EFFECTS OF DIFFERENCES IN DIETARY PROTEIN AND VARYING THE INTERVAL FROM COLLECTION OF BOVINE EMBRYOS TO FREEZING ON EMBRYO QUALITY AND VIABILITY

by

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ABSTRACT

High levels of dietary protein may be detrimental to reproductive performance in cattle. The objective of Exp. 1 was to determine the effects of differences in dietary protein on the production and quality of bovine embryos collected from superovulated donors. Angus cows were randomly assigned to receive one of three experimental diets: a daily ration of 5.7 kg poultry litter, 2.0 kg hay, 3.1 kg corn, and 0.5 kg peanut hulls (LITTER; n = 15); a daily ration of 6.2 kg peanut hulls, 2.2 kg soybean meal, 2.0 kg hay, 0.5 kg corn, and 0.4 kg dicalcium phosphate (SBM; n = 15); or a daily ration of 6.2 kg peanut hulls, 2.0 kg hay, and 3.1 kg corn (CON; n = 19). Diets differed in the amount of total, soluble and degradable protein, but were comparable in energy. After 30 d on the diets, all cows were treated to induce superovulation (28.8 mg FSH/cow, Folltropin®) and synchronize estrus. After the detection of estrus each cow was inseminated with semen from one of four Holstein bulls. Embryos were collected 7 d after estrus and evaluated for quality (according to the International Embryo Transfer Society (IETS) standards) and stage of development. Prior to treatment to induce superovulation, blood samples were collected 6 h after feeding. Samples were analyzed to assess dietary effects on plasma urea nitrogen (PUN). Mean levels of PUN were higher ($P < 0.01$) in cows fed the LITTER or SBM diet (16.3 mg/dL, LITTER; 21.8 mg/dL, SBM; 9.7 mg/dL, CON) than in cows fed the CON diet. Additionally, concentration of PUN was higher in cows fed SBM than in those fed LITTER ($P < 0.01$). An average of 9.2 transferable embryos (Grade 1, 2 and 3) was collected from each cow and there were no significant differences in the number of transferable embryos collected among groups (9.2, LITTER; 9.3, SBM; 9.1, CON). The number of degenerate embryos or unfertilized ova did not differ among dietary groups. High-protein diets elevated PUN, but did not affect the number or quality of embryos collected from superovulated donors.

Cryopreservation of bovine embryos is an important aspect of a successful embryo transfer program. The objective of Exp. 2 was to evaluate the post-thaw viability of bovine embryos collected in Exp. 1 in an *in vitro* culture system after the embryos had been held at room temperature or refrigerated for 2 to 12 h prior to freezing. Upon embryo recovery, each embryo was randomly assigned to be placed in holding media for 2, 6 or 12 h prior to freezing. During this interval, one-half of the embryos were maintained in a refrigerated environment (5 °C), while the remaining half of the embryos were held at room temperature (20.5 to 22 °C) until freezing. Immediately prior to freezing, embryos were removed from the holding media, transferred to a well containing ethylene glycol (10%) in ovum culture media and loaded individually into a 0.25-mL plastic straw. Straws were then placed in a freezer unit (-6 °C) and seeded to induce ice crystal formation through all columns of the straw. The temperature of the freezer was then decreased 0.6 °C/min to –32 °C, and straws were loaded into canes and plunged into a liquid nitrogen tank (-196 °C). After storage, each straw was exposed to a 5-s air thaw and placed in a water bath at 35 °C for 20 s. Each embryo was then washed to remove excess ethylene glycol prior to *in vitro* culture. Embryos were individually cultured in Ham's F-10 media supplemented with 4% fetal bovine serum for 72 h. Embryos were evaluated at 24 h intervals throughout the culture period and assigned a stage of development and quality grade score (according to IETS standards). The percentage of embryos that developed to the expanded blastocyst stage and hatched from the zona pellucida was greater for embryos held 2 or 6 h prior to freezing ($P < 0.05$) than for embryos held for 12 h after collection before being frozen (62.9, 52.0 and 31.1%, respectively). The percentage of embryos that degenerated during *in vitro* culture was lower for embryos held 2 or 6 h prior to freezing (20.4 and 26.6%; $P < 0.05$) than for embryos held for 12 h before freezing (50.8%). Furthermore, embryo quality grade was more desirable for embryos held for 2 or 6 h (1.5 and 1.7; $P < 0.05$) than for those held for 12 h before freezing (2.1). The semen used to inseminate donors and the diet fed to donors for 4 wk prior to embryo collection did not influence the proportion of embryos that hatched or degenerated during the 72 h of *in vitro* culture. Additionally, holding embryos in a refrigerated environment from the time of collection until freezing did not enhance embryonic development during post-thaw culture. Thus, embryonic viability may be impaired when embryos are held longer than 6 h following embryo recovery before being frozen; however, the storage temperature during the interval from collection to freezing does not influence embryonic development post-thaw.

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LIST OF ABBREVIATIONS

Abbreviations used in this paper are defined in alphabetical order.

AI	artificial insemination
CIDR	controlled internal drug-releasing device
CP	crude protein
DEG	degenerate
DMI	dry matter intake
E_2	estradiol
EG	ethylene glycol
ET	embryo transfer
FBS	fetal bovine serum
FSH	follicle stimulating hormone
GLY	glycerol
IETS	International Embryo Transfer Society
IVF	*in vitro* fertilized/*in vitro* fertilization
LH	luteinizing hormone
LN_2	liquid nitrogen
NPN	non-protein nitrogen
OCM	ovum culture media
P_4	progesterone
PBS	phosphate buffered saline
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PGL	plasma glucose
ppm	parts per million
PUN	plasma urea nitrogen
RDP	rumen degradable protein
RUP	rumen undegradable protein
SBM	soybean meal
TDN	total digestible nutrients
TPP	total plasma protein
UFO	unfertilized ova

CHAPTER I. LITERATURE REVIEW

A. Importance of Poultry Litter as a Protein Source for Cattle

I. Poultry Industry

Consumer demand for poultry products has increased in recent years. In 1997, total meat consumption (red meat, poultry and fish) amounted to 86.36 kg/person. On average, each person consumed 9.55 kg less red meat and 14.09 kg more poultry as compared to 1970 (USDA/ERS, 1999). In order to meet this demand for poultry products, production has shifted from smaller, diversified, family-run operations to larger, highly specialized operations. Compared to other livestock industries, such as swine or cattle, the poultry industry has the smallest proportion of small operators. Changes in the number of poultry operations and poultry production in the United States from 1982 to 1997 are depicted in Table 1-1 (USEPA, 2001). Although the number of operations decreased during that time-span, larger operations have more than compensated for the decline in poultry operations with increased production per operation.

Table 1-1. Broiler Operations and Production in the United States from 1982 to 1997

Year	Operations (No. of farms)	Production (No. of birds)
1982	30,100	3,516,095,408
1987	27,645	4,361,198,301
1992	23,949	5,427,532,921
1997	23,937	6,741,476,153

Taken from: USEPA, 2001

While poultry production has risen since the 1980s, a greater amount of manure and litter has been generated annually. During a typical 47-d grow-out period, a broiler will produce approximately 0.94 kg of litter (manure mixed with wood shavings or other bedding material) or approximately 0.02 $kg \bullet d^{-1} \bullet bird^{-1}$ (U.S. Poultry and Egg Association, 2002). Hence, a typical broiler house with a capacity for 22,000 birds will produce 120 tons of litter per year (USEPA, 2001). Since most of the water evaporates from the manure that is excreted by the birds, a high amount of litter is recoverable when houses are cleaned out.

Due to increased litter production, environmentalists and governmental agencies began to question the management practices of poultry producers. A survey conducted by the National Chicken Council and the U.S. Poultry and Egg Association provided insight to the environmental

management practices of 16,311 poultry growers across the nation (Starkey, 2002). The average respondent to the survey was from a poultry farm that consisted of 3.20 houses/farm with a one-time capacity of 63,807 birds/farm. Due to the dryness and transportability of poultry litter, most growers were able to move more than half of this valuable commodity to off-site users. Poultry litter has been used as a natural fertilizer in many areas of the country. Additionally, beef producers have used poultry litter as a low-cost, alternative feedstuff for cows due to its high nutritive value.

II. Uses of Poultry Litter for Beef Cattle

History on Uses of Animal Wastes. Animal wastes possess substantial nutritional value. Prior to the last 50 yr, manure was used extensively as fertilizers because of the convenience of disposal while maintaining soil fertility and providing nutrients for crops (Bhattacharya and Taylor, 1975). However, the incorporation of intensive confinement systems complicated this practice. Large, concentrated livestock operations, such as cattle feedlots, poultry farms and dairies, are often developed on small areas of land that are in close proximity to lakes and streams. This makes the application of wastes as fertilizers problematic due to insufficient land close to these systems for disposal of large amounts of waste. Additionally, the availability of low cost fertilizers coupled with increasing environmental concerns has reduced the economic efficiency of using animal wastes as fertilizers (Fontenot, 1996). Thus, a more economical method for recycling animal wastes is as a nutrient source for animal production.

Over 100 million tons of animal waste (dry matter) is produced in the United States each year (Fontenot, 1996). While approximately 50% of all animal wastes can be collected, poultry litter is relatively dry and nearly completely recoverable. This makes transporting poultry litter away from the main site of production to beef producers economical, and beef producers are able to utilize litter as a valuable feed source (Stephenson et al., 1990). Furthermore, poultry litter is higher in crude protein (CP) and digestible energy as compared to swine or cattle wastes (Fontenot et al., 1983). Therefore, poultry litter can be utilized in cattle feeding programs to supply nutrients at relatively low costs.

Nutritive Value of Poultry Litter. Poultry litter has been used as a feed source for different classes of beef cattle, and the utilization of poultry litter for animal production continues today. Due to increased interests in environmental quality, the beef industry has capitalized on feeding this valuable resource (Fontenot, 1991). Poultry litter has been used as an

inexpensive feed source for cattle while enhancing the quality of the environment. Diets containing poultry litter can be formulated for beef cows, lactating cows, growing calves or finished cattle. It is recommended that these rations be mixed with ground grain to ensure adequate consumption of the feed (Kunkle et al., 1997).

Poultry litter consists of a mixture of manure, bedding material, spilled feed and feathers. Common bedding materials used in broiler houses consist of wood-shavings, sawdust, rice hulls or peanut hulls (Evers et al., 1996). In a typical year, poultry producers will raise five to six flocks of broilers in a single broiler house. After each flock is removed, a small amount of "cake" (litter that has become saturated with water due to spillage around bird watering systems) must be removed. All litter is removed from each house at least once per year (Jacob et al., 1997), and this poultry litter can be processed and used as an economical source of feed for cattle.

The nutrient content of various sources of poultry litter differ due to varying management strategies incorporated by poultry producers. These strategies include the type of bedding material used, the number of flocks of birds housed on the litter, house management, litter removal method and moisture content of the litter (Hopkins and Poore, 2001). Because of the variation in management, litter should be tested to determine the nutritive content prior to feeding. The average and range in nutrient content of 106 samples of poultry litter analyzed from various poultry farms in Alabama are depicted in Table 1-2 (Stephenson et al., 1990).

A description of the nutritive value of poultry litter, according to Jacob et al. (1997), is as follows.

- Moisture: The moisture of poultry litter influences the quality of the feed. If the moisture content of litter is less than 12% (too dry), it will not go through the heat cycle properly during deep stacking and be dusty when fed. However, if the moisture content of poultry litter is greater than 25% (too wet), litter may be difficult to mix with other feeds. Additionally, deep stacking may generate too much heat and denature the protein in the litter.
- Total Digestible Nutrients (TDN): Poultry litter is approximately 60% TDN and is comparable in energy to good quality hay. A comparison of percent CP and TDN of hays and poultry litter is illustrated in Table 1-3 (McKinnon, 1996).

Table 1-2. Nutrient Content of Poultry Litter in Alabama

Components, Dry Basis	Average	Range
Moisture, %	19.5	4.70-39
Dry matter, %	80.5	61-95
Ash, %	24.7	9-54
TDN, %	50.0	36-64
Nitrogen, %	4.0	2-6
Crude protein, %	24.9	15-38
Bound nitrogen, %	15.0	5-64
Crude fiber, %	23.6	11-52
Calcium, %	2.3	0.81-6.13
Phosphorus, %	1.6	0.56-3.92
Potassium, %	2.3	0.73-5.17
Magnesium, %	0.52	0.19-0.88
Sulfur, %	0.50	0.22-0.83
Copper, ppm	473	25-1,003
Iron, ppm	2,377	529-12,604
Manganese, ppm	348	125-667
Zinc, ppm	315	106-669

Taken from: Stephenson et al., 1990

Table 1-3. Nutrient Content of Hays and Poultry Litter

Ingredient	Crude Protein (%)	TDN (%)
Fair quality, first cut grass hay	10.5	54.0
Poor quality, first cut grass hay	8.0	49.0
Deep stacked poultry litter	28.0	60.0
80:20 litter:ground corn mixture	24.0	66.0

Taken from: McKinnon, 1996

- CP: Quality poultry litter should contain 20 to 30% CP. Approximately 50% of this CP is in the form of non-protein nitrogen (NPN), mainly uric acid, which is produced by poultry. The digestibility of CP in poultry litter is 65 to 75%.

- Crude Fiber: The fiber content of poultry litter mainly comes from the bedding material used in poultry houses. This makes poultry litter higher in crude fiber as compared to swine waste (Fontenot, 1999). However, as increasing numbers of flocks are grown on the bedding material and litter, the total fiber content of the litter is reduced.
- Ash: Ash content is an indicator of the quality of the poultry litter. Ash consists of the minerals from feed, manure, bedding material and soil. If litter is to be utilized as a feed source, the percentage of soil in the litter should be low. Ash levels greater than 25% result in poor feed consumption and poor animal performance.

Bhattacharya and Fontenot (1966) performed three metabolism trials with 10 yearling wethers to study the protein and energy value of peanut-hull-based and wood-shaving-based broiler litters fed at two different levels. Two rations containing peanut-hull broiler litter were substituted for 25 and 50% of a 1:1 ratio of ground alfalfa hay and ground corn, respectively, while two rations containing wood-shaving broiler litter were substituted at 25 and 50% of a 1:1 ratio of ground alfalfa hay and ground corn, respectively. Apparent digestibility of the litter rations, calculated by difference, is shown in Table 1-4. The apparent digestibility of CP was not different between kinds or levels of litter fed, with an average digestion coefficient of 72.5% CP. There was a significant difference in crude fiber digestibility between levels of litter, but not between kinds of litter. As the amount of litter in the ration increased from 25 to 50%, the crude fiber digestibility decreased. No differences were detected in digestibility of dry matter or energy content between kinds or levels of litter fed. This study showed that both peanut-hull and wood-shaving poultry litters are effective feed sources for ruminants.

Hopkins and Poore (2001) evaluated deep-stacked broiler litter as a protein source for dairy replacement heifers. Fifty Holstein heifers were assigned to one of the following treatments for 105 d. Treatments 1 through 4 are represented as the percentage of supplemental CP from soybean meal (SBM) or deep-stacked broiler litter in each ration, respectively: 1) 100 and 0%; 2) 67 and 33%; 3) 33 and 67%; and 4) 0 and 100%. Treatment 5 consisted of 67% deep-stacked broiler litter and 33% from a rumen undegradable protein (RUP) source. Each ration was formulated to contain 15% CP and 68% TDN on a dry matter basis. Results from this experiment are shown in Table 1-5. Dry matter and CP intake as well as feed efficiency (full average daily gain/dry matter intake) were not significantly different among treatments. Average

daily weight gains decreased linearly as the level of litter feeding increased. Additionally, no significant differences were detected for changes in hip height or body condition score. These results indicate that deep-stacked broiler litter can be used as a low-cost source of protein for dairy heifers.

Poultry litter has an economical benefit as a low-cost protein supplement for heifers and cows. Due to varying strategies in managing poultry houses, the nutritive value of poultry litter may differ. However, poultry litter provides a source of CP, and if fed with ground grain, such as corn, this mixture can provide enough energy to meet the requirements for a mature beef cow.

Table 1-4. Apparent Digestion Coefficients[a] of Peanut-Hull and Wood-Shaving Broiler Litter

Kind of Litter	Peanut-Hull Poultry Litter		Wood-Shaving Poultry Litter	
	Level of Litter in Ration (%)			
Apparent Digestibility (%)	25	50	25	50
Crude Protein	73.1	73.0	73.5	70.4
Crude Fiber	71.5[b]	66.8[c]	70.0[b]	66.1[c]
Dry Matter	62.5	63.4	66.1	61.5
Energy	64.7	63.1	64.8	63.6

Taken from: Bhattacharya and Fontenot, 1966
[a] Calculated by difference.
[b, c] Means in each row with different superscripts differ ($P < 0.01$)

Table 1-5. Least Squares Means for Intake as Affected by Level of Broiler Litter Feeding

	Supplemental Crude Protein from Deep-Stacked Broiler Litter					Effect
Intake	0%	33%	67%	100%	67% + RUP	Linear
DM, kg/d	10.12	10.22	10.40	9.36	9.13	NS
CP, kg/d	1.53	1.54	1.55	1.40	1.39	NS
Full ADG, kg/d	1.37	1.40	1.30	1.17	1.26	0.01
Full ADG/DMI, kg/kg	0.14	0.14	0.13	0.14	0.14	NS
Hip Height Change, cm	8.32	9.08	10.29	9.27	8.45	NS
Change in BCS[a]	0.64	0.73	0.60	0.45	0.48	NS

Taken from: Hopkins and Poore, 2001
[a] BCS: Body Condition Score (five point scale where 1 = emaciated to 5 = obese)

Processing Methods. When poultry litter is processed to eliminate potentially harmful pathogens, litter can be utilized as a safe source of CP and energy for cattle. Deep stacking and ensiling are common methods for processing litter. A stack depth of 1.8 to 2.5 m is usually required when deep-stacking litter. This results in a temperature of at least 54 °C in the stack within 5 d (Hopkins and Poore, 2001). Poultry litter can be ensiled alone or in combination with other ingredients, such as corn silage. If ensiled alone, the moisture level of the litter should be about 40% to ensure proper processing of the litter (Fontenot, 1996). Processing poultry litter by deep stacking or ensiling eliminates pathogens without adversely affecting the nutritive content of the poultry litter (McCaskey and Martin, 1988).

