion (Rettmer et al., 1992a; Mann and Lamming, 1995a) in an action that likely involves luteinization of the dominant follicle (Thatcher et al., 1989; Rettmer et al., 1992a; Ryan et al., 1994). In some cases, extended estrous cycle length (Lynch et al., 1999) and increased progesterone secretion also results (Rettmer et al., 1992a; Stevenson et al., 1993; Ryan et al., 1994; Willard et al., 2003). Improvement of fertility has been seen by administration of GnRH or its analogues at day 11-14 in nulliparous beef heifers (Rettmer et al., 1992b) and lactating dairy cows (Macmillan et al., 1986; Lajili et al., 1991; Sheldon et al., 1993; Drew and Peters, 1994; Willard et al., 2003; López-Gatius et al., 2005a). In contrast to these positive results, there was no favorable effect of similar treatments of GnRH or GnRH analogues on pregnancy rates in other studies (Jubb et al., 1990; Stevenson et al., 1993; Ryan et al., 1994; Bartolome et al., 2005). In a meta-analysis of published results, Peters et al. (2000) concluded that the overall effect of GnRH administration betweendDay 11 and 14 after anticipated ovulation was positive, but that results were not consistent between studies.

It is possible that GnRH treatment is more effective at increasing pregnancy rate per insemination during periods of heat stress than in cool weather because circulating concentrations of progesterone can be reduced in cows subjected to heat stress (Wolfenson et al., 2000). In addition, the anti-luteolytic process may be compromised because heat stress can decrease growth of the filamentous stage conceptus (Biggers et al., 1987) and increase uterine prostaglandin-$F_{2\alpha}$ secretion from the uterus (Wolfenson et al., 1993). Beneficial effects of GnRH treatment at day 11-12 after insemination on fertility have been observed in lactating dairy cows during heat stress (Willard et al., 2003; López-Gatius et al., 2005a). The purpose of the present series of experiments was to evaluate the effectiveness of GnRH treatment at either day 11, 14 or 15 after anticipated ovulation for improving fertility of lactating cows and heifers and determine whether the beneficial effect of GnRH was greater during summer than winter.

Materials and Methods

Experiment 1 - GnRH Administration at Day 11 after Anticipated Ovulation in Heifers Subjected to Timed Artificial Insemination during Heat Stress

The experiment was conducted at a commercial dairy located in Trenton, Florida (29°37' N 82°49' W) from July to September, 2003 using 149 Holstein heifers. The heifers ranged in age from 13-23 mo (mean=539 d, SD=76) and ranged in weight from 316 to 448 kg (mean=360 kg, SD=32). Heifers were maintained on grass pasture with supplemental grass hay. Heifers were randomly allocated to one of four treatments in a 2 x 2 factorial design with main effects of timing of insemination (protocol A vs B) and treatment (vehicle vs GnRH). The experiment was replicated twice with between 70 and 79 heifers per replicate. Heifers were subjected to timed artificial insemination (TAI) based on a protocol published previously (Martinez et al., 2002ab). On Day -10 of the

protocol (Day 0 equals the day of anticipated ovulation), heifers received 100 μg (i.m.) of GnRH (Fertagyl, equivalent to 50 μg /ml gonadorelin diacecetate tetrahydrate; (Intervet Inc. Millsboro, DE) and an unused intravaginal progesterone-releasing device insert (EAZI-BREED CIDR® insert, 1.38 g of progesterone, Pfizer Animal Health, New York, NY, USA). At Day -3, CIDR devices were removed and 25 mg (i.m.) of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; 5 ml Lutalyse®, Pfizer Animal Health, New York, NY, USA) was administered. A second 100 μg GnRH injection was given 48 h after CIDR withdrawal (Day -1). Regardless of estrus behavior, heifers in protocol A were inseminated 24 h after the second GnRH injection (d 0) and heifers in protocol B were inseminated at the same time as the second GnRH injection (d -1). Two individuals conducted all inseminations and semen from one sire was used for all heifers. Heifers from each synchronization treatment protocol were randomly allocated to receive either 100 μg of GnRH, (i.m.) or an equivalent volume (2 ml) of vehicle (9 mg/ml of benzyl alcohol and 7.47 mg/ml of sodium chloride in water) at Day 11 after anticipated ovulation.

On the day of insemination and on Day 11 after anticipated ovulation, a 10-ml blood sample was collected via coccygeal or jugular venipuncture into heparinized tubes (Becton Dickinson, Franklin Lakes, NJ) to measure the proportion of heifers successfully synchronized. An animal was considered synchronized if progesterone concentrations were lower than 1 ng/ml on the day of insemination and greater than 1 ng/ml on Day 11 after anticipated ovulation. A third blood sample was collected in a subset of 76 heifers at Day 15 after anticipated ovulation (i.e., 4 d after the injection of GnRH or vehicle) to determine the effect of GnRH treatment on serum concentrations of progesterone. Pregnancy was diagnosed by palpation per rectum at Day 44-51 after insemination.

Blood samples were stored on ice (~2-4 h) until centrifugation at 2,000 x g for 20 min at 4 ^{o}C to obtain plasma. Plasma was stored at -20 ^{o}C until assayed for progesterone concentrations using a progesterone radioimmunoassay kit (Coat-a-Count®; Diagnostic Products Corp., Los Angeles, CA). The sensitivity of the assay was 0.1 ng/ml and the intrassay and interassay CV were each 6%.

Experiment 2 - GnRH Administration at Day 11 after Anticipated Ovulation in Lactating Cows Subjected to Timed Artificial Insemination

This study took place at the University of Florida Dairy Research Unit (Hague, Florida; 29^{o}46' N 82^{o}25' W). A total of 244 primiparous and multiparous lactating Holstein cows housed in freestall barns equipped with a fan–and-sprinkler system were used. Cows were fed a total mixed ration (TMR) to meet or exceed requirements recommended for lactating dairy cows, were milked three times a day, and received bovine somatotropin (Posilac, Monsanto Corp., St. Louis, MO) according to manufacturer's recommendation. Cows were subjected to the OvSynch TAI program (Schmitt et al., 1996a; Pursley et al., 1998); 100 μg (i.m.) GnRH (Fertagyl equivalent to 50 μg /ml gonadorelin diaecetate tetrahydrate, Intervet, Millsboro, DE) was injected at Day 0 of the protocol, 25 mg (i.m.) $PGF_{2\alpha}$ (5 ml of Lutalyse®, Pfizer Animal Health, New York, NY, USA) was given at Day 7, 100 μg (i.m.), GnRH was again injected, i.m., at Day 9, and cows were inseminated 16 h later (the day of anticipated ovulation). At the time of insemination (from January - September, 2004), 244 cows were between 76 and 594 days in milk (DIM; mean= 176, SD= 114). Multiple individuals conducted inseminations (n=7) and multiple AI sires were used (n=45).

Cows were randomly assigned within pair to receive 100 μg (i.m.) GnRH or an equivalent volume (2 ml) of vehicle (9 mg/ml benzyl alcohol and 7.47 mg/ml sodium

chloride in water) at Day 11 after anticipated ovulation (i.e., 11 d after insemination). Rectal temperature was recorded in a subset of cows (n=134) on the afternoon of Day 11 after TAI at 1500 – 1600 h. Pregnancy was diagnosed by rectal palpation at ~Day 46 after insemination.

Experiment 3 - GnRH Administration at Day 14 after Anticipated Ovulation in Lactating Cows Subjected to Timed Artificial Insemination

This study was conducted at two different locations using lactating Holsteins. Farm 1 was the University of Florida Dairy Research Unit at Hague, Florida while farm 2 was a commercial dairy in Chiefland, Florida (29°30' N 82°52' W). Cows from farm 1 (n=307) were inseminated from February - November 2004 and cows in farm 2 (n=170) were inseminated from June - October 2004. At both farms, primiparous and multiparous cows were used. At farm 1, 307 cows were TAI between 76 – 590 DIM (mean= 187, SD= 102). Multiple individuals conducted inseminations (n=7) and multiple AI sires were used (n=42). At farm 2, 170 cows were used for first service after calving using seven different sires and one inseminator. The TAI protocol was designed to achieve insemination at 60 $\pm$ 3 d in milk. Cows in both farms were housed in freestall barns equipped with fans and sprinklers, were fed a TMR, were milked three times a day, and received Posilac® (Monstanto, St. Louis, MO) according to manufacturer's directions.

Cows in farm 1 were subjected to an OvSynch protocol as described for Experiment 2. Cows for farm 2 were subjected to a TAI protocol that incorporated a pre-synchronization with $PGF_{2\alpha}$ (Moreira et al., 2001) and the CIDR-Synch ovulation synchronization protocol (Portaluppi and Stevenson, 2005). Cows received two injections of 25 mg $PGF_{2\alpha}$ (i.m.) (Lutalyse) 14 d apart starting on Day 21-27 DIM. Twelve days after the second $PGF_{2\alpha}$ injection, a timed ovulation synchronization protocol

was initiated. Cows received 100 μg (i.m.) GnRH (2 ml of Cystorelin®; Merial Limited, Iselin, NJ, USA) and an unused EAZI-BREED CIDR® intravaginal progesterone-releasing device insert. Seven days later, CIDR devices were removed and 25 mg (i.m.) $PGF_{2\alpha}$ was given. Cows received a second 100 μg (i.m.) injection of GnRH at 72 h after CIDR withdrawal. Estrus was detected using tail chalk or KaMar estrus detection patches (KAMAR Inc., Steamboat Springs, CO, USA). Cows observed in estrus at 24 or 48 h after CIDR removal were inseminated at estrus. Cows not observed in estrus were inseminated at 72 h after CIDR withdrawal. Ovulation was anticipated to occur 72 h after CIDR withdrawal. All animals received the GnRH injection at 72 h regardless of estrus behavior. Cows were also randomly assigned within pair to receive either 100 μg (i.m.) GnRH (2 ml of Cystorelin®; Merial Limited, Iselin, NJ, USA), or vehicle (as for experiment 2) at 14 d after anticipated ovulation. Pregnancy was diagnosed by rectal palpation at ~Day 45 after insemination.

Rectal temperature was recorded in a subset of 100 cows in Farm 1 and 39 cows in Farm 2 at 1500 h of Day 14 after anticipated ovulation.

Experiment 4 - GnRH Administration at Day 14 after Anticipated Ovulation in Lactating Cows Subjected to Timed Artificial Insemination During Heat Stress

This study took place at the University of Florida Dairy Research Unit with inseminations in April to June, 2005. A total of 137 primiparous and multiparous lactating Holstein cows ranging in DIM from 78 to 566 d (mean= 185, SD= 110) were subjected to an OvSynch protocol as described for Experiment 2. Multiple individuals conducted inseminations (n=4) and multiple AI sires were used (n=22).

Cows were randomly assigned within pair to receive 100 μg (i.m.) GnRH or an equivalent volume (2 ml) of vehicle (9 mg/ml benzyl alcohol and 7.47 mg/ml sodium

chloride in water) at Day 14 after anticipated ovulation (i.e., 14 d after insemination). Pregnancy was diagnosed by rectal palpation at ~Day 46 after insemination.

Experiment 5 - GnRH Administration at Day 14 or Day 15 after Detected Estrus

This study took place at a commercial dairy in Chiefland, Florida. A total of 296 primiparous and multiparous lactating Holstein cows inseminated at detected estrus were used. Cows were inseminated from April – August, 2005. At the time of insemination, cows were between 51 and 235 DIM (mean= 122, SD= 40).

Estrus was detected using tail chalk or KaMar estrus detection patches (KAMAR Inc., Steamboat Springs, CO, USA). Estrus detection patches were visually monitored twice (morning and afternoon) daily by the inseminator. When cows were first diagnosed in estrus in the afternoon, insemination was performed the next morning. When estrus was first detected in the morning, cows were inseminated at that time. Cows were bred by one inseminator and 31 different sires used. Every other day of the experiment, cows were selected to receive injections at Day 14 or 15 after insemination. Within each day, cows were randomly assigned within a pair to receive 100 μg (i.m.) GnRH or an equivalent volume (2 ml) of vehicle (9 mg/ml benzyl alcohol and 7.47 mg/ml sodium chloride in water). Pregnancy was diagnosed by rectal palpation at ~Day 45 after insemination.

Statistical Analysis

Data on pregnancy rate were analyzed by logistic regression with the LOGISTIC and GENMOD procedures of SAS (SAS for Windows, Release 8.02; SAS Inst., Inc., Cary, NC). For the LOGISTIC procedure, a backward stepwise logistic model was used. Variables were continuously removed from the model by the Wald statistic criterion if the significance was greater than 0.20. The Wald χ^2 statistic was used to determine the

significance of each main effect that remained in the reduced model. The adjusted odds ratio (AOR) estimates and the 95% Wald confidence intervals from logistic regression were obtained for each variable that remained in the final statistical model following the backward elimination. Data were also analyzed by PROC GENMOD and *P* values for significant treatment effects are reported from this analysis. The full mathematical model for experiment 1 included main effects of inseminator, treatment, protocol, replicate, replicate x protocol, replicate x treatment, replicate x inseminator, protocol x treatment, protocol x inseminator, treatment x inseminator. The full mathematical model for experiment 2 included the effects of season of insemination (January to March vs April to September), treatment, and season x treatment. For experiment 3, the full mathematical model included the effects of farm, treatment, season of insemination (warm vs cool season; farm 1 = October to March vs April to September; farm 2 = June to September vs October to November), and season x treatment, season x farm, and treatment x farm. In addition, a subset of data composed of cows from farm 2 only was analyzed where the additional factor of estrus detection (yes or no) was included in the model. For experiment 4, the full mathematical model included the effects of treatment, month of insemination, parity (1 vs others), sire, DIM at insemination class (<150 d vs > 150 d), parity x treatment, DIM class x treatment and month x treatment. For experiment 5, the full mathematical model included the effects of treatment, season of insemination (April and May vs June to August), parity (1 vs > 1), number of services (1, 2 and >2), DIM at insemination class (<150 d vs > 150 d) and interactions of main effects with treatment. Since interactions were not significant, data were reanalyzed with main effects only.

Data on rectal temperatures were analyzed by least-squares analysis of variance using the GLM procedure of SAS. The model included effects of season (Exp.2) or season, farm and farm x season (Exp. 3).

A meta-analysis was performed using Mantel-Haenszel procedures available using software downloaded from http://www.pitt.edu/~super1/lecture/lec1171/index.htm. Three analyses were performed – using all experiments, experiments with GnRH treatment at Day 11, and experiments with GnRH treatment at Day 14 or 15.

Results

Experiment 1 - GnRH Administration at Day 11 after Anticipated Ovulation in Heifers Subjected to Timed Artificial Insemination During Heat Stress

Based on progesterone concentrations measured at insemination and at Day 11 after anticipated ovulation, estrous cycles of 137/149 (92%) of the heifers were successfully synchronized. Pregnancy rate was not significantly affected by GnRH treatment or insemination protocol. This is true whether all heifers were considered (Table 1) or only those successfully synchronized (results not shown). There was also no effect ($P > 0.10$) of GnRH treatment at Day 11 on concentrations of plasma progesterone on Day 15. Values were 3.5 ± 0.19 ng/ml for heifers receiving vehicle and 3.6 ± 0.19 ng/ml for heifers receiving GnRH.

Experiment 2 - GnRH Administration at Day 11 after Anticipated Ovulation in Lactating Cows Subjected to Timed Artificial Insemination

Treatment with GnRH did not significantly ($P > 0.10$) affect pregnancy rate per insemination (Table 2). This was true for inseminations in both cool seasons (January to March) and warm season (April to September) (results not shown). There was also no significant difference in pregnancy rate between seasons.

Rectal temperatures were higher ($P < 0.001$) for cows in the warm season (least-squares means + SEM; 39.3 + 0.07 oC) than for cows in the cool season (38.9 + 0.07 oC).

Experiment 3 - GnRH Administration at Day 14 after Anticipated Ovulation in Lactating Cows Subjected to Timed Artificial Insemination

Injection of GnRH increased pregnancy rates at both farms (treatment, $P < 0.02$; treatment x farm, non-significant) (Table 3). While pregnancy rates were lower in summer than winter ($P < 0.05$), the effect of GnRH was apparent in both seasons and the season x treatment interaction was not significant.

Cows in farm 2 were monitored for estrus. No cows were seen in estrus at 24 h after $PGF_{2\alpha}$, 4.7% (8/171) were detected in estrus at 48 h, 32.2% (55/171) at 72 h, and 63.1% (108/171) were not detected in estrus. Cows in estrus at 48 h were inseminated at that time while other cows (those seen in estrus at 72 h and those not seen in estrus) were inseminated at 72 h. There was an estrus detection class (detected in estrus vs not detected) x treatment interaction ($P < 0.03$) on pregnancy rate per insemination that reflected the fact that GnRH was effective at increasing pregnancy rate for those cows displaying estrus [3/29 (10%) for control and 12/34 (35%) for GnRH[but had no effect for those cows not displaying estrus [7/54 (13%) for control and 4/54 (8%) for GnRH].

Rectal temperatures were higher ($P < 0.01$) for cows in the warm season (least-squares means $\pm$ SEM: 39.4 $\pm$ 0.06 ^{o}C) than for cows in the cool season (39.1 $\pm$ 0.11 ^{o}C) and higher ($P < 0.001$) for farm 2 (39.5 $\pm$ 0.10 ^{o}C) than for farm 1 (39.1 $\pm$ 0.07 ^{o}C), but there was no farm x season interaction.

Experiment 4 - GnRH Administration at Day 14 after Anticipated Ovulation in Lactating Cows Subjected to Timed Artificial Insemination During Heat Stress

Treatment with GnRH did not significantly affect pregnancy rate (Table 4). Pregnancy rate was higher ($P<0.02$) for cows inseminated at or before 150 DIM (30.3%,

20/66) than for cows inseminated after 150 DIM (12.7%, 9/71). There were no other significant main effects or interactions of GnRH treatment with other effects.

Experiment 5 - GnRH Administration at Day 14 or Day 15 after Detected Estrus

Overall, pregnancy rate was higher (P<0.0001) for cows inseminated in April and May (55/171, 32.2%) than for animals inseminated in June, July or August (12/125, 9.6%). There were, however, no other significant main effects or interactions of GnRH treatment with other effects. Pregnancy rates were 25.6% (32/125) for cows receiving vehicle at day 14 or 15, 20.7% (19/92) for cows receiving GnRH at Day 14, and 20.3% (16/79) for cows receiving GnRH at Day 15.

Overall Effectiveness of GnRH Treatment as Determined by Meta-Analysis

When data from multiple experiments were considered together by meta-analysis, there was no significant effect of GnRH on pregnancy rate. This was the case when all experiments were considered (odds ratio=0.97; 95% CI=0.63, 1.50), or whether experiments with GnRH treatment on Day 11 (odds ratio=0.87; 95% CI=0.50, 1.50) or Day 14 or 15 (odds ratio=1.06; 95% CI=0.68, 1.65) were considered separately.

Discussion

Overall, there was no significant effect of GnRH treatment on pregnancy rate. In particular, GnRH treatment at Day 11 after anticipated ovulation did not improve pregnancy rate of heifers or lactating cows in any experiment, whether animals were exposed to heat stress or not. Moreover, GnRH did not consistently improve fertility when given at Day 14 after anticipated ovulation or at Days 14 or 15 after insemination. In one experiment (experiment 3), administration of GnRH at Day 14 after anticipated ovulation in cows subjected to TAI increased pregnancy rate of lactating cows in

summer and winter at two locations. However, this positive effect could not be replicated either in lactating cows subjected to TAI or for cows inseminated at standing estrus.

The variability in response to GnRH is reminiscent of the results of the meta-analysis of published studies performed by Peters et al. (2000) in which inconsistency between studies was noted. Variability in results could reflect either error in estimates of treatment effects because of small numbers of experimental units or variability in biological responses to GnRH. The number of animals used for the present studies varied and could have been too small in some studies to detect significant differences or have lead to sampling errors that obscured the magnitude or direction of the treatment differences. However, meta-analysis of the entire data set, involving 1303 cows, indicated that there was no overall effect of GnRH.

It is also possible that herds differ between each other or over time in the predominant biological response to GnRH treatment. Presumably, beneficial effects of GnRH post-insemination on fertility are related to its actions to cause LH release. Treatment with GnRH at Day 11-15 of the estrous cycle can decrease function of the dominant follicle (Thatcher et al., 1989; Rettmer et al., 1992a; Ryan et al., 1994; Mann and Lamming, 1995a) and increase progesterone secretion (Rettmer et al., 1992a; Stevenson et al., 1993; Ryan et al., 1994; Willard et al., 2003). The reduction in estradiol-17β secretion caused by GnRH should delay luteolysis and conceivably allow a slowly-developing conceptus additional time to initiate secretion of interferon-τ. Low progesterone secretion may also compromise fertility in dairy cattle (Mann and Lamming, 1999; Lucy, 2001) and an increase in progesterone secretion caused by GnRH may facilitate embryonic development. Whether a herd responds to GnRH by undergoing

follicular changes may depend upon the characteristics of follicular growth because a follicle must reach 10 mm in diameter to ovulate in response to LH (Sartori et al., 2001). Perhaps, herds that do not respond to GnRH with an increase in fertility are herds where many cows have lower follicular growth or follicular wave characteristics that do not result in sufficient follicular development at the time of injection.

One example of the potential importance of follicular dynamics in determining responses to GnRH is the expected response to GnRH treatment at Day 11 after anticipated ovulation. In the current studies, injection of GnRH at Day 11 after anticipated ovulation did not increase pregnancy rates in either lactating Holstein cows or nulliparous heifers. For lactating cows, the absence of an effect of GnRH at Day 11 was seen in both summer and winter. This result, which agrees with other studies in which injection of GnRH at Day 11 does not affect fertility (Stevenson et al., 1993; Jubb et al., 1990), is in contrast to other studies indicating that GnRH treatment at Day 11 can increase fertility of heifers (Rettmer et al., 1992b) and lactating cows (Sheldon and Dobson, 1993; Willard et al., 2003). One factor that could influence the effectiveness of GnRH treatment at Day 11 is the number of follicular waves that an individual animal expresses. Animals with estrous cycles characterized by three follicular waves have larger second-wave dominant follicles at Day 11 of the estrous cycle than animals with two-wave cycles (Ginther et al., 1989; Savio et al., 1990; Ko et al., 1991) and thus the preponderance of cycle type (two-wave vs three-wave) within a herd may determine effectiveness of GnRH treatment at Day 11. There is variation from study to study in the relative frequency of three-wave vs two-wave cycles, at least among Holstein heifers (Ginther et al., 1989; Knopf et al., 1989; Rajamahendran et al., 1991; Gong et al., 1993),

and this variation is evidence for herd-to-herd variation in frequency of follicular wave patterns.

Even in animals with three-wave follicular cycles, Day 11 would appear to not be an optimal time of the estrous cycle for using GnRH to cause luteinization because the second-wave dominant follicle is smaller at Day 11 than at 14-15 in heifers (Ginther et al., 1989; Ko et al., 1991) and lactating cows (Ko et al., 1991). Results from a limited number of cows in Experiment 3 suggested that the effectiveness of GnRH at Day 14 after anticipated ovulation depends upon whether cows are detected in estrus. Presumably, ovulation occurred on average sooner for cows in estrus at 48 and 72 h after prostaglandin than for cows not detected in estrus (which contains cows that had not initiated estrus by 72 h as well as some cows in which estrus occurred by 72 h but was not detected). Among those detected in estrus, GnRH injection improved fertility from 10.3% to 35.3%. Among animals not detected in estrus, however, there was no difference in pregnancy rate between animals treated with vehicle (13.0%) or GnRH (7.6%). It is likely that GnRH did not affect pregnancy rate in the cows not detected in estrus because this group included cows that were anovulatory at insemination or that were not successfully synchronized; GnRH would be unlikely to increase pregnancy rate in these animals.

It was hypothesized that beneficial effects of GnRH would be greater during heat stress because this condition can decrease growth of the filamentous stage conceptus (Biggers et al., 1987), increase uterine prostaglandin $F_{2\alpha}$ secretion from the uterus (Wolfenson et al., 1993) and reduce circulating concentrations of progesterone (Wolfenson et al., 2000). Beneficial effects of GnRH treatment at Day 11-12 after

insemination on fertility have been observed in lactating dairy cows during heat stress (Willard et al., 2003; López-Gatius et al., 2005a). There was no evidence, however, that GnRH was more effective during the summer. In particular, the increase in pregnancy rate caused by injection of GnRH at Day 14 during experiment 3 was similar for cows inseminated in summer and winter. In other experiments conducted during the summer, GnRH was without beneficial effect.

In experiment 1, there were no differences in pregnancy rates for Holstein heifers inseminated either at second GnRH injection (24.4%) or 24 after GnRH (19.8%). This result is similar to results of Pursley et al. (1998) who reported little difference in pregnancy rates and no differences in calving rates between lactating cows inseminated at 0, 8, 16, or 24 h after the second GnRH injection of the OvSynch regimen. The pregnancy rates achieved with heifers in experiment 1 were low compared to other studies in which heifers received a similar ovulation synchronization program (Martinez et al., 2002ab). The low fertility was not a result of delayed puberty or unresponsiveness to the synchronization protocol because 92% of the heifers had both low progesterone concentrations during the expected periovulatory period and high progesterone concentrations at the predicted luteal phase of the cycle. It is possible that some of these heifers classified as synchronized experienced short estrous cycles (Schmitt et al., 1996b; Moreira et al., 2000a). The experiment was conducted during the summer and it is also possible that heat stress reduced fertility. Although fertility in Holstein heifers does not always decline during the summer (Ron et al., 1984; Badinga et al., 1985), there is one report (Donovan et al., 2003) that heifers from a dairy farm in north central Florida inseminated in summer were more than four times less likely to become pregnant to first

insemination than heifers inseminated during the rest of the year. It is also possible that the one sire used to inseminate all heifers was not a fertile bull.

In conclusion, injection of GnRH at Day 11-15 after anticipated ovulation or insemination did not consistently increase pregnancy rates in heifers or lactating cows. The fact that GnRH administration was effective in one study indicates that such a treatment may be useful for increasing pregnancy rate in some herds or situations. More work will be required to describe factors that could identify which groups of cows would be most likely to benefit from GnRH treatment.

Table 2-1. Descriptive statistics, adjusted odds ratio (AOR) estimates, and 95% Wald confidence intervals (CI) for effect of GnRH administration at Day 11 after anticipated ovulation and ovulation synchronization protocol on pregnancy rates of heifers during heat stress.

	Pregnancy rate				
	Proportion [1]	%	AOR	95% Wald CI	*P*-value 2
GnRH Treatment [3]					
GnRH	20/78	25.6	1.29	0.59 – 2.83	0.41
Vehicle	14/71	19.7			
Protocol [4]					
B	20/79	25.3	1.34	0.61 – 2.95	0.41
A	14/70	20.0			

[1] Data represent the number of females pregnant at Day 44-51 after insemination / total number of females inseminated.
[2] Derived from PROC GENMOD.
[3] Wald chi-square statistic =0.54 (N.S).
[4] Wald chi-square statistic = 0.40 (N.S.)

Table 2-2. Descriptive statistics, adjusted odds ratio (AOR) estimates, and 95% Wald confidence intervals (CI) for effect of GnRH administration at Day 11 after anticipated ovulation and season of insemination on pregnancy rates of lactating cows subjected to timed artificial insemination.

	Pregnancy rate				
	Proportion [1]	%	AOR	95% Wald CI	*P*-value 2
GnRH Treatment [3]					
GnRH	26/121	21.5	0.66	0.37 – 1.18	0.16
Vehicle	36/123	29.3			
Season [4]					
January – March	30/103	29.1	1.38	0.77 – 2.48	0.27
April - September	32/141	22.7			

[1]Data represent the number of females pregnant at ~d 45 after insemination / total number of females inseminated.
[2] Derived from PROC GENMOD.
[2] Wald chi-square statistic =1.50 (N.S).
[4] Wald chi-square statistic = 1.38 (N.S.)

Table 2-3. Descriptive statistics, adjusted odds ratio (AOR) estimates, and 95% Wald confidence intervals (CI) for effect of GnRH administration at Day 14 after anticipated ovulation and season of insemination on pregnancy rates of lactating cows subjected to timed artificial insemination.

	Pregnancy rate				
	Proportion [1]	%	AOR	95% Wald CI	*P*-value 2
GnRH Treatment [3]					
GnRH	49/241	20.3	1.76	1.07 – 2.89	0.02
Vehicle	30/236	12.7			
Season [4]					
Oct, Nov, Feb, March	40/187	21.4	1.76	1.08 – 2.87	0.02
May - September	39/290	13.5			

[1] Data represent the number of females pregnant at ~Day 45 after insemination / total number of females inseminated.
[2] Derived from PROC GENMOD.
[3] Wald chi-square statistic =4.94 (P=0.026).
[4] Wald chi-square statistic = 5.12 (P=0.024)

Table 2-4. Descriptive statistics, adjusted odds ratio (AOR) estimates, and 95% Wald confidence intervals (CI) for effect of GnRH administration at Day 14 after anticipated ovulation and Days in milk (<150 d vs > 150) at insemination on pregnancy rates of lactating cows subjected to timed artificial insemination during heat stress.

	Pregnancy rate				
	Proportion [1]	%	AOR	95% Wald CI	*P*-value 2
GnRH Treatment [3]					
GnRH	11/73	15.1	0.43	0.18 – 1.04	0.05
Vehicle	18/64	28.1			
Days in milk at insemination [4]					
< 150 d	20/66	30.3	3.11	1.27 – 7.62	0.02
> 150 d	9/71	12.7			

[1] Data represent the number of females pregnant at ~Day 45 after insemination / total number of females inseminated.
[2] Derived from PROC GENMOD.
[3] Wald chi-square statistic =3.55 (P=0.060).
[4] Wald chi-square statistic = 6.12 (P=0.013)

CHAPTER 3
EFFECT OF TRANSFER OF ONE OR TWO IN VITRO-PRODUCED EMBRYOS AND POST-TRANSFER ADMINISTRATION OF GONADOTROPIN RELEASING HORMONE ON PREGNANCY RATES OF HEAT-STRESSED DAIRY CATTLE

Introduction

The in vitro produced (IVP) embryo is different from the embryo produced in vivo in terms of morphology (Iwasaki et al., 1992; Massip et al., 1995; Crosier et al., 2001), gene expression (Bertolini et al., 2002a; Lazzari et al., 2002; Lonergan et al., 2003), metabolism (Khurana et al., 2000b), and incidence of chromosomal abnormalities (Iwasaki et al., 1992; Viuff et al., 2000). Not surprisingly, pregnancy rates achieved following transfer of an IVP embryo are often less than what is obtained following transfer of an embryo produced by superovulation and calves born as the result of in vitro production are more likely to experience developmental defects (Hasler et al., 2003). Problems associated with the transfer of IVP embryos have limited the realization of the potential of these embryos for enhancing genetic improvement and reproductive performance of lactating dairy cattle (Rutledge, 2001; Hansen and Block et al., 2004).

One method that might be useful for increasing pregnancy rates in dairy cattle recipients that receive an IVP embryo is to transfer two embryos into the uterine horn ipsilateral to the CL. Such a treatment might increase pregnancy rate because the likelihood is increased that the cow receives at least one embryo competent for sustained development. In addition, the transfer of two embryos into the ipsilateral uterine horn is likely to increase the amounts of interferon-τ and other embryonic signaling molecules in the uterus needed to maintain pregnancy and prevent luteolysis. Co-transfer of

embryonic vesicles to increase trophoblastic signals has been reported to increase pregnancy rates in embryo transfer recipients (Heyman et al., 1987). For the current experiment, both embryos were transferred into the uterine horn ipsilateral to the CL because of the requirement for the antiluteolytic signal in cattle to be locally administered (Del Campo et al., 1977; 1983). In a recent study with a small number of transfers (n=10 to 28 recipients), there was a tendency for higher calving rate for recipients that received two embryos in the uterine horn ipsilateral to the CL as compared to recipients that received one embryo (Bertolini et al., 2002b). Anderson et al. (1979) found a tendency for pregnancy rates to be higher in cows that received two embryos in the same uterine horn (unilateral transfer) than for cows that received two embryos distributed in both uterine horns (bilateral transfer); the opposite was true for heifers. In other studies, transfer of embryos to create two pregnancies in the uterine horn ipsilateral to the CL has produced a similar pregnancy rate as bilateral twins and single pregnancies (Sreenan and Diskin, 1989; Reichenbach et al., 1992) or reduced pregnancy rate as compared to bilateral transfer (Rowson et al., 1971).

Another treatment that has potential for increasing pregnancy rates in embryo transfer recipients is injection of GnRH at Day 11 after the anticipated day of ovulation. Such a treatment was shown to increase pregnancy rates in heat-stressed, lactating cows following insemination (Sheldon and Dobson, 1993; Willard et al., 2003) and embryo transfer (Block et al., 2003). Treatment with GnRH or its analogues at Day 11 to12 of the estrous cycle has been reported to increase progesterone secretion (Ryan et al., 1994; Willard et al., 2003) and inhibit function of the dominant follicle (Savio et al., 1990; Ryan et al., 1994) to possibly delay luteolysis.

The purpose of the current pair of experiments was to examine the effectiveness of unilateral transfer of twin embryos and treatment with GnRH at Day 11 after the anticipated day of ovulation for increasing pregnancy rates in dairy cattle recipients that received IVP embryos. Experiments were performed during periods of heat stress because embryo transfer offers benefits as a method for increasing pregnancy rate as compared to AI in females subjected to heat stress (Rutledge, 2001).

Materials and Methods

Experiment 1 - Single or Twin Transfer of IVP Embryos into Crossbred Dairy Recipients

The experiment was conducted at a commercial dairy located in Santa Cruz, Bolivia (17°48' S, 63°10' W) from November – December, 2004. Data on minimum and maximum air temperatures during the experiment collected by Servicio Nacional de Meteorología e Hidrología (http://www.senamhi.gov.bo/meteorologia/) for Santa Cruz are presented in Figure 1. Females receiving embryos included 32 virgin crossbred heifers sired by Simmental, Gyr, or Brown Swiss bulls and Holstein or Holstein crossbred dams and 26 lactating, crossbred cows with the proportion of Holstein varying from 1/2 to 15/16. The heifers ranged in age from 363 to 2070 d (mean = 850 d and median = 664 d; SD = 421 d) and ranged in weight from 247 to 430 kg (mean = 310 kg and median = 288 kg; SD = 52.3 kg). Animals were maintained on grass pasture until two weeks prior to the start of the synchronization program when they also received a supplement of 6 kg/head/d of spent brewers' grain. The cows ranged in age from 820 to 4075 d (mean = 2083 d and median = 1670 d; SD = 986 d), were maintained on grass pasture, and received 11 kg of brewers' grains and 2 kg of a soybean-based concentrate mixture before each milking. Cows were milked two times per day and ranged from 110

to 417 d in milk (mean =190 d and median = 170 d; SD = 75 d). Milk yield per day across all days of lactation ranged from 5.9 to 21.1 kg/d (mean = 12.5 kg/d and median = 12.6 kg/d; SD = 3.8 kg/d).

Recipients were synchronized for timed embryo transfer using a modified OvSynch protocol (Portaluppi and Stevenson, 2005) with the inclusion of a controlled intravaginal drug releasing device (EAZI-BREED CIDR® insert, 1.38 g of progesterone, Pfizer Animal Health, New York, NY, USA). On Day -10 (Day 0 equals the day of anticipated ovulation), females received 100 μg (i.m.) of GnRH (1 ml of Profertil®; Tortuga Cia. Zootécnica Agrária, São Paulo, Brazil) and an intravaginal progesterone-releasing device insert that had been used one time previously. On Day -3, CIDR devices were removed and females received 150 μg (i.m.) of $PGF_{2\alpha}$ (2 ml of Prostaglandina Tortuga, Tortuga Cia. Zootécnica Agrária). On Day 0, 100 μg (i.m.) of GnRH was administered. Behavioral symptoms of estrus were monitored about 5 times each day for 3 d following CIDR removal and $PGF_{2\alpha}$ injection. On Day 6 after anticipated ovulation, all females, including those not seen in estrus, were examined per rectum for the presence of a CL using an Aloka 210 ultrasound unit equipped with a 5 MHz linear array probe (Aloka, Wallingford, CT, USA). A group of females having a CL (n=32 heifers and n=26 cows) were randomly selected within recipient type (heifers or cows) to receive one (n=31 females) or two (n=27 females) embryos on Day 7 after anticipated ovulation. For embryo transfer, an epidural block of 5 ml of lidocaine hydrochloride (2% w/v; Sparhawk Laboratories Inc., Lenexa, KS, USA) was administered to each recipient, and one or two IVP embryos were deposited into the uterine horn ipsilateral to the ovary containing the CL. One technician conducted all transfers.

A total of 85 blastocysts (72 at Day 7 after insemination and 13 and Day 8 after insemination) were transferred in this experiment. Of these, six were produced by Transova (Sioux City, IA, USA) using Holstein oocytes and a Holstein sire and were cultured in Synthetic Oviductal Fluid (SOF) medium. Embryos were shipped overnight in a portable incubator to Gainesville, FL, USA on Day 4 after insemination. Embryos were transferred to fresh microdrops of a modified SOF (Fischer-Brown et al., 2002) prepared by Specialty Media (Phillipsburg, NJ, USA) and cultured at 38.5^{o}C in a humidified atmosphere of 5% O2 and 5% (v/v) CO2 (balance N_2). The remainder were produced using oocytes obtained from ovaries of a variety of breeds collected at a local abattoir located at a travel distance of approximately 1.5 h from the Gainesville laboratory. Procedures, reagents, and media formulation for oocyte maturation, fertilization, and embryo culture were as previously described (Roth and Hansen, 2005) with some modifications. Cumulus-oocyte complexes were matured for approximately 22 h at 38.5°C in an atmosphere of 5% (v/v) CO2 in humidified air and then inseminated with a cocktail of Percoll-purified spermatozoa from three different bulls of various breeds. At 8 – 12 h post-insemination (hpi), putative zygotes were denuded of cumulus cells by suspension in Hepes-TALP medium (Caisson, Rexburg, ID, USA) containing 1000 units/ml hyaluronidase type IV (Sigma, St Louis, MO, USA) and vortexed in a microcentrifuge tube for 5 min. Presumptive zygotes were then placed in groups of ~30 in 50 µl microdrops of KSOM-BE2 (Soto et al., 2003) (Caisson, Rexburgh, ID, USA) at 38.5°C in an atmosphere 5% (v/v) CO2 in air.

Regardless of method of production, embryos greater than 16 cells in appearance were collected at 1300 h on Day 6 or 7. Embryos were placed in groups of 21 to 65 into

2 ml cryogenic vials (Nalge Company, Rochester, NY, USA) filled to the top with KSOM-BE2 that was pre-warmed and equilibrated in 5% (v/v) CO2 in air. Embryos produced by Transova were kept separately from those produced using ovaries from the local abattoir. Vials containing embryos were placed in a portable incubator (Minitube of America, Verona, WI, USA) that had been pre-warmed to 39^{o}C for 24 h prior to use. Embryos were shipped by air and arrived at Santa Cruz de la Sierra, Bolivia, at 1100 h the next day (Day 7 or 8 after in vitro insemination) and transported by ground to the farm.

Embryos were transferred over a time span from 1300 h and 2000 h. One or two embryos were loaded into 0.25 cc straws in Hepes-TALP (Caisson) containing 10% (v/v) bovine steer serum (Pel-Freez, Rogers, AR, USA) and 100 μM 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA). Embryos were transferred to recipients that were palpated the day before and had a detectable CL. Recipients were randomly assigned to receive one or two embryos, and all embryos were transferred into the ipsilateral horn to the CL. Pregnancy diagnosis was performed by rectal palpation at Day 64 and 127 post-transfer, and the number of fetuses was recorded on Day 127. Data collected at calving included length of gestation (with the day of transfer being considered Day 7 of gestation), occurrence of dystocia (defined as needing assistance), sex, weight and viability of each calf, and occurrence of retained placenta (failure of the placenta to be expelled within 12 h after calving). Calf survival until Day 7 of age was also recorded.

Experiment 2 - Administration of GnRH on Day 11 after Anticipated Ovulation in Lactating Recipients that Received an IVP Embryo

This study took place at a commercial dairy located in Bell, FL, USA (29^{o} 45’ N 82^{o} 51’ W) from June to October, 2004. Data on minimum and maximum air

temperatures and average relative humidity collected by the Florida Automated Weather Service (http://fawn.ifas.ufl.edu) for Alachua, FL, USA are presented in Figure 1. A total of 87 multiparous, lactating Holstein cows in late lactation were used as recipients. Cows were fed a total mixed ration to meet or exceed requirements recommended for lactating dairy cows, milked three times a day, and received bovine somatotropin (Posilac®, 500 mg sometribove zinc, Monsanto, St. Louis, MO, USA) according to manufacturer's directions. Cows were housed in a dry lot with access to a permanent shade structure without fans or sprinklers and with access to a cooling pond.

Cows were prepared for embryo transfer in groups of 6 to 18; a total of 10 replicates were completed. To synchronize recipients for timed embryo transfer, cows received 100 μg (i.m.) of GnRH (2 ml of Cystorelin®; Merial Limited, Iselin, NJ, USA), on Day –10; 25 mg (i.m.) of $PGF_{2\alpha}$, on Day -3; and 100 μg (i.m.) of GnRH, on Day 0 (i.e., the day of anticipated ovulation). On Day 7 after anticipated ovulation, all cows were palpated per rectum for the presence of a CL. Cows that had a palpable CL received an epidural block of 5 ml of lidocaine (2%, w/v), and a single embryo was transferred to the uterine horn ipsilateral to the ovary containing the CL. Recipients were randomly assigned to receive 100 μg (i.m) of GnRH or vehicle (9 mg/ml of benzyl alcohol and 7.47 mg/ml of sodium chloride in water) on Day 11 after anticipated ovulation.

The embryos used for transfer were produced in the Gainesville laboratory using oocytes of various breeds and a pool of semen from three bulls of various breeds as described for Experiment 1. A different pool of semen was used for each replicate. Presumptive zygotes were cultured in groups of ~30 in 50 μl microdrops of modified SOF (Fischer-Brown et al., 2002) containing 100 ng/ml of insulin-like growth factor-1

(Upstate Biotechnology, Lake Placid, NY, USA). Embryos were cultured at 38.5°C in a humidified atmosphere of 5% (v/v) O2 and 5% (v/v) CO2 with the balance N2. On Day 7 after insemination, blastocysts were harvested and transported to the farm in 2 ml cryogenic vials (20 to 25 embryos/tube) filled to the top with pre-warmed Hepes-TALP. Tubes containing embryos were placed in a portable incubator (Minitube of America, Verona, WI, USA) that had been pre-warmed to 39^{o}C for 24 h prior to use. Embryos were transported to the farm and loaded in 0.25 cc straws prior to transfer into recipients. Pregnancy was diagnosed by rectal palpation at Day 45 to 53 after anticipated ovulation.

Statistical Analysis

Categorical data were analyzed by logistic regression using the LOGISTIC procedure of SAS for Windows (Version 9, SAS Institute Inc., Cary, NC, USA) with a backward stepwise logistic model. Variables were continuously removed from the model by the Wald statistic criterion if the significance was greater than 0.2. The full statistical model for Experiment 1 included treatment (one embryo or two embryos), parity (cows vs heifers), estrus (observed in estrus vs not observed) and treatment x parity on pregnancy rate, pregnancy loss, calving rate, calf mortality and twinning rate. The only variable in the final mathematical model for Experiment 2 was GnRH treatment as other effects (replicate and replicate x treatment) were not significant. The adjusted odds ratio estimates and the 95% Wald confidence intervals (CI) from logistic regression were obtained for each variable that remained in the final statistical model following the backward elimination. Data were also analyzed with the GENMOD procedure of SAS to determine the significance of each effect that remained in the reduced model; P values for logistic regression analyses reported in the tables are derived from these analyses. Data for gestation length and calf birth weight were analyzed by analysis of variance using

Proc GLM. The full statistical model included the effects of treatment, parity and treatment x parity. The $\chi 2$ test was used to determine whether the sex ratio of calves differed from the expected 1:1 ratio.