Bakshi and Fontenot (1998) evaluated various methods of processing broiler litter to be used as a livestock feed. Poultry litter was deep-stacked at 30 or 40% moisture or ensiled at 40% moisture for 6 wk and evaluated for pathogenic organisms and nutrient utilization in wether lambs. Processing of poultry litter by either method (at all moisture levels) completely eliminated all coliform bacteria. Elimination of these pathogens could have been due to their sensitivity to temperatures above 42 °C. Additionally, the apparent digestibility of nutrients and nitrogen-retention in the litter-supplemented diets fed to the lambs were similar to those of the SBM fed lambs. These researchers concluded that total and fecal coliforms can by eliminated by deep stacking at different moisture levels or by ensiling while the nitrogen from deep-stacked or ensiled litter could be utilized efficiently by wether lambs.

Health Aspects. Poultry litter should be processed in such a way that minimizes or prevents contamination by bacteria (Pugh et al., 1994). Processing poultry litter by deep stacking or ensiling effectively destroys potential harmful pathogens, as described above. However, attention must be given to the concentration of copper and other metals in the litter. While practicing veterinarians have reported few cases of copper toxicity in cattle fed poultry litter, copper toxicosis may occur if litter with 300 to 400 parts per million (ppm) of copper is fed for an extended time (Pugh et al., 1994). Analysis of each batch of litter by a forage testing laboratory can ensure the nutritive quality of the feed while determining the concentration of various minerals and metals that could cause health problems if not fed properly. Medicinal residues that are fed to broilers may appear in poultry litter. Webb and Fontenot (1975) reported that if litter containing these residues was fed to cattle, a 5 d withdrawal from litter resulted in no

detectable tissue residues. Thus, proper management of poultry litter by beef producers before feeding litter to livestock should eliminate potential health problems.

Economic Evaluation. Poultry litter can be used effectively in a cow-feeding program as a low-cost protein supplement or roughage substitute or extender (Heidel, 1996). Thin, gestating cows or cows with calves can be fed an 80% poultry litter, 20% ground grain mixture to meet the protein and energy requirement of these cattle. If a hay source is available, a 60% poultry litter, 40% ground grain mixture can be used as a supplement to the hay, thereby extending the amount of hay that can be used by the beef producer (Heidel, 1996).

Since the moisture content of poultry litter is low, handling, processing and storing litter is relatively easy. Due to the ease of handling litter, it can be transported distances up to 300 km at a price of about $30/ton (Fontenot, 1996). Litter can be stored in a facility with a permanent roof to prevent exposure to the weather; however, if a covered facility is not available, litter can be stockpiled in a bunker and covered with plastic to reduce damage caused by rain. Additionally, if the poultry litter has completed the heating cycle, it can be stored in barrels or bags (Leech, 1996). Thus, poultry litter can be implemented into beef cattle feeding programs as a low-cost feed source that is worth more than it costs.

III. Effects of Feeding High Protein Rations on Reproduction

Importance of Superovulation. Recent developments in the field of reproductive biology have furthered our understanding of ovarian follicular dynamics in cattle. Similar to males, females have a reproductive potential in excess of what is realized by natural mating (D'Occhio et al., 1999). Optimizing the reproductive performance of superior females allows for increased genetic improvement in cattle. Effective utilization of superovulation requires control of the dominant follicle for manipulation of the estrous cycle, thus permitting superovulatory treatments to be initiated at an optimal time to increase follicular growth and embryo production.

Approximately 20 to 30 follicles are stimulated to grow in a series of follicular waves during each estrous cycle (D'Occhio et al., 1999). These follicular waves result from a surge in systemic follicle stimulating hormone (FSH) that recruit follicular growth, and FSH peaks when the largest follicles of the wave emerge at about 4 mm in diameter (Ginther et al., 1997). Following this surge, FSH concentration declines to its nadir and the dominant follicle and largest subordinate follicle grow at comparable rates (Ginther et al., 1996). These two follicles begin to deviate in diameter at the end of the common growth phase (about 8.5 mm in diameter),

and deviation is characterized by continued growth of the largest follicle to become the dominant follicle coupled with a reduction of growth of the second largest follicle to become the largest subordinate follicle (Ginther et al., 1997). The dominate follicle is the first follicle to develop luteinizing hormone (LH) receptors and become LH-dependent, and upon the subsequent LH surge, this LH-dependent follicle will ovulate and result in the emergence of a new follicular wave (Driancourt, 2001). During each follicular wave, the dominant follicle inhibits the continued growth of smaller follicles by increasing inhibin production, which suppresses the release of FSH (Singh and Adams, 1998). However, removal of this dominant follicle allows for a new follicular wave to emerge due to a surge in FSH. Rouillier et al. (1996) reported an increase in the population of follicles recruited following exogenous FSH stimulation when the dominant follicle was removed than when the dominant follicle remained intact. Therefore, removal of the inhibitory effects of the dominant follicle allows for the initiation of superovulatory treatments to promote growth of multiple follicles.

Kim et al. (2001) studied the effects of dominant follicle removal before superovulation on follicular growth, ovulation and embryo production in Holstein cows. Follicular growth during superovulation occurred earlier in the dominant follicle removal (DFR) group than in the dominant follicle intact (control) group due to an increase in the number of medium (6 to 9 mm) and large follicles (≥ 10 mm) on d 1 to 2 and d 3 to 4 of superovulation, respectively. The number of total ova (7.7 ±1.3 vs 3.9 ± 1.0) and transferable embryos (4.6 ± 0.9 vs 2.3 ± 0.8) collected were greater in the DFR group than the control group, respectively. Thus, removal of the dominant follicle prior to the initiation of superovulatory treatment promoted follicular growth and increased ovulations, resulting in a greater number of ova and transferable embryos collected.

A practical method for synchronization of follicular waves eliminates the dominant follicle through mechanical or chemical manipulation (Shaw and Good, 2000). Baracaldo et al. (2000) compared superovulatory responses in cattle when superovulatory treatment followed synchronization of follicular wave emergence. This was accomplished by using cattle at random stages of the estrous cycle, and ablation of the two largest follicles per pair of ovaries, ablation of all follicles ≥ 5 mm or administration of 5 mg estradiol (E_2) plus 100 mg progesterone (P_4) were utilized to synchronize follicular wave emergence. The number of follicles ≥ 8 mm on the day of insemination was not different among groups. In addition, no differences were detected among

groups in the number of unovulated follicles ≥ 8 mm, total ova/embryos, fertilized ova or transferable embryos collected. These results suggest that $E_2 + P_4$ treatment is as effective as either ablation treatment in synchronizing follicular wave emergence and superovulation in cattle. In addition, treatments were administered to cattle at random stages of the estrous cycle and effectively synchronized follicular wave emergence.

Effective utilization of superovulation requires the removal of the dominant follicle. This can be accomplished by physical or chemical manipulation, but administration of $E_2 + P_4$ treatment requires a single injection and has been proven to effectively remove the dominant follicle and its suppressive effects. A new follicular wave then emerges, and superovulatory treatments can then be initiated to optimize follicular growth and the number of embryos that can be recovered at embryo collection.

Effect of Nutrition on Superovulation. The relationship between nutrition and reproduction is complex, and superovulatory responses can be quite variable and inconsistent (Boland et al., 2001). Nutrition of donors used for superovulation may affect embryo production. The type of diet fed prior to superovulation can influence the response to superovulatory treatment and subsequent embryo quality. Ad-libitum concentrate intake reduced the superovulatory response in heifers and negatively influenced the quality of embryos collected following superovulation in one trial (Yaakub et al., 1999). Additionally, heifers offered only 3 kg of concentrate daily produced a greater numbers of transferable embryos than heifers fed ad-libitum concentrate daily (4.8 ± 0.7 vs 2.8 ± 0.4, respectively). These results indicate that high concentrate intake may negatively influence the production of transferable embryos.

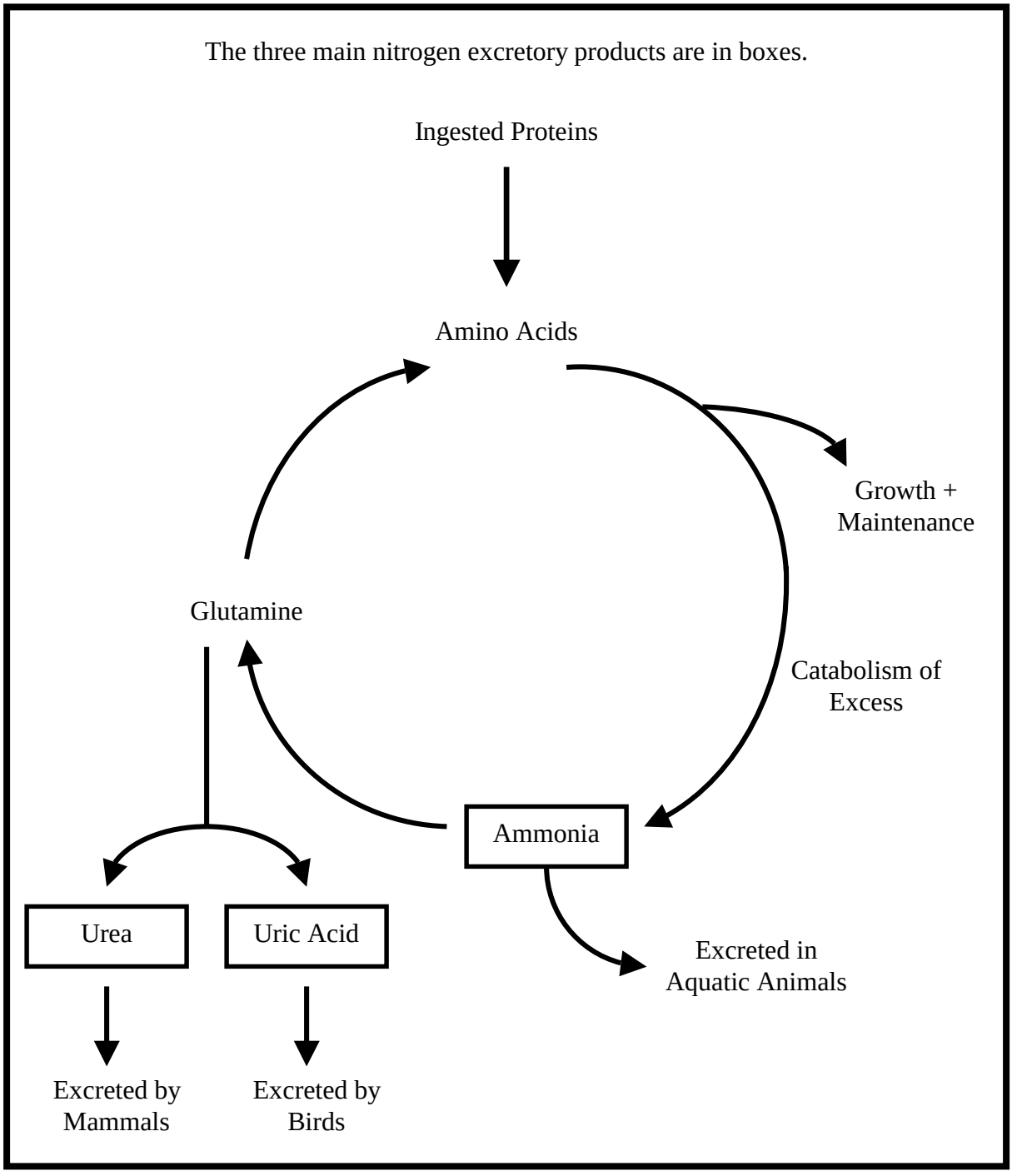
Nolan et al. (1998) reported that follicle numbers after superovulation were greater in heifers offered a low energy diet (9.6 Mcal$\bullet$kg$^{-1}\bullet$hd$^{-1}\bullet$d^{-1}) than in heifers offered a higher energy diet (28.6 Mcal$\bullet$kg$^{-1}\bullet$hd$^{-1}\bullet$d^{-1}; 13.5 ± 2.4 vs 9.6 ± 1.2, respectively). This difference was due to a larger number of follicles 7 to 10 mm in size following eight injections of FSH. However, there was no effect of nutrition on ovulation rate after FSH injections (14.4 ± 1.9 vs 16.3 ± 3.0 for high and low energy diets, respectively). Additionally, the number of embryos recovered was not different between heifers on low (11.3 ± 2.4) or high (10.4 ± 1.3) energy diets. Following 24 h of *in vitro* culture, heifers fed the low energy diet had a higher blastocyst yield and blastocyst cell number as compared to heifers offered the higher energy diet. Therefore, dietary energy intake during superovulation may need to be controlled to optimize the production of quality

embryos (Nolan et al., 1998). Furthermore, because the number of follicles that grow in response to superovulation can be influenced by dietary intake (Boland et al., 2001), it may be difficult to predict the number of follicles that grow and ovulate in response to superovulatory treatment if dietary energy intake is variable.

Effect of High Protein Diets on Reproduction. Animals excrete a variety of nitrogen waste products, mainly ammonia, urea and uric acid. A major determinant of the mode of nitrogen excretion in animals is the availability of water in the environment. A general overview of nitrogen metabolism and excretion in animals is illustrated in Figure 1-1. As animals consume a meal, ingested proteins are hydrolyzed to amino acids that can be used to form new proteins for growth and basic protein turnover. Those amino acids not used in protein production are catabolised, yielding ammonia as a byproduct. The ammonia is either excreted or detoxified in the liver, and converted to urea or uric acid. The environment where the animal lives dictate which nitrogenous byproduct is excreted. For example, aquatic animals use 400 mL of water to dilute 1 gm of ammonia to keep ammonia concentrations below toxic levels. Thus, these animals must live in water, and ammonia is excreted as their major nitrogen waste product. As compared to aquatic animals, mammals excrete either urea or uric acid and require 10 and 50 times less water than ammonia for excretion, respectively (Wright, 1995). In birds, uric acid is the major nitrogenous product that is excreted in its manure. As mentioned earlier, cattle are capable of utilizing the uric acid found in poultry litter (Heidel, 1996), allowing cattle producers to feed poultry litter as an alternative source of CP. However, the level and solubility of dietary protein may influence the reproductive performance in cattle.

Conception and the establishment of pregnancy involve a series of interrelated events of the female reproductive tract: follicular development resulting in ovulation, subsequent fertilization of the oocyte, embryo transport and development, maternal recognition of pregnancy and implantation (Butler, 1998). Various factors may intercede at one or more of these steps that could result in reproductive failure. One multifaceted factor is the effect of nutrition on reproduction (Blanchard et al., 1990). Feeding diets high in CP may result in reduced conception rates in cattle. The amount of rumen degradable protein (RDP) and RUP fed in relation to requirements must also be considered when formulating rations (Butler, 1998). Exceeding recommended amounts of RDP or RUP in the diet may increase concentrations of ammonia, urea or other toxic products, thereby impairing reproductive efficiency.

Figure 1-1. General Overview of Nitrogen Metabolism and Excretion in Animals



Taken from: Wright, 1995

Blanchard et al. (1990) studied the effect of rumen degradability of dietary CP on fertilization rate and embryo quality in lactating Holstein cows. The two diets were formulated

to be isocaloric and isonitrogenous, and each contained 16% CP. However, Diet 1 consisted of 73% RDP while Diet 2 consisted of 64% RDP. Cows were treated to induce superovulation, inseminated and ova/embryos were recovered non-surgically 7 d following breeding. Excellent, good and fair quality embryos were considered to be transferable, whereas, unfertilized ova (UFO) and poor and degenerate quality embryos were considered to be non-transferable. No differences were detected between cows fed the two diets for mean numbers of fertilized, unfertilized, or transferable ova/embryos collected. However, cows fed a lower RDP diet had a greater percentage of fertilized ova recovered than cows fed the high RDP diet (79.2 ± 4.8 vs 54.8 ± 9.7%, respectively). More cows fed the high RDP diet failed to yield any transferable ova than those cows fed the low RDP diet (7/19 vs 1/19, respectively). These results suggest that feeding excess amounts of RDP may result in fertilization failure or poor embryonic development (Blanchard et al., 1990).

In a similar study, conducted by Garcia-Bojalil et al. (1994), non-lactating Holstein cows were randomly assigned to a diet with a CP content of 12.3 or 27.4%. After 35 d on the experimental diets, estrus was synchronized in all cows and superovulatory treatments were initiated. Cows were inseminated upon detected estrus, and embryos were recovered non-surgically 6.5 d after breeding. Cows fed the high CP diet had a greater concentration of plasma urea nitrogen (PUN) than those cows on the low CP diet (21.3 vs 9.8 ± 0.6 mg/dL, respectively). However, no differences were detected in the numbers of normal embryos, abnormal embryos or UFO collected from cows on either dietary treatment. Additionally, the numbers and percentages of transferable and non-transferable embryos were not different between cows on the two diets. The authors concluded that excess intake of CP failed to influence the numbers of embryos produced in non-lactating, Holstein cows.

The differences in the results reported by Blanchard et al. (1990) and Garcia-Bojalil et al. (1994) exemplify the lack of understanding of the effects of high protein intake on reproduction. A possible explanation for differing results may be the difference in the energy status of the cows used in the studies. Blanchard et al. (1990) utilized lactating Holstein cows, whereas, Garcia-Bojalil et al. (1994) used non-lactating Holstein cows. The interaction of increased intake of RDP and energy status during lactation may have affected embryonic development differently in the two studies. Elrod and Butler (1993) fed Holstein heifers a ration that either met (Normal) or exceeded (High) RDP requirements and measured fertilization rate and embryo viability. The

fertilization rate between treatments was similar for the Normal and High diets (32/39 vs 32/41, respectively). However, embryonic mortality after d 20 of pregnancy was observed in 7 of 41 heifers fed High RDP. This embryonic death loss could be due to an altered uterine environment where the embryo was developing. Jordan et al. (1983) reported that diets high in CP altered concentrations of urea in uterine secretions. Therefore, diets with a high CP content, particularly those with a high fraction of RDP, may impair fertilization and embryonic development by altering the uterine environment.