Results

Experiment 1 - Single or twin transfer of IVP embryos

Pregnancy and calving rates

Data are summarized in Table 1. At Day 64 of gestation, the pregnancy rate tended to be higher (P=0.07) for cows than for heifers. While there were no significant effects of number of embryos transferred or parity x number transferred, heifers that received two embryos tended to have lower pregnancy rates than those that received a single embryo (20% for two embryos vs 41% for one embryo) while there was no difference in pregnancy rate due to number of embryos transferred to cows (50% for two embryos vs 57% for one embryo).

Pregnancy losses between Day 64 and 127 occurred in one group only – cows receiving two embryos. In that group, pregnancy rate was 50% at Day 64 but decreased to 17% at Day 127. There was no difference in pregnancy rates at Day 127 between cows and heifers, but recipients that received two embryos had lower pregnancy rates (17% for cows and 20% for heifers) than recipients that received one embryo (57% for cows and 41% for heifers, $P < 0.03$).

Pregnancy loss after Day 127 occurred in one female only. In particular, a cow receiving a single embryo gave birth to a stillborn calf at 251 d of gestation. Like for pregnancy rate at Day 127, there was no difference in calving rate between cows and heifers, but recipients that received two embryos had lower calving rates (17% for cows

and. 20% for heifers) than recipients that received one embryo (50% for cows and 41% for heifers, $P < 0.03$).

Estrus was detected at 24, 48 or 72 h after prostaglandin injection in 21/32 heifers (8 at 24 h after injection and 13 at 48 h) and 19/26 cows (1 at 24 h after injection, 14 at 48 h and 4 at 72 h). While not statistically different ($P=0.11$), there was a tendency for pregnancy rates to be lower for animals not detected in estrus. For example, pregnancy rates at Day 127 for animals receiving one embryo was 55% (11/20) for animals in estrus vs 36% (4/11) for animals not observed in estrus. Pregnancy rates at Day 127 for animals receiving two embryos were 25% (5/20) for animals in estrus vs 0% (0/7) for animals not observed in estrus.

Characteristics of gestation, parturition, and calves

Gestation length was affected by recipient type x number of embryos transferred ($P<0.05$; Table 2). For cows, gestation length was slightly longer for those receiving one embryo as compared to those receiving two embryos while the opposite was true for heifers. Two of 5 females calving that received two embryos produced twin calves. There was no significant effect of recipient type or number of embryos transferred on dystocia or incidence of retained placenta (Table 2). Sex ratio (including the one stillborn calf) was in favor of males with 15 males compared to 7 female calves born (68% male; Table 3). This ratio tended to be different from the expected 1:1 ratio ($P<0.10$).

While there were no significant differences, there was a tendency for calf mortality at birth to be greater for heifers receiving two embryos than for other groups (Table 3). None of the cows lost their calf at birth and only 1 of 7 heifers receiving a single embryo experienced calf death at birth. In contrast, 2 of 3 heifers receiving two embryos experienced calf loss. One heifer had twin fetuses and both were born dead as a result of

complications with calving. Another heifer gave birth to a single calf that was born dead as a result of complications with calving. The calf from the third heifer was born alive. All calves born alive were alive 7 d later.

Experiment 2 - Administration of GnRH on Day 11 after Anticipated Ovulation

Administration of GnRH at Day 11 after anticipated ovulation had no effect ($P>0.10$) on pregnancy rates. Recipients treated with GnRH had a pregnancy rate of 17.8% (8/45) while those recipients that received placebo had a pregnancy rate of 16.7% (7/42). The odds ratio was 1.08 with 95% Wald confidence interval of 0.23 and 3.30.

Discussion

The purpose of the experiments described here was to examine two strategies for increasing pregnancy rates in heat-stressed dairy recipients that receive an IVP embryo. Neither approach, transferring two embryos into the uterine horn ipsilateral to the CL or injection of GnRH at Day 11 after anticipated ovulation, increased pregnancy rates.

Results of Experiment 1 indicated that the transfer of two embryos into recipients led to pregnancy loss and that such loss occurred earlier for heifers than for cows. There was a distinct difference in pregnancy rate between heifers that received one or two embryos as early as Day 64 of gestation. Among cows, in contrast, there were no differences in pregnancy rate at this stage of gestation between recipients that received one or two embryos. By Day 127, however, cows that received two embryos experienced substantial mid-to-late fetal loss and pregnancy rate and subsequent calving rate was lower for this group than for cows that received a single embryo.

The most likely explanation for the increased frequency of pregnancy loss in recipients receiving two embryos is uterine crowding, with the effects of crowding occurring sooner in gestation for nulliparous animals than for multiparous animals.

Similar results were obtained in another study (Anderson et al., 1979). In that study, calving rates and twinning rates were similar for cow recipients regardless of whether twin transfers were performed via bilateral or unilateral placement. For heifers, in contrast, calving rate and twinning rate was lower for unilateral twin transfers than for bilateral transfers. Using heifers, Rowson et al. (1971) also found lower embryonic survival rates and twinning rates for recipients of unilateral twin transfers than for recipients of bilateral transfers.

It is evident, however, that uterine capacity can vary between herds of cattle. Thus, there were no differences in pregnancy success between recipients of twin embryos placed unilaterally or bilaterally for heifers (Sreenan and Diskin 1989; Reichenbach et al., 1992) or cows (Sreenan and Diskin 1989). Similarly, embryonic survival rate for beef cows selected for twinning was similar for those having unilateral or bilateral multiple ovulations (Echternkamp et al., 1990). In lactating dairy cows, in contrast, the likelihood of a twin pregnancy resulting from multiple ovulation going to term was higher if ovulations occurred bilaterally than if unilateral ovulations occurred (López-Gatius et al., 2005b). Perhaps, identification of the biological processes controlling uterine capacity will lead to new approaches for increasing the efficacy of producing twins in cattle.

In an earlier study, administration of GnRH at Day 11 after anticipated ovulation tended to increase pregnancy and calving rates in lactating Holstein recipients (Block et al., 2003). The management of these cows was similar to those in Experiment 2. In both studies, recipients were exposed to heat stress and received an IVP embryo using a timed embryo transfer protocol. Effectiveness of treatment with GnRH or its analogues at 11 to12 d after estrus for inseminated cows has yielded variable results, as some reports

indicated a positive effect (Sheldon and Dobson, 1993; Willard et al., 2003) while others indicated no effect (Ryan et al., 1994). One factor that could influence the effectiveness of GnRH treatment at Day 11 is the number of follicular waves that a female experiences during an estrous cycle. Females with estrous cycles characterized by three follicular waves have larger second-wave dominant follicles at Day 11 than females with two-wave cycles (Ginther et al., 1989; Savio et al., 1990; Ko et al., 1991). Given that a follicle must reach 10 mm in diameter to ovulate in response to LH (Sartori et al., 2001), the preponderance of cycle type (two-wave vs three-wave) within a herd may determine effectiveness of GnRH treatment at Day 11. Finally, it remains possible that failure to observe an effect of GnRH treatment was because the number of animals per group was low. The pitfalls associated with interpretation of experiments with low numbers has been discussed (Amann, 2005) and could be responsible for the variation in results for trials to test effects of GnRH on pregnancy rates in embryo transfer recipients.

Estrus is difficult to detect in lactating dairy cows because of the short duration of estrus and the large proportion of cows that do not display intense mounting activity (Dransfield et al., 1998). This problem, which is exacerbated by heat stress (Thatcher et al., 1986), makes embryo transfer in lactating cows inefficient if recipient selection is based solely on estrus detection. The first report of a timed embryo transfer protocol, where ovulation was synchronized using an OvSynch protocol, was by Ambrose et al. (1999). The suitability of timed embryo transfer as a method for preparing recipients was demonstrated in Experiment 1 because calving rates were 50 and 41% for cow and heifer recipients that received a single embryo, respectively. Similarly, using beef recipients, a pregnancy rate of 49% was achieved using timed embryo transfer (Bo et al., 2002). In

contrast, pregnancy rate at Day 45 of gestation in Experiment 2 was only 17%. Low pregnancy rates have been reported in other studies with timed embryo transfer using lactating, heat-stressed recipients with pregnancy rates at ~ 45 d of gestation following timed embryo transfer ranging from 11 – 26% (Ambrose et al., 1999; Al-Katanani et al., 2002a; Block et al., 2003). The reason for the differences in pregnancy rates between Experiment 1 and 2 cannot be deduced because of the large number of variables between studies including nutrition, housing, level of milk yield, stage of lactation, breed, synchronization protocol, and embryo culture protocol.

Despite the effectiveness of timed embryo transfer, there was a tendency for pregnancy rates in Experiment 1 to be higher for those recipients detected in estrus. Most of the animals not detected in estrus likely ovulated after the last GnRH injection because embryos were only transferred to recipients with a detectable CL. Nonetheless, some cows in this group probably were not synchronized with respect to predicted ovulation time.

Transfer of IVP embryos has been associated with large calf syndrome, increased rates of fetal loss, sex ratio skewed towards the male and increased rate of dystocia and calf mortality (see Hasler et al., 2000; Hansen and Block, 2004; Farin et al., 2004 for review). There are also reports of prolonged gestation length (Kruip and den Dass, 1997; Rerat et al., 2005). In Experiment 1, most characteristics of the fetus and calf that were measured in females receiving one embryo were within normal ranges including gestation length, rates of fetal loss, calf birth weight, and calf survival at birth and within the first 7 d of age. The incidence of dystocia among females receiving one calf was 21% and it is difficult to determine whether this value is high because of the particular mating

combinations used (embryos of diverse genotypes transferred into females of several different genotypes). In a study with Holsteins bred by artificial insemination, the frequency of difficult births ranged from 6 to 18% (Djemali et al., 1987).

The one abnormality identified was a skewed sex ratio with 68% of the calves being male. While previous work suggests that the altered sex ratio among IVP embryos is due to toxic effects of concentrations of glucose in excess of 1 mM on female embryos (Kimura et al., 2005), the concentration of glucose in the medium used for culture here (KSOM-BE2) contains only 0.2 mM glucose (Soto et al., 2003). Others have found a tendency for male embryos to become blastocysts sooner in development when cultured in KSOM than female embryos (Nedambale et al., 2004b). Differences in sex ratio have been seen as early as between the eight-cell and morula stages of development (Block et al., 2003). While it is possible that selection of most embryos for transport done on Day 6 after insemination exacerbated the skewed sex ratio, Block et al. (2003) reported that 64% of calves born as a result of transfer of IVP embryos cultured in modified KSOM were male even though embryos were harvested for transfer on Day 8 after insemination. In conclusion, results indicate that unilateral transfer of two embryos to increase pregnancy rate is unwarranted. The fact that fetal loss occurred sooner for heifers than cows points out the importance of uterine capacity as a limiting factor for maintenance of fetal development of two conceptuses. There was also no evidence that GnRH treatment at Day 11 after anticipated ovulation improves pregnancy rate. Finally, the suitability of timed embryo transfer as a method for preparing recipients for transfer was evident by the high pregnancy and calving rates achieved with crossbred females that received a single embryo. Additional research is warranted to reduce incidence of skewed sex ratio.

While sexed semen could be used to control sex ratio (Wilson et al., 2005), it is likely that the underlying biological causes of altered sex ratio affect other aspects of embryo physiology also.

Table 3-1. Effect of recipient type and number of embryos transferred per recipient on pregnancy rates and losses.

Recipient type	Pregnancy rate, d 64 of gestation[ab]	Pregnancy rate, d 127 of gestation[ac]	Pregnancy loss between Day 64 and 127 of gestation[d]	Calving rate[e,f]	Pregnancy loss between Day 127 and calving[g]
Lactating cow – single embryo	8/14 (57%)	8/14 (57%)	0/8 (0%)	7/14 (50%)	1/8 (13%)[h]
Lactating cow – two embryos	6/12 (50%)	2/12 (17%)	4/6 (66%)	2/12 (17%)	0/2 (0%)
Nulliparous heifer – single embryo	7/17 (41%)	7/17 (41%)	0/7 (0%)	7/17 (41%)	0/7 (0%)
Nulliparous heifer – two embryos	3/15 (20%)	3/15 (20%)	0/3 (0%)	3/15 (20%)	0/3 (0%)

[a] Data are the proportion of animals pregnant of those that received embryos and, in parentheses, the percent pregnant.
[b] Logistic regression indicated effect of recipient type (P=0.07). The odds ratio estimate was 0.38 (heifer/cow) (95% Wald CI = 0.13, 1.14; Wald Chi-Square statistic = 2.96, P=0.08).
[c] Logistic regression indicated an effect of number of embryos transferred (P<0.03). The odds ratio estimate was 4.13 (one embryo/two embryos) with a 95% Wald CI of 1.243, 13.690. Wald Chi-Square statistic = 5.36; P<0.03).
[d] Data are the proportion of pregnant recipients at Day 64 that lost their pregnancy by Day 127 of gestation and, in parentheses, the percent pregnancy loss.
[e] Data are the proportion of animals that calved of those that received embryos and, in parentheses, the percent pregnant.
[f] Logistic regression indicated an effect of number of embryos transferred (P<0.03). The odds ratio estimate was 3.62 (one embryo/two embryos) with a 95% Wald CI of 1.090, 12.047. Wald Chi-Square statistic = 4.41; P<0.04).
[g] Data are the proportion of pregnant recipients at Day 127 that lost their pregnancy before calving and, in parentheses, the percent pregnancy loss.
[h] One cow expelled a stillborn calf at 251 d of gestation.

Table 3-2. Effect of recipient type and number of embryos transferred per recipient on characteristics of pregnancy and parturition.

Recipient type	Gestation length, d[a]	Twin pregnancies[b]	Dystocia[c]	Retained placenta[d]
Lactating cow – single embryo	282 ± 3	0/7 (0%)	2/7 (29%)	4/7 (57%)
Lactating cow – two embryos	274 ± 5	1/2 (50%)	0/2 (0%)	1/2 (50%)
Nulliparous heifer – single embryo	276 ± 3	0/7 (0%)	1/7 (14%)	5/7 (71%)
Nulliparous heifer – two embryos	284 ± 4	1/3 (33%)	1/3 (33%)	2/3 (67%)

[a] Data are least-squares means ± SEM. Gestation length was affected by recipient type x number of embryos transferred ($P<0.05$).

[b] Data are the proportion of pregnancies in which twin calves were born and, in parentheses, the percent pregnant. Logistic regression indicated an effect of number of embryos transferred ($P<0.02$).

[c] Data are the proportion of pregnancies in which dystocia was recorded at birth and, in parentheses, the percent cows experiencing dystocia.

[d] Data are the proportion of cows calving that experienced retained placenta and, in parentheses, the percent cows experiencing retained placenta.

Table 3-3. Effect of recipient type and number of embryos transferred per recipient on characteristics of calves born.

Recipient type	Sex ratio (M:F)[a]	Calf birth weight, kg[b]	Calf mortality at birth[c]	Calf mortality to Day 7 of age[d]
Lactating cow – single embryo	5:3[e]	34 ± 3	0/7 (0%)	0/7 (0%)
Lactating cow – two embryos	2:1	25 ± 5	0/3 (0%)	0/3 (0%)
Nulliparous heifer – single embryo	4:3	26 ± 3	1/7 (14%)[f]	0/6 (0%)
Nulliparous heifer – two embryos	4:0	25 ± 5	3/4 (75%)[g]	0/1 (0%)

[a] The overall sex ratio of 15 male and 7 females tended to be different ($P<0.10$) than the expected 1:1 ratio.

[b] Data are least-squares means ± SEM.

[c] Data are the proportion of calves that were born dead and, in parentheses, the percent born dead.

[d] Data are the proportion of calves born alive that died before d 7 of live and, in parentheses, the percent death before Day 7.

[e] Data includes the stillborn calf at 251 d of gestation

[f] One calf was stillborn from a cow not experiencing dystocia.

[g] One heifer had twin fetuses and both were born dead as a result of complications with calving. The other two heifers gave birth to a single calf. One calf was born alive and the other was born dead as a result of complications with calving.

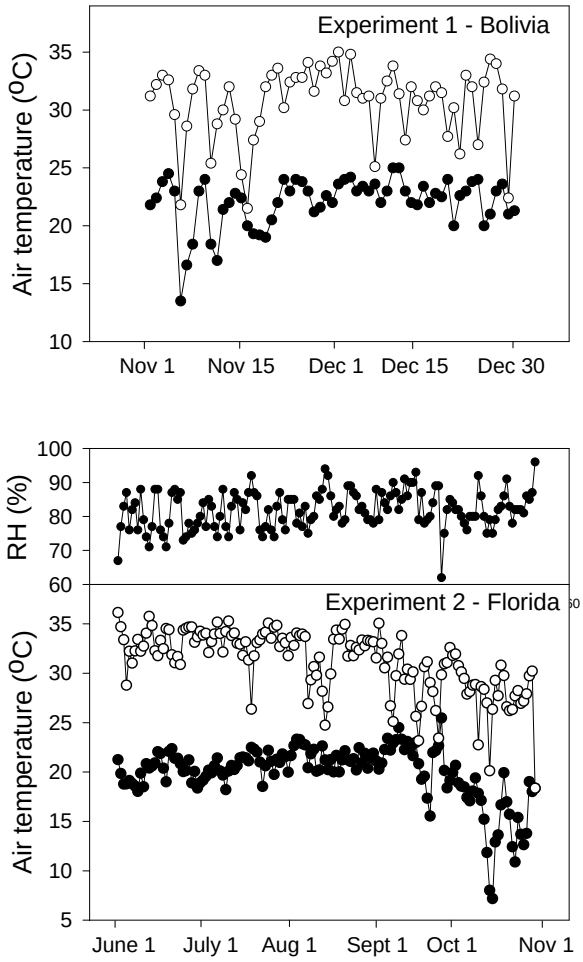

Figure 3-1. Maximum (open circles) and minimum (closed circles) daily air temperatures and relative humidities (RH) during the experiments.

CHAPTER 4
EFFECTS OF HYALURONIC ACID IN CULTURE AND CYTOCHALASIN B TREATMENT BEFORE FREEZING ON SURVIVAL OF CRYOPRESERVED BOVINE EMBRYOS PRODUCED IN VITRO

Introduction

In vitro production of embryos is an important tool for improving genetic merit and fertility of cattle and is an indispensable component of other technologies such as somatic cell cloning and transgenesis (Hansen and Block, 2004). One limitation to the widespread use of in vitro produced embryos in the cattle industry is the poor survivability of in vitro produced embryos to cryopreservation. In vitro survival rates following thawing (Pollard and Leibo, 1993; Enright et al., 2000; Khurana and Niemann, 2000a; Diez et al., 2001; Guyader-Joly et al., 1999) and pregnancy rates following thawing and transfer (Hasler et al., 1995; Agca et al., 1998; Ambrose et al., 1999; Al-Katanani et al., 2002a) are consistently lower for embryos produced in vitro when compared to embryos produced in vivo by superovulation.

The poor survival of the in vitro produced embryo is associated with culture-induced changes in ultrastructure (Rizos et al., 2002), gene expression (Bertolini et al., 2002a; Lazzari et al., 2002; Lonergan et al., 2003), and metabolism (Krisher et al., 1999; Khurana and Niemann, 2000b) that make it distinct from the embryo produced in vivo. Among the metabolic changes are an increase in lipid content (Abe et al., 1999; Rizos et al., 2002) and this condition has been linked to poor freezability. Mechanical delipidation (Tominaga et al., 2000; Diez et al., 2001) and addition of inhibitors of fatty acid synthesis (De la Torre-Sanchez et al., 2005) can improve survival following cryopreservation.

In the current study, two approaches for enhancing survival of bovine embryos following cryopreservation were evaluated. The first was to culture embryos in the presence of hyaluronic acid. This unsulphated glycosaminoglycan is present in follicular, oviductal and uterine fluids in several species including cattle (Lee and Ax, 1984). Receptors for hyaluronic acid (CD44) have been reported on the bovine oocyte, cumulus cell, and preimplantation stage embryo (Valcarcel et al., 1999). Addition of hyaluronic acid to culture medium has been reported to increase blastocyst re-expansion and hatching after freezing (Stojkovic et al., 2002; Lane et al., 2003). The second approach was to determine whether altering the cytoskeleton before cryopreservation would enhance embryo survival. The rationale for this treatment is that cryoinjuries such as intracellular ice formation and osmotic shock induce irreversible disruption in microtubules and microfilaments (Kuwayama et al., 1994; Fair et al., 2001) and that temporary depolymerization of actin microfilaments before cryopreservation could reduce cytoskeletal damage and plasma membrane fracture caused by alterations in cytoskeletal architecture (Dobrinsky, 1996). Addition of cytochalasin B to cause actin depolymerization had no effect on survival of eight-cell embryos in the mouse (Prather and First, 1986) but enhanced survival of expanded and hatched blastocysts without effecting survival of morula and early blastocysts in the pig (Dobrinsky et al., 2000).

Materials and Methods

Embryo Production

Procedures, reagents, and media formulation for oocyte maturation, fertilization, and embryo culture were as previously described (Roth and Hansen, 2005) with some modifications. Briefly, cumulus oocyte complexes (COCs) were harvested from ovaries of a variety of breeds collected at a local abattoir located at a travel distance of

approximately 1.5 h from the laboratory. The COCs were matured in Tissue Culture Medium-199 with Earle's salts supplemented with 10% (v/v) steer serum, 2 μg/mL estradiol 17-β, 20 μg/ml follicle stimulating hormone, 22 μg/ml sodium pyruvate, 50 μg/ml gentamicin and an additional 1 mM glutamine for approximately 22 h at 38.5°C in an atmosphere of 5% (v/v) CO_2 in humidified air. Insemination with a cocktail of Percoll-purified spermatozoa from three different bulls was performed in In Vitro Fertilization – Tyrode's Albumin Lactate solution. At 8 – 12 h post-insemination (hpi), putative zygotes were denuded of cumulus cells by suspension in Hepes-TALP medium containing 1000 units/ml hyaluronidase type IV (Sigma, St Louis, MO, USA) and vortexing in a microcentrifuge tube for 5 min. Presumptive zygotes were then placed in groups of ~30 in 50 μl microdrops of a modified Synthetic Oviductal Fluid (SOF) prepared as described by Fisher-Brown et al. (2002). Embryos were cultured at 38.5°C in a humidified atmosphere of 5% (v/v) CO_2, 5% O_2, and with the balance N_2. Blastocysts were collected for cryopreservation on day 7 after insemination.