IV. Summary

Poultry litter has a great economic value when used as a feedstuff for cows; however, poultry litter must be processed in order to eliminate harmful pathogens prior to feeding. Feed ingredients in broiler diets and the number of flocks housed on the litter can influence the quality of the litter. Thus, an analysis of the litter should be conducted prior to feeding to ensure that the litter is of acceptable quality. When managed properly by beef producers, poultry litter can be effectively utilized as a protein supplement or hay substitute for cattle. Though the influence of nutrition on reproduction is not well understood, feeding rations that are high in CP content, especially those containing high fractions of RDP may result in a decreased fertilization rate or impaired embryonic development.

V. Rationale for Experiment 1

Researchers have studied the effects of feeding diets high in CP or RDP on reproduction. However, the results are often variable and inconsistent. Several researchers have also analyzed the impact of utilizing poultry litter for growing and finishing cattle; however, the effects of poultry litter on reproduction are not well documented. Since poultry litter is relatively dry and easy to collect and transport, it can be utilized as an inexpensive source of protein for cows. Therefore, the objective of Exp. 1 was to determine the effects of differences in dietary protein on the production and quality of embryos collected from superovulated Angus cows.

B. Embryo Collection and Cryopreservation of Bovine Embryos.

I. Practical Importance of Embryo Collection

Method of Embryo Collection. Commercial bovine embryo transfer (ET) increased during the late 1970s and was spurred on by the high prices and demand for several exotic breeds of cattle. During that time, embryo recoveries involved a mid-ventral surgical technique with the donor placed under general anesthesia. This practice was not widely utilized by the dairy industry because the udder interfered with access to the reproductive tract. Furthermore, complications following surgery sometimes led to loss or impairment of fertility (Hasler, 1992). However, the development of a new technique utilizing Foley catheters allowed practitioners to recover bovine embryos using an efficient, non-traumatic, non-surgical technique. The application of non-surgical embryo recovery resulted in the rapid incorporation of ET in the dairy industry. With continued refinement of embryo collection, ET became an on-farm practice, resulting in a greater than 100% annual increase in the number of ET calves registered in the Holstein breed during the 1980s (Hasler, 1992). Today, the ET industry has grown into an international industry with more than 500,000 embryos recovered and transferred or frozen annually.

The primary goal of ET in cattle has been to increase the number of progeny from valuable females. This provides cattle producers with many opportunities for genetic improvement while increasing marketing options. Typically, embryos are collected 7 d following breeding. A close synchrony must exist between the stage of pregnancy when the embryo is recovered and the stage of the estrous cycle of the recipient at the time of transfer to optimize pregnancy rates. Lester et al. (1999) reported that a higher pregnancy rate (58.2 vs 47.9%) was achieved with a close synchrony between the embryo and recipient (±0 to 12 vs ±12 to 24 h, respectively), but this was not a statistically significant difference. Following the transfer of fresh or frozen-thawed embryos to recipients, Spell et al. (2001) reported a decrease in recipient pregnancy rate from 83% with fresh embryos to 69% with frozen-thawed embryos. Although pregnancy rates are typically higher when transferring fresh embryos, most cattle producers do not have sufficient numbers of cattle for the transfer of fresh embryos to synchronized recipients. Therefore, cryopreservation of bovine embryos has become a crucial element to the ET industry, as more than 50% of cattle embryos are frozen following recovery (Hasler, 2001).

II. Cryopreservation of Bovine Embryos

Fundamental Aspects of Cryopreservation of Bovine Embryos. Pregnancy rates in cattle receiving a frozen-thawed embryo have consistently exceeded 50%. It is important to understand the physiological principles of cryobiology to continue improving these pregnancy rates (Robertson, 2000). In order to avoid cellular damage, cryoprotectants are added to freezing solutions for several reasons: 1) The freezing point of the solution is reduced; 2) They are highly soluble in water; and 3) At high concentrations, they are relatively non-toxic to the embryo. The molecular weight of the cryoprotectant dictates whether it penetrates into the embryo quickly (ethylene glycol (EG) and glycerol (GLY) are examples with molecular weights of 62 and 92, respectively) or is a non-penetrating cryoprotectant (sucrose or galactose that have molecular weights of 344 and 180, respectively). Until recently, GLY was a common cryoprotectant for freezing bovine embryos. However, EG has become a widely used cryoprotectant with pregnancy rates of recipients receiving an EG frozen-thawed embryo being comparable to those of cattle receiving a GLY frozen-thawed embryo. As embryos are frozen, both cryoprotectants (EG and GLY) effectively displace water in the cells of the embryo and stabilize membranes of the embryonic cells.

When embryos are placed in GLY or EG, osmotic changes occur, resulting in the withdrawal of water from the embryo due to the greater extracellular osmolarity. When embryos are placed into a hypertonic solution, water moves across the membrane more quickly than the solute (Leibo, 1986). Consequently, the intracellular contents increase to the osmolarity of the extracellular solution. As the cryoprotectant begins to enter the embryo, water will re-enter the embryo to maintain osmotic equilibrium between intracellular and extracellular solutions until the concentrations of the cryoprotectant are equilibrated inside and outside of the zona pellucida. Typically, embryos are loaded into 0.25 mL plastic straws after being placed in the cryoprotectant and the equilibrium process is completed in the straw.

After the required time for equilibration of the embryo in the cryoprotectant has elapsed (10 min), straws are placed in a freezer chamber at –6 °C. Straws are then seeded and remain at –6 °C for 5 to 10 min. Seeding results in the formation of an ice crystal in a super-cooled solution. Touching the straw with a cold object that has been previously dipped in liquid nitrogen (LN_2,) such as a cotton swab, results in the initial formation of an ice crystal. Typically, EG and GLY constitute 10% of the freezing solution with the remainder consisting of phosphate

buffered saline (PBS) or ovum culture media (OCM; Robertson, 2000). As the PBS or OCM forms ice surrounding the embryo, salt ions are left in the extracellular fluid, resulting in a greater concentration of solute surrounding the embryo, thereby causing more water to be drawn from the embryo. With continued decreasing temperatures, the ion concentration continues to increase due to the freezing of additional water molecules, continuing the dehydration of the embryo. Ultimately, sufficient water is removed from the embryo so that when intracellular ice forms, cell membranes will not be disrupted by the expansion of water molecules inside the embryo (Mazur, 1980). Between –30 to –40 °C, the embryo has shrunk to 60 to 70% of its original volume and is then plunged in LN_2, resulting in the inner cell mass of the embryo freezing due to this rapid plunge. At this temperature range, sufficient water is left to maintain cellular integrity of the embryo, but not enough to cause severe expansion and intracellular damage. Once embryos are plunged in LN_2 (-196 °C), essentially all metabolic activity has ceased, yet the embryo can be preserved with little or no loss of viability post-thaw.

While it is crucial to understand the physiology of the freezing process of bovine embryos, it is equally important to understand the process of thawing embryos. Since embryos actually freeze following the direct plunge into LN_2, they should also be rapidly thawed to prevent recrystallization of small ice crystals inside the embryo (Robertson, 2000). If embryos are frozen in GLY, they must be "washed" to remove the cryoprotectant, because if GLY remains in the embryo, it could be lethal. If these embryos are rapidly diluted in PBS, they will swell as water moves into the embryo to achieve equilibrium. Thus, embryos can be successfully frozen and thawed, but may be killed due to this osmotic change during dilution. Therefore, GLY embryos are often diluted using a stepwise dilution. This results in GLY diffusing from the embryo while the embryo returns to its isotonic volume (Leibo, 1986). Removing GLY from the embryo requires microscopes and other laboratory equipment for handling embryos. On the contrary, EG-frozen embryos can be directly transferred to recipients following thawing. This eliminates the need for laboratory equipment, making this thawing method effective for on-farm utilization. Thus, embryos frozen in GLY or EG can be effectively cryopreserved with little or no change in post-thaw viability.

Importance of Cryopreservation of Bovine Embryos. Cryopreservation of mammalian embryos has become an integral part of ET. In recent years, cryopreservation has become a routine procedure as the birth of offspring from laboratory, domestic and wild species

has resulted from the transfer of cryopreserved embryos to recipient females (Leibo et al., 1996). Development of methods for successful cryopreservation of embryos has expanded the scope of the ET industry. This enables cattle producers to market or purchase genetics from valuable females. In addition, embryos may be frozen for logistical purposes to improve management efficiency (Leibo, 1986). Therefore, ET allows for the widespread distribution of genetics from superior females, and in some cases, a way to enhance reproductive management.

While mammalian spermatozoa were successfully frozen in the 1950s, it was nearly 25 yr later before attempts to freeze mammalian embryos were successful (Mazur, 1980). During this time, researchers determined that the presence of a cryoprotectant enabled embryos to be cooled to –196 °C, maintained in the frozen state and returned to normal temperatures with acceptable pregnancy rates. Early studies utilized GLY as a cryoprotectant for embryo freezing, but to ensure post-thaw viability, it was mandatory that embryos cryopreserved in GLY be "washed" at room temperature immediately after thawing in order to remove the GLY prior to transfer (Arreseigor et al., 1998). Ethylene glycol was discovered to be another effective cryoprotectant for bovine embryos, and embryos frozen in EG could be directly transferred to recipients after thawing (Robertson, 2000). This method was beneficial in that no laboratory equipment was needed for direct transfer of embryos frozen in EG. This finding made freezing embryos in EG an acceptable procedure for utilization under on-farm conditions (Dochi et al., 1998). Embryos frozen in EG were placed in a holding media containing 1.5 M EG (10%), aspirated into straws, seeded at –6 to –7 °C to induce ice formation under controlled conditions, cooled (0.3 to 0.5 °C/min to 32 to 35 °C) and then plunged into LN_2 (Nibart and Humblot, 1997). This procedure was effective in cryopreserving bovine embryos without compromising viability of the embryo.

While most storage methods involve the use of low temperatures (-196 °C), Moore and Bilton (1973) studied the effects of storage on viability of sheep ova cooled to 5 °C. Fertilized ova were collected from mature, medium-wool Merino ewes that were superovulated. Following mating to fertile rams, ova/embryos were removed 2 or 5 d later by flushing the fallopian tubes and uterine horns. Embryos were then cooled to 5 °C at a rate of 0.25 or 1 °C/min and stored for 10 min, 60 min or 6 h before *in vitro* culture for 2 d. The majority of embryos collected on d 2 were at the two-cell stage of development, and after storage, re-warming and culture, 75 of the 103 embryos (73%) were found to be at the eight-cell stage. Additionally, 21 embryos were cultured immediately after collection and not stored at 5 °C (control). Fifteen embryos in the

control group developed to the eight-cell stage (71%). Similarly, with embryos collected on d 5, 57 of 88 embryos stored at 5 °C (65%) and 11 of 16 embryos that had not been stored at 5 °C (69%) developed to blastocysts in culture. Therefore, cooling and storage of d 2 or 5 sheep embryos at 5 °C prior to *in vitro* culture had no effect on subsequent development (Moore and Bilton, 1973).

Pettit (1985) retrospectively analyzed the freezing program of a commercial bovine ET company. Practitioners recorded the time between embryo collection and freezing. Pregnancy rates recorded relative to time between the collection of embryos and the onset of freezing are depicted in Table 1-6. There was a significant difference among the five collection-to-freezing groups for recipient pregnancy rate. Embryos that were frozen 1.5 to 3 h following collection resulted in a 66% pregnancy rate when transferred to recipients, whereas, embryos held in holding media for 5 h or longer prior to freezing resulted in pregnancy rates of less than 50% following transfer to recipients. Wright (1985) evaluated the time from collection to freezing for another commercial ET program. At that time, over 75% of embryo collections for this company were conducted on-farm, but embryos were frozen at a central facility. On average, collection of donors required 1 to 12 h for each farm visit, and the travel time from the farm to the ET facility where embryos were frozen was 1 to 4 h. Therefore, embryos collected on-farm for freezing were held from 2 to 16 h before freezing in the laboratory. Pregnancy rates of recipients receiving an embryo frozen between 2 and 16 h after collection are illustrated in Figure 1-2. These results indicate a marked difference in pregnancy rate due to the increase in the interval from embryo collection to freezing of bovine embryos.

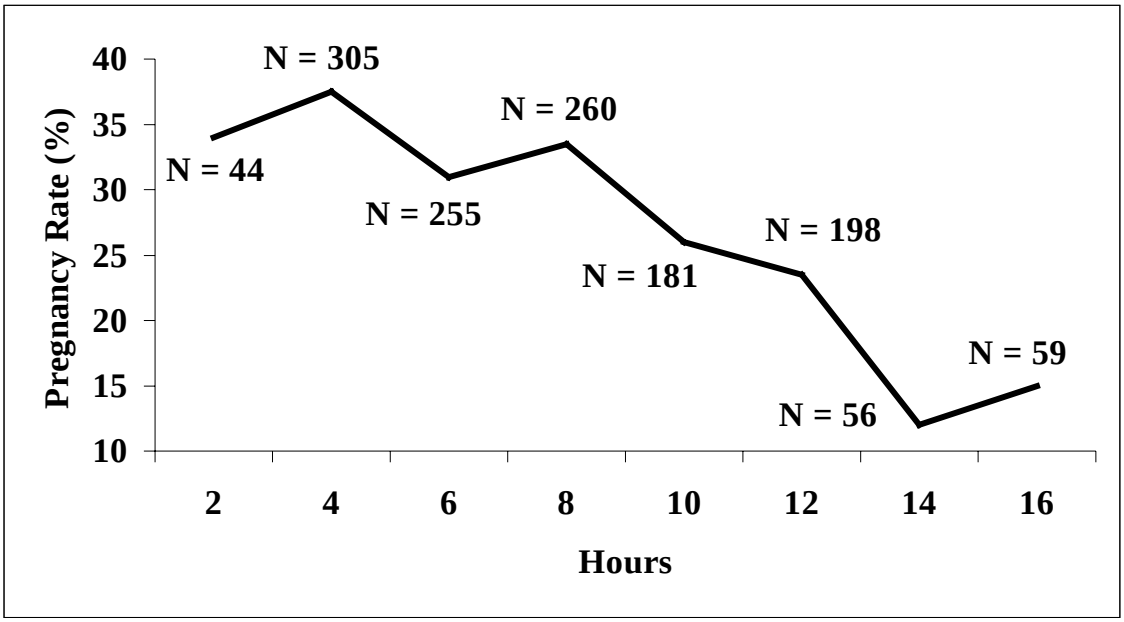
Table 1-6. Pregnancy Rates relative to Time between Embryo Collection and Freezing

Time from Collection to Freezing (h)	Total No. of Embryos Thawed	No. of Pregnancies	Pregnancy Rate (%)[a]
1.5 to 3.0	119	79	66
3.1 to 4.0	166	93	56
4.1 to 5.0	114	57	50
5.1 to 7.0	102	46	45
7.1 to 9.0	28	13	46

Taken from: Pettit, 1985

[a] Difference in pregnancy rate among groups ($P < 0.005$)

Figure 1-2. Pregnancy Rates due to Varying Intervals from Collection to Freezing of Bovine Embryos



Taken from: Wright, 1985

A retrospective analysis of data performed by Hasler (2001) revealed that embryo stage of development and quality grade influenced pregnancy rate following transfer of fresh and frozen embryos to recipient cattle. Evaluation of embryos was conducted using the standards reported by Lindner and Wright (1983). One study included all embryos transferred by personnel at Em Tran, Inc., Elizabethtown, PA, (EmT) and Em Tran-West, Turlock, CA, (EmT-W) from 1987 to 1988 and 1991 to 1992. The effects of fresh and frozen-thawed embryo grade and stage of development on the pregnancy rate of recipients are depicted in Table 1-7. Pregnancy rates following the transfer of Grade 1, 2 or 3 embryos (fresh or frozen) to recipients differed; however, no significant differences in pregnancy rates were detected for fresh or frozen-thawed embryos that differed in their stage of development when transferred to recipients. Another study reported by Hasler (2001) involved embryos frozen at EmT during 1988 to 1995 and exported to Holland Genetics in the Netherlands for transfer to recipients. As shown in Table 1-8, embryo grade affected the pregnancy rate of recipients as the transfer of Grade 1 and 2 embryos resulted in a higher pregnancy rate than the transfer of Grade 3 embryos (57.8, 54.3 and 37.9%, respectively). In this study, the effect of embryo stage was also significant. The

transfer of early blastocysts resulted in a higher pregnancy rate (59.7%) than transfer of morulae or mid-blastocysts (54.3 and 52.1%, respectively).

Table 1-7. Effects of Fresh and Frozen-thawed Embryo Grade and Stage of Development on Pregnancy of Recipients

Embryo Grade	Fresh		Frozen	
	No. Transfers	% Pregnant	No. Transfers	% Pregnant
1	4163	73.2[a]	2482	62.8[a]
2	3156	68.3[b]	2329	56.8[b]
3	1641	56.3[c]	454	43.6[c]
4	61	47.5[c]	22	36.4[b,c]
Embryo Stage				
M	5633	66.9	3576	57.7
EB	1978	70.3	1140	61.2
MB	995	70.9	478	57.9
XB	391	71.4	93	50.5
HB	25	56.0	---	---

Taken from: Hasler, 2001

[a,b,c] Means in columns with different superscripts differ ($P < 0.05$)

M = morula; EB = early blastocyst; MB = mid-blastocyst; XB = expanded blastocyst; HB = hatched blastocyst

Table 1-8. Effect of Embryo Grade and Stage of Development on Pregnancy Rate of Frozen-thawed Embryos

Embryo Grade	No. Transfers	% Pregnant
1	1723	57.8[a]
2	788	54.3[a]
3	468	37.9[b]
Embryo Stage		
M	1997	54.3[c]
EB	494	59.7[d]
MB	257	52.1[c]

Taken from: Hasler, 2001

[a,b,c,d] Means in columns with different superscripts differ (a vs b: $P < 0.001$, c vs d: $P < 0.05$)

M = morula; EB = early blastocyst; MB = mid-blastocyst

The distribution of the number of embryos transferred by stage of development and embryo grade from a study reported by Wright (1981) is depicted in Table 1-9. Wright (1981) revealed that pregnancy rates following transfer of morulae (44%) or advanced morulae (53%) to recipients were not significantly different; however, pregnancy rates following the transfer of morulae to recipients were significantly lower than those following the transfer of embryos in the three blastocysts stages (early blastocyst, blastocyst and advanced blastocysts with a pregnancy rate of 65, 66 and 64%, respectively). Additionally, pregnancy rates were significantly different among cows that received a Grade 1, 2, or 3 embryo (64, 45 and 33%, respectively). Although some variation exists in the results of different trials, it appears that embryo grade and stage of development may influence the pregnancy rate of fresh and frozen-thawed embryos.