Experimental Design and Embryo Manipulation

The experiment was a 2 x 2 factorial design to test main effects of hyaluronic acid during culture (+ or -) and cytochalasin B before cryopreservation (+ or -). Data on development were obtained from 18 replicates using 5022 oocytes while data on cryopreservation were obtained from 7 replicates using a total of 197 blastocysts.

Following insemination and transfer to fresh microdrops, embryos cultured without hyaluronic acid were cultured in SOF for 7 days beginning after insemination. Embryos treated with hyaluronic acid were cultured in SOF until day 5 when all embryos were transferred to a fresh microdrop of SOF containing 6 mg/ml hyaluronic acid from *Streptococcus zooepidemicus* (Sigma).

Blastocysts and expanded blastocysts were harvested on the morning of day 7 after insemination and washed twice in holding medium consisting of Hepes-TALP (Parrish et al., 1989) containing 10% (v/v) fetal calf serum (FCS). Embryos treated with cytochalasin B were incubated for 10 min at 38.5oC in air while in Hepes-TALP containing 10% (v/v) FBS and 7.5 µg/ml cytochalasin B (Sigma) in a 1.5 ml microcentrifuge tube (Tominaga et al., 2000). Cytochalasin B was initially dissolved in DMSO at a concentration of 5 mg/ml and was then added to HEPES-TALP to achieve a final concentration of 7.5 µg/ml. Control embryos were incubated similarly in HEPES-TALP containing 10% (v/v) FBS.

Cryopreservation

Procedures for freezing were modified from those reported elsewhere (Hasler et al., 1995; Enright et al., 2000). In brief, blastocysts were transferred in groups of 10 to a fresh 100 µl microdrop of Hepes-TALP containing 10% FCS at 38.5^{o}C for the time it took to harvest all embryos (~ 10 min). Next, embryos in groups of 5 - 8 per treatment (hyaluronic acid or control) were randomly selected to receive cytochalasin B treatment before freezing or not as described above. Afterwards, each group of 5 – 8 embryos was placed in a 50 µl microdrop of 10% (v/v) glycerol in Dulbecco's phosphate-buffered saline (DPBS) containing 0.4% (w/v) bovine serum albumin (freezing medium) in a grid plate over a slide warmer at 30^{o}C. Within 10 min, embryos were loaded in a 50 µl volume into 0.25 ml plastic straws (Agtech, Manhattan, KS). Up to 8 embryos were loaded in each straw. Two columns of 50 µl freezing medium separated by air bubbles were always placed above and below the column of embryos. Straws were transferred to a freezing chamber (Cryologic Model CL5500 (Mulgrave, Victoria, Australia) for 2 min at -5^{o}C and then ice crystals were induced by touching the straw where the top column of medium

resided with a cotton plug that had been immersed in liquid nitrogen. After an additional 3 min at -5°C, embryos were cooled to -32°C at a rate of -0.6°C/min. After 2 min at -32°C, straws were directly immersed in liquid N_2 and stored until thawing (4 days – 1 week later).

Thawing and Determination of Survival

Straws containing embryos were thawed by warming for 10 sec in air at room temperature and 20 sec in a 32°C water bath. All subsequent steps before culture were performed with media prewarmed to ~30°C and with dishes placed on a slide warmer set at 30°C. Embryos were then expelled into an empty petri dish and immediately transferred to a fresh 60 µl drop of DPBS containing 6.6% (v/v) glycerol and 0.3 M sucrose in an grid dish. After 5 min, embryos were sequentially transferred to DPBS containing 3.3% (v/v) glycerol and 0.3 M sucrose for 5 min and DPBS + 0.3 M sucrose for 5 min. Embryos were then washed three times in HEPES-TALP + 10 % (v/v) FCS and placed into culture in groups of 5- 8 in 25 µl microdrops of SOF containing 10% (v/v) FCS. Culture was at 38.5°C in a humidified atmosphere of 5% (v/v) CO_2, 5% O_2, and 90% N_2. Re-expansion was determined at 48 h after thawing and hatching at 72 h.

Statistical Analysis

The proportion of oocytes that cleaved and the proportion of embryos that developed to the blastocyst stage on day 7 and day 8 were determined for each replicate. Treatment effects were determined by least-squares analysis of variance using the proc GLM procedure of SAS (SAS for Windows 90, Cary, NC). The model included the main effects of replicate and treatment. Data for the proportion of frozen/thawed embryos that re-expanded and on the proportion that hatched by 72 h of culture were analyzed using the CATMOD procedure of SAS. The initial model included all main effects and two-

way interactions. After removing nonsignificant effects, the final model included replicate, hyaluronic acid, preparation prior to freezing (none, cytochalasin B), and the interaction of hyaluronic acid and preparation before freezing.

Results

Effect of Hyaluronic Acid on Embryonic Development

As shown in Table 1, addition of hyaluronic acid at day 5 after insemination caused a slight reduction in the yield of blastocysts on day 7 and day 8 after insemination regardless of whether data were expressed as the proportion of oocytes developing to the blastocyst stage ($P < 0.05$) or the proportion of cleaved embryos developing to the blastocyst stage ($P < 0.01$). Of the blastocysts that were recovered, 62-68% were recovered at day 7 and the balance at day 8. There was no effect of hyaluronic acid on the proportion of blastocysts collected at day 7 (Table 4-1).

Survival after Cryopreservation

Overall, cytochalasin B increased the percent of embryos that re-expanded following thawing ($P < 0.0001$) and that hatched following thawing ($P < 0.05$) (Table 4-2). Re-expansion rates were 51.2% (22/43) for embryos treated with cytochalasin B and 18.2% (8/44) for embryos not subjected to cytochalasin B. Hatching rates were 39.5% (17/43) for embryos treated with cytochalasin B and 4.5% (2/44) for embryos not subjected to cytochalasin B.

While there was no significant effect of hyaluronic acid on cryosurvival, there was a tendency ($P=0.09$) for a hyaluronic acid x cytochalasin B interaction affecting percent of blastocysts that hatched following thawing. This interaction reflects the fact that hyaluronic acid increased the percent hatching for embryos not subjected to cytochalasin B treatment and decreased percent hatched for embryos subjected to cytochalasin B.

Discussion

Of the two treatments evaluated for enhancing cryosurvival of in vitro produced bovine embryos, cytochalasin B treatment was the most effective as determined by an improvement in both embryo re-expansion and hatching. The rationale for this treatment is to reduce cellular injury caused by disruption in microtubules and microfilaments (Kuwayama et al., 1994; Fair et al., 2001) and to increase flexibility of the plasma membrane to allow it to tolerate forces associated with freezing that lead to membrane damage. In other studies, addition of cytochalasin B had no effect on survival of eight-cell embryos in the mouse (Prather and First, 1986), enhanced survival of expanded and hatched pig blastocysts without effecting survival of morula and early blastocysts (Dobrinsky et al., 2000), and improved survival of in vivo derived bovine blastocysts subjected to vitrification (Dobrinsky et al., 1995).

For embryos not exposed to cytochalasin B, there was a tendency for those cultured in hyaluronic acid to have a higher re-expansion rate and hatching rate than embryos cultured without hyaluronic acid. Both Stojkovic et al. (2002) and Lane et al. (2003) reported improved survival rates to freezing when embryos were cultured in hyaluronic acid; such a beneficial effect has not always been observed (Furnus et al., 1998). Surprisingly, embryos cultured in hyaluronic acid were less likely to survive freezing than control embryos when the cytochalasin B treatment was applied. Perhaps physiological changes induced by hyaluronic acid cause the embryo to be less able to adjust to the cellular actions of cytochalasin B. Those changes are potentially numerous because hyaluronic acid acts to affect cell function through several means including signaling through cell surface receptors, modifying the biophysical properties of extracellular and pericellular matrices by attracting water, and by interacting physically

with a variety of ions and other molecules (Laurent, 1987; Ruoslahti and Yamaguchi, 1991; Hardingham and Fosang, 1992; Yasuda et al., 2002; Toole et al., 2005). One possible mechanism by which hyaluronic acid could increase embryo survival to freezing is by increasing the total number of cells in the embryo (Stojkovic et al., 2002; Jang et al., 2003; Kim et al., 2005)

One unexpected finding was the reduction in the percentage of embryos that became blastocysts caused by hyaluronic acid. In other studies, hyaluronic acid either had no effect (Stojkovic et al., 2002; Lane et al., 2003) or caused an increase in blastocyst yield (Furnus et al., 1998; Jang et al., 2003). Differences in origin and concentration of hyaluronic acid could explain some of this difference between studies. Hyaluronic acid can be isolated from different sources (ex., bacteria, rooster comb, and umbilical cord) and preparations can differ in protein, endotoxin, and nucleotide content (Shiedlin et al., 2004). Stojkovic et al. (2002) reported that preliminary results indicated that embryo development in vitro was dependent upon the origin of the commercially-available hyaluronic acid. However, embryos cultured with hyaluronic acid experienced a change in culture medium at day 5 whereas control embryos did not. Such a difference could have obscured beneficial effects of hyaluronic acid although another paper indicates no effect of changing culture medium at 72 hpi on blastocyst yield in cattle (Ikeda et al., 2000).

The percent of embryos that underwent hatching after freezing in glycerol and thawing has varied from 0% (Enright et al., 2000) 22% (Diez et al., 2001; Nedambale et al., 2004a), 32% (Guyader-Joly et al., 1999) and 69% (Hasler et al., 1997). The best survival achieved in this study was for embryos cultured without hyaluronic acid and

treated with cytochalasin B. In this group, 51.2% of cryopreserved embryos were capable of re-expansion and 39.5% hatched. It is likely that the percent hatching can be further improved by modifying post-thaw culture-conditions. Massip et al. (1993) found hatching rates for frozen/thawed, in vitro produced embryos were 41% when culture was performed in the presence of bovine oviductal epithelial cells while hatching rate using other culture conditions not involving co-culture was 0-6%. Nonetheless, one would not expect optimal pregnancy rates to be achieved following direct transfer of embryos frozen in glycerol even with the inclusion of cytochalasin B treatment. Rather, it is suggested that pregnancy rates following transfer of embryos cryopreserved using slow-freezing procedures can be optimized by selecting embryos for transfer based on development in culture shortly after thawing.

In contrast to the poor survival of in vitro-produced embryos frozen using conventional slow-freezing techniques, several experiments indicate that cryosurvival can be enhanced by using vitrification (Vajta, 2000). It remains to be tested whether survival of embryos produced in vitro after vitrification can be improved by cytochalasin B treatment. There was a beneficial effect of cytochalasin B treatment on cryosurvival of embryos derived in vivo following vitrification (Dobrinsky et al., 1995).

In conclusion, cytochalasin B treatment before freezing improved cryosurvival of bovine embryos produced in vitro and subjected to slow-freezing in glycerol. Such a treatment could be incorporated into methods for cryopreservation of bovine embryos provided post-transfer survival is adequate. In contrast, culture with hyaluronic acid was of minimal benefit - the increased cryosurvival in the absence of cytochalasin B was not sufficient to allow an adequate number of embryos to survive.

Table 4-1. Effect of hyaluronic acid added at day 5 after insemination on production of blastocysts at day 7 and 8 after insemination[a,b].

Culture medium	Number of oocytes	Percent cleaved	Blastocysts/oocyte (%) [c]	Blastocysts/cleaved embryo (%) [c]	Percent of total blastocysts that were collected at day 7
Control	1935	76.0 ± 0.9	36.0 ± 1.2*	47.2 ± 1.3**	68.8 ± 2.4
Hyaluronic acid	3087	77.7 ± 0.9	31.5 ± 1.2	40.7 ± 1.3	62.2 ± 2.4

[a] n=18 replicates
[b] Means within a column that differ significantly are indicated by * ($P < 0.05$) and ** ($P < 0.01$)
[c] Includes blastocysts collected at day 7 and those collected at day 8.

Table 4-2. Effect of culture in hyaluronic acid and treatment with cytochalasin B on survival after cryopreservation. [a]

Culture medium	Cytochalasin treatment	Re-expansion by 72 h[b]	Hatching by 72 h[c]
Control	Control	8/44 (18.2%)	2/44 (4.5%)
Control	Cytochalasin B	22/43 (51.2%)	17/43 (39.5%)
Hyaluronic acid	Control	16/55 (29.0%)	7/55 (12.7%)
Hyaluronic acid	Cytochalasin B	26/55 (47.3%)	12/55 (21.8%)

[a] Data are the fraction of embryos, and in parentheses, percent. Number of replicates was 7.
[b] Effect of cytochalasin B ($P < .0001$).
[c] Effect of cytochalasin B ($P < 0.05$), hyaluronic acid ($P < 0.10$), and the cytochalasin B x hyaluronic acid interaction ($P = 0.09$).

CHAPTER 5
GENERAL DISCUSSION

As alluded to at the beginning of this thesis, there has been a precipitous decline in fertility of dairy cows over the last 10-40 years in North America (Butler, 1998), Ireland (Roche, 2000), Spain (López-Gatius et al., 2003), and the United Kingdom (Royal et al., 2000). In addition, heat stress can compromise fertility in lactating dairy cows (Putney et al., 1989b; Al-Katanani et al., 1999). The purpose of the present series of experiments described in the thesis was to 1) evaluate strategies for enhancing fertility after AI using GnRH treatment (Chapter 2) and 2) further develop ET using in vitro produced embryos as a tool for increasing fertility by testing whether pregnancy rate could be improved by transfer of twin embryos (Chapter 3) and whether the developmental competence of embryos after cryopreservation could be improved by hyaluronan or cytochalasin B treatment (Chapter 4). Results indicated no consistent benefit of injection of GnRH at Day 11-15 after anticipated ovulation or insemination on pregnancy rates in heifers or lactating cows. While unilateral transfer of two embryos was not shown to be an effective treatment for increasing pregnancy rate in recipients, the high pregnancy rates achieved in this study point to the potential usefulness of ET as a tool for enhancing fertility. Large-scale use of embryo transfer will require the ability to freeze embryos successfully. Results suggest that treatment of embryos with cytochalasin B before freezing is a promising tool for enhancing survival of embryos following cryopreservation. A large number of studies have been performed to test the effect of GnRH administration after expected ovulation on fertility of cattle. Previous results indicated that GnRH was

sometimes effective at increasing pregnancy rate, but this beneficial effect was often not observed (Peters et al., 2000). Despite this knowledge, we chose to reevaluate the effectiveness of GnRH treatment because of a report that GnRH treatment at Day 11 after estrus increases pregnancy rates in lactating cows exposed to heat stress (Willard et al., 2003). Accordingly, it was hypothesized in Chapter 2 that the beneficial effect of GnRH treatment would be greater during the summer than winter. This may be so because the antiluteolytic process may be compromised by heat stress because of decreased growth of the filamentous stage conceptus (Biggers et al., 1987) and increased uterine PGF2α secretion from the uterus (Wolfenson et al., 1993).

Overall, the results of GnRH treatment were generally negative. For treatment at Day 11, a positive effect of GnRH on fertility was never seen. This was the case for heifers and lactating cows subjected to AI or whether animals were exposed to heat stress or not (Chapter 2; experiment 1 and 2). Treatment of lactating recipients with GnRH at Day 11 also failed to increase pregnancy rate during heat stress in ET recipients (Chapter 3, experiment 2). Effectiveness of treatment with GnRH or its analogues at 11 to 12 d after estrus for inseminated, heat-stressed lactating cows has yielded variable results, as some reports indicated a positive effect (Sheldon et al., 1993; Willard et al., 2003), while others indicated no effect (Jubb et al., 1990). Also, administration of GnRH at Day 11 after anticipated ovulation tended to increase pregnancy and calving rates in lactating Holstein embryo transfer recipients exposed to heat stress (Block et al., 2003).

One factor that could influence the effectiveness of GnRH treatment at Day 11 is the number of follicular waves that a female experiences during an estrous cycle. Females with estrous cycles characterized by three follicular waves have larger second-wave

dominant follicles at Day 11 than females with two-wave cycles (Ginther et al., 1989; Savio et al., 1990; Ko et al., 1991). Given that a follicle must reach 10 mm in diameter to ovulate in response to LH (Sartori et al., 2001), the preponderance of cycle type (two-wave vs three-wave) within a herd may determine effectiveness of GnRH treatment at Day 11.

In one experiment (Chapter 2; experiment 3), administration of GnRH at Day 14 after anticipated ovulation in cows subjected to TAI increased pregnancy rates of lactating cows in the summer and winter at two locations. In the following year, though, GnRH failed to improve fertility when treatment was administered either at day 14 in cows subjected to TAI (experiment 4) or at day 14 or 15 in cows previously diagnosed coming in estrus (experiment 5). It is important to recognize that GnRH treatment should improve fertility only when triggering luteinization or ovulation of developing (estrogenic) follicles. Thus, there are at least two possible reasons for a lack in response upon GnRH treatment at day 14 or 15. One possibility relates to the timing of ovulation relative to the GnRH treatment and whether these animals failed to ovulate after being diagnosed as coming into estrus. Although after observing estrus one does not expect ovulation to fail, this expression does not necessarily mean that subsequent ovulation occurred (López-Gatius et al., 2005b) and insemination after a false identified estrus often occurs (Heersche and Nebel 1994). According to López-Gatius et al. (2005b), the risk of cows failing to ovulate (12%) during the summer was greater than in the cool period (3%).

During experiment 3 all cows received a GnRH injection at 72 h following PGF2α to insure an ovulation of the synchronized dominant follicle. Perhaps, the positive

GnRH effect observed during experiment 3 was masked in the following experiment because cows did not receive an additional GnRH dose at estrus to ensure subsequent ovulation. According to Lopez-Gatius et al. (2005a), there is evidence demonstrating the benefits upon GnRH treatment when given on the day of insemination compared to controls (30.8% vs. 20.6%), but conception rates were greater if cows received an additional dose at day 12 post-insemination (35.4%). On the other hand, when GnRH treatment took place on day 15 to ensure a responsive (estrogenic) dominant follicle would ovulate at the time of GnRH treatment, it failed to improve fertility as well. Similarly, in a recent study (Bartolome et al., 2005) there was no effect of GnRH treatment on pregnancy rates of lactating cows when administered either on day 15 or day 5 and 15 after TAI.

It remains possible that inconsistency in effects of GnRH treatment is caused in part by the low number of animals per treatment group. The pitfalls associated with interpretation of experiments with low numbers has been discussed (Dransfield et al., 1998) and could be responsible for the variation in results for trials to test effects of GnRH on pregnancy rates in embryo transfer recipients and for inseminated cows.

With an existent variation among trials regarding the use of GnRH at day 11-15 post-insemination, one could speculate that such inconsistency regarding treatment is due to the fact that herds of cattle determine the result that an experiment achieves. However, our results indicate that such a hypothesis is not likely because when an experiment was replicated the next year using the same herd, GnRH treatment once again proved to be inconsistent in improving pregnancy rates.

According to Thatcher et al. (2005), hCG results in a more prolonged rise in LH activity than is achieved following GnRH treatment. Perhaps the likelihood of ovulating or luteinizing the dominant follicles present at the time of treatment would be higher using hCG. Although low numbers of inseminated animals were used (n=8; n=49) hCG treatment on d 14 after estrus improved pregnancy rates (Rajamahendran and Sianangama, 1992; Sianangama and Rajamahendran, 1992). Use of hCG warrants further investigation for any additional effect or response during the summer to enhance pregnancy rates of lactating cows.

Recent work has focused on use of ET to bypass early embryonic death (Putney et al., 1989b; Ambrose et al., 1999; Al-Katanani et al., 2002a). Given that ET can be more effective at increasing pregnancy rates than AI for lactating cows during periods of heat stress (Putney et al., 1989b; Ambrose et al., 1999; Drost et al., 1999; Al-Katanani et al., 2002a), the potential benefit of ET can be realized. For ET to become an economical alternative to AI on a wide scale basis in commercial herds, embryos must be inexpensive to produce (Hansen and Block et al., 2004). Although embryos produced using IVP systems are relatively inexpensive as compared to embryos produced by superovulation, pregnancy rates achieved following transfer of an IVP embryo are often less than what is obtained following transfer of an embryo produced by superovulation (Hasler et al., 1995; Agca et al., 1998; Ambrose et al., 1999; Al-Katanani et al., 2002a). In addition, IVP embryos are less likely to survive freezing than superovulated embryos (Hasler et al., 2003), likely due to their increased lipid content (Abe et al., 1999; Rizos et al., 2002). Accordingly, the second approach for the thesis focused on improvements in ET by

comparing pregnancy rates following the transfer of two embryos compared to one and by increasing the viability of embryos that were cryopreserved.

The first effort was to determine whether transfer of two IVP embryos into the uterine horn ipsilateral to the CL could increase pregnancy rates during periods of heat stress. It was hypothesized that such a treatment might increase pregnancy rates because the likelihood is increased that the cow receives at least one embryo competent for sustained development. In addition, the transfer of two embryos into the ipsilateral uterine horn is likely to increase the amounts of interferon-τ and other embryonic signaling molecules in the uterus needed to maintain pregnancy and prevent luteolysis.

Transferring two embryos into the uterine horn ipsilateral to the CL failed to increase pregnancy rates. Instead, the transfer of two embryos into recipients led to pregnancy loss, which occurred earlier for heifers than for cows. The most likely explanation for the increased frequency of pregnancy loss in recipients receiving two embryos is uterine crowding, with the effects of crowding occurring sooner in gestation for nulliparous animals than for multiparous animals. Regardless of whether twin transfers were performed via bilateral or unilateral placement, similar results were obtained in another study (Anderson et al., 1979). In contrast, calving rate and twinning rate in heifers was lower for unilateral twin transfers than for bilateral transfers. Similarly, Rowson et al. (1971) also found lower embryonic survival rates and twinning rates for recipients of unilateral twin transfers than for recipients of bilateral transfers in heifers.