Table 1-9. Effect of Embryo Stage and Grade on Pregnancy Rate

Stage of Development	No. Transferred	No. Pregnant	% Pregnant
M	362	158	44[a]
AM	503	269	53[a]
EB	521	339	65[b]
B	531	349	66[b]
AB	369	238	64[b]
Embryo Grade			
1	1748	1122	64[a]
2	438	198	45[b]
3	100	33	33[c]
Total	2286	1353	59

Taken from: Wright, 1981

[a,b,c] Pregnancy rates with different superscripts differ ($P < 0.05$)

M = morula; AM = advanced morula; EB = early blastocyst; B = blastocyst; AB = advanced blastocyst

Even though it took many years to develop techniques that allowed practitioners to successfully freeze bovine embryos, these advancements have made cryopreservation of embryos a common practice. Embryos can be routinely collected from superovulated donors and transferred directly or cryopreserved, depending on the availability of recipients. Embryos frozen in EG can be thawed and transferred directly to recipients, allowing this procedure to be

utilized on-farm without complicated embryo handling procedures. The synchrony of the recipient and the developmental stage of the embryo as well as the quality grade may influence the pregnancy rate of recipients receiving a frozen-thawed embryo.

III. Culture of Bovine Embryos

Following the collection, cryopreservation and thawing of bovine embryos, the best test of embryo viability would be the number of pregnancies that develop after transferring embryos to synchronized recipients. The ultimate determinant of viability would be the birth of live offspring (Wright and Ellington, 1995). However, these tests require a large number of recipients, making this option unfeasible in many experimental situations. Therefore, morphological classification systems have been used to estimate expected pregnancy rates, and *in vitro* culture systems have been devised to test viability of frozen-thawed embryos in research situations.

Morphological evaluations have been developed in an effort to predict the viability of embryos. Examples of these non-invasive, microscopic evaluation criteria include embryo shape, number and compactness of cells, size of the perivitelline space and number of extruded cells (Wright and Ellington, 1995). Lindner and Wright (1983) developed a four-category system to evaluate quality of individual embryos using the following criteria: 1) Excellent – an ideal embryo that is spherical, symmetrical with cells of uniform size, color and texture; 2) Good – embryo with trivial imperfections, such as a few extruded blastomeres, irregular shape, or few vesicles; 3) Fair – embryo with definite but not severe problems, such as the presence of extruded blastomeres, vesiculation, and few degenerated cells; and 4) Poor – embryo with severe problems, having numerous extruded blastomeres, degenerated cells, cells of varying sizes and large, numerous vesicles, but is a viable-appearing embryo mass. These quality grades were correlated with pregnancy rates from a commercial ET center. The relationship between embryo quality and pregnancy rate of recipients are depicted in Table 1-10. No differences were found in pregnancy rate among cows receiving excellent (45%) or good (44%) quality embryos; however, pregnancy rates following the transfer of embryos of these two quality grades to recipients differed from that following transfer of fair or poor quality embryos to recipients (27 and 20%, respectively). These results suggest that there may be more factors involved in determining pregnancy rate than the morphological criteria used to assess embryo quality, as some excellent and good quality embryos did not produce pregnancies, whereas, pregnancy

resulted in some recipients following the transfer of some poor embryos (Lindner and Wright, 1983).

Table 1-10. Effect of Embryo Quality on Pregnancy Rate

Embryo Quality	No. Transferred	No. Pregnant	% Pregnant
Excellent	292	130	45[a]
Good	292	128	44[a]
Fair	149	40	27[b]
Poor	50	10	20[b]

Taken from: Lindner and Wright, 1983

[a,b] Percentages in the same column with different superscripts differ ($P < 0.001$)

While evaluating morphological characteristics of embryos may be an indicator of viability, this is a subjective evaluation. Hence, continued development of embryos in culture has been proposed to be a better indicator of embryo viability. *In-vitro* culture systems allow embryonic development to be monitored microscopically. As a byproduct of the increased interest in developing *in vitro* fertilized (IVF) embryos for ET, many culture systems have been established to enable growth of developing embryos. Many researchers have cultured IVF embryos to the morula or blastocyst stage (O'Doherty et al., 1997; Bavister et al., 1992). However, embryonic development following the blastocyst stage can be sustained in culture until the embryo escapes ("hatches") from the enclosing zona pellucida. This event serves as a valuable developmental endpoint, because not all blastocysts continue development and hatch, perhaps due to developmental incompetence (Bavister, 1995).

An essential component of embryo culture is the type of media used to support development. Rajamahendran et al. (1985) compared *in vitro* development of bovine morulae in Ham's F-10 and Dulbecco's phosphate buffered saline (D-PBS) media supplemented with 10% normal steer serum (SS). Non-lactating Holstein cows (n = 15) were superovulated, and 53 excellent or good quality embryos were recovered and used for *in vitro* culture. Each embryo was randomly assigned to be cultured in Ham's F-10 or D-PBS. Embryo development was recorded at 12 h intervals and assigned a value of 0 to 5 at each inspection based on the following system: 0 = no development (morula); 1 = early blastocyst; 2 = blastocyst; 3 = expanded blastocyst; 4 = hatching blastocyst; and 5 = hatched blastocyst. All embryos developed to the expanded blastocyst stage; however 19 of 27 embryos (70.4%) cultured in

Ham's F-10 continued development to the hatched blastocyst stage, whereas, only 3 of 26 embryos (11.5%) cultured in D-PBS developed to hatched blastocysts. In addition, Canseco et al. (1988) reported that Ham's F-10 media supplemented with SS allowed for increased embryonic development, whereas, supplementing Ham's F-10 with immunoglobulins alone failed to support embryonic growth. These results suggest that Ham's F-10 media supplemented with 10% SS can promote bovine embryonic development to the hatched blastocyst stage of development.

In addition to establishing an effective culture system, embryo density and media volume can influence embryonic development. Canseco et al. (1992) investigated the optimum volume of media/embryo and number of embryos/drop of media needed to support development of early murine embryos to the hatched blastocyst stage. Embryos were grouped into numbers of 1, 5, 10 or 20 and cultured in 5, 10, 20 or 40 μL drops using a long term mouse embryo culture media under silicone oil at 37.5 °C in a humidified atmosphere of 5% CO_2 and 95% air for 120 h. Embryos were evaluated at 24 h intervals and scored 0 to 9 according to the following system: 0 = degenerate; 1 = two-cell; 2 = four-cell; 3 = eight-cell; 4 = compact morula; 5 = early blastocyst; 6 = blastocyst; 7 = expanded blastocyst; 8 = hatching blastocyst; and 9 = hatched blastocyst. The mean development score of mouse embryos cultured in various sized drops of media during the culture period is depicted in Table 1-11. Embryos cultured in 10 μL drops had a higher developmental score (6.1 ± 0.3) than embryos cultured in 20 (4.9 ± 0.3) or 40 μL (4.5 ± 0.3) drops. Developmental scores for embryos cultured in 5 μL drops (5.6 ± 0.3) was similar to those cultured in 10 or 20 μL drops, but was higher than embryos cultured in 40 μL drops. Embryos cultured in groups of 5, 10 or 20 resulted in higher developmental scores than those embryos cultured alone (Table 1-12). The highest development score was achieved when 5 embryos were cultured in 10 μL drops. Similarly, O'Doherty et al. (1997) reported that bovine embryos cultured in groups were more developmentally competent as compared to singly cultured embryos. Thus, embryo numbers and media volume should be controlled to optimize embryo development during *in vitro* culture.

Table 1-11. Mean (± SE) Development Score for Mouse Embryos Cultured in Various Sized Drops over Time

	Culture Drop Volume (μL)			
Time in Culture (h)	**5**	**10**	**20**	**40**
24	1.0 ± 0.03^{c}	1.0 ± 0.03^{c}	1.2 ± 0.03^{b}	1.6 ± 0.03^{a}
48	3.7 ± 0.07^{a}	3.7 ± 0.07^{a}	3.4 ± 0.07^{b}	3.8 ± 0.07^{a}
72	5.1 ± 0.19^{a}	5.4 ± 0.19^{a}	4.7 ± 0.19^{a}	5.0 ± 0.19^{a}
96	5.5 ± 0.23^{a}	5.9 ± 0.23^{a}	5.1 ± 0.23^{a}	5.1 ± 0.23^{a}
120	$5.6 \pm 0.27^{a,b}$	6.1 ± 0.27^{a}	$4.9 \pm 0.27^{b,c}$	4.5 ± 0.27^{c}

Taken from: Canseco et al., 1992

^{a,b,c} Means in the same row with different superscripts differ ($P < 0.01$)

Table 1-12. Mean (± SE) Development Score for Mouse Embryos Cultured in Varying Numbers over Time

	Number of Embryos per Drop			
Time in Culture (h)	**1**	**5**	**10**	**20**
24	1.3 ± 0.03^{a}	1.2 ± 0.03^{a}	1.2 ± 0.03^{a}	1.2 ± 0.03^{a}
48	3.7 ± 0.07^{a}	3.7 ± 0.07^{a}	3.4 ± 0.07^{b}	3.8 ± 0.07^{a}
72	4.8 ± 0.19^{a}	5.3 ± 0.19^{a}	5.1 ± 0.19^{a}	5.1 ± 0.19^{a}
96	4.7 ± 0.24^{b}	5.6 ± 0.24^{a}	$5.5 \pm 0.24^{a,b}$	5.8 ± 0.24^{a}
120	3.8 ± 0.27^{b}	5.5 ± 0.27^{a}	5.6 ± 0.27^{a}	6.2 ± 0.27^{a}

Taken from: Canseco et al., 1992

^{a,b,c} Means in the same row with different superscripts differ ($P < 0.01$)

IV. Summary

Although it took many years to develop methods that could be used to successfully freeze bovine embryos, cryopreservation of embryos has become a common practice. Embryos collected from superovulated donors can be transferred directly or cryopreserved, depending on the availability of recipients. Embryos frozen in EG can be thawed and transferred directly to recipients, making this a practical procedure able to be utilized on-farms. Embryo grade and stage of development may influence the pregnancy rate of recipients receiving a frozen-thawed

embryo; therefore, these morphological features are often used to estimate or predict pregnancy rates following ET.

The ultimate test of embryonic viability is the birth of offspring; however, in many experiments this is not feasible. Thus, various culture systems have been established that are effective in promoting embryonic development. Ham's F-10 media supplemented with a protein source supports embryonic growth through the development of hatched blastocysts. Monitoring development of embryos through the hatched blastocyst stage is an effective test for assessing viability in experimental trials. Factors to consider when designing systems for embryo culture are embryo density and media volume.

V. Rationale for Experiment 2

The development of techniques whereby embryos can be non-surgically collected and cryopreserved has allowed for the continued growth of the ET industry. Typically, embryos are frozen within 2 to 3 h following collection. Studies on the interval from embryo collection to freezing were conducted several decades ago with varying results between and within species. With the incorporation of new techniques and procedures for cryopreserving embryos, it is possible that embryos could remain in holding media for longer periods of time before being frozen following collection. This would enable practitioners to recover embryos from more donors at a time without having to search, clean and freeze embryos that have already been collected. Additionally, this would allow practitioners to freeze embryos once or twice a day rather than at 2 to 3 h intervals. Thus, the objective of Exp. 2 was to determine the effects of the interval from embryo collection to freezing on post-thaw viability when embryos were held at either room temperature or in a refrigerated environment from the time of collection to freezing.

CHAPTER II. EFFECTS OF DIFFERENCES IN DIETARY PROTEIN ON THE PRODUCTION AND QUALITY OF BOVINE EMBRYOS FROM SUPEROVULATED DONORS

A. ABSTRACT

High levels of dietary protein may be detrimental to reproductive performance in cattle. The objective of Exp. 1 was to determine the effects of differences in dietary protein on the production and quality of bovine embryos collected from superovulated donors. Angus cows were randomly assigned to receive one of three experimental diets: a daily ration of 5.7 kg broiler litter, 2.0 kg hay, 3.1 kg corn, and 0.5 kg peanut hulls (LITTER; n = 15); a daily ration of 6.2 kg peanut hulls, 2.2 kg soybean meal, 2.0 kg hay, 0.5 kg corn, and 0.4 kg dicalcium phosphate (SBM; n = 15); or a daily ration of 6.2 kg peanut hulls, 2.0 kg hay, and 3.1 kg corn (CON; n = 19). Diets differed in the amount of total, soluble and degradable protein, but were comparable in energy. After 30 d on the diets, all cows were treated to induce superovulation (28.8 mg FSH/cow, Folltropin®) and synchronize estrus. After the detection of estrus each cow was inseminated with semen from one of four Holstein bulls. Embryos were collected 7 d after estrus and evaluated for quality (according to IETS standards) and stage of development. Prior to treatment to induce superovulation, blood samples were collected 6 h after feeding. Samples were analyzed to assess dietary effects on plasma urea nitrogen (PUN). Mean levels of PUN were higher ($P < 0.01$) in cows fed the LITTER or SBM diet (16.3 mg/dL, LITTER; 21.8 mg/dL, SBM; 9.7 mg/dL, CON) than in cows fed the CON diet. Additionally, concentration of PUN was higher in cows fed SBM than in those fed LITTER ($P < 0.01$). An average of 9.2 transferable embryos (Grade 1, 2 and 3) was collected from each cow and there were no significant differences in the number of transferable embryos collected among groups (9.2, LITTER; 9.3, SBM; 9.1, CON). The number of degenerate embryos or unfertilized ova did not differ among dietary groups. High-protein diets elevated PUN, but did not affect the number or quality of embryos collected from superovulated donors.

B. Materials and Methods – Experiment 1

Animals and Treatments. Forty-nine non-lactating Angus cows, ranging in age from 2 to 9 yr and exhibiting 18- to 24- d estrous cycles, were used as embryo donors and were randomly assigned to receive one of three experimental diets on an as fed basis: a daily ration consisting of 5.7 kg poultry litter, 2.0 kg chopped hay, 3.1 kg ground corn and 0.5 kg peanut-hulls (LITTER; n = 15); a daily ration consisting of 6.2 kg peanut-hulls, 2.2 kg SBM, 2.0 kg chopped hay, 0.5 kg ground corn and 0.4 kg dicalcium phosphate (SBM; n = 15); or a daily ration consisting of 6.2 kg peanut-hulls, 2.0 kg chopped hay and 3.1 kg ground corn (CON; n = 19). Diets differed in the amount of total, soluble and degradable protein, but were comparable in energy content (Table 2-1). Crude and degradable protein levels were higher in the LITTER and SBM diets than in the CON diet; however, the level of soluble protein was higher in the LITTER diet than in the SBM and CON diets. As compared to the protein and TDN requirements for a mature, non-lactating Angus cow (Jurgens, 1997), the CON diet met or exceeded the amount of crude protein (133%) and TDN (118%) needed for maintenance. Additionally, the CON diet met or exceeded the calcium (116%) and phosphorus (100%) maintenance requirements. All diets were formulated so that the calcium:phosphorus ratio was as close to 1:1 as possible. For the duration of the experiment, donors were housed in a dry lot. Donors were fed their respective diets for 30 d prior to the initiation of estrus synchronization and superovulatory treatments. Donors were weighed at the beginning of feeding, at the time of estrus synchronization and prior to embryo recovery to determine if changes in body weight occurred during the feeding period.

Table 2-1. Nutrient Composition of the Three Diets used in Experiment 1 (dry matter basis)

Feed	CP (%)	Soluble CP (%)	Degradable CP (%)	TDN (%)	Ca (%)	P (%)
CON	9.2	29.0	49.9	57.5	0.22	0.19
SBM	17.9	27.9	64.8	55.1	1.05	0.93
LITTER	16.4	78.0	71.1	65.6	1.28	1.09

Synchronization and Superovulation. Cows were fitted with a controlled internal drug-releasing (CIDR) device (Vetrepharm Canada Inc., Ontario, Canada) containing 1.9 g of P_4 on d 0 (Figure 2-1). Coincident with CIDR insertion, each cow received an intramuscular injection

containing 50 mg of P_4 and 2.5 mg of E_2. Injections of FSH (Folltropin-V®, Vetrepharm Canada Inc.) containing 28.8 mg equivalent of NIH-FSH-PI were administered in decreasing doses at 12 h intervals over 4-d beginning on the evening of d 4 and ending the morning of d 8 when the CIDR was removed (Table 2-2). Lutalyse® (Pharmacia, Upjohn, Kalamazoo, MI) was injected on the evening of d 7 (37.5 mg) and the morning of d 8 (25 mg). Cows were fitted with Heat Watch transmitters (DDX, Inc., Denver, CO) to determine the time of estrus. After detection of estrus, each cow was bred twice via artificial insemination (AI; +12 h with two units of semen and +18 h with one unit) by one of two technicians using semen from one of four Holstein bulls. Technicians and semen from each of the four bulls were randomly assigned to donors across treatments.