It is evident that uterine capacity can vary between herds of cattle. Thus, there were no differences in pregnancy success between recipients of twin embryos placed

unilaterally or bilaterally for heifers (Sreenan et al., 1989, Reichenbach et al., 1992) or cows (Sreenan et al., 1989). Similarly, embryonic survival rate for beef cows selected for twinning was similar for those having unilateral or bilateral multiple ovulations (Echternkamp et al., 1990). In lactating dairy cows, in contrast, the likelihood of a twin pregnancy resulting from multiple ovulations going to term was higher if ovulations occurred bilaterally than if unilateral ovulations occurred (López-Gatius and Hunter, 2005). Perhaps, identification of the biological processes controlling uterine capacity will lead to new approaches for increasing the efficacy of producing twins in cattle.

An additional limitation to the widespread use of IVP embryos in cattle is their poor survival following cryopreservation. In vitro survival rates following thawing (Pollard and Leibo, 1993; Enright et al., 2000; Khurana and Niemann, 2000a; Diez et al., 2001; Guyader-Joly et al., 1999) and pregnancy rates following thawing and transfer (Hasler et al., 1995; Agca et al., 1998; Ambrose et al., 1999; Al-Katanani et al., 2002a) are consistently lower for IVP embryos when compared to embryos produced in vivo by superovulation.

The percent of embryos that underwent hatching after freezing in glycerol and thawing has varied from 0% (Enright et al., 2000), 22% (Diez et al., 2001; Nedambale et al., 2004a), 32% (Guyader-Joly et al., 1999), and 69% (Hasler et al., 1997). Of the two treatments evaluated for enhancing cryosurvival of IVP bovine embryos, cytochalasin B treatment was the most effective as determined by an improvement in embryo re-expansion and hatching rates. In this treatment, 51.2% of cryopreserved embryos were capable of re-expansion and 39.5% hatched. Nonetheless, one would not expect optimal

pregnancy rates to be achieved following direct transfer of embryos frozen in glycerol even with the inclusion of cytochalasin B treatment.

In contrast to the poor survival of IVP embryos frozen using conventional slow-freezing techniques, several experiments indicated that embryo survival can be enhanced following vitrification (Vajta, 2000). It remains to be tested whether survival of embryos produced in vitro after vitrification can be improved by cytochalasin B treatment. Rather, it is suggested that pregnancy rates following transfer of embryos cryopreserved using slow-freezing procedures can be optimized by selecting embryos for transfer based on development in culture shortly after thawing.

Indeed, fertility issues will continue to drive new ideas for developing strategies to improve or at least reduce undesirable conception and pregnancy rates in any cattle operation. Efficiency among cattle operations is of major interest and ET has the potential to be the vehicle that can help overcome some fertility issues associated with oocyte developmental competence, fertilization, and early embryonic development. However, the potential this reproductive technology has is underestimated when pregnancy rates continue to be less than AI during the absence of heat stress. Further research that identifies embryos that are more likely to survive following transfer and establish a pregnancy is warranted.

In conclusion, GnRH treatment did not consistently increase pregnancy rates when administered at Day 11-15 after insemination and is not recommended as a fertility-enhancing treatment. Similarly, transfer of two embryos to the uterine horn ipsilateral to the CL was not an effective method for increasing pregnancy rates in recipients. Transfer of cryopreserved embryos may be enhanced by treatment of embryos with cytochalasin B

since this molecule increased in vitro survival. Since several experiments indicate that cryosurvival can be enhanced using vitrification (Vajta, 2000), it remains to be tested whether survival of IVP embryos after vitrification can be improved by cytochalasin B treatment.

LIST OF REFERENCES

Abe H, Yamashita S, Itoh T, Satoh T, Hoshi H. Ultrastructure of bovine embryos developed from in vitro-matured and fertilized oocytes: comparative morphological evaluation of embryos cultured either in serum-free medium or in serum supplemented medium. Mol Reprod Dev 1999; 53:325-35.

Abilay TA, Johnson HD, Madan M. Influence of environmental heat on peripheral plasma progesterone and cortisol during the bovine estrous cycle. J Dairy Sci 1975; 58:1836-40.

Achard D, Gilbert M, Bénistant C, Slama SB, DeWitt DL, Smith WL and Lagarde M. Eicosapentaenoic and docosahexaenoic acids reduce PGH synthase 1 expression in bovine aortic endothelial cells. Bioch Biophy Res Comm 1997; 241:513–18.

Adam CL, Findlay PA, Moore HA. Effects of insulin-like growth factors-I on luteinizing hormone secretion in sheep. Anim Reprod Sci 1997; 50:45-56.

Agca Y, Monson RL, Northey DL, Abas Mazni O, Schaeffer DM, Rutledge JJ. Transfer of fresh and cryopreserved IVP bovine embryos: normal calving, birth weight and gestation lengths. Theriogenology 1998; 50:147-62.

Ahlswede L, Lotthammer KH. Studies on a specific vitamin A-unrelated effect of beta carotene on the fertility of cattle. 5. Studies of organs (ovaries, corpora lutea, liver, fatty tissues, uterine secretion, adrenal glands--determination of weight and content] Dtsch Tierarztl Wochenschr 1978; 85:7-12.

Ahmad N, Townsend EC, Dailey RA, Inskeep EK. Relationships of hormonal patterns and fertility to occurrence of two or three waves of ovarian follicles, before and after breeding, in beef cows and heifers. Anim Reprod Sci 1997; 49:13-28.

Akordor FY, Stone JB, Walton JS, Leslie KE, Buchanan-Smith JG. Reproductive performance of lactating Holstein cows fed supplemental β-carotene. J Dairy Sci 1986; 69:2173-8.

Al-Katanani YM, Webb DW, Hansen PJ. Factors affecting seasonal variation in 90 day non-return rate to first service in lactating Holstein cows in a hot climate.J Dairy Sci 1999; 82:2611-5.

Al-Katanani YM, Drost M, Monson RL, Rutledge JJ, Krininger-III CE, Block J, Thatcher WW, Hansen PJ. Pregnancy rates following timed embryo transfer with fresh or vitrified in vitro produced embryos in lactating dairy cows under heat stress conditions. Theriogenology 2002a; 58:171-82.

Al-Katanani YM, Paula-Lopes FF, Hansen PJ. Effect of season and exposure to heat stress on oocyte competence in Holstein cows. J Dairy Sci 2002b; 85:390-6.

Alvarez RH, da Silva MV, de Carvalho JB, Binelli M. Effects of inbreeding on ovarian responses and embryo production from superovulated Mantiqueira breed cows. Theriogenology 2005; 64:1669-76.

Amann RP. Weaknesses in reports of "fertility" for horses and other species. Theriogenology 2005; 63:698-715.

Ambrose JD, Drost M, Monson RL, Rutledge JJ, Liebfried-Rutledge ML, Thatcher MJ, Kassa T, Binelli M, Hansen PJ, Chenoweth PJ, Thatcher WW. Timed embryo transfer in heat-stressed dairy cattle: A field trial with IVF-derived embryos. J Dairy Sci 1997; 80:239 (Abstr.).

Ambrose JD, Drost M, Monson RL, Rutledge JJ, Leibfried-Rutledge ML, Thatcher MJ, Kassa T, Binelli M, Hansen PJ, Chenoweth PJ, Thatcher WW. Efficacy of timed embryo transfer with fresh and frozen in vitro produced embryos to increase pregnancy rates in heat-stressed dairy cattle. J Dairy Sci 1999; 82:2369-76.

Anderson GB, Cupps PT, Drost M. Induction of twins in cattle with bilateral and unilateral embryo transfer. J Anim Sci 1979; 49:1037-42.

Aréchiga CF, Vázquez-Flores S, Ortíz O, Hernández-Cerón J, Porras A, McDowell LR, Hansen PJ. Effect of injection of β-carotene or vitamin E and selenium on fertility of lactating dairy cows. Theriogenology 1998a; 50:65-76.

Aréchiga, CF, Staples CR, McDowell LR, Hansen PJ. Effects of timed insemination and supplemental β-carotene on reproduction and milk yield of dairy cows under heat stress. J Dairy Sci 1998b; 81, 390-402.

Armstrong JD, Goodall EA, Gordon FJ, Rice DA, McCaughey WJ. The effects of levels of concentrate offered and inclusion of maize gluten or fish meal in the concentrate on reproductive performance and blood parameter of dairy cows. Anim Prod 1990; 50:1–10.

Badinga L, Thatcher WW, Diaz T, Drost M, Wolfenson D. Effect of environmental heat stress on follicular steroidogenesis and development in lactating Holstein cows. Theriogenology 1993; 39:797-810.

Badinga L, Thatcher WW, Wilcox CJ, Morris G, Entwistle K, Wolfenson D. Effect of season on follicular dynamics and plasma concentrations of estradiol-17α, progesterone and luteinizing hormone in lactating Holstein cows. Theriogenology 1994; 42:1263–74.

Barkema HW, Schukken YH, Lam TJGM, Beiboer ML, Wilmink H, Benedictus G, Brand A. Incidence of clinical mastitis in dairy herds grouped in three categories by bulk milk somatic cell counts. J Dairy Sci 1998; 81:411-9.

Barker AR, Schrick FN, Lewis MJ, Dowlen HH, Oliver SP. Influence of clinical mastitis during early lactation on reproductive performance of Jersey cows. J Dairy Sci 1998; 81:1285-90.

Barros CM, Newton GR, Thatcher WW, Drost M, Plante C, Hansen PJ. The effect of bovine interferon-τ I1 on pregnancy rate in heifers. J Anim Sci 1992; 70:1471-7.

Bartolome JA, Melendez P, Kelbert D, Swift K, McHale J, Hernandez J, Silvestre F, Risco CA, Arteche AC, Thatcher WW, Archbald LF. Strategic use of gonadotrophin-releasing hormone (GnRH) to increase pregnancy rate and reduce pregnancy loss in lactating dairy cows subjected to synchronization of ovulation and timed insemination. Theriogenology 2005; 63:1026-37.

Bauman DE, Currie WB. Partitioning of nutrients during pregnancy and lactation: a review of mechanisms involving homeostasis and homeorhesis. J Dairy Sci 1980; 63:1514–29.

Beam SW, Butler WR. Energy balance and ovarian follicle development prior to the first ovulation in dairy cows receiving three levels of dietary fat. Biol Reprod 1997; 56:133–42

Beam SW, Butler WR. Energy balance, metabolic hormones, and early postpartum follicular development in dairy cows fed prilled lipid. J Dairy Sci 1998; 81:121-31.

Beam SW, Butler WR. Effects of energy balance on follicular development and first ovulation in postpartum dairy cows. J Reprod Fertil 1999; 54: 411-24.

Berman A, Folman Y, Kaim M, Mamen M, Herz Z, Wolfenson D, Arieli A, Graber Y. Upper critical temperatures and forced ventilation effects for high-yielding dairy cows in a subtropical environment. J Dairy Sci 1985; 68:1488-95.

Bertolini M, Beam SW, Shim H, Bertolini LR, Moyer AL, Famula TR, Anderson GB. Growth, development, and gene expression by in vivo- and in vitro-produced day 7 and 16 bovine embryos. Mol Reprod Dev 2002a; 63:318-28.

Bertolini M, Mason JB, Beam SW, Carneiro GF, Sween ML, Kominek DJ, Moyer AL, Famula TR, Sainz RD, Anderson GB. Morphology and morphometry of in vivo- and in vitro-produced bovine concepti from early pregnancy to term and association with high birth weights. Theriogenology 2002b; 58:973-94.

Biggers BG, Geisert RD, Wetteman RP, Buchanan DS. Effects of heat stress on early embryonic development in the beef cow. J Anim Sci 1987; 64:1512-18.

Bilby TR, Guzeloglu A, Kamimura S, Pancarci SM, Michel F, Head HH, Thatcher WW. Pregnancy and bovine somatotropin in nonlactating dairy cows: I. Ovarian, conceptus, and insulin-like growth factor system responses. J Dairy Sci 2004; 87:3256-67.

Binelli M, Thatcher WW, Mattos R, Baruselli PS. Antiluteolytic strategies to improve fertility in cattle. Theriogenology 2001; 56:1451-63.

Block J, Drost M, Monson RL, Rutledge JJ, Rivera RM, Paula-Lopes FF, Ocon OM, Krininger CE III, Liu J, Hansen PJ. Use of insulin-like growth factor-I during embryo culture and treatment of recipients with gonadotropin-releasing hormone to increase pregnancy rates following the transfer of in vitro-produced embryos to heat-stressed, lactating cows. J Anim Sci 2003; 81:1590-602.

Bo GA, Baruselli PS, Moreno D, Cutaia L, Caccia M, Tribulo R, Tribulo H, Mapletoft RJ. The control of follicular wave development for self-appointed embryo transfer programs in cattle. Theriogenology 2002; 57:53-72.

Boyd LJ, Seath DM, Olds D. Relationship between level of milk production and breeding efficiency in dairy cattle. J Dairy Sci 1954; 13:89.

Breuel KF, Spitzer JC, Henricks DM. Systemic progesterone concentration following human chorionic gonadotropin administration at various times during the estrous cycle in beef heifers. J Anim Sci 1989; 67:1564-72.

Breukink HJ, Wensing TH. Pathophysiology of the liver in high yielding dairy cows and its consequences for health and production. Bovine Practitioner 1998; 32:74-8.

Britt JH, Scott RG, Armstrong JD, Whitacre MD. Determinants of estrous behavior in lactating Holstein cows. J Dairy Sci 1986; 69:2195-202.

Bruckental I, Dori D, Kaim M, Lehrer H, Folman Y. Effects of source and level of protein on milk yield and reproductive performance of high-producing primiparous and multiparous dairy cows. Anim Prod 1989; 48:319–29.

Bulman DC, Lamming GE. Milk progesterone levels in relation to conception, repeat breeding and factors influencing acyclicity in dairy cows. J Reprod Fertil 1978; 54:447-58.

Burke JM, Staples CR, Risco CA, De La Sota RL, Thatcher WW. Effect of ruminant grade menhaden fish meal on reproductive and productive performance of lactating dairy cows. J Dairy Sci 1996; 80:3386–98.

Butler WR. Nutritional interactions with reproductive performance in dairy cattle. Anim Reprod Sci. 2000; 60:449-57.

Butler WR. Review: effect of protein nutrition on ovarian and uterine physiology in dairy cattle. J. Dairy Sci. 1998; 81:2533–39.

Butler WR, Smith RD. Interrelationships between energy balance on postpartum reproductive function in dairy cattle. J Dairy Sci 1989; 7:767–83.

Carlson JC, Wu XM, Sawada M. Oxygen radicals and the control of ovarian corpus luteum function. Free Radic Biol Med 1993; 14:79-84.

Chassagne M, Barnouin J, Chacornac JP. Biological predictors for early clinical mastitis occurrence in Holstein cows under field conditions in France. Prev Vet Med 1998; 16:29-38.

Chase CC, Kirby CJ, Hammond AC, Olson TA, Lucy MC. Patterns of ovarian growth and development in cattle with a growth hormone receptor deficiency. J Anim Sci 1998; 76:212-9.

Chebel RC, Santos JE, Reynolds JP, Cerri RL, Juchem SO, Overton M. Factors affecting conception rate after artificial insemination and pregnancy loss in lactating dairy cows. Anim Reprod Sci 2004; 84:239-55.

Chenault JR, Kratzer DD, Rzepkowsky RA, Goodwin MC. LH and FSH response of Holstein heifers to fertirelin acetate, gonadorelin and buserelin. Theriogenology 1990; 34:81-98.

Cole JA, Hansen PJ. Effects of administration of recombinant bovine somatotropin on the responses of lactating and nonlactating cows to heat stress. J Am Vet Med Assoc 1993; 203:113-7.

Crosier A, Farin PW, Dykstra MJ, Alexander JE, Farin CE. Ultrastructural morphometry of bovine blastocysts produced in vivo or in vitro. Biol Reprod 2001; 64:1375-85.

Currie EJ. The influence of milk yield on fertility in dairy cattle. J Dairy Sci 1956; 23:301-9.

De la Sota RL, Lucy MC, Staples CR, Thatcher WW. Effects of recombinant bovine somatotropin (sometribove) on ovarian function in lactating and nonlactating dairy cows. J Dairy Sci 1993; 76:1002-13.

De La Torre-Sanchez J, Gardner D, Preis K, Seidel Jr. G. Regulation of glucose metabolism to decrease lipid content of in vitro-produced bovine embryos. Reprod Fertil Dev 2005; 218 [Abstract].

Del Campo MR, Rowe RF, French LR, Ginther OJ. Unilateral relationship of embryos and the corpus luteum in cattle. Biol Reprod 1977; 16:580-5.

Del Campo MR, Rowe RF, Chaichareon D, Ginther OJ. Effect of the relative locations of embryo and corpus luteum on embryo survival in cattle. Reprod Nutr Dev 1983; 23:303-8.

Deluyker HA, Gay JM, Weaver LD, Azari AS. Change of milk yield with clinical diseases for a high producing dairy herd. J Dairy Sci 1991; 74:436-45.

de Vries A, Risco CA. Trends and seasonality of reproductive performance in Florida and Georgia dairy herds from 1976 to 2002. J Dairy Sci 2005; 88:3155-65.

Diaz T, Schmitt EJ, de la Sota RL, Thatcher MJ, Thatcher WW. Human chorionic gonadotropin-induced alterations in ovarian follicular dynamics during the estrous cycle of heifers. J Anim Sci 1998; 76:1929-36.

Diez C, Heyman Y, Le Bourhis D, Guyader-Joly C, Degrouard J, Renard, JP. Delipidating in vitro-produced bovine zygotes: Effect on further development and consequences for freezability. Theriogenology 2001; 55: 923-36.

Dinnyés A, Wallace GA, Rall WF. Effect of genotype on the efficiency of mouse embryo cryopreservation by vitrification or slow freezing methods. Mol Reprod Dev 1995; 40:429-35.

Djemali M, Berger PJ, Freeman AE. Ordered categorical sire evaluation for dystocia in Holsteins. J Dairy Sci 1987; 70:2374-84.

Dobrinsky JR, Overström EW, Duby RT, Johnson LA, Duffy P, Roche JF, Boland MP. Effect of cytoskeletal stabilization on the development of bovine embryos cryopreserved by vitrification. Theriogenology 1995; 43:199 (Abstract).

Dobrinsky JR. Cellular approach to cryopreservation of embryos. Theriogenology 1996; 45:17-26.

Dobrinsky JR, Pursel VG, Long CR, Johnson LA. Birth of piglets after transfer of embryos cryopreserved by cytoskeletal stabilization and vitrification. Biol Reprod 2000; 62:564-70.

Donaghy AJ, Baxter RC. Insulin-like growth factor bioactivity and its modification in growth hormone resistant states. Baillieres Clin Endocrinol Metabol 1996; 10:421–446.

Donovan DC, Schingoethe DJ, Baer RJ, Ryali J, Hippen AR, Franklin ST. Influence of dietary fish oil on conjugated linoleic acid and other fatty acids in milk fat from lactating dairy cows. J Dairy Sci 2000;83: 2620-8.

Dransfield MB, Nebel RL, Pearson RE, Warnick LD. Timing of insemination for dairy cows identified in estrus by a radiotelemetric estrus detection system. J Dairy Sci 1998; 81:1874-82.

Drost M, Thatcher JD, Cantrell CK, Wolfsdorf KE, Hasler JF, Thatcher WW. Conception rates after artificial insemination or transfer of frozen/thawed embryos to lactating cows during summer. J Dairy Sci 1994; 77:380 (Abstract).

Drew SB, Peters AR. Effect of buserelin on pregnancy rates in dairy cows. Vet Rec 1994; 134:267–69.

Dunlap SE, Vincent CK. Influence of postbreeding thermal stress on conception rate in beef cattle. J Anim Sci 1971; 32: 1216-8.

Ealy AD, Drost M, Hansen PJ. Developmental changes in embryonic resistance to adverse effects of maternal heat stress in cows. J Dairy Sci 1993; 76:2899–905.

Ealy AD, Arechiga CF, Bray DR, Risco CA, Hansen PJ. Effectiveness of short-term cooling and vitamin E for alleviation of infertility induced by heat stress in dairy cows. J Dairy Sci 1994; 77:3601-7.

Ealy AD, Hansen PJ. Induced thermotolerance during early development of murine and bovine embryos. J Cell Physiol 1994; 160:463-8.

Ealy AD, Howell JL, Monterroso VH, Arechiga CF, Hansen PJ. Developmental changes in sensitivity of bovine embryos to heat shock and use of antioxidants as hermoprotectants. J Anim Sci 1995; 73:1401-7.

Ealy A D, Drost M, Barros CM, Hansen PJ. Thermoprotection of preimplantation bovine embryos from heat shock by glutathione and taurine. Cell Biol Int Rep 1992; 16:125 [abstract].

Echternkamp SE, Gregory KE, Dickerson GE, Cundiff LV, Koch RM, Van Vleck LD. Twinning in cattle: II. Genetic and environmental effects on ovulation rate in puberal heifers and postpartum cows and the effects of ovulation rate on embryonic survival. J Anim Sci 1990; 68:1877-88.

Edwards JL, Hansen PJ. Differential responses of bovine oocytes and preimplantation embryos to heat shock. Mol Reprod Dev 1997; 46:138-145.

Enright BP, Lonergan P, Dinnyes A, Fair T, Ward FA, Yang X, Boland MP. Culture of in vitro produced bovine zygotes in vitro vs in vivo: Implications for early embryo development and quality. Theriogenology 2000; 54:659-73.

Erickson PJ, Murphy MR, Clark JH. Supplementation of dairy cow diets with calcium salts of longchain fatty acids and nicotinic acid in early lactation. J Dairy Sci 1992; 75:1078–89.

Fahy GM, MacFarlane DR, Angell CA, Meryman HT. Vitrification as an approach to cryopreservation. Cryobiology 1984; 21:407-26.

Fair T, Lonergan P, Dinnyes A, Cotell DC, Hyttel P, Ward FA, Boland MP. Ultrastructure of bovine blastocysts following cryopreservation: Effects of method of blastocyst production. Mol Reprod Dev 2001; 58: 186-95.

Falconer DS. 1981. Introduction to Quantitative Genetics. 2nd ed. Longman House, Harlow, Essex, UK.

Farin CE, Farin PW, Piedrahita JA. Development of fetuses from in vitro-produced and cloned bovine embryos. J Anim Sci 2004; 82:53-62.

Farin PW, Piedrahita JA, Farin CE. Errors in development of fetuses and placentas from in vitro-produced bovine embryos. Theriogenology 2006; 30 (in press).

Faust MA, McDaniel AB, Robinson OW, Britt JH. Environmental and yield effects on reproduction in primiparous Holsteins. J Dairy Sci 1988; 71:3092.

Ferguson J D, Sklan D, Chalupa WV, Kronfeld DS. Effects of hard fats on in vitro and in vivo rumen fermentation, milk production, and reproduction in dairy cows. J Dairy Sci 1990; 73:2864–79.

Fischer-Brown A, Monson R, Parrish J, Rutledge J. Cell allocation in bovine embryos cultured in two media under two oxygen concentrations, Zygote 2002; 10:341–8.

Fleischer P, Metzner M, Beyerbach M, Hoedemaker M, Klee W. The relationship between milk yield and the incidence of some diseases in dairy cows. J Dairy Sci 2001; 84:2025-35.

Fonseca FA, Britt JH, McDaniel BT, Wilk JC, Rakes AH. Reproductive traits of Holsteins and Jerseys. Effects of age, milk yield, and clinical abnormalities on involution of cervix and uterus, ovulation, estrous cycles, detection of estrus, conception rate, and days open. J Dairy Sci 1983; 66:1128-47.

Fourichon C, Seegers H, Malher X. Effect of disease on reproduction in the dairy cow: a meta-analysis. Theriogenology 2000; 53:1729–1759.

Fujitani Y, Kasai K, Ohtani S, Nishimura K, Yamada M, Utsumi K. Effect of oxygen concentration and free radicals on in vitro development of in vitro-produced bovine embryos. J Anim Sci 1997; 75:483-9.

Gaines Wl. Milk yield in relation to recurrence of conception. J Dairy Sci 1927; 10:117.

Garcia-Bojalil CM, Staples CR, Risco CA, Savio JD, Thatcher WW. Protein degradability and calcium salts of long-chain fatty acids in the diets of lactating dairy cows: reproductive responses. J Dairy Sci 1998; 81:1385-95.

Garcia-Bojalil CM. 1993. Reproductive, productive, and immunological responses by Holstein dairy cows fed diets varying in concentration and ruminal degradability of protein and supplemented with ruminally-inert fat. Ph.D. Diss., Univ. Florida, Gainesville.

Garrett JE, Geisert RD, Zavy MT, Morgan GL. Evidence for maternal regulation of early conceptus growth and development in beef cattle Reprod Fertil 1988a; 84:437–46.

Garrett JE. Geisert RD. Zavy NIT, Gries LK, Wetteman RP. Buchanan DS. Effect of exogenous progesterone on prostaglandin $F_{2\alpha}$. release and the interestrous interval in the bovine. Prostaglandins 1988b; 36:85-96.

Ginger, R., Faisser, D., Busato, A., Blum, J. and Kupfer, U. Blood parameters during early lactation and their relationship to ovarian function in dairy cows. Reprod Domest Anim 1997; 32:313-9.

Ginther OJ. Effect of progesterone on length of estrous cvcle in cattle. Am J Vet Res 1970; 31:493-6.

Ginther OJ, Knopf L, Kastelic JP. Temporal associations among ovarian events in cattle during oestrus cycles with two and three follicular waves. J Reprod Fertil 1989; 87:223-30.

Ginther OJ, Wiltbank MC, Fricke PM, Gibbons JR, Kot K. Selection of the dominant follicle in cattle. Biol Reprod 1996; 55:1187–94.

Gong JG, Bramley TA, Webb R. The effect of recombinant bovine somatotrophin on ovarian follicular growth and development in heifers. J Reprod Fertil 1993; 97:247-54.

Gossen N, Feldmann M, Hoedemaker M. [Effect of parenteral supplementation with β-carotene in the form of an injection solution (Carofertin) on the fertility performance of dairy cows] Dtsch Tierarztl Wochenschr 2004;111:14-21.

Gossen N, Hoedemaker M.[Effect of beta-carotin serum concentration on the reproductive performance in dairy cows] Berl Munch Tierarztl Wochenschr 2005; 118:326-33.

Green LE, Hedges VJ, Schukken YH, Blowey RW, Packington AJ. The impact of clinical lameness on the milk yield of dairy cows. J Dairy Sci 2002; 85:2250-6.

Gröhn YT, Wilson DJ, Gonzalez RN, Hertl JA, Schulte H, Bennett G, Schukken YH. Effect of pathogen-specific clinical mastitis on milk yield in dairy cows. J Dairy Sci 2004; 87:3358-74.

Grummer RR, Carroll DJ. Effects of dietary fat on metabolic disorders and reproductive performance of dairy cattle. J Anim Sci 1991; 69:3838–52

Guyader-Joly C, Ponchon S, Durand M, Heyman Y, Renard JP, Ménézo Y. Effect of lecithin on in vitro and in vivo survival of in vitro produced bovine blastocysts after cryopreservation. Theriogenology 1999; 52:1193-202.

Gwazdauskas FC, Thatcher WW, Kiddy CA, Paape MJ, Wilcox, CJ. Hormonal pattern during heat stress following tham salt induced luteal regression in heifers. Theriogenology 1981; 16:271–285.

Hansen L, Heins B, Seykora T. Crossbreeding: Why the interest? What to expect. Proceedings Florida Dairy Production Conference, Gainesville, May 3, 2005: 42:14-20.

Hansen LB. Consequences of selection for milk yield from a geneticist's viewpoint. J Dairy Sci 2000; 83:1145-50.

Hansen PJ, Aréchiga CF. Strategies for managing reproduction in the heat-stressed dairy cow. J. Anim. Sci. 1999; 77:36-50.

Hansen PJ, Drost M, Rivera RM, Paula-Lopes FF, Al-Katanani YM, Krininger III CE, Chase CC Jr. Adverse impact of heat stress on embryo production: Causes and strategies for mitigation. Theriogenology 2001; 55:91-103.

Hansen PJ, Block J. Towards an embryocentric world: the current and potential uses of embryo technologies in dairy production. Reprod Fertil Dev 2004; 16:1-14.

Hansel W. Plasma hormone concentrations associated with early embryo mortality in heifers. J Reprod Fertil 1981; 30:231–9.

Hardingham TE, Fosang AJ. Proteoglycans: many forms and many functions. FASEB J 1992; 6:861-70.

Harrison JH, Hancock DD, St Pierre N, Conrad HR, Harvey WR. Effect of prepartum selenium treatment on uterine involution in the dairy cow. J Dairy Sci 1986; 69:1421-5.

Harrison RO, Ford SP, Young JW, Conley AJ, Freeman AE. Increased milk production versus reproductive and energy status of high producing dairy cows. J Dairy Sci 1990; 73:2749-58.

Hasler JF, Henderson WB, Hurtgen PJ, Jin ZQ, McCauley AD, Mower SA, Neely B, Skuey LS, Stokes, JE, Trimmer SA. Production, freezing and transfer of bovine IVF embryos and subsequent calving results. Theriogenology 1995; 43:141-52.

Hasler, JF, Hurtgen PJ, Jin ZQ, Stokes JE. Survival of IVF-derived bovine embryos frozen in glycerol or ethylene glycol. Theriogenology 1997; 48:563-79.

Hasler JF. In-vitro production of cattle embryos: problems with pregnancies and parturition. Hum Reprod 2000; 15:47-58.

Hasler, JF. The current status and future of commercial embryo transfer in cattle. Anim Reprod Sci 2003a; 79:245-64.

Hasler JF, Bilby CR, Collier RJ, Denham SC, Lucy MC. Effect of recombinant bovine somatotropin on superovulatory response and recipient pregnancy rates in a commercial embryo transfer program. Theriogenology 2003b; 59:1919-28.

Hawkins DE, Niswender KD, Oss GM, Moeller CL, Odde KG, Sawyer HR, Niswender GD. An increase in serum lipids increases luteal lipid content and alters the disappearance rate of progesterone in cows. J Anim Sci 1995; 73:541–5.

Heersche Jr G, Nebel RL. Measuring efficiency and accuracy of detection of estrus. J Dairy Sci 1994; 77:2754–61.

Hernandez J, Shearer JK, Webb DW. Effect of lameness on the calving-to-conception interval in dairy cows. J Am Vet Med Assoc 2001; 15:1611-4.

Heyman Y, Chesné P, Chupin D, Ménézo Y. Improvement of survival rate of frozen cattle blastocysts after transfer with trophoblastic vesicles. Theriogenology 1987; 27:477-84.

Hillers JK, Senger PL, Darlington RL, Fleming WN. Effects of production, season, age of cow, days dry, and days in milk on conception to first service in large commercial dairy herds. J Dairy Sci 1984; 67:861.

Hommeida A, Nakao T, Kubota H. Luteal function and conception in lactating cows and some factors influencing luteal function after first insemination. Theriogenology 2004; 62:217-25.

Howell JL, Fuquay JW, Smith AE. Corpus luteum growth and function in lactating Holstein cows during spring and summer. J Dairy Sci 1994; 77:735–9.

Ikeda K, Takahashi Y, Katagiri S. Effect of medium change on the development of in vitro matured and fertilized bovine oocytes cultured in medium containing amino acids. J Vet Med Sci 2000; 62:121-3.

Ingraham RH, Gillette DD, Wagner WD. Relationship of temperature and humidity to conception rate of Holstein cows in subtropical climate. J Dairy Sci 1974;57:476-81.

Inskeep EK. Factors that affect fertility during estrous cycles with short luteal phases in postpartum cows. J Reprod Fertil 1995; 49: 493–503.

Ireland JJ, Roche JF. Effect of progesterone on basal LH and episodic LH and FSH secretion in heifers. J Reprod Fertil 1982;64:295-302.

Ishii T, Matsuki S, Iuchi Y, Okada F, Toyosaki S, Tomita Y, Ikeda Y, Fujii J. Accelerated impairment of spermatogenic cells in SOD1-knockout mice under heat stress. Free Radic Res 2005; 39:697-705.

Iwasaki S, Hamano S, Kuwayama M, Ushijima H, Nagaoka S, Nakahara T. Developmental changes in the incidence of chromosomal anomalies of bovine embryos fertilized in vitro. J Exp Zool 1992; 261:79-85.

Jang G, Lee BC, Kang SK, Hwang WS. Effect of glycosaminoglycans on the preimplantation development of embryos derived from in vitro fertilization and somatic cell nuclear transfer. Reprod Fertil Dev 2003; 30:179-85.

Jones JI, Clemmons DR. Insulin-like growth factors and their binding proteins: biological actions. Endocrine Reviews 1995; 16:3–34.

Jorritsma R, Jorritsma H, Schukken YH, Wentink GH. Relationships between fatty liver and fertility and some periparturient diseases in commercial Dutch dairy herds. Theriogenology 2000; 54:1065–74.

Jousan FD, Drost M, Hansen PJ. Factors associated with early and mid-to-late fetal loss in lactating and non-lactating Holstein cattle in a hot climate. J Anim Sci 2005; 83:1017-22.

Jubb TF, Abhayaratne D, Malmo J, Anderson GA. Failure of an intramuscular injection of an analogue of GnRH 11 to 13 days after insemination to increase pregnancy rates in dairy cattle. Aust Vet J 1990; 67:359–61.

Juengel JL, Garverick HA, Johnson AL, Youngquist RS, Smith MF. Apoptosis during luteal regression in cattle. Endocrinology 1993; 132:249–54.

Kadarmideen HN, Thompson R, Simm G. Linear and threshold model genetic parameter estimates for disease, fertility and production traits in UK dairy cattle. J Anim Sci 2000; 71:411–9.

Kappel LC, Ingraham RH, Morgan EB, Dixon JM, Zeringue L, Wilson D, Babcock DK. Selenium concentrations in feeds and effects of treating pregnant Holstein cows with selenium and vitamin E on blood selenium values and reproductive performance. Am J Vet Res 1984;45:691-4.

Kelton DF, K D Lissemore, Martin RE. Recommendations for recording and calculating the incidence of selected clinical diseases of dairy cattle. J Dairy Sci 1998; 81:2502–9.

Kerbler TL, Buhr MM, Jordan LT, Leslie KE, Walton JS. Relationship between maternal plasma progesterone concentration and interferon-τ synthesis by the conceptus in cattle. Theriogenology 1997; 47:703–14.

Khurana N, Niemann, H. Effects of cryopreservation on glucose metabolism and survival of bovine morulae and blastocysts derived in vitro or in vivo. Theriogenology 2000a; 54:313-26.

Khurana NK, Niemann H. Energy metabolism in preimplantation bovine embryos derived in vitro or in vivo. Biol. Reprod 2000b; 62:847-56.

Kim H, Lee G, Hyun S, Nam D, Lee S, Jeong Y, Kim S, Kim J, Kang S, Lee B, Hwang W. Embryotropic effect of glycosaminoglycans and receptors in development of porcine pre-implantation embryos. Theriogenology 2005; 63:1167-80.

Kimura M, Nakao T, Moriyoshi M, Kawata K. Luteal phase deficiency as a possible cause of repeat breeding in dairy cattle. Br Vet J 1987; 143:560–6.

Kimura K, Spate LD, Green MP, Roberts RM. Effects of D-glucose concentration, D-fructose, and inhibitors of enzymes of the pentose phosphate pathway on the development and sex ratio of bovine blastocysts. Mol Reprod Dev 2005; 72:201-7.

Kinsel ML, Marsh WE, Ruegg PL, Etherington WG. Risk factors for twinning in dairy cows. J Dairy Sci 1998; 81:989-93.

Knopf L, Kastelic JP, Schallenberger E, Ginther OJ. Ovarian follicular dynamics in heifers: Test of two-wave hypothesis by ultrasonically monitoring individual follicles. Domest Anim Endocrinol 1989; 6:111-19.

Ko JCH, Kastelic JP, Del Campo MR, Ginther OJ. Effects of a dominant follicle on ovarian follicular dynamics during the oestrous cycle in heifers. J Reprod Fertil 1991; 91:511-19.

Krisher RL, Lane M, Bavister BD. Developmental competence and metabolism of bovine embryos cultured in semi-defined and defined culture media. Biol Reprod 1999; 60:1345-52.

Kruip TAM, den Daas JHG. In vitro produced and cloned embryos: Effects on pregnancy, parturition and offspring. Theriogenology 1997; 47:43–52.

Kuwayama M, Fujikawa S, Nagai T. Ultrastructure of IVM-IVF bovine blastocysts vitrified after equilibration in glycerol 1,2-propanediol using 2-step and 16-step procedures. Cryobiology 1994; 31:415-22.

Laben RC, Shanks RD, Berger PJ, Freeman AE. Factors affecting milk yield and reproductive performance. J Dairy Sci 1982; 65:1004.

Lafrance M, Goff AK. Effects of progesterone and oestradiol-17 beta on oxytocin-induced release of prostaglandin $F_{2\alpha}$ in heifers. J Reprod Fertil 1988; 82:429-36.

Lajili H, Humblot P, Thibier M. Effects of PGF2 alpha treatment on conception rates of dairy cows treated with a GnRH agonist 12 to 14 days after artificial insemination. Theriogenology 1991; 36:335-47.

Lamming GE, Darwash AO. Effect of inter-luteal interval on subsequent luteal phase length and fertility in postpartun dairy cows. Biol Reprod 1995; 53:63.

Lammoglia MA, Willard ST, Hallford DM and Randel RD. Effects of dietary fat on follicular development and circulating concentrations of lipids, insulin, progesterone, estradiol 17b, 13,14-dihydro-15-ketoprostaglandin $F_{2\alpha}$, and growth hormone in estrous cyclic Brahman cows. J Anim Sci 1997; 75:1591–600

Lane M, Maybach JM, Hooper K, Hasler JF, Gardner DK. Cryo-survival and development of bovine blastocysts are enhanced by culture with recombinant albumin and hyaluronan. Mol Reprod Dev 2003; 64:70-78.

Larson SF, Butler WR, Currie WB. Reduced fertility associated with low progesterone postbreeding and increased milk urea nitrogen in lactating cows. J Dairy Sci 1997; 80:1288-95.

Laurent TC. Biochemistry of hyaluronan. Acta Otolaryngol 1987; 442:7-24.

Lazzari G, Wrezycki C, Herrmann D, Duchi R, Kruip T, Niemann H, Galli C. Cellular and molecular deviations in bovine in vitro-produced embryos are related to the large offspring syndrome. Biol Reprod 2002; 67:767-75.

Lee CN, Ax RL. Concentration and composition of glycosaminoglycans in the female bovine reproductive tract. J Dairy Sci 1984; 67:2006-9.

Lemaster JW, Seals RC, Hopkins FM, Schrick FN. Effects of administration of oxytocin on embryonic survival in progestogen supplemented cattle. Prostaglandins Other Lipid Mediat 1999; 57:259-68.

Leeuwenberg BR, Hudson NL, Moore LG, Hurst PR, McNatty KP. Peripheral and ovarian IGF-I concentrations during the ovine oestrous cycle. J Endocrinol 1996; 148: 281-9.

Levine L, Worth N. Eicosapentaenoic acid – its effects on arachidonic acid metabolism by cells in culture. J Allergy and Clin Immun 1984; 74:430–6.

Loeffler SH, de Vries MJ, Schukken YH. The effects of time of disease occurrence, milk yield, and body condition on fertility of dairy cows. J Dairy Sci 1999; 82:2589-604.

Lonergan P, Rizos D, Gutierrez-Adan A, Moreira PM, Pintado B, De La Fuente J, Boland MP. Temporal divergence in the pattern of messenger RNA expression in bovine embryos cultured from the zygote to blastocyst stage in vitro or in vivo. Biol Reprod 2003; 69:1424-31.

López-Gatius F. Is fertility declining in dairy cattle? A retrospective study in northeastern Spain. Theriogenology 2003;60:89-99.

López-Gatius F, Santolaria P, Yaniz J, Fenech M, Lopez-Bejar M. Risk factors for postpartum ovarian cysts and their spontaneous recovery orpersistence in lactating dairy cows. Theriogenology. 2002; 58:1623-32.

López-Gatius F, Hunter RH. Spontaneous reduction of advanced twin embryos: its occurrence and clinical relevance in dairy cattle. Theriogenology 2005; 63:118-25.

López-Gatius F, Santolaria P, Martino A, Delétang F, De Rensis F. The effects of GnRH treatment at the time of AI and 12 days later on reproductive performance of high producing dairy cows during the warm season in northeastern Spain. Theriogenology 2005a; (in press).

López-Gatius F, Lopez-Bejar M, Fenech M, Hunter RH. Ovulation failure and double ovulation in dairy cattle: risk factors and effects. Theriogenology 2005b; 63:1298-307.

López H, Satter LD, Wiltbank MC. Relationship between level of milk production and estrous behavior of lactating dairy cows. Anim Reprod Sci 2004; 81:209-23.

López H, Caraviello DZ, Satter LD, Fricke PM, Wiltbank MC. Relationship between level of milk production and multiple ovulations in lactating dairy cows. J Dairy Sci 2005; 88:2783-93.

Loucopoulos A, Ferin M. Gonadotropin-releasing hormone and its clinical applications. Obstet Gynecol Annu 1984; 13:275-88.

Lucy MC. Reproductive loss in high-producing dairy cattle: where will it end? J Dairy Sci. 2001; 84:1277-93.

Lucy MC, Berk J, Staples CR, Head HH, Sota RLD, Thatcher WW. Follicular dynamics, plasma metabolites, hormones and insulin-like growth factor I (IGF-1) in lactating cows with positive or negative energy balance during the preovulatory period. Reprod Nutr Dev 1992a, 32:331-41.

Lucy MC, De la Sota RL, Staples CR, Thatcher WW. Ovarian follicular populations in lactating dairy cows treated with recombinant bovine somatotropin (sometribove) or saline and fed diets differing in fat content and energy. J Dairy Sci 1993; 76:1014–27.

Lucy MC, Gross TS, Thatcher WW. Effect of intravenous infusion of soybean oil emulsion on plasma concentrations of 15-keto-13,14- dihydro-prostaglandin $F_{2\alpha}$ and ovarian function in cycling Holstein heifers. In International Atomic Energy Agency Livestock Reproduction in Latin America 1990; 119–32.

Lucy MC, Staples CR, Michel FM, Thatcher WW. Effect of feeding calcium soaps to early post-partum dairy cows on plasma prostaglandin $F_{2\alpha}$, luteinizing hormone, and follicular growth. J Dairy Sci 1991a; 74:483–9.

Lucy MC, Staples CR, Michel FM, Thatcher WW. Energy balance and size and number of ovarian follicles detected by ultrasonography in early post-partum cows. J Dairy Sci 1991b; 74:473–82.

Lucy MC, Staples CR, Thatcher WW, Erickson PS, Cleale RM, Firkins JL, Clark JH, Murphy MR, Brodie BO. Influence of diet composition, dry matter intake, milk production and energy balance on time of postpartum ovulation and fertility in dairy cows. Anim Prod 1992b; 54:323–31.

Lucy MC, Stevenson JS. Gonadotropin-releasing hormone at estrus: luteinizing hormone, estradiol, and progesterone during the periestrual and postinsemination periods in dairy cattle. Biol Reprod 1986; 35:300-11.

Lucy MC, Weber WJ, Baumgard LH, Seguin BS, Koenigsfeld AT, Hansen LB. Reproductive endocrinology of lactating dairy cows selected for increased milk production. J. Dairy Sci 1998; 81:246 (abstract).

Lukaszewska J, Hansel W. Corpus luteum maintenance during early pregnancy in the cow. J Reprod Fertil 1980; 59:485-93.

Lynch PR, Macmillan KL, Taufa VK. Treating cattle with progesterone as well as a GnRH analogue affects oestrous cycle length and fertility. Anim Reprod Sci 1999; 56:189-200.

Macmillan KL, Taufa VK, Day AM. Effects of an agonist of GnRH (buserelin) in cattle. III. Pregnancy rates after a post-insemination injection during metoestrus or dioestrus. Anim Reprod Sci 1986; 11:1–10.

Macmillan KL, Thatcher WW. Effects of an agonist of gonadotropin-releasing hormone on ovarian follicles in cattle. Biol Reprod 1991; 45:883-9.

Mahanna WC, Hardie AR, Tyler WJ. Variation in health related expenditures of Holstein-Friesian cattle. J Daiy Sci 1979; 62:86 (abstract).