Figure 2-1. Diagram of the estrus synchronization and AI protocol for donor cows

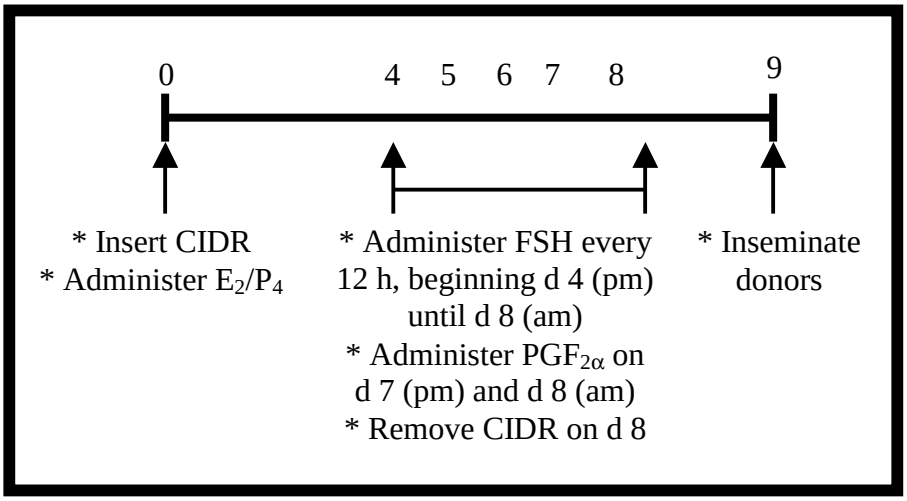


Table 2-2. Injection Scheme for FSH Administration (mg equivalent of NIH-FSH-PI)

	Day of Estrus Synchronization Treatment							
	4	**5**		**6**		**7**		**8**
Time	**PM**	**AM**	**PM**	**AM**	**PM**	**AM**	**PM**	**AM**
FSH Dose	4.8	4.8	4.0	4.0	3.2	3.2	2.4	2.4

Embryo Collection. Embryos were collected non-surgically 7 d after AI by one technician, and these collections occurred on one of 4 consecutive days. The order and day of

collection was randomized across treatments. Embryos were flushed from the uterus using approximately 400 mL of Emcare™, a buffered saline solution containing 0.4% albumin and Kanamycin Sulphate (25 mg/L; Immuno-Chemical Products, Ltd., Auckland, NZ). Embryo flushing was performed using intermittent gravity flow through a two-way, round-tip Foley catheter introduced through the cervix. The recovered effluent was filtered through a 75-μm embryo filter (PETS, Inc., Canton, TX) and washed into a flat search dish (PETS, Inc.). Embryos were washed four times in holding media (OCM; ECHM-500, Immuno-Chemical Products, Ltd.) before being presented for embryo evaluation.

Embryo Evaluation. Embryos were evaluated with an Olympus SZH-ILLK stereomicroscope (Olympus Optical Co., Ltd., Japan; 10 to 70x) by one technician. Immediately after collection, one of nine developmental stages (Table 2-3) was assigned to each embryo (Stage 1 to Stage 9) based on the International Embryo Transfer Society (IETS) guidelines (1998). In addition, each embryo was assigned one of three quality grades (Grade 1 to Grade 3; Table 2-4) or was classified as a degenerate embryo or UFO according to IETS guidelines (1998).

Blood Collection and Analysis. Blood samples were collected via venipuncture of the coccygeal vein at the start of estrus synchronization (d 0) and prior to the recovery of embryos (d 15) with sample collections occurring 2 h prior to feeding and 6 h after feeding on each day. Samples were collected in heparinized evacuated tubes, centrifuged to collect plasma and stored at –20 °C until assayed for relative concentrations of PUN, total plasma protein (TPP) and plasma glucose (PGL). Each assay was performed using a SYNCHRON CX SYSTEMS CS MULTI Calibrator (Beckman Instruments, Inc., Brea, CA). Concentration of PUN was measured following the procedures of Talke and Schubert (1965) and Tiffany et al. (1972) with a coefficient of variation of 1.06%. Concentration of TPP was measured following the method of Hiller et al. (1976) with a coefficient of variation of 0.64%. Concentration of PGL was measured following the hexokinase method (Beckman Instruments, Inc., 1995) with a coefficient of variation of 1.11%.

Statistical Analysis. Differences in body weight at the beginning of feeding, at the time of estrus synchronization and prior to embryo recovery as well as the number of embryos collected, embryo quality grades and concentrations of PUN, TPP and PGL were analyzed by analysis of variance using the MIXED model procedure of SAS (2001). Analysis of embryo traits (embryos collected, embryo quality grade and stage of development) was performed with diet and date of embryo collection as main effects. Embryos were collected on four consecutive days, and date of embryo collection was used as a blocking factor in assigning cows from each treatment a collection date. Body weight and levels of PUN, TPP and PGL were analyzed with diet as the only main effect. Variation in pre- and post-feeding mean concentrations of PUN, TPP and PGL among groups fed the CON, SBM or LITTER diets were determined using Tukey's least square means analysis. Each analysis of variance table for Exp. 1 can be found in Appendix A.

Table 2-3. Classification of Bovine Embryos based on Stage of Development

Name	IETS Stage	Description
Ovum	1	Unfertilized.
2-12 Cell	2	Fertilized ovum, but is less than 16 cells.
Early Morula	3	Contains 16 or more cells that are distinct individuals and have not coalesced.
Compact Morula	4	Cells have coalesced to form a compact mass.
Early Blastocyst	5	Embryo has formed a small blastocoele up to a blastocoele that half-fills the embryonic mass.
Blastocyst	6	Blastocoele is highly prominent but not enough to fill the zona pellucida and begin stretching the zona.
Expanded Blastocyst	7	Overall diameter of embryo increases, with a thinning of the zona pellucida.
Hatched Blastocyst	8	Undergoing the process of hatching or may have completely shed the zona pellucida.
Hatched Expanding Blastocyst	9	This is a Stage 8 embryo that has re-expanded.

Table 2-4. Classification of Bovine Embryos based on Quality

Grade	Quality	Description
1	Excellent to Good	Symmetrical and spherical mass with blastomeres that are uniform in size, color, and density. At least 85% of the cellular material is in an intact, viable embryo mass.
2	Fair	Moderate irregularities in shape of the embryo mass, or size, color, and density of individual cells. At least 50% of the cellular material should be intact, viable embryo mass.
3	Poor	Major irregularities in shape of the embryo mass, or size, color, and density of individual cells. At least 25% of the cellular material should be intact, viable embryo mass.
DEG	Degenerate	Degenerating or dead embryos with less than 25% of the total cellular material in one viable mass.
UFO	Unfertilized	Oocytes that were not fertilized at insemination.

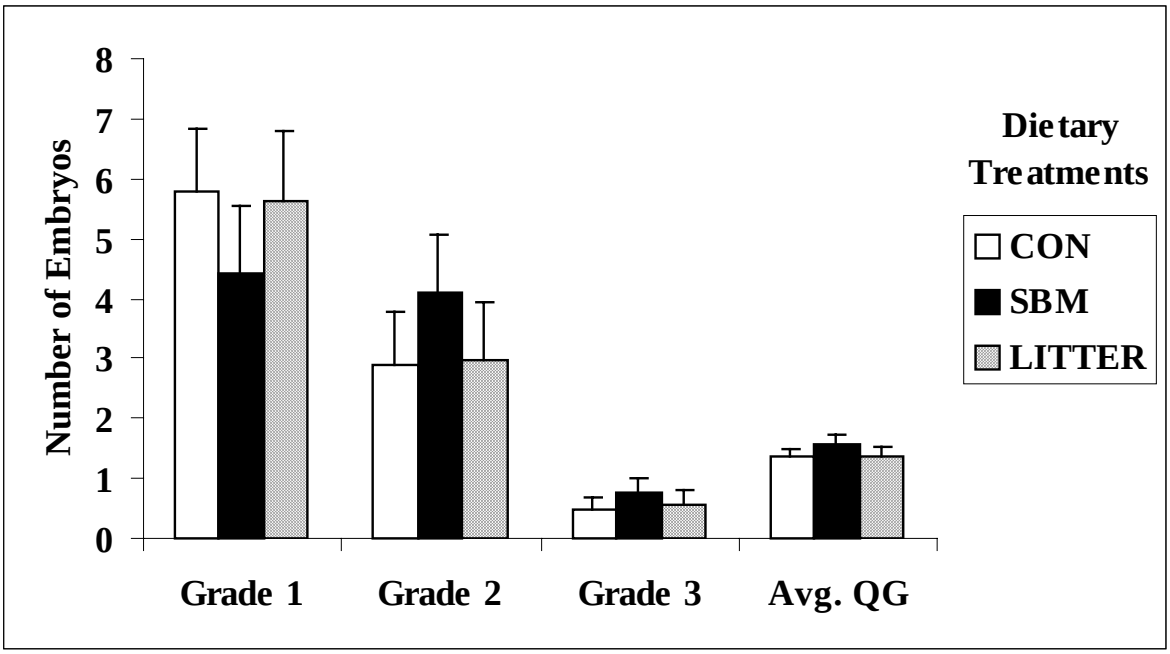
C. Results

Changes in Body Weight. No differences were detected for weight changes among cows assigned to one of the three diets. Mean weights recorded at the initiation of feeding for cows fed the CON, SBM or LITTER diet were 595.8 ± 20.6, 600.6 ± 23.1 and 569.6 ± 23.1 kg, respectively. The final weight, taken prior to embryo recovery, for cows on the CON, SBM or LITTER diet were 597.2 ± 20.7, 599.5 ± 23.3 and 581.4 ± 23.3 kg, respectively.

Superovulation and Embryo Quality. One donor from each treatment group failed to produce embryos, and their data was not included in the statistical analysis. There were no significant differences in the mean number of ova/embryos recovered from donors that were assigned to one of the three diets (17.7 ± 1.9, 17.9 ± 2.1 and 21.9 ± 2.2 ova/embryos collected from cows fed the CON, SBM and LITTER diet, respectively). In addition, no differences were detected among donors assigned to the CON, SBM or LITTER diet for the number of Stage 4 (4.1 ± 1.0, 7.0 ± 1.1 and 4.7 ± 1.1, respectively), Stage 5 (4.9 ± 1.1, 1.9 ± 1.2 and 3.8 ± 1.3, respectively) or Stage 6 embryos recovered (0.1 ± 0.1, 0.0 ± 0.1 and 0.4 ± 0.2, respectively).

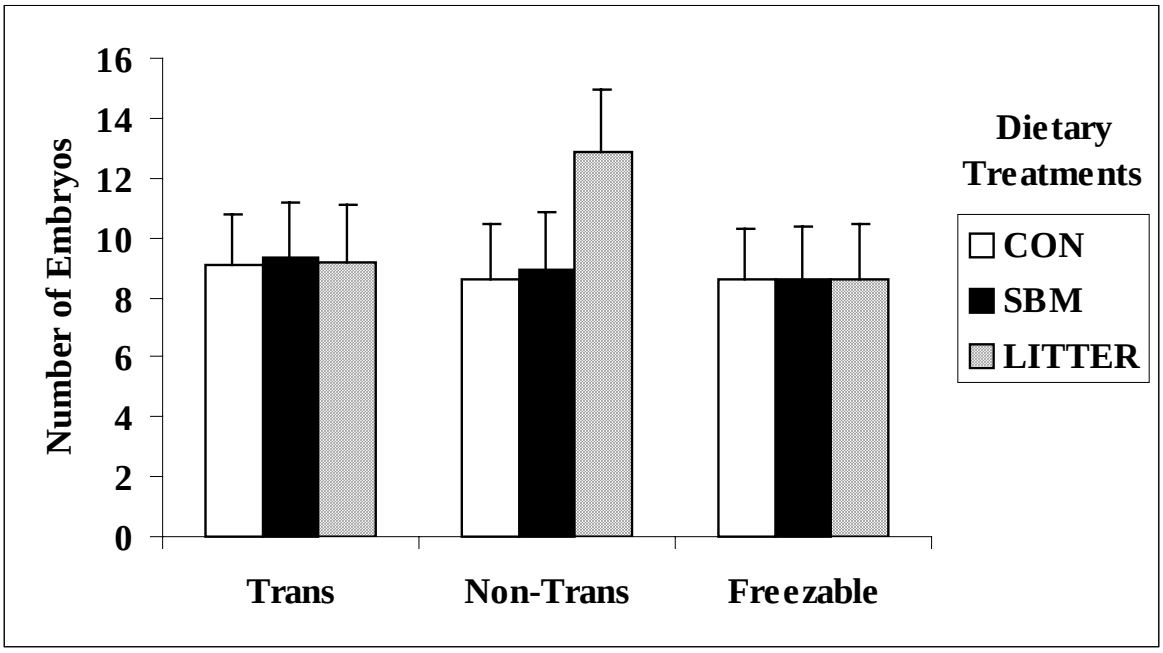
The average number of Grade 1, 2 and 3 embryos collected, as well as the average quality grade for embryos collected from each cow that received the CON, SBM or LITTER diet, is shown in Figure 2-2. Average quality grade (Avg. QG) of embryos collected from each cow was determined using the following formula: Avg. QG = ((No. Grade 1 embryos ∗ 1) + (No. Grade 2 embryos ∗ 2) + (No. Grade 3 embryos ∗ 3)) / Total embryos collected from each cow. No differences were detected among groups of donors fed the three experimental diets for the number of Grade 1, 2 or 3 embryos recovered or in the Avg. QG of embryos collected. In addition, there were no differences among cows receiving the CON, SBM or LITTER diet for the number of degenerate embryos (1.4 ± 0.7, 2.2 ± 0.7 and 2.2 ± 0.7, respectively) or UFO collected/donor (7.2 ± 1.8, 6.8 ± 2.0 and 10.8 ± 2.1, respectively. Finally, no differences were detected among cows fed one of the three diets for the number of embryos considered transferable (Grade 1 + Grade 2 + Grade 3), non-transferable (degenerate embryos + UFO) or freezable (Grade 1 + Grade 2; Figure 2-3).

Figure 2-2. Average Numbers (± SE) of Grade 1, 2 and 3 Embryos and Average Quality Grade* for Dietary Treatments



* Average Quality Grade (Avg. QG) = ((No. Grade 1 embryos ∗ 1) + (No. Grade 2 embryos ∗ 2) + (No. Grade 3 embryos ∗ 3)) / Total embryos collected for each cow

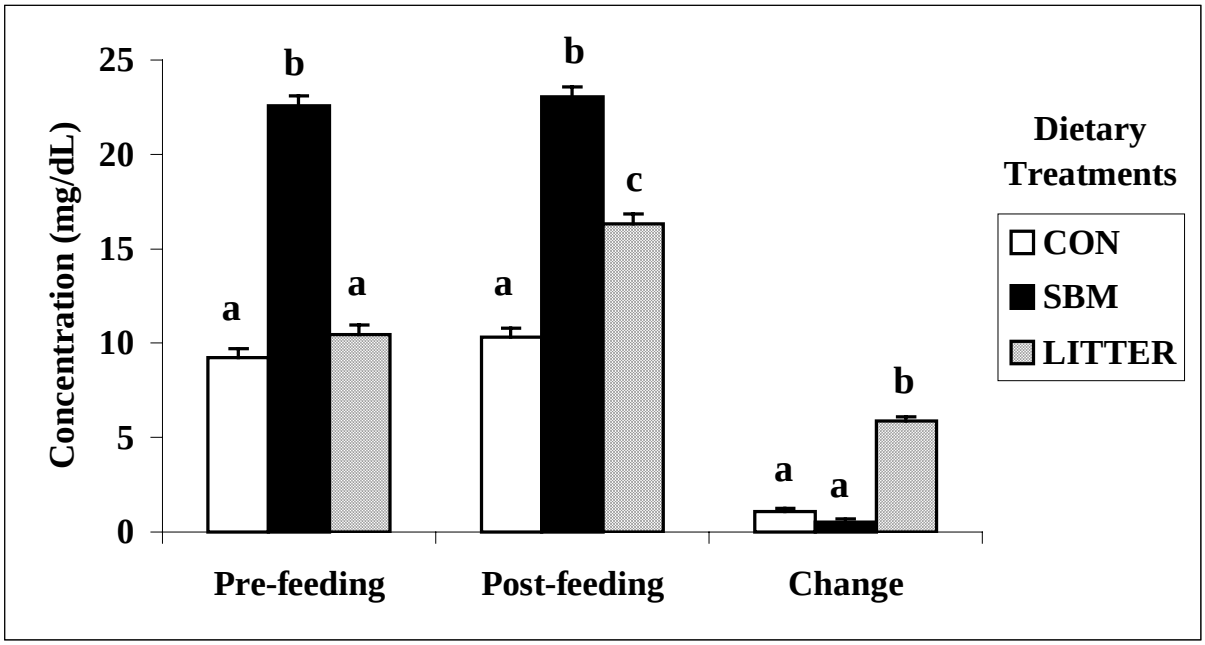
Figure 2-3. Average Numbers (± SE) of Transferable, Non-Transferable and Freezable Embryos* for Dietary Treatments



* Transferable = Grade 1 + Grade 2 + Grade 3 embryos; Non-Transferable = degenerate embryos + UFO; Freezable = Grade 1 + Grade 2 embryos

Blood Analysis. Blood samples were collected on the day of estrus synchronization and prior to embryo recovery for analysis of PUN, TPP and PGL concentrations. The concentration of PUN pre- and post-feeding, as well as the change in PUN concentration on the day of estrus synchronization, is depicted in Figure 2-4. Concentration of PUN was significantly different for blood samples collected from cows that received the CON, SBM or LITTER diet prior to feeding (9.3 ± 0.5, 22.6 ± 0.5 and 10.4 ± 0.5 mg/dL, respectively) as well as after feeding (10.3 ± 0.5, 23.1 ± 0.5 and 16.4 ± 0.5 mg/dL, respectively). Prior to feeding, only cows fed the SBM diet had elevated PUN levels. At 6 h after feeding, however, the concentration of PUN was higher in cows fed either the SBM or LITTER diet. The mean change in PUN concentration that occurred following the daily feeding was greater ($P < 0.001$) for cows fed the LITTER diet (+5.9 ± 0.2 mg/dL) than for cows fed the SBM or CON diet (+0.5 ± 0.2 and +1.1 ± 0.2 mg/dL, respectively).