Malayer JR, Hansen PJ. Differences between Brahman and Holstein cows in heat-shock induced alterations of protein synthesis and secretion by oviducts and uterine endometrium. J Anim Sci 1990; 68:266-80.

Mann GE, Lamming GE. Effects of treatment with buserelin on plasma concentrations of oestradiol and progesterone and cycle length in the cow. Br Vet J 1995a; 151:427-32.

Mann GE, Lamming GE. Progesterone inhibition of the development of the luteolytic signal in cows. J Reprod Fertil 1995b; 104:1–5.

Mann GE, Mann SL, Lamming GE. The inter-relationship between the maternal hormone environment and the embryo during the early stages of pregnancy in the cow. J Reprod Fertil 1996; 17:21.

Mann GE, Lamming GE, Payne JH. Role of early luteal phase progesterone in control of the timing of the luteolytic signal. J Reprod Fertil 1998; 113:47–51.

Mann GE, Lamming GE. The influence of progesterone during early pregnancy in cattle. Reprod Domest Anim 1999; 34:269-74.

Mann GE, Lamming GE. Relationship between maternal endocrine environment, early embryo development and inhibition of luteolytic mechanism in cows. Reproduction 2001; 121:175–80.

Margolin Y, Behrman HR. Xanthine oxidase and dehydrogenase activities in rat ovarian tissues. Am J Physiol 1992; 262:173-8.

Martinez MF, Kastelic JP, Adams GP, Cook RB, Olson WO, Mapletoft RJ. The use of progestins in regimens for fixed-time artificial insemination in beef cattle. Theriogenology 2002b; 57:1049–59.

Martinez MF, Kastelic JP, Adams GP, Mapletoft RJ. The use of a progesterone-releasing device (CIDR-B) or melengestrol acetate with GnRH, LH, or estradiol benzoate for fixed-time AI in beef heifers. J Anim Sci. 2002a; 80:1746-51.

Martino A, Songsasen N, Leibo SP. Development into blastocysts of bovine oocytes cryopreserved by ultra-rapid cooling. Biol Reprod 1996a; 54:1059-69.

Martino A, Pollard JW, Leibo SP. Effect of chilling bovine oocytes on their developmental competence. Mol Reprod Dev 1996b; 45:503-12.

Massip A, Mermillod P, Wils C, Dessy F. Effects of dilution procedure and culture conditions after thawing on survival of frozen bovine blastocysts produced in vitro. J Reprod Fertil 1993; 97:65-9.

Massip A, Mermillod P, Dinnyes A. Morphology and biochemistry of in vitro produced bovine embryos: implications for their cryopreservation. Hum Reprod 1995; 10:3004-11.

Mattos R, Staples CR, Thatcher WW. Effects of dietary fatty acids on reproduction in ruminants. Rev Reprod 2000; 5:38-45.

Mattos R, Guzeloglu A, Thatcher WW. Effect of polyunsaturated fatty acids on secretion of PGF2α from bovine endometrial (BEND) cells stimulated with phorbol 12, 13 dibutyrate (PDBu). Theriogenology 2001; 55:326.

Mattos R, Staples CR, Arteche A, Wiltbank MC, Diaz FJ, Jenkins TC, Thatcher. The effects of feeding fish oil on uterine secretion of PGF2alpha, milk composition, and metabolic status of periparturient Holstein cows. J Dairy Sci 2004; 87:921-32.

McAllister AJ, Lee AJ, Batra TR, Lin CY, Roy GL, Vesely JA, Wauthy JM, Winter KA. The influence of additive and nonadditive gene action on lifetime yields and profitability of dairy cattle. J Dairy Sci 1994; 77:2400-14.

McNatty KP, Heath DA, Lundy T, Filder AE, Quirke L, O'Connell A, Smith P, Groom N, Tisdall DJ. Control of early ovarian follicular development. J Reprod Fertil 1999; 54:3–16.

Mee MO, Stevenson JS, Scoby RK, Folman Y. Influence of gonadotropin-releasing hormone and timing of insemination relative to estrus on pregnancy rates of dairy cattle at first service. J Dairy Sci 1990; 73:1500-7.

Mee MO, Stevenson JS, Alexander BM, Sasser RG. Administration of GnRH at estrus influences pregnancy rates, serum concentrations of LH, FSH, estradiol-17 beta, pregnancy-specific protein B, and progesterone, proportion of luteal cell types, and in vitro production of progesterone in dairy cows. J Anim Sci 1993; 71:185-98.

Melendez P, Bartolome J, Archbald LF, Donovan A. The association between lameness, ovarian cysts and fertility in lactating dairy cows. Theriogenology 2003; 59:927-37.

Meyer MD, Hansen PJ, Thatcher WW, Drost M, Badinga L, Roberts RM, Li J, Ott TL, Bazer FW. Extension of corpus luteum lifespan and reduction of uterine secretion of prostaglandin $F_{2\alpha}$ of cows in response to recombinant interferon-τ. J Dairy Sci 1995; 78:1921-31.

Milvae RA, Hansel W. Concurrent uterine venous and ovarian arterial prostaglandin $F_{2\alpha}$ concentrations in heifers treated with oxytocin. J Reprod Fenil 1980; 60:7- 15.

Monty DE, Racowsky C. In vitro evaluation of early embryo viability and development in summer heat-stressed, superovulated dairy cows. Theriogenology 1987; 28:451-465.

Morales-Roura JS, Zarco L, Hernandez-Ceron J, Rodriguez G. Effect of short-term treatment with bovine somatotropin at estrus on conception rate and luteal function of repeat-breeding dairy cows. Theriogenology 2001; 55:1831-41.

Moreira F, de la Sota RL, Diaz T, Thatcher WW. Effect of day of the estrous cycle at the initiation of a timed artificial insemination protocol on reproductive responses in dairy heifers. J Anim Sci 2000a; 78:1568-76.

Moreira F, Risco CA, Pires MF, Ambrose JD, Drost M, Thatcher WW. Use of bovine somatotropin in lactating dairy cows receiving timed artificial insemination. J Dairy Sci. 2000b; 83:1237-47.

Moreira F, Orlandi C, Risco CA, Mattos R, Lopes F, Thatcher WW. Effects of presynchronization and bovine somatotropin on pregnancy rates to a timed artificial insemination protocol in lactating dairy cows. J Dairy Sci 2001; 84:1646-859.

Moreira F, Badinga L, Burnley C, Thatcher WW. Bovine somatotropin increases embryonic development in superovulated cows and improves post-transfer pregnancy rates when given to lactating recipient cows. Theriogenology 2002; 57:1371-87.

Murakami M, Otoi T, Sumantri C, Suzuki T. Effects of centrifugation and lipid removal on the cryopreservation of in vitro produced bovine embryos at the eight-cell stage. Cryobiology 1998; 36:206-12.

Murphy LJ, Bell GI, Friesen HG. Tissue distribution of insulin-like growth factor-I and II messenger ribonucleic acid in the adult rat. Endocrinology 1987; 120: 1279-282.

Nebel RL, McGilliard ML. Interactions of high milk yield and reproductive performance in dairy cows. J Dairy Sci 1993; 76:3257-68.

Nebel RL, Jobst SM, Dransfield MBG, Pandolfi SM, Bailey TL. Use of radio frequency data communication system, HeatWatch®, to describe behavioral estrus in dairy cattle. J Dairy Sci 1997; 80:179 (Abstr.).

Nedambale TL, Dinnyes A, Groen W, Dobrinsky JR, Tian XC, Yang X. Comparison on in vitro fertilized bovine embryos cultured in KSOM or SOF and cryopreserved by slow freezing or vitrification. Theriogenology 2004a; 62:437-49.

Nedambale TL, Dinnyes A, Yang X, Tian XC. Bovine blastocyst development in vitro: timing, sex, and viability following vitrification. Biol Reprod 2004b; 71:1671-6.

Newton G R, Martinod S, Hansen PJ, Thatcher WW, Siegenthaler B, Gerber C, Voirol MJ. Effect of bovine interferon on acute changes in body temperature and serum progesterone concentration in heifers. J Dairy Sci 1990; 73:3439.

Nielen MYH, Schukken DT, Scholl HJ, Wilbrink HJ, Brand A. Twinning in dairy cattle: a study of risk factors and effects. Thaiogenology 1989; 32:845.

Niki E, Noguchi N, Tsuchihashi H, Gotoh N. Interaction among vitamin C, vitamin E, and beta-carotene. Am J Clin Nutr 1995; 62:1322-6.

O'Callaghan DO, Boland MP. Nutritional effects on ovulation, embryo development and the establishment of pregnancy in ruminants. J Anim Sci 1999; 68:299–314.

O'Connor ML, Senger PL, 1997: Estrus detection. In: Youngquist, RS (ed.), Current Therapy in Large Animal Theriogenology, Chapter 34. W.B. Saunders Co., Philadelphia, USA, p. 285.

Oldick BS, Staples CR, Thatcher WW, Gyawu P. Abomasal infusion of glucose and fat – effects on digestion, production, and ovarian and uterine functions of cows. J Dairy Sci 1997; 80:1315–28.

Olds D, Cooper T, Thrift FA. Relationships between milk yield and fertility in dairy cattle. J Dairy Sci 1979; 62:1140-4.

Palasz AT, Mapletoft RJ. Cryopreservation of mammalian embryos and oocytes: recent advances. Biotechnol Adv 1996; 14:127-49.

Parrish JJ, Susko-Parrish JL, Critser ES, Eyestone WH, First NL. Bovine in vitro fertilization with frozen-thawed semen. Theriogenology 1986; 25:591-600.

Paula-Lopes FF, Al-Katanani YM, Majewski AC, McDowell LR, Hansen PJ. Manipulation of antioxidant status fails to improve fertility of lactating cows or survival of heat-shocked embryos. J Dairy Sci 2003; 86:2343-51.

Pecsok SR, McGilliard ML, Nebel RL. Conception rates. 1. Derivation and estimates for effects of estrus detection on cow profitability. J Dairy Sci 1994; 77:3008-15.

Pell JM, Saunders JC, Gilmour RS. Differential regulation of transcription initiation from insulin-like growth factor-I (IGF-I) leader exons and of tissue IGF-I expression in response to changed growth hormone and nutritional status in sheep. Endocrinology 1993; 132:1797–1807.

Perry GA, Smith MF, Lucy MC, Green JA, Parks TE, MacNeil MD, Roberts AJ, Geary TW. Relationship between follicle size at insemination and pregnancy success. Proc Natl Acad Sci U S A 2005; 102:5268-73.

Peters AR, Martinez TA, Cook AJ. A meta-analysis of studies of the effect of GnRH 11-14 days after insemination on pregnancy rates in cattle. Theriogenology 2000; 54:1317-26.

Petersson KJ, Strandberg E, Gustafsson H, Berglund B. Environmental effects on progesterone profile measures of dairy cow fertility. Anim Reprod Sci 2005; 8: [in press].

Picton H, Briggs D, Gosden R. The molecular basis of oocyte development. Mol Cell Endocrinol 1998; 145:27–37.

Plaizier JC, Lissemore KD, Kelton D, King GJ. Evaluation of overall reproductive performance of dairy herds. J Dairy Sci 1998; 81:1848-54.

Pollard JW, Leibo SP. Comparative cryobiology of in vitro and in vivo derived bovine embryos. Theriogenology 1993; 39:287.

Portaluppi MA, Stevenson JS. Pregnancy rates in lactating dairy cows after presynchronization of estrous cycles and variations of the Ovsynch protocol. J Dairy Sci 2005; 88:914-21.

Prather RS, First NL. Effect of cytochalasin B and demecolcine on freeze-thaw survival of murine embryos in vitro. J Exp Zool 1986; 239:37-40.

Pryce JE, Coffey MP, Simm G. The relationship between body condition score and reproductive performance. J Dairy Sci 2001; 84:1508-15.

Pursley JR, Mee MO, Wiltbank MC. Synchronization of ovulation in dairy cows using PGF2a and GnRH. Theriogenology 1995; 44:915.

Pursley JR, Silcox RW, Wiltbank MC. Effect of time of artificial insemination on pregnancy rate, calving rates, pregnancy loss, and gender ratio after synchronization of ovulation in lactating dairy cows. J Dairy Sci 1998; 81:2139-44.

Pushpakumara PG, Gardner NH, Reynolds CK, Beever DE, Wathes DC. Relationships between transition period diet, metabolic parameters and fertility in lactating dairy cows. Theriogenology 2003;60:1165-85.

Putney DJ, Drost M, Thatcher WW. Embryonic development in superovulated dairy cattle exposed to elevated ambient temperature between days 1 to 7 post insemination. Theniogenology 1988a; 30:195-209.

Putney DJ, Thatcher WW, Drost M, Wright JW, DeLorenzo MA. Influence of environmental temperature on reproductive performance of bovine embryo donors and recipients in the southwest region of the United States. Theriogenology 1988b; 30:905.

Putney DJ, Malayer JR, Gross TS, Thatcher WW, Hansen PJ, Drost M. Heat stress-induced alterations in the synthesis and secretion of proteins and prostaglandins by cultured bovine conceptuses and uterine endometrium. Biol Reprod. 1988c; 39:717-28.

Putney DJ, Mullins S, Thatcher WW, Drost M, Gross TS. Embryonic development in superovulated dairy cattle exposed to elevated ambient temperatures between the onset of estrus and insemination. Anim Reprod Sci 1989a; 19:37–51.

Putney DJ, Drost M, Thatcher WW. Influence of summer heat stress on pregnancy rates of lactating dairy cattle following embryo transfer or artificial insemination. Theriogenology 1989b; 31:765−78.

Purcell SH, Beal WE, Gray KR. Effect of a CIDR insert and flunixin meglumine, administered at the time of embryo transfer, on pregnancy rate and resynchronization of estrus in beef cattle. Theriogenology 2005; 64:867-78.

Quintal-Franco JA, Kojima FN, Melvin EJ, Lindsey BR, Zanella E, Fike KE, Wehrman ME, Clopton DT, Kinder JE. Corpus luteum development and function in cattle with episodic release of luteinizing hormone pulses inhibited in the follicular and early luteal phases of the estrous cycle. Biol Reprod 1999; 61:921-6.

Rabiee AR, Macmillan KL, Schwarzenberger F. Progesterone metabolism in ovariectomised non-lactating Holstein-Friesian cows treated with progesterone with two levels of feed intake. Anim Reprod Sci 2001; 66:35-46.

Rajamahendran R, Taylor C. Follicular dynamics and temporal relationships among body temperature, oestrus, the surge of luteinizing hormone and ovulation in Holstein heifers treated with norgestomet. J Reprod Fertil 1991; 92:461-67.

Rajamahendran R, Sianangama PC. Effect of human chorionic gonadotrophin on dominant follicles in cows: formation of accessory corpora lutea, progesterone production and pregnancy rates. J Reprod Fertil 1992; 95:577-84.

Rakes AH, Owens MP, Britt JH, Whitlow LW. Effects of adding beta-carotene to rations of lactating cows consuming different forages. J Dairy Sci 1985; 68:1732-7.

Reichenbach HD, Liebrich J, Berg U, Brem G. Pregnancy rates and births after unilateral or bilateral transfer of bovine embryos produced in vitro. J Reprod Fertil 1992; 95:363-70.

Rerat M, Zbinden Y, Saner R, Hammon H, Blum JW. In vitro embryo production: growth performance, feed efficiency, and hematological, metabolic, and endocrine status in calves. J Dairy Sci 2005; 88:2579-93.

Rettmer I, Stevenson JS, Corah LR. Endocrine responses and ovarian changes in inseminated dairy heifers after an injection of a GnRH agonist 11 to 13 days after estrus. J Anim Sci 1992a; 70:508-17.

Rettmer I, Stevenson JS, Corah LR. Pregnancy rates in beef cattle after administering a GnRH agonist 11 to 14 days after insemination. J Anim Sci 1992b; 70:7-12.

Risco CA, Donovan GA, Hernandez J. Clinical mastitis associated with abortion in dairy cows. J Dairy Sci 1999; 82:1684–9.

Rivera RM, Hansen PJ. Development of cultured bovine embryos after exposure to increased temperatures in the physiological range. Reproduction 2001; 121:107-15.

Rivlin J, Mendel J, Rubinstein S, Etkovitz N, Breitbart H. Role of hydrogen peroxide in sperm capacitation and acrosome reaction. Biol Reprod 2004; 70:518-22.

Rizos D, Fair T, Papadopoulos S, Boland MP, Lonergan P. Developmental, qualitative, and ultrastructural differences between ovine and bovine embryos produced in vivo or in vitro. Mol Reprod Dev. 2002; 62:320-7.

Roberts JS, McCracken JA. Does PGF2α released from the uterus by oxvtocin mediate the oxvtocic action of oxytocin? Biol Reprod 1976; 15:457-463.

Robinson NA, Leslie KE, Walton JS. Effect of treatment with progesterone on pregnancy rate and plasma concentrations of progesterone in Holstein cows. J Dairy Sci 1989; 72:202-7.

Robinson RS, Mann GE, Lamming GE, Wathes DC. Expression of oxytocin, oestrogen and progesterone receptors in uterine biopsy samples throughout the oestrous cycle and early pregnancy in cows. Reproduction 2001; 122:965-79.

Rocha A, Randel RD, Broussard JR, Lim JM, Blair RM, Roussel JD, Godke R_A, Hansel W. High environmental temperature and humidity decrease oocyte quality in Bos taurus but not in Bos indicus cows. Theriogenology 1998; 49:657-65.

Roche JF, Mackey D, Diskin MD. Reproductive management of postpartum cows. Anim Reprod Sci 2000; 60:703-12.

Roman-Ponce H, Thatcher WW, Wilcox CJ. Hormonal interrelationships and physiological responses of lactating dairy cows to shade management system in a tropical environment. Theriogenology 1981; 16:139–154.

Ron M, Bar-Anan R, Wiggans GR. Factors affecting conception rate of Israeli Holstein cattle. J Dairy Sci 1984; 67:854–60.

Rose MT, Weekes TE, Rowlinson P. Individual variation in the milk yield response to bovine somatotropin in dairy cows. J Dairy Sci 2004;87:2024-31.

Rosenberg M, Folman Y, Herz Z, Flamenbaum I, Berman A, Kaim M. Effect of climatic conditions on peripheral concentrations of LH, progesterone and oestradiol-17 beta in high milk-yielding cows. J Reprod Fertil 1982; 66:139-46.

Roth Z. 1998. Immediate and delayed effect of heat stress on ovarian follicular development and function in dairy cows. MSc Thesis, Fac. Agric., Hebrew Univ., Rehovot, Israel, in Hebrew, with English abstract.

Roth Z, Meidan R, Braw-Tal R, Wolfenson D. Immediate and delayed effects of heat stress on follicular development and its association with plasma FSH and inhibin concentration in cows. J Reprod Fertil 2000; 120:83–90.

Roth Z, Arav A, Bor A, Zeron Y, Braw-Tal R, Wolfenson D. Improvement of quality of oocytes collected in the autumn by enhanced removal of impaired follicles from previously heat-stressed cows. Reproduction 2001; 122:737–744.

Roth Z, Hansen PJ. Involvement of apoptosis in disruption of developmental competence of bovine oocytes by heat shock during maturation. Biol Reprod 2004; 71:1898-906.

Roth Z, Hansen PJ. Disruption of nuclear maturation and rearrangement of cytoskeletal elements in bovine oocytes exposed to heat shock during maturation. Reproduction 2005; 129:235-44.

Rowson LE, Lawson RA, Moor RM. Production of twins in cattle by egg transfer. J Reprod Fertil 1971; 25:261-8.

Royal M, Mann GE, Flint AP. Strategies for reversing the trend towards subfertility in dairy cattle. Vet J 2000; 160:53-60.

Royal MD, Flint AP, Woolliams JA. Genetic and phenotypic relationships among endocrine and traditional fertility traits and production traits in Holstein-Friesian dairy cows. J Dairy Sci 2002; 85:958-67.

Rueda BR, Tilly KI, Hansen TR, Hoyer PB, Tilly JL. Expression of superoxide dismutase, catalase and glutathione peroxidase in the bovine corpus luteum: evidence supporting a role for oxidative stress in luteolysis. Endocrine 1995; 3:227–32.

Ruoslahti E, Yamaguchi Y. Proteoglycans as modulators of growth factor activities. Cell 1991; 64:867-9.

Rutledge, JJ. Use of embryo transfer and IVF to bypass effects of heat stress. Theriogenology 2001; 55:105-11.

Ryan DP, Snijders S, Condon T, Grealy M, Sreenan J, O'Farrell KJ. Endocrine and ovarian responses and pregnancy rates in dairy cows following the administration of a gonadotrophin releasing hormone analog at the time of artificial insemination or at mid-cycle post insemination. Anim Reprod Sci 1994; 34:179-91.

Sangsritavong S, Combs DK, Sartori R, Armentano LE, Wiltbank MC. High feed intake increases liver blood flow and metabolism of progesterone and estradiol-17beta in dairy cattle. J Dairy Sci. 2002; 85:2831-42.

Santos JE, Thatcher WW, Pool L, Overton MW. Effect of human chorionic gonadotropin on luteal function and reproductive performance of high-producing lactating Holstein dairy cows. J Anim Sci 2001; 79:2881-94.

Santos JEP, Cerri RLA, Ballou MA, Higginbotham GE, Kirk JH. Effect of timing of first clinical mastitis occurrence on lactational and reproductive performance on Holstein dairy cows. Anim Reprod Sci 2004a; 80:31–45.

Santos JE, Juchem SO, Cerri RL, Galvao KN, Chebel RC, Thatcher WW, Dei CS, Bilby CR. Effect of bST and reproductive management on reproductive performance of Holstein dairy cows. J Dairy Sci 2004b; 87:868-81.

Sartori R, Fricke PM, Ferreira JC, Ginther OJ, Wiltbank MC. Follicular deviation and acquisition of ovulatory capacity in bovine follicles. Biol Reprod 2001; 65:1403-9.

Sartori R, Rosa GJM, Wiltbank MC. Ovarian structures and circulating steroids in heifers and lactating cows in summer and lactating and dry cows in winter. J Dairy Sci 85; 2002:2813–22.

Sartori R, Haughian JM, Shaver RD, Rosa GJ, Wiltbank MC. Comparison of ovarian function and circulating steroids in estrous cycles of Holstein heifers and lactating cows. J Dairy Sci 2004; 87:905-20.

Savio JD, Boland MP, Roche JF. Development of dominant follicles and length of ovarian cycles in post-partum dairy cows. J Reprod Fertil 1990; 88:581-91.

Schmitt EJ, Diaz T, Drost M, Thatcher WW. Use of a gonadotropin-releasing hormone agonist or human chorionic gonadotropin for timed insemination in cattle. J Anim Sci 1996a; 74:1084-91.

Schmitt EJ, Barros CM, Fields PA, Fields MJ, Diaz T, Kluge JM, Thatcher WW. A cellular and endocrine characterization of the original and induced corpus luteum after administration of a gonadotropin-releasing hormone agonist or human chorionic gonadotropin on day five of the estrous cycle. J Anim Sci 1996b; 74:1915-29.

Schneider B H, Sklan D, Chalupa W, Kronfeld DS. Feeding calcium salts of fatty acids to lactating cows. J Dairy Sci 1988; 71:2143–50.

Schukken YH, Grommers FJ, Van de Geer D, Erb HN, Brand A. Risk factors for clinical mastitis in herds with a low bulk milk somatic cell count. 1. Data and risk factors for all cases. J Dairy Sci 1990; 73:3463-71.

Scott TA, Shaver RD, Zepeda L, Yandell B, Smith TR. Effects of rumen-inert fat on lactation, reproduction, and health of high producing Holstein herds. J Dairy Sci 1995; 78:2435–51.

Segerson EC Jr, Murray FA, Moxon AL, Redman DR, Conrad HR. Selenium/vitamin E: role in fertilization of bovine ova. J Dairy Sci 1977; 60:1001-5.

Segerson EC, Ganapathy SN. Fertilization of ova in selenium/vitamin E-treated ewes maintained on two planes of nutrition. J Anim Sci 1980; 51:386-94.

Sheldon IM, Dobson H. Effects of gonadotrophin releasing hormone administered 11 days after insemination on the pregnancy rates of cattle to the first and later services. Vet Rec 1993; 133:160-3.

Sianangama PC, Rajamahendran R. Effect of human chorionic gonadotropin administered at specific times following breeding on milk progesterone and pregnancy in cows Theriogenology 1992; 38:85-96.

Shaw DW, Farin PW, Washburn SP, Britt JH. Effect of retinol palmitate on superovulation rate and embryo quality in superovulated cattle.Theriogenology 1995; 44:51-8.

Shiedlin A, Bigelow R, Christopher W, Arabi S, Yang L, Maier RV, Wainwright N, Childs A, Miller R. Evaluation of hyaluronan from different sources: Streptococcus zooepidemicus, rooster comb, bovine vitreous, and human umbilical cord. Biomacromolecules 2004; 5:2122-27.

Shilton KMF, Gayerie D, Hunter MG, Parkinson TJ, Lamming GE. Luteal inadequacy during the early luteal phase of sub-fertile cows. J. Reprod. Fertil 1990; 90:1–10.

Silvia WJ, Taylor ML. Relationship between uterine secretion of prostaglandin $F_{2\alpha}$, induced by oxytocin and endogenous concentrations of estradiol and progesterone at three stages of the bovine estrous cycle. J Anim Sci 1989; 67:2347-53.

Sirois J, Fortune JE. Ovarian follicular dynamics during the estrous cycle in heifers monitored by real-time ultrasonography. Biol Reprod 1988; 39:308–17.

Skarzynski DJ, Okuda K. Sensitivity of bovine corpora lutea to prostaglandin $F_{2\alpha}$ is dependent on progesterone, oxytocin, and prostaglandins. Biol Reprod 1999; 60:1292-8.

Sklan D, Bogin E, Avidar Y, Gur-arie S. Feeding calcium soaps of fatty acids to lactating cows: effect on production, body condition, and blood lipids. J. Dairy Res 1989; 56:675–81.

Sklan D, Moallem U, Folman Y. Effect of feeding calcium soaps of fatty acids on production and reproductive responses in high producing lactating cows. J Dairy Sci 1991; 74:510–517.

Sklan, D., M. Kaim, U. Moallem, and Y. Folman. Effect of dietary calcium soaps on milk yield, body weight, reproductive hormones, and fertility in first parity and older cows. J Dairy Sci 1994; 77:1652–60.

Smith JW, Legates JE. Relation of days open and days dry to lactation milk and fat yields. J Dairy Sci 1962; 45:1192-6.

Spalding RW, Everett RW, Foote RH. Fertility in New York artificial inseminated Holstein herds in dairy herd improvement. J Dairy Sci 1975; 58:718-23.

Son J, Grant RJ, Larson LL. Effects of tallow and escape protein on lactational and reproductive performance of dairy cows. J Dairy Sci 1996; 79:822–30.

Soto P, Natzke RP, Hansen PJ. Identification of possible mediators of embryonic mortality caused by mastitis: Actions of lipopolysaccharide, prostaglandin $F_{2\alpha}$, and the nitric oxide generator, sodium nitroprusside dihydrate, on oocyte maturation and embryonic development in cattle. Am J Reprod Immunol 2003; 50:263-72.

Spencer TE, Bazer FW. Temporal and spatial alterations in uterine estrogen receptor and progesterone receptor gene expression during the estrous cycle and early pregnancy in the ewe. Biol Reprod 1995; 53:1527-43.

Spencer TE, Ott TL, Bazer FW. τ-Interferon: pregnancy recognition signal in ruminants. Proc Soc Exp Biol Med 1996; 213:215–29.

Spencer TE, Bazer FW. Biology of progesterone action during pregnancy recognition and maintenance of pregnancy. Front Biosci 2002; 7:1879–98.

Sreenan JM, Diskin MG. Early embryonic mortality in the cow: its relationship with progesterone concentration. Vet Rec 1983; 112:517-21.

Sreenan JM, Diskin MG. Effect of a unilateral or bilateral twin embryo distribution on twinning and embryo survival rate in the cow. J Reprod Fertil 1989; 87:657-64.

Short TH, Lawlor TJ, Everett RW. Inbreeding in US. Holsteins and its effect on yield and type traits. J Dairy Sci 1992; 75:154 [Abstr].

Stahl TJ, Conlin BJ, Seykora AJ, Steuernagel GR. Characteristics of Minnesota dairy farms that significantly increased milk production from 1989-1993. J Dairy Sci 1999; 82:45-51.

Staples CR, Thatcher WW, Clark JH. Relationship between ovarian activity and energy status during the early postpartum period of high producing dairy cows. J Dairy Sci 1990; 73:938-47.

Staples CR, Burke JM, Thatcher WW. I nfluence of supplemental fats on reproductive tissues and performance of lactating cows. J Dairy Sci 1998; 81:856-71.

Stevenson JS, Schmidt MK, call EP. Factors affecting reproductive performance of Dairy cows first inseminated after five weeks postpartum. J Dairy Sci 1983; 66:1148.

Stevenson JS, Call EP, Scoby RK, Phatak AP. Double-insemination and gonadotropin-releasing hormone treatment of repeat-breeding dairy cattle. J Dairy Sci 1990; 73:1766.

Stevenson JS, Phatak AP, Rettmer I, Stewart RE. Postinsemination administration of Receptal: Follicular dynamics, duration of cycle, hormonal responses, and pregnancy rates. J Dairy Sci 1993; 76:2536–47.

Stojkovic M, Kolle S, Peinl S, Stojkovic P, Zakhartchenko V, Thompson JG, Wenigerkind H, Reichenbach HD, Sinowatz F, Wolf E. Effects of high concentrations of hyaluronan in culture medium on development and survival rates of fresh and frozen-thawed bovine embryos produced in vitro. Reproduction 2002; 124:141-153.

Stott GH, Wiersma F, Woods JM. Reproductive health program for cattle subjected to high environmental temperatures. J Am Vet Med Assoc. 1972 1; 161:1339-44.

Stowe HD, Thomas JW, Johnson T, Marteniuk JV, Morrow DA, Ullrey DE. Responses of dairy cattle to long-term and short-term supplementation with oral selenium and vitamin E1. J Dairy Sci 1988;71:1830-9.

Stronge AJ, Sreenan JM, Diskin MG, Mee JF, Kenny DA, Morris DG. Post-insemination milk progesterone concentration and embryo survival in dairy cows. Theriogenology 2005; 64:1212-24.

Taylor VJ, Cheng Z, Pushpakumara PG, Beever DE, Wathes DC. Relationships between the plasma concentrations of insulin-like growth factor-I in dairy cows and their fertility and milk yield. Vet Rec. 2004; 155:583-8.

Thatcher WW, Chenault JR. Reproductive physiological responses of cattle to exogenous prostaglandin $F_{2\alpha}$. J Dairy Sci 1976; 59:1366-75.

Thatcher WW, Collier RJ. Effects of climate on bovine reproduction. In: D. A. Morrow (Ed.) Current Therapy in Theriogenology 1986: 301−309. WB Saunders, Philadelphia.

Thatcher WW, Macmillan KL, Hansen PJ, Drost M. Concepts for regulation of corpus luteum function by the conceptus and ovarian follicles to improve fertility. Theriogenology 1989; 31:149-64.

Thatcher WW, Driancourt MA, Terqui M, Badinga L. Dynamics of ovarian follicular development in cattle following hysterectomy and during early pregnancy. Domest Anim Endocrinol 1991; 8:223-34.

Thatcher WW, Staples CR, Danet-Desnoyers G, Oldick B, Schmitt EP. Embryo health and mortality in sheep and cattle. J Anim Sci 1994; 72:16.

Thatcher WW, Meyer MD, Danet-Desnoyers G. Maternal recognition of pregnancy. J Reprod Fertil 1995; 49:15-28.

Thatcher WW, De la Sota RL, Schmitt EJ, Diaz TC, Badinga L, Simmen FA, Staples CR, Drost M. Control and management of ovarian follicles in cattle to optimize fertility. Reprod Fertil Dev 1996; 8:203-17.

Thatcher WW, Binelli M, Burke JM, Staples CR, Ambrose JD, Coelho S. Antiluteolytic signals between conceptus and endometrium. Theriogenology 1997; 47:131–140

Thatcher WW, Mattos R, Moreira F, Binelli M, Ambrose JD. Experimental manipulation of follicular growth. Reprod Domest Anim 2000; 6:27–33.

Thatcher WW, Moreira F, Santos JE, Mattos RC, Lopes FL, Pancarci SM, Risco CA. Effects of hormonal treatments on reproductive performance and embryo production. Theriogenology 2001; 55:75-89.

Thatcher WW, Moreira F, Pancarci SM, Bartolome JA, Santos JE. Strategies to optimize reproductive efficiency by regulation of ovarian function. Domest Anim Endocrinol 2002; 23:243-54.

Thissen JP, Ketelslegers JM, Underwood LE. Nutritional regulation of the Insulin-like growth factors. Endocr Rev 1994; 15:80-101.

Thomas MG, Williams GL. Metabolic hormone secretion and FSH-induced superovulatory responses of beef heifers fed dietary fat supplements containing predominantly saturated and polyunsaturated fatty acids. Theriogenology 1996; 45:451–8.

Thompson JR, Everett RW, Hammerschmidt NL. Effects of inbreeding on production and survival in Holsteins. J Dairy Sci 2000a; 83:1856-64.

Thompson JR, Everett RW, Wolfe CW. Effects of inbreeding on production and survival in Jerseys. J Dairy Sci 2000b;83:2131-8.

Tominaga K, Shimizu M, Ooyama S, Izaike Y. Effect of lipid polarization by centrifugation at different developmental stages on post-thaw survival of bovine in vitro produced 16-cell embryos. Theriogenology 2000; 53:1669-80.

Toole BP, Zoltan-Jones A, Misra S, Ghatak S. Hyaluronan: a critical component of epithelial-mesenchymal and epithelial-carcinoma transitions. Cells Tiss Organs 2005; 179:66-72.

Townson DH, Tsang PC, Butler WR, Frajblat M, Griel LC Jr, Johnson CJ, Milvae RA, Niksic GM, Pate JL. Relationship of fertility to ovarian follicular waves before breeding in dairy cows. J Anim Sci 2002; 80:1053-8.

Trout JP, McDowell LR, Hansen PJ. Characteristics of the estrous cycle and antioxidant status of lactating Holstein cows exposed to heat stress. J Dairy Sci 1998; 81:1244–50.

Twagiramungu H, Guilbault LA, Proulx J, Villeneuve P, Dufour JJ. Influence of an agonist of gonadotropin-releasing hormone (Buserelin) on estrus synchronization and fertility in beef cows. J Anim Sci 1992; 70:1904-10.

Ullah G, Fuquay JW, Keawkhong T, Clark BL, Pogue DE, Murphey EJ. Effect of gonadotropin-releasing hormone at estrus on subsequent luteal function and fertility in lactating Holsteins during heat stress. J Dairy Sci 1996; 79:1950-3.

Vajta G. Vitrification of the oocytes and embryos of domestic animals. Anim Reprod Sci 2000; 60:357-64.

Valcarcel A, de Matos DG, Furnus CC. The hyaluronic acid receptor (CD44) is expressed in bovine oocytes and preimplantation stage embryos. Theriogenology 1999; 51:193 (Abstract).

Van Cleef, J, Macmillan KL, Thatcher WW, Lucy MC. Estrous synchronization and fertility in heifers treated with CIDR before and after insemination. J Anim Sci 1989; 65:383 (Abstract).

Van Wagtendonk-de Leeuw AM, Den Daas JHG, ThAM Kruip, Rall WF. Comparison of the efficacy of conventional slow freezing and rapid cryopreservation methods for bovine embryos. Cryobiology 1995; 32:157–67.

Vasconcelos JL, Silcox RW, Rosa GJM, Pursley JR, Wiltbank MC. Synchronization rate, size of the ovulatory follicle, and pregnancy rate after synchronization of ovulation beginning on different days of the estrous cycle in lactating dairy cows. Theriogenology 1999; 52:1067–78.

Vasconcelos JL, Sartori R, Oliveira HN, Guenther JG, Wiltbank MC. Reduction in size of the ovulatory follicle reduces subsequent luteal size and pregnancy rate. Theriogenology 2001; 56:307-14.

Vasconcelos JL, Sangsritavong S, Tsai SJ, Wiltbank MC. Acute reduction in serum progesterone concentrations after feed intake in dairy cows. Theriogenology 2003; 60:795-807.

Vicini JL, Buonomo FC, Veenhuizen JJ, Miller MA, Clemmons DR, Collier RJ. Nutrient balance and stage of lactation affect responses of insulin, insulin-like growth factors I and II, and insulin-like growth factor-binding protein 2 to somatotropin administration in dairy cows. J Nutr 1991;121:1656-64.

Villa-Godoy A, Ireland JJ, Wortman JA, Ames NK, Hughes TL, Fogwel RL. Effect of ovarian follicles on luteal regression in heifers. J Anim Sci 1985; 60:519-27.

Villarroel A, Martino A, BonDurant RH, Deletang F, Sischo WM. Effect of post-insemination supplementation with PRID on pregnancy in repeat-breeder Holstein cows. Theriogenology 2004; 61:1513-20.

Viuff D, Greve T, Avery B, Hyttel P, Brockhoff PB, Thomsen PD. Chromosome aberrations in in vitro-produced bovine embryos at days 2-5 post-insemination. Biol Reprod 2000; 63:1143-48.

Wall E, Brotherstone S, Kearney JF, Woolliams JA, Coffey MP. Impact of nonadditive genetic effects in the estimation of breeding values for fertility and correlated traits. J Dairy Sci 2005; 88:376-85.

Wang JY, Larson LL, Owen FG. Effect of beta-carotene supplementation on reproductive performance of dairy heifers. Theriogenology 1982; 18:461-73.

Wang X, Quinn PJ. The location and function of vitamin E in membranes. Mol Membr Biol 2000; 17:143-56.

Wang X, Quinn PJ. Vitamin E and its function in membranes. Prog Lipid Res. 1999; 38:309-36.

Webb R, Garnsworthy PC, Gong JG, Armstrong DG. Control of follicular growth: local interactions and nutritional influences. J Anim Sci 2004; 82:63-74.

Wehrman ME, Welsh TH, Williams GL. Diet-induced hyperlipidemia in cattle modifies the intrafollicular cholesterol environment, modulates ovarian follicular dynamics, and hastens the onset of postpartum luteal activity. Biol Reprod 1991; 45:514–22.

Westwood CT, Lean IJ, Garvin JK. Factors influencing fertility of Holstein dairy cows: a multivariate description. J Dairy Sci 2002; 85:3225-37.

Wiggans GR, Ernst CA. Effect of genetic merit of sire for milk yield and herd yield level on reproductive traits. J Dairy Sci 1987; 70:232.

Wiggans GR, VanRaden PM, Zuurbier J. Calculation and use of inbreeding coefficients for genetic evaluation of United States dairy cattle. J Dairy Sci 1995; 78:1584-90.

Willard S, Gandy S, Bowers S, Graves K, Elias A, Whisnant C. The effects of GnRH administration postinsemination on serum concentrations of progesterone and pregnancy rates in dairy cattle exposed to mild summer heat stress. Theriogenology 2003; 59:1799-810.

Wilson SJ, Marion RS, Spain JN, Spiers DE, Keisler DH, Lucy MC. Effects of controlled heat stress on ovarian function of dairy cattle: 1. Lactating cows. J. Dairy Sci. 1998a 81:2124–31.

Wilson SJ, Kirby CJ, Koenigsfeld AT, Keisler DH, Lucy MC. Effects of controlled heat stress on ovarian function of dairy cattle: 2. Heifers. J Dairy Sci 1998b; 81:2132–38.

Wilson RD, Weigel KA, Fricke PM, Rutledge JJ, Leibfried-Rutledge ML, Matthews DL, Schutzkus VR. In vitro production of Holstein embryos using sex-sorted sperm and oocytes from selected cull cows. J Dairy Sci 2005; 88:776-82.

Wiltbank MC, Fricke PM, Sangsritavong S, Sartori R, Ginther OJ. Mechanisms that prevent and produce double ovulations in dairy cattle. J Dairy Sci 2000; 83:2998-3007.

Wise ME, Armstrong DV, Huber JT, Hunter R. Wiersma F. Hormonal alterations in the lactating dairy cow in response to thermal stress. J Dairy Sci 1988; 71:2480–85.

Wolfenson D, Bartol FF, Badinga L, Barros CM, Marple DN, Cummings K, Wolfe D, Lucy MC, Spencer TE, Thatcher WW. Secretion of PGF2α and oxytocin during hyperthermia in cyclic and pregnant heifers. Theriogenology 1993; 39:1129−41.

Wolfenson D, Thatcher WW, Savio JD, Badinga L, Lucy MC. The effect of a GnRH analogue on the dynamics of follicular development and synchronization of estrus in lactating dairy cows. Theriogenology 1994; 42:633-44.

Wolfenson D, Thatcher WW, Badinga L, Savio JD, Meidan R, Lew BJ, Braw-Tal R, Berman A. Effect of heat stress on follicular development during the estrous cycle in lactating dairy cattle. Biol Reprod 1995; 52:1106–1113.

Wolfenson D, Lew BJ, Thatcher WW, Graber Y, Meidan R. Seasonal and acute heat stress effects on steroid production by dominant follicles in cows. Anim Reprod Sci 1997; 47:9-19.

Wolfenson D, Roth Z, Meidan R. Impaired reproduction in heat-stressed cattle: basic and applied aspects. Anim Reprod Sci 2000; 60-61: 535-47.

Wolfenson D, Inbar G, Roth Z, Kaim M, Bloch A, Braw-Tal R. Follicular dynamics and concentrations of steroids and gonadotropins in lactating cows and nulliparous heifers. Theriogenology 2004; 15:1042-55.

Wright S. Coefficients of inbreeding and relationship. Am. Naturalist 1922; 56:330-338.

Yasuda M, Nakano K, Yasumoto K, Tanaka Y. CD44: functional relevance to inflammation and malignancy. Histol Histopathol 2002; 17:945-50.

Younas M, Fuquay JW, Smith AE ,Moore AB. Estrous and endocrine responses of lactating Holsteins to forced ventilation during summer. J Dairy Sci 1993; 76:430–6.

Young C W, Seykora AJ. Estimates of inbreeding and relationship among registered Holstein females in the United States. J Dairy Sci 1996; 79:502–5.

Yoshioka K, Suzuki C, Arai S, Iwamura S, Hirose H. Gonadotropin-releasing hormone in third ventricular cerebrospinal fluid of the heifer during the estrous cycle. Biol Reprod 2001; 64:563–70.

Zingg HH, Rozen F, Breton C, Larcher A, Neculcea J, Chu K, Russo C, Arslan A. Gonadal steroid regulation of oxytocin and oxytocin receptor gene expression. Adv Exp Med Biol 1995; 395:395-404.

Zulu VC, Sawamukai Y, Nakada K, Kida K, Moriyoshi M. Relationship among insulin-like growth factor-I, blood metabolites and postpartum ovarian function in dairy cows. J Vet Med Sci. 2002a; 64:879-85.

Zulu VC, Nakao T, Sawamukai Y. Insulin-like growth factor-I as a possible hormonal mediator of nutritional regulation of reproduction in cattle. J Vet Med Sci. 2002b; 64:657-65.

BIOGRAPHICAL SKETCH

C. Moisés Franco was born in 1979 in Santa Cruz, Bolivia. He is the youngest of three brothers (Inj. Oscar Antonio Franco; Inj. Jorge Mauricio Franco) and three sisters (Dra. Rosario Franco; María Isabel Franco; Arq. Erika Lorena Franco). He is the son of Antonio V. Franco Monasterio (may god bless his soul) and Mercedes Yolanda Vaca El-Hage. He graduated from La Salle High School in the same city in 1997 and enrolled the next year in the Department of Animal Science at the University of Arkansas in Fayetteville, USA, where he received his Bachelor of Science degree in animal science in 2001. During 2002 he did an internship in the Scottish Agricultural College with Dr Tom McEvoy. He enrolled in the graduate program of the Department of Animal Sciences at the University of Florida under supervision of Dr. Peter J. Hansen in January, 2003. He is currently a Master of Science candidate. Upon completion of his degree, he will open an embryo transfer company. In the near future he plans to resume his studies through pursuit of the Doctor of Philosophy degree at the University of Florida under Dr P.J. Hansen.