Figure 2-4. Means (± SE) and Change in Concentration of PUN at Estrus Synchronization for Cows receiving the Dietary Treatments

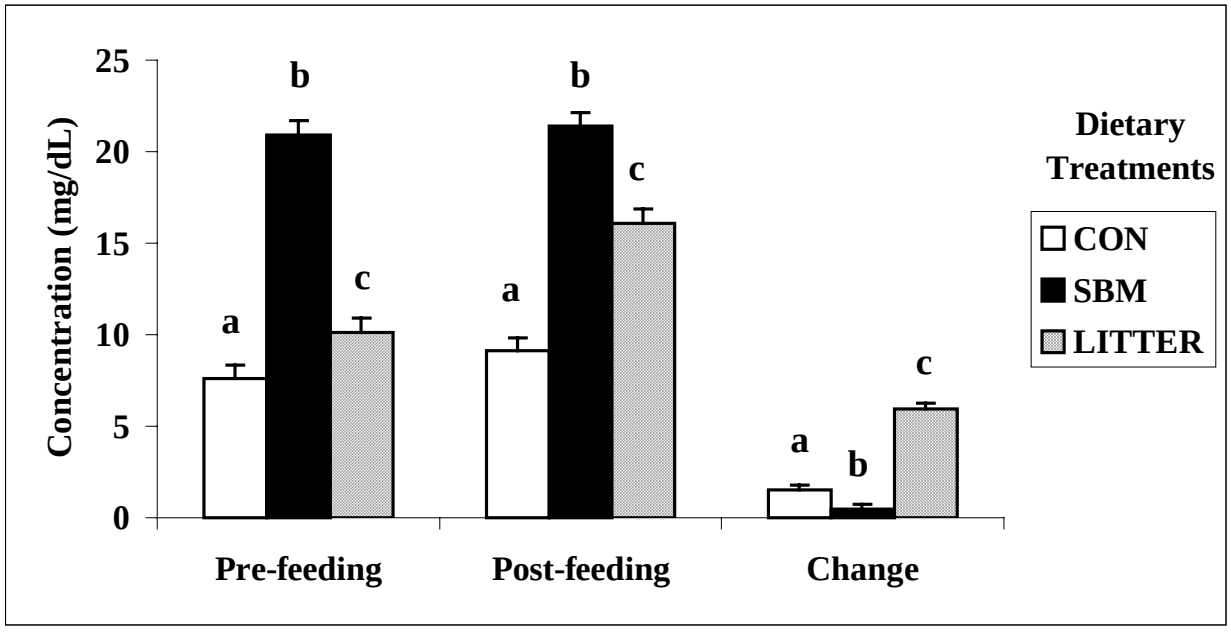


[a,b,c] Means within category (Pre-feeding, Post-feeding, Change) with different superscripts differ ($P < 0.001$)

The concentration of PUN pre- and post-feeding and changes in PUN concentration from before until after feeding were determined for a second time prior to embryo recovery. The concentration of PUN for blood samples collected from donors prior to embryo recovery were

similar to those recorded earlier in the experiment and are depicted in Figure 2-5. Concentrations of PUN were different ($P < 0.001$) among cows fed one of the three diets prior to feeding (CON, SBM or LITTER with 7.6 ± 0.7, 20.9 ± 0.8 and 10.1 ± 0.8 mg/dL, respectively) as well as 6 h after feeding ($P < 0.001$; CON, SBM or LITTER with 9.2 ± 0.7, 21.4 ± 0.8 and 16.1 ± 0.8 mg/dL, respectively). The PUN concentration for cows receiving the SBM diet was highest before and after feeding. Blood samples collected from cows fed the LITTER diet had higher PUN levels than cows fed the CON diet. In addition, the pre- and post-feeding change in PUN concentration was greatest for cows on the LITTER diet ($P < 0.001$; $+6.0 \pm 0.3$ mg/dL) and lowest among cows fed the SBM diet ($+0.5 \pm 0.3$ mg/dL).

Figure 2-5. Means (± SE) and Change in Concentration of PUN Prior to Embryo Recovery for Cows receiving the Dietary Treatments



[a,b,c] Means within category (Pre-feeding, Post-feeding, Change) with different superscripts differ ($P < 0.05$)

Changes in the concentration of TPP on the day of estrus synchronization and prior to embryo collection are presented in Table 2-5. Significant differences in mean plasma protein levels were detected in blood collected before feeding for cows receiving the CON, SBM or LITTER diet (6.8 ± 0.1, 7.2 ± 0.1 and 6.6 ± 0.1 g/dL, respectively). Additionally, significant differences for mean TPP after feeding were detected for cows fed the CON, SBM or LITTER

diet (6.8 ± 0.1, 7.1 ± 0.1 and 6.8 ± 0.1 g/dL, respectively). Cows fed the SBM diet had higher TPP levels before and after feeding than cows fed either the CON or LITTER diet. Differences ($P < 0.05$) for the mean change in TPP were detected between cows fed the LITTER or SBM diet (+0.2 ± 0.1 and –0.1 ± 0.1 g/dL, respectively).

Table 2-5. Means (± SE) and Concentration (g/dL) of TPP Pre- and Post-Feeding at Estrus Synchronization and Prior to Embryo Recovery

	Dietary Treatments		
At Estrus Synchronization	**CON**	**SBM**	**LITTER**
Pre-feeding	6.8 ± 0.1^{a}	7.2 ± 0.1^{b}	6.6 ± 0.1^{a}
Post-feeding	6.8 ± 0.1^{a}	7.1 ± 0.1^{b}	6.8 ± 0.1^{a}
Change	0.0 ± 0.1	-0.1 ± 0.1^{a}	$+0.2 \pm 0.1^{b}$
Prior to Embryo Recovery	**CON**	**SBM**	**LITTER**
Pre-feeding	6.7 ± 0.1^{a}	7.2 ± 0.1^{b}	6.6 ± 0.1^{a}
Post-feeding	6.9 ± 0.1^{a}	7.3 ± 0.1^{b}	6.7 ± 0.1^{a}
Change	+0.2 ± 0.1	+0.1 ± 0.1	+0.1 ± 0.1

[a,b,c] Means within rows with different superscripts differ ($P < 0.05$)

For the mean concentration of TPP before feeding prior to embryo recovery, differences were found ($P < 0.001$) for cows fed the CON, SBM or LITTER diet (6.7 ± 0.1, 7.2 ± 0.1 and 6.6 ± 0.1 g/dL, respectively). The mean concentration of plasma protein at 6 h following feeding differed ($P < 0.001$) for cows assigned to the CON, SBM or LITTER diet (6.9 ± 0.1, 7.3 ± 0.1 and 6.7 ± 0.1 g/dL, respectively). Similar to the mean concentration of TPP collected at estrus synchronization, cows fed the SBM diet had elevated levels of TPP than cows that received either the CON or LITTER diet. However, no differences were detected for the mean change in TPP for cows fed the CON, SBM or LITTER diet (+0.2 ± 0.1, +0.1 ± 0.1 and +0.1 ± 0.1 g/dL, respectively) in blood samples collected prior to embryo recovery.

Means and changes in the concentration of PGL on the day of estrus synchronization and prior to embryo collection are presented in Table 2-6. Differences among cows that received the CON, SBM or LITTER diet were found for PGL before feeding ($P < 0.05$; 62.3 ± 0.8, 65.9 ± 0.9 and 62.5 ± 0.9 mg/dL, respectively). No differences in the mean concentration of PGL were

noted among cows fed the CON, SBM or LITTER diet after feeding (61.2 ± 1.3, 61.7 ± 1.5 and 64.2 ± 1.5 mg/dL, respectively). Cows assigned to the SBM diet had elevated levels of PGL prior to feeding; however, cows fed the LITTER diet had higher levels of PGL after feeding than cows that received the CON or SBM diet. The mean change in PGL following feeding differed ($P < 0.05$) between cows on the LITTER or SBM diet (+1.7 ± 1.7 and –4.2 ± 1.3 mg/dL, respectively), but not for cows on the CON diet (-1.1 ± 1.2 mg/dL) for blood samples collected on the day of estrus synchronization.

Table 2-6. Means (± SE) and Concentration of PGL (mg/dL) Pre- and Post-Feeding at Estrus Synchronization and Prior to Embryo Recovery

	Dietary Treatments		
At Estrus Synchronization	**CON**	**SBM**	**LITTER**
Pre-feeding	62.3 ± 0.8[a]	65.9 ± 0.9[b]	62.5 ± 0.9[a]
Post-feeding	61.2 ± 1.3	61.7 ± 1.5	64.2 ± 1.5
Change	-1.1 ± 1.2	-4.2 ± 1.3[a]	1.7 ± 1.7[b]
Prior to Embryo Recovery	**CON**	**SBM**	**LITTER**
Pre-feeding	62.9 ± 1.0[a]	66.3 ± 1.1[b]	65.0 ± 1.1
Post-feeding	61.8 ± 0.9	63.8 ± 1.0	63.8 ± 1.0
Change	-1.1 ± 1.1	-2.5 ± 1.2	-1.2 ± 1.2

[a,b] Means within rows with different superscripts differ ($P < 0.05$)

Mean concentration of PGL prior to feeding differed ($P < 0.05$) among cows fed the CON, SBM or LITTER diet prior to embryo recovery (62.9 ± 1.0, 66.3 ± 1.1 and 65.0 ± 1.1 mg/dL, respectively). For the mean concentration of PGL collected 6-h following feeding, no differences were found among cows fed the CON, SBM or LITTER diet (61.8 ± 0.9, 63.8 ± 1.0 and 63.8 ± 1.0 mg/dL, respectively). Prior to embryo recovery, cows fed either the SBM or LITTER diet had higher levels of PGL than cows assigned to the CON diet. However, the magnitude of this difference was reduced following feeding. No differences were detected among cows fed the CON, SBM or LITTER diet in the mean change in PGL (-1.1 ± 1.1, -2.5 ± 1.2 and -1.2 ± 1.2 mg/dL, respectively) for samples collected prior to embryo collection.

D. Discussion

The impact of nutrition on reproduction is a complex matter, and one area where nutrition can greatly influence reproduction is at the level of embryo production. The nutritional intake of each donor must be taken into consideration to optimize responses to superovulation and embryo production. Researchers have studied reproductive responses when various levels of CP and RDP have been incorporated into cow and heifer diets (Blanchard et al., 1990; Garcia-Bojalil et al., 1994). Results from these studies have varied, and this may be attributed to the energy status of the animals during the experimental period. Additionally, various nutrition-related factors could have interceded during follicular growth, fertilization or embryonic development that could reduce fertility or impair embryonic growth (Butler, 1998). The purpose of Exp. 1 was to determine the effects of differences in the amount and source of dietary protein on the production and quality of embryos recovered from superovulated donors. Differences in dietary protein intake and nitrogen metabolism were accomplished by feeding poultry litter or SBM as the main source of dietary CP and comparing the physiological response of the cows to those fed a low-protein diet. Specifically, the goal was to evaluate the effects of diets differing in the amount of CP and protein solubility on the number and quality of embryos recovered following superovulation of non-lactating Angus cows.

In the current study, an average of 9.2 transferable embryos (Grade 1 + Grade 2 + Grade 3) were collected from each donor. In the past, our laboratory has averaged 8.5 transferable embryos/collection from superovulated Angus cows. The average percentage of UFO recovered from donors in each of the three groups ranged from 38 to 49%. Lerner et al. (1986) reported that 37% of the ova collected from 476 Holstein cows that were superovulated and flushed in a commercial ET facility were unfertilized. In a follow-up study Hasler (1992) indicated an additional 150 donors superovulated multiple times yielded 11,537 ova, 36% of which were unfertilized. Therefore, the numbers of embryos collected and the rate of UFO recorded in this study are comparable to those of previous reports.

Collection and evaluation of embryos in the current study revealed no differences in the total numbers of ova/embryos recovered among cows in the three dietary treatments. In addition, there were no differences detected for embryonic stage of development, Avg. QG, number of degenerate embryos or UFO recovered per donor in this experiment. Furthermore, no differences were observed among cows in the dietary treatments in numbers of transferable or

freezable embryos collected. These results are similar to those of Garcia-Bojalil et al. (1994) who reported that non-lactating Holstein cows fed diets with a CP content of 12.3 or 27.4% produced comparable numbers of normal or abnormal embryos and UFO. Additionally, the numbers and percentages of transferable and non-transferable embryos recovered were not different between the two CP dietary treatments. Therefore, when combined with the results of other research, the current study indicates that the intake of different amounts and types of dietary protein does not influence the numbers or quality of embryos recovered from non-lactating Angus cows following superovulation. The effects of the three dietary treatments on subsequent embryo viability and developmental capacity following *in vitro* culture are discussed in Chapter III.

Feeding a diet containing 50% poultry litter was as effective as feeding an isonitrogenous diet containing SBM or a lower-protein diet with similar energy density for maintaining the weight of the donors and in optimizing the number of quality embryos recovered following superovulation of non-lactating Angus cows. Over the past several decades, researchers have determined that feeding poultry litter to beef cattle can have a beneficial effect on growth and development. Fontenot (1991) stated that poultry litter could be fed to cattle as an inexpensive source of protein while enhancing environmental quality. Since most poultry litter can be recovered during the clean-out period of poultry houses and can be economically transported over long distances, litter can be a practical addition to a cow nutrition program. Most studies investigating the effects of feeding poultry litter to cattle have focused on the impact on growth and development or evaluated the ability to maintain cow weight and productivity when litter was fed to cows as a substitute for hay. This study demonstrates that cattle producers can feed poultry litter to cows without incurring negative effects on embryo production or embryo quality.

Although no differences were detected in embryo numbers or the quality of these embryos at recovery, metabolic differences were observed among cows fed the LITTER, SBM or CON diet. Differences in the concentrations of PUN, TPP and PGL were recorded during the pre- and post-feeding periods. Both the SBM and LITTER diets were formulated to provide a level of protein to the cows that was in excess of that required for maintenance. In addition, fractions of soluble and degradable CP were greater in the LITTER diet than for the SBM and CON diets. Prior to feeding, the concentration of PUN for blood samples collected from cows fed the SBM diet was significantly higher than samples collected from cows assigned to the

LITTER or CON diet. The elevated nitrogen levels for cows fed the SBM diet could be attributed to an excess of protein supplied in the SBM diet.

As a meal is consumed, proteins are partitioned into soluble or insoluble pools in rumen fluid and into pools that are degradable or undegradable by rumen micro-organisms. Generally, proteins soluble in the rumen are more rapidly and completely degradable than those proteins that are insoluble. Proteins can be broken down into amino acids, and this process could have occurred in a number of ways. Degradable protein could have been broken down into peptide chains, amino acids or microbial protein, and the amino acids and microbial protein could be converted into ammonia. This ammonia could then be absorbed across the rumen wall and detoxified in the liver to urea that is then excreted in the urine. However, ammonia not absorbed across the rumen wall and amino acids not converted into ammonia could have been transformed into microbial protein for utilization in the small intestine. This microbial protein could then be broken down to form amino acids in the small intestine, with a fraction of these amino acids being converted to ammonia and detoxified to form urea by the liver. In contrast, some amino acids in the small intestine could be utilized to repair or maintain body and reproductive tract tissues. Undegradable protein in the rumen could also be broken down in the small intestine to amino acids that are used for tissue maintenance and repair. Protein that is not digestible would then be excreted in the feces (Chalupa, 1984).

Since the SBM diet fed in this experiment contained less soluble CP than the LITTER diet, more CP may have escaped degradation by rumen micro-organisms in cows fed the SBM diet, thus being degraded into amino acids in the small intestine. Prior to feeding, cows fed the SBM diet had a higher PUN concentration than cows fed the LITTER or CON diet, and this could have been due to the SBM diet being less soluble and degradable in CP than the other two diets. Following feeding, the change in PUN concentration was significantly greater for cows receiving the LITTER diet than cows fed the SBM or CON diet. Smith (1973) cited a study by Looper and Stallcup (1958) that demonstrated that ammonia was released from poultry litter at nearly the same rate as urea when incubated in ruminal fluid. Blood samples collected from cows fed the SBM diet before and after feeding had a higher PUN concentration than cows fed the CON or LITTER diet; however, the change in PUN concentration between feeding was greater for cows fed the LITTER diet.

Concentrations of TPP were higher in blood collected from cows assigned to the SBM diet than for cows fed the LITTER or CON diet. The higher TPP in samples collected from cows assigned to the SBM diet may be attributed to an excess of amino acids supplied in the diet as well as the reduced solubility of CP. Concentrations of TPP were comparable in blood samples collected from cows fed the LITTER or CON diet, indicating that the LITTER diet provided sufficient protein to these donors.

As proteins are broken down and hydrolyzed into amino acids, the carbon skeletons from these amino acids can be used as an energy source (Chalupa, 1984). Overfeeding protein in the SBM diet may have increased the availability of amino acids whose carbon skeletons were used to form glucose. This may have been responsible for the higher concentration of PGL before feeding in cows fed the SBM diet. To accurately measure changes in glucose concentrations following the meal, blood samples could have been taken more frequently as well as sooner after feeding. However, due to the dynamics of this experiment, it took the cows approximately 3 h to consume the meal. Blood samples were collected at 6 h post-feeding with the principal goal of characterizing PUN concentrations, as PUN levels peak around this time following feeding (Butler, 1998).

After an animal begins to consume a meal, glucose levels are expected to increase, thus causing pancreatic β cells to synthesize and secrete insulin that results in increased glucose uptake in muscle and adipose tissue (Sherwood, 1997). This causes circulating glucose concentrations to decrease below normal base-line levels. After insulin secretion ceases, glucose concentration increases to basal levels. During this experiment, blood samples were not taken frequently enough following consumption of the meal to accurately characterize changes in PGL concentrations. Concentration of PGL was higher in serum collected from cows that received the SBM diet prior to feeding than cows fed the LITTER or CON diet. Due to the overfeeding of protein to cows receiving the SBM diet, excess amino acids may have been available for deamination, thus providing additional carbon skeletons for the formation of glucose.

Nutrition can potentially influence numerous “sites of action” in the reproductive system of the donor. The objective of Exp. 1 was to characterize differences in dietary protein on the production and quality of embryos produced by superovulated donors. Rations that contained SBM, poultry litter or peanut-hulls as the main dietary source of protein did not influence the number or quality of embryos recovered from superovulated Angus cows. Thus, donors can be

fed a high protein ration, consisting of SBM or poultry litter, without compromising the number or quality of embryos collected 7 d following breeding. From an economical standpoint, cattle producers can feed poultry litter ($.18/kg protein) as the main source of dietary protein at a much lower cost than feeding SBM ($.51/kg protein). It should be noted that metabolic differences were detected among cows fed diets containing excess protein (SBM or LITTER). However, although different amounts and sources of dietary protein altered the concentrations of PUN, TPP and PGL, they failed to influence the number or quality of embryos recovered.

E. Implications

Feeding rations that consisted of poultry litter, SBM or peanut-hulls as the main source of dietary protein did not alter the number or quality grade of embryos recovered from superovulated Angus cows. On the contrary, metabolic differences were observed among serum samples collected before and after feeding for cows receiving the three diets. While the concentration of PUN increased following feeding for those cows fed poultry litter, no detrimental embryonic effects were observed at embryo recovery.

CHAPTER III. EFFECTS OF VARYING THE INTERVAL FROM COLLECTION OF BOVINE EMBRYOS TO FREEZING ON EMBRYO QUALITY AND VIABILITY

A. ABSTRACT

Cryopreservation of bovine embryos is an important aspect of a successful embryo transfer program. The objective of Exp. 2 was to evaluate the post-thaw viability of bovine embryos collected in Exp. 1 in an *in vitro* culture system after the embryos had been held at room temperature or refrigerated for 2 to 12 h prior to freezing. Upon embryo recovery, each embryo was randomly assigned to be placed in holding media for 2, 6 or 12 h prior to freezing. During this interval, one-half of the embryos were maintained in a refrigerated environment (5 °C), while the remaining half of the embryos were held at room temperature (20.5 to 22 °C) until freezing. Immediately prior to freezing, embryos were removed from the holding media, transferred to a well containing ethylene glycol (10%) in ovum culture media and loaded individually into a 0.25-mL plastic straw. Straws were then placed in a freezer unit (-6 °C) and seeded to induce ice crystal formation through all columns of the straw. The temperature of the freezer was then decreased 0.6 °C/min to –32 °C, and straws were loaded into canes and plunged into a liquid nitrogen tank (-196 °C). After storage, each straw was exposed to a 5-s air thaw and placed in a water bath at 35 °C for 20 s. Each embryo was then washed to remove excess ethylene glycol prior to *in vitro* culture. Embryos were individually cultured in Ham's F-10 media supplemented with 4% fetal bovine serum for 72 h. Embryos were evaluated at 24 h intervals throughout the culture period and assigned a stage of development and quality grade score (according to IETS standards). The percentage of embryos that developed to the expanded blastocyst stage and hatched from the zona pellucida was greater for embryos held 2 or 6 h prior to freezing ($P < 0.05$) than for embryos held for 12 h after collection before being frozen (62.9, 52.0 and 31.1%, respectively). The percentage of embryos that degenerated during *in vitro* culture was lower for embryos held 2 or 6 h prior to freezing (20.4 and 26.6%; $P < 0.05$) than for embryos held for 12 h before freezing (50.8%). Furthermore, embryo quality grade was more desirable for embryos held for 2 or 6 h (1.5 and 1.7; $P < 0.05$) than for those held for 12 h before freezing (2.1). The semen used to inseminate donors and the diet fed to donors for 4 wk prior to embryo collection did not influence the proportion of embryos that hatched or degenerated during the 72 h of *in vitro* culture. Additionally, holding embryos in a refrigerated environment

from the time of collection until freezing did not enhance embryonic development during post-thaw culture. Thus, embryonic viability may be impaired when embryos are held longer than 6 h following embryo recovery before being frozen; however, the storage temperature during the interval from collection to freezing does not influence embryonic development post-thaw.

B. Materials and Methods – Experiment 2

Animals and Treatments. Fifty non-lactating Angus cows exhibiting normal estrous cycles were used to produce embryos in this experiment (Chapter II, Section B). This experiment was a 2 x 3 factorial design in which embryos were maintained at one of two holding temperatures from collection until freezing and the interval from embryo collection to freezing was varied from 2 to 12 h. The interval from embryo collection to freezing (2, 6 or 12 h) was randomly assigned to embryos collected from each donor. In addition, one-half of the embryos from each donor were assigned to remain at room temperature (20.5 – 22 °C) in holding media (OCM), while the remaining half were refrigerated (5 °C; Whirlpool ET1 MTK/ET1 MTM, Benton Harbor, MI) in OCM until they were frozen.

Synchronization and Superovulation. Synchronization and superovulation procedures were those described for Exp. 1 (Chapter II, Section B).

Embryo Evaluation. Embryos were evaluated according to the procedures of IETS (1998) described above. In addition to evaluation after collection, each embryo was re-evaluated for stage of development and assigned a quality grade immediately prior to freezing, after thawing and during embryo culture (see below).

Embryo Freezing. After the collection and grading of embryos, each embryo was placed individually in a six-well culture dish containing 1 mL OCM until the time of freezing. The interval from collection to freezing was varied, as embryos from each donor were randomly assigned to one of three collection to freezing intervals: 2, 6 or 12 h. In addition, one-half of the embryos were randomly assigned to remain at room temperature (20.5 to 22 °C), while the remaining half were refrigerated (5 °C) until the initiation of the freezing process.

Ethylene glycol (10%; Immuno-Chemical Products Ltd., Auckland, NZ) in OCM was used as the cryoprotectant for the freezing of embryos. Immediately prior to freezing, each embryo was removed from OCM, transferred to a well containing EG, and loaded individually into a 0.25-mL plastic straw. This was done according to the following procedure: 1) A column of EG was drawn into the straw followed by a small column of air; 2) A larger column of EG containing the embryo was drawn into the straw followed by a small column of air; and 3) A final column of EG was drawn into the straw until the first column of EG came into contact with the cotton plug at the end of the straw. An extension was inserted into the straw that contained the embryo identification code. Eight to nine min after each straw was loaded, they were placed

in the freezer at –6 °C and remained there for at least one minute to allow each column of EG to equilibrate to the temperature. Straws were then seeded and left in the freezer for 8 min to allow the ice crystal induced by seeding to travel through all columns. The temperature of the freezer was then decreased 0.6 °C/min to –32 °C. Frozen straws were then loaded into canes and plunged into a LN_2 tank where they were stored (-196 °C) for 75 to 105 d until *in vitro* culture.

***In Vitro* Culture.** Prior to thawing embryos in preparation for *in vitro* culture, the drops of media to be used for embryo culture were prepared. The volume of each drop was 10 µL, and the media contained a mixture of F-10 Nutrient Mixture (HAM) with L-glutamine (GIBCO; Carlsbad, CA) that was supplemented with 4% fetal bovine serum (FBS; GIBCO; Carlsbad, CA). Two drops were placed in a 35-mm petri dish (Fisher Scientific; Suwanee, GA) and overlaid with 2 mL of embryo-tested mineral oil (Sigma-Aldrich, St. Louis, MO). These petri dishes were placed in an incubator (Forma Scientific, Inc., Marietta, OH) and allowed to equilibrate to 39 °C in a humidified atmosphere of 5% carbon dioxide in air overnight.

After storage in LN_2, each straw containing an embryo was exposed to a 5-sec air thaw. The straw was then placed in a water bath at 35 °C for 20 sec. Each embryo was removed from the straw in preparation for re-hydration and washing to remove excess EG. Each embryo was individually placed into Well 1 of a six-well culture dish, and the following is a description of the contents through which the embryo passed during the re-hydration/washing procedure used in this experiment. Well 1 contained 1.5 mL of OCM and was used to collect the embryo following discharge from the straw. The embryo was transferred immediately to Well 2 that contained 0.5 mL of OCM and was allowed to re-hydrate for 1 min. At this time, the embryo was evaluated for stage of development and quality grade. The embryo was then transferred to Well 3 that contained 0.5 mL of OCM where it remained for 1 min. Wells 4 and 5 contained 0.5 mL of F-10 Nutrient Mixture (HAM) with 25 mM HEPES buffer and L-glutamine (GIBCO; Carlsbad, CA). These wells served as additional washing steps as well as to hold the embryo in media with greater buffering capacity. Embryos were exposed to the media in these two wells and then immediately transferred to the final well. Well 6 contained the culture media used for the experiment, Ham's F-10 media supplemented with 4% FBS. This served as the final washing step.

Embryos were evaluated using an Olympus SZH-ILLK stereomicroscope for morphological development at 24-h intervals for 72 h. Each petri dish containing the embryos

was removed from the incubator, and the embryos in that dish were immediately evaluated for stage of development and quality grade as described in Chapter II, Section B. Endpoints for Exp. 2 were whether the embryo developed to an expanded blastocyst and hatched from the zona pellucida (Figure 3-1), developed past the early blastocyst stage (Stage 5; Figure 3-2), or degenerated (Figure 3-3). At the end of the culture period, Stage 8 and 9 embryos were considered to have hatched, whereas, Stage 6 or 7 embryos were classified as having developed. Embryos that failed to develop during culture (i.e., embryos that remained at Stage 4 or 5) were classified as having degenerated.

Statistical Analysis. Differences in embryonic development and embryo quality grade during or at the end of culture were analyzed by analysis of variance using the GLM procedure of SAS (2001). Diet of the donor (diet), semen used for breeding donors (bull), interval from embryo collection to freezing (time) and the holding temperature for embryos prior to freezing (temp) were included as main effects. Variation within main effects with more than one degree of freedom (df) was determined using Tukey's least square means analysis. In a preliminary analysis of the data, the following two-way interactions were tested and determined not to be statistically significant: bull•diet, bull•temp, bull•time, diet•time, diet•temp and time•temp. Therefore, the final statistical model analyzed only the main effects. Differences in embryo development or quality grade due to the effects of the interval from collection to freezing or holding temperature were analyzed using the variation within cow as the error term with 405 df. Because each cow was randomly assigned to receive only one diet and to be inseminated with semen from only one bull, effects of diet and bull on embryo development and embryo quality grade are nested within cow. Therefore, differences in embryo development and quality grade due to the effects of diet and bull were analyzed using the variation among cows nested within bull•diet (df = 41) as the error term. Each analysis of variance table for Exp. 2 can be found in Appendix B.

Figure 3-1. Example of an Embryo that Hatched from the Zona Pellucida

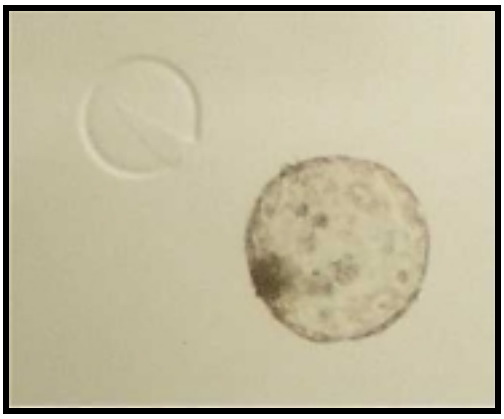


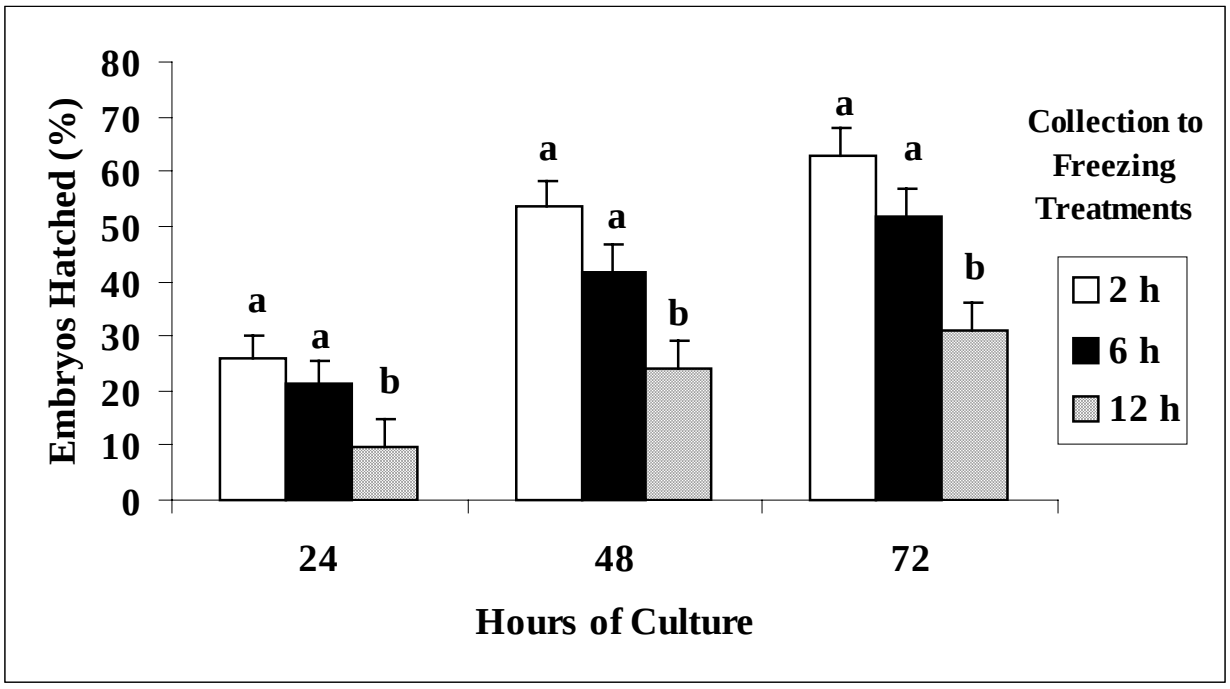
Figure 3-2. Example of an Embryo that Developed during *In Vitro* Culture

Figure 3-3. Example of an Embryo that Degenerated during *In Vitro* Culture

C. Results

The percentages of embryos that hatched during *in vitro* culture, when embryos were held for 2, 6 or 12 h prior to freezing, are illustrated in Figure 3-4. Hatching percentage after 24, 48 and 72 h of *in vitro* culture were significantly different among embryos held for 2, 6 or 12 h prior to freezing. At each time of evaluation, embryos that were held for 2 or 6 h from the time of collection until freezing had a higher hatching percentage than embryos held for 12 h ($P < 0.05$). At 72 h of *in vitro* culture, the hatching percentage of embryos held for 6 h until freezing was 11% lower than embryos that were frozen after being held for 2 h prior to freezing; however, this difference was not significant ($P < 0.12$).

Figure 3-4. The Percentage of Embryos that Hatched (± SE) based on the Time Interval from Embryo Collection to Freezing Treatments

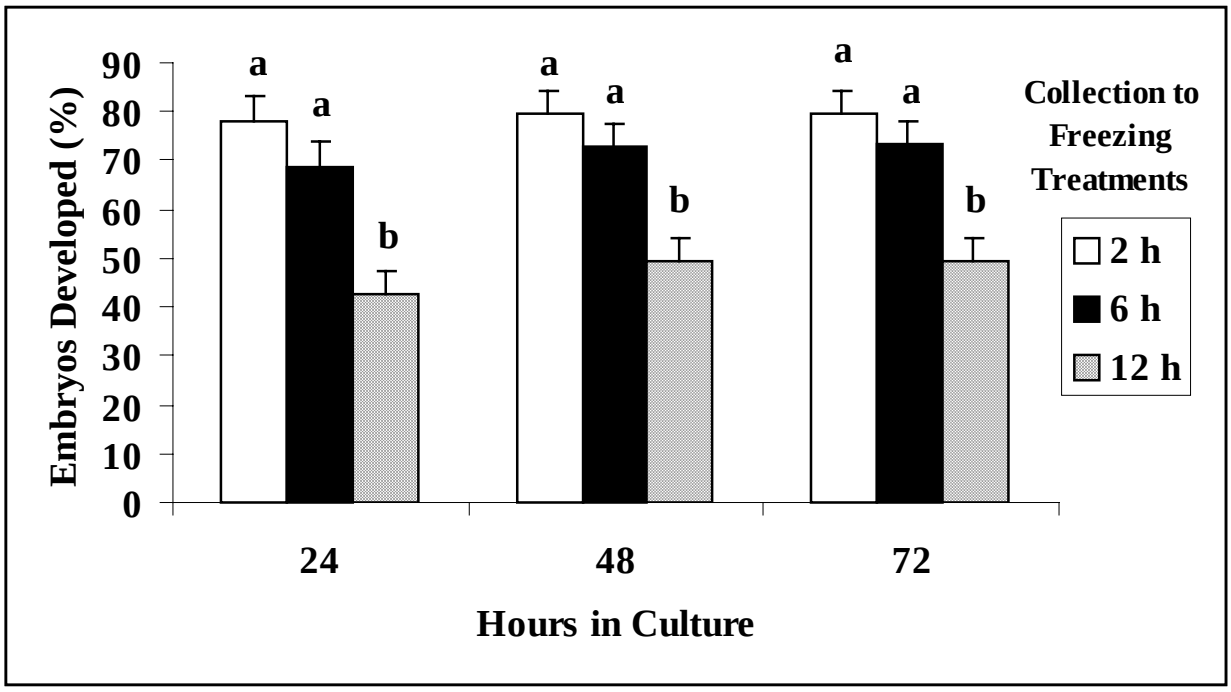


[a,b] Means within category (24, 48, 72) with different superscripts differ ($P < 0.05$)

The percentage of embryos held for 2, 6 or 12 h prior to freezing that developed past the early blastocyst stage (Stage 5) during *in vitro* culture is depicted in Figure 3-5. Differences were detected in the percentage of embryos that developed after 24, 48 and 72 h of *in vitro* culture among embryos held for 2, 6 or 12 h prior to freezing. At each time of evaluation, the percentage of embryos that developed was lower for embryos held for 12 h until freezing than

embryos held for 2 or 6 h prior to freezing ($P < 0.05$). Embryos held for 2 h until freezing had a higher percentage that developed past the early blastocyst stage (6%) than embryos that were frozen after being held for 6 h prior to freezing, but this difference was not significant ($P < 0.46$).

Figure 3-5. The Percentage of Embryos that Developed (± SE) based on the Time Interval from Embryo Collection to Freezing Treatments

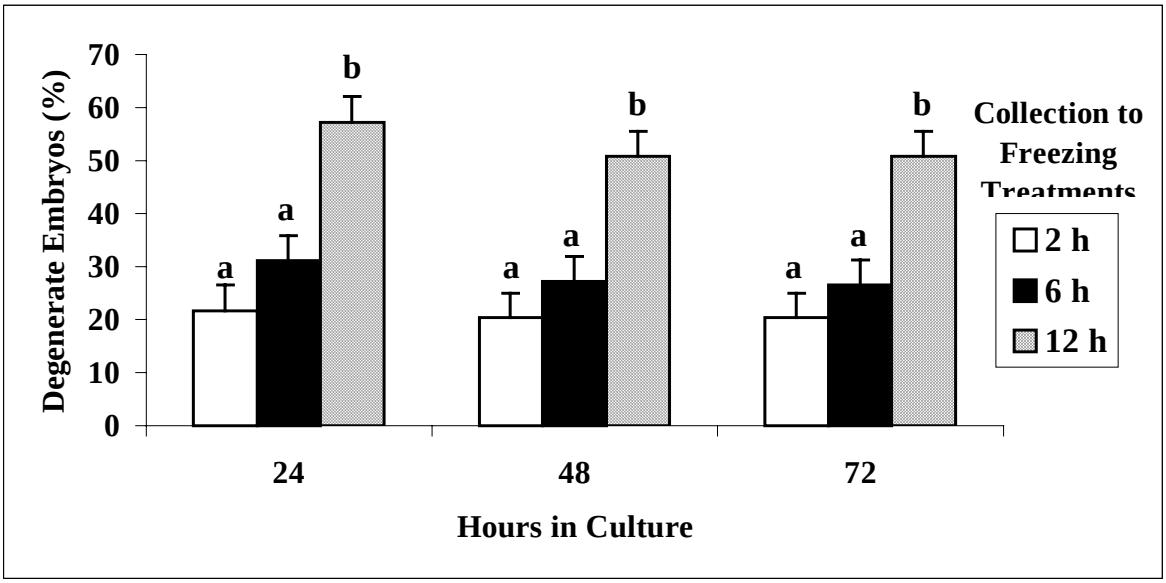


[a,b] Means within category (24, 48, 72) with different superscripts differ ($P < 0.05$)

The percentages of embryos that failed to exhibit any development and were classified as degenerate during *in vitro* culture are depicted in Figure 3-6. The percentage of degenerate embryos at 24, 48 and 72 h of *in vitro* culture differed ($P < 0.001$) for the three collection to freezing intervals. A greater percentage of embryos held for 12 h from collection to freezing were classified as degenerate at each evaluation during *in vitro* culture.

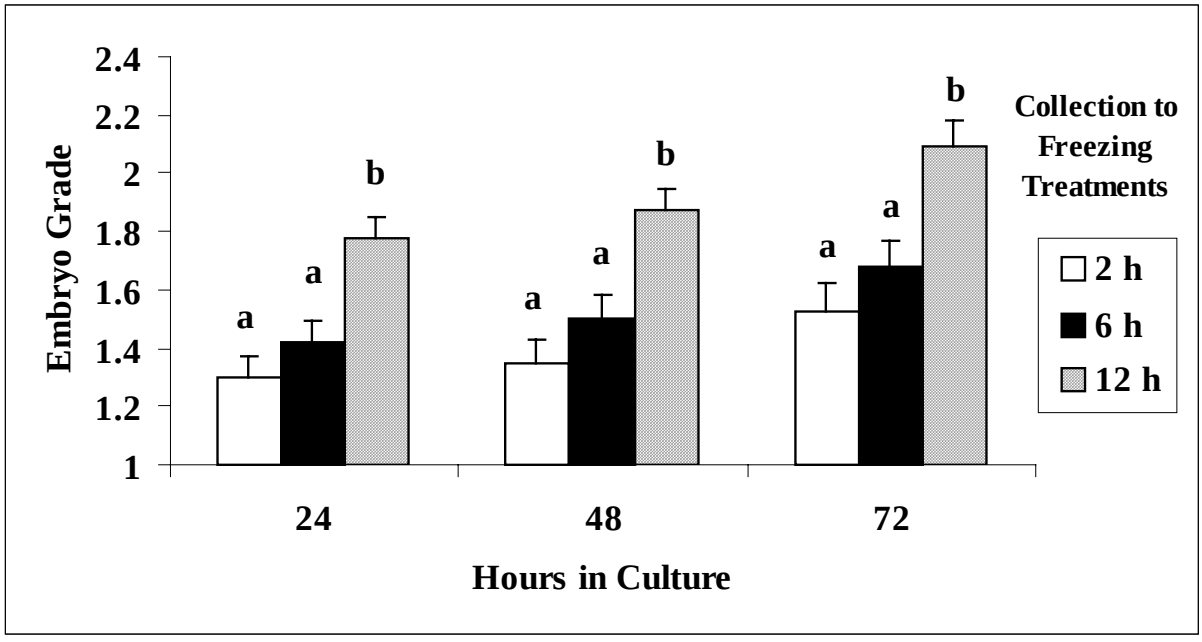
Quality grade for embryos that were evaluated at 24, 48 and 72 h of *in vitro* culture differed ($P < 0.001$) for the embryo collection to freezing intervals (Figure 3-7). Embryos held for 12 h before being frozen had a higher numerical embryo quality grade (less desirable) than for those held for 2 or 6 h before the initiation of freezing. Embryos held for 2 h before freezing had a lower quality grade (more desirable) than embryos held for 6 or 12 h until freezing.

Figure 3-6. The Percentage of Embryos that Degenerated (± SE) based on the Time Interval from Embryo Collection to Freezing Treatments



[a,b] Means within category (24, 48, 72) with different superscripts differ (P < 0.001)

Figure 3-7. Quality Grade of Embryos (± SE) During *In Vitro* Culture based on the Time Interval from Embryo Collection to Freezing Treatments



[a,b] Means within category (24, 48, 72) with different superscripts differ (P < 0.05)

The percentage of embryos that developed to expanded blastocysts and hatched, that showed some evidence of development or that degenerated during *in vitro* culture, as well as the quality grade for embryos held at room temperature (20.5 to 22 °C) or that were refrigerated (5 °C) prior to freezing, is depicted in Table 3-1. No differences were detected in hatching percentage at 24 and 72 h of *in vitro* culture for embryos that were held at room temperature or were refrigerated. However, a difference in the effects of the two holding temperatures was detected ($P < 0.05$) for hatching percentage at 48 h of *in vitro* culture, as those embryos held at room temperature had a greater hatching rate (44.2 ± 4.4 and $35.2 \pm 4.5\%$). Thus, embryos held at room temperature during collection to freezing had a similar hatching percentage as embryos that were refrigerated except for one isolated time.

No differences were detected at 24, 48 or 72 h of *in vitro* culture in the percentage of embryos that degenerated among the embryos that were held at room temperature or were refrigerated prior to freezing. Average embryo quality grade after 24 h of *in vitro* culture was slightly more desirable (1.4 ± 0.1) for embryos held at room temperature than those that were refrigerated (1.6 ± 0.1) during the period from embryo collection to freezing ($P < 0.06$). However, no differences were detected for embryo quality grade between the two holding temperatures at 48 or 72 h of *in vitro* culture. Other than two isolated instances (percentage of embryos that hatched at 48 h of culture and average quality grade at 24 h of culture), the temperature at which the embryos were held from the time of embryo collection until freezing did not influence the percentage of embryos that developed or the quality grade of those embryos during *in vitro* culture.

Differences in the percentage of embryos that developed or degenerated during the *in vitro* culture period as well as differences in quality grade of embryos produced using semen from each of the four bulls are depicted in Table 3-2. No differences among bulls were detected for the percentage of embryos that hatched at 24, 48 or 72 h of *in vitro* culture. Likewise, no differences in embryo quality grade due to bull effects were detected at 24, 48 and 72 h of *in vitro* culture. There was a 35% difference between two bulls in the proportion of embryos that developed to expanded blastocysts and hatched from the zona pellucida at 72 h of *in vitro* culture. Those two bulls also had a 19.1% difference in the percentage of embryos that failed to show any sign of development during the 72 h of culture. Despite the magnitude of these

differences, the effect of the bull used to inseminate donors was not significant for the percentage of embryos that hatched ($P < 0.36$) or were classified as degenerate ($P < 0.20$).

Table 3-1. Differences (± SE) in the Percentage of Embryos that Hatched, Developed or Degenerated and Quality Grade for Embryos Held at Room Temperature or Refrigerated

	Embryos that Hatched[^] (%)	
***In Vitro* Culture Period (h)**	**Room Temperature**	**Refrigerated**
24	18.6 ± 3.7	19.0 ± 3.7
48	44.2 ± 4.4[a]	35.2 ± 4.5[b]
72	50.9 ± 4.5	46.4 ± 4.5
	Embryos that Developed[*] (%)	
***In Vitro* Culture Period (h)**	**Room Temperature**	**Refrigerated**
24	47.2 ± 4.9	41.8 ± 4.9
48	25.1 ± 4.5	29.9 ± 4.5
72	19.0 ± 4.0	18.6 ± 4.1
	Embryos that Degenerated[+] (%)	
***In Vitro* Culture Period (h)**	**Room Temperature**	**Refrigerated**
24	34.2 ± 4.3	39.2 ± 4.3
48	30.8 ± 4.2	34.9 ± 4.2
72	30.2 ± 4.2	35.0 ± 4.2
	Average Embryo Quality Grade[•]	
***In Vitro* Culture Period (h)**	**Room Temperature**	**Refrigerated**
24	1.4 ± 0.1[c]	1.6 ± 0.1[d]
48	1.5 ± 0.1	1.6 ± 0.1
72	1.8 ± 0.1	1.8 ± 0.1

[a,b] Differences exist in rows ($P < 0.05$)
[c,d] Differences exist in rows ($P < 0.06$)
[^] Hatched = embryos that developed to expanded blastocysts and hatched from the zona pellucida
[*] Developed = embryos that grew past the early blastocyst stage (Stage 5)
[+] Degenerated = embryos that failed to show signs of development
[•] Average Embryo Quality Grade = morphological evaluation based on criteria in Table 15

Table 3-2. Differences (± SE) in the Percentage of Embryos that Hatched, Developed or Degenerated and Quality Grade for Bull Effects

***In Vitro* Culture Period (h)**	**Embryos that Hatched[^] (%)**			
	Bull 1	**Bull 2**	**Bull 3**	**Bull 4**
24	21.6 ± 5.1	16.6 ± 5.1	8.9 ± 4.9	28.2 ± 9.3
48	50.7 ± 6.1	35.7 ± 6.1	30.5 ± 5.9	42.0 ± 11.2
72	62.0 ± 6.2	47.2 ± 6.1	37.1 ± 6.0	48.3 ± 11.3
	Embryos that Developed[*] (%)			
***In Vitro* Culture Period (h)**	**Bull 1**	**Bull 2**	**Bull 3**	**Bull 4**
24	53.2 ± 6.7	54.1 ± 6.7	43.9 ± 6.5	26.7 ± 12.3
48	25.0 ± 6.2	41.3 ± 6.2	26.0 ± 6.0	17.7 ± 11.4
72	13.8 ± 5.6	29.8 ± 5.5	19.5 ± 5.3	12.1 ± 10.1
	Embryos that Degenerated[+] (%)			
***In Vitro* Culture Period (h)**	**Bull 1**	**Bull 2**	**Bull 3**	**Bull 4**
24	25.1 ± 5.9	29.4 ± 5.8	47.2 ± 5.6	45.2 ± 10.7
48	24.3 ± 5.7	23.0 ± 5.7	43.5 ± 5.5	40.4 ± 10.5
72	24.3 ± 5.7	23.0 ± 5.7	43.4 ± 5.5	39.6 ± 10.5
	Average Embryo Quality Grade[•]			
***In Vitro* Culture Period (h)**	**Bull 1**	**Bull 2**	**Bull 3**	**Bull 4**
24	1.4 ± 0.1	1.4 ± 0.1	1.6 ± 0.1	1.6 ± 0.2
48	1.4 ± 0.1	1.5 ± 0.1	1.8 ± 0.1	1.6 ± 0.2
72	1.5 ± 0.1	1.6 ± 0.1	1.9 ± 0.1	2.0 ± 0.2

[^] Hatched = embryos that developed to expanded blastocysts and hatched from the zona pellucida
[*] Developed = embryos that grew past the early blastocyst stage (Stage 5)
[+] Degenerated = embryos that failed to show signs of development
[•] Average Embryo Quality Grade = morphological evaluation based on criteria in Table 15

No differences were detected among cows fed one of the three diets for the percentage of embryos that developed to the expanded blastocyst stage and hatched, developed past the early blastocyst stage (Stage 5) or degenerated at 24, 48, or 72 h of *in vitro* culture (Table 3-3). However, there was a noticeable difference (-14 and -17%) among cows that received the LITTER diet and cows that received either the SBM or CON diet for the percentage of embryos that hatched. Despite the magnitude of this difference, it was not statistically significant

(P < 0.29). Furthermore, after 24, 48 or 72 h of *in vitro* culture, differences in embryo quality and the percentage of degenerate embryos collected from cows assigned to one of the three diets were observed, but not determined to be statistically significant. The difference (+15 and +20%) among cows that received the LITTER diet and cows that received the SBM or CON diet in the percentage of degenerate embryos at 72 h of culture was sizable, but this difference was not significant (P < 0.19). Conversely, the difference in average embryo quality grade tended to be significant (P < 0.06).

Table 3-3. Differences (± SE) in the Percentage of Embryos that Hatched, Developed or Degenerated and Quality Grade for Diet Effects

	Embryos that Hatched[^] (%)		
***In Vitro* Culture Period (h)**	**CON**	**SBM**	**LITTER**
24	16.0 ± 4.6	17.5 ± 4.1	23.0 ± 7.3
48	47.2 ± 5.5	40.7 ± 4.9	31.2 ± 8.7
72	55.4 ± 5.6	52.6 ± 5.0	38.0 ± 8.8
	Embryos that Developed[*] (%)		
***In Vitro* Culture Period (h)**	**CON**	**SBM**	**LITTER**
24	57.3 ± 6.1	45.7 ± 5.4	30.4 ± 9.6
48	28.9 ± 5.7	28.9 ± 5.0	24.7 ± 8.9
72	20.7 ± 5.1	17.9 ± 4.5	17.8 ± 7.9
	Embryos that Degenerated[+] (%)		
***In Vitro* Culture Period (h)**	**CON**	**SBM**	**LITTER**
24	26.7 ± 5.3	36.7 ± 4.7	46.7 ± 8.4
48	23.9 ± 5.2	30.4 ± 4.6	44.1 ± 8.2
72	23.9 ± 5.2	29.6 ± 4.6	44.2 ± 8.2
	Average Embryo Quality Grade[•]		
***In Vitro* Culture Period (h)**	**CON**	**SBM**	**LITTER**
24	1.4 ± 0.1	1.6 ± 0.1	1.6 ± 0.1
48	1.4 ± 0.1	1.6 ± 0.1	1.7 ± 0.1
72	1.5 ± 0.1	1.7 ± 0.1	2.1 ± 0.2

[^] Hatched = embryos that developed to expanded blastocysts and hatched from the zona pellucida
[*] Developed = embryos that grew past the early blastocyst stage (Stage 5)
[+] Degenerated = embryos that failed to show signs of development
[•] Average Embryo Quality Grade = morphological evaluation based on criteria in Table 15

D. Discussion

Cryopreservation is an important aspect of a successful bovine ET program. It is essential that embryos be frozen and thawed without a loss of viability. Typically, practitioners strive to initiate the freezing process of bovine embryos within 4 h after embryo collection. Early researchers attempted to freeze embryos 12 to 15 h following collection (Wright, 1985), but the pregnancy rate following the transfer of embryos to recipients was compromised. Improvements in techniques for freezing embryos, especially the use of different cryoprotective agents (EG), have been achieved since those experiments were performed. Therefore, the purpose of Exp. 2 was to determine the effects of the interval from embryo collection to freezing (2, 6 or 12 h) on post-thaw viability when embryos were held at room temperature (20.5 to 22 °C) or in a refrigerated environment (5 ° C) from the time of collection to freezing. The experiment was performed using the most current technology. A reliable embryo culture system was developed to evaluate the post-thaw viability of bovine embryos that had been held at room temperature or in a refrigerated environment for 2, 6 or 12 h from the time of embryo collection to freezing.

Evaluation of embryos during *in vitro* culture revealed differences in the percentage of embryos that developed or degenerated as well as differences in embryo quality grade due to differences in the holding time prior to freezing. At 24, 48 and 72 h of culture, embryos held for 2 or 6 h prior to freezing had a higher percentage that developed to expanded blastocysts and hatched from the zona pellucida than embryos held 12 h prior to freezing. On the contrary, a higher percentage of embryos held 12 h from collection to freezing degenerated during culture. Embryos held 6 h from collection to freezing had a similar hatching percentage and similar incidence of degeneration as those embryos held 2 h following embryo recovery. Embryos frozen 2 to 6 h after collection resulted in a higher hatching percentage as well as a lower percentage that degenerated than when embryos were frozen 12 h following collection. These data clearly indicate that holding embryos longer than 6 h before freezing may compromise post-thaw viability.

The results of the current experiment are similar to those of Wright (1985) who analyzed differences in pregnancy rate of recipients relative to the length of time that embryos were held before freezing. Wright (1985) reported a steady decline in pregnancy rate in cows that received an embryo that was held longer than 4 h after collection to freezing. Likewise, Pettit (1985)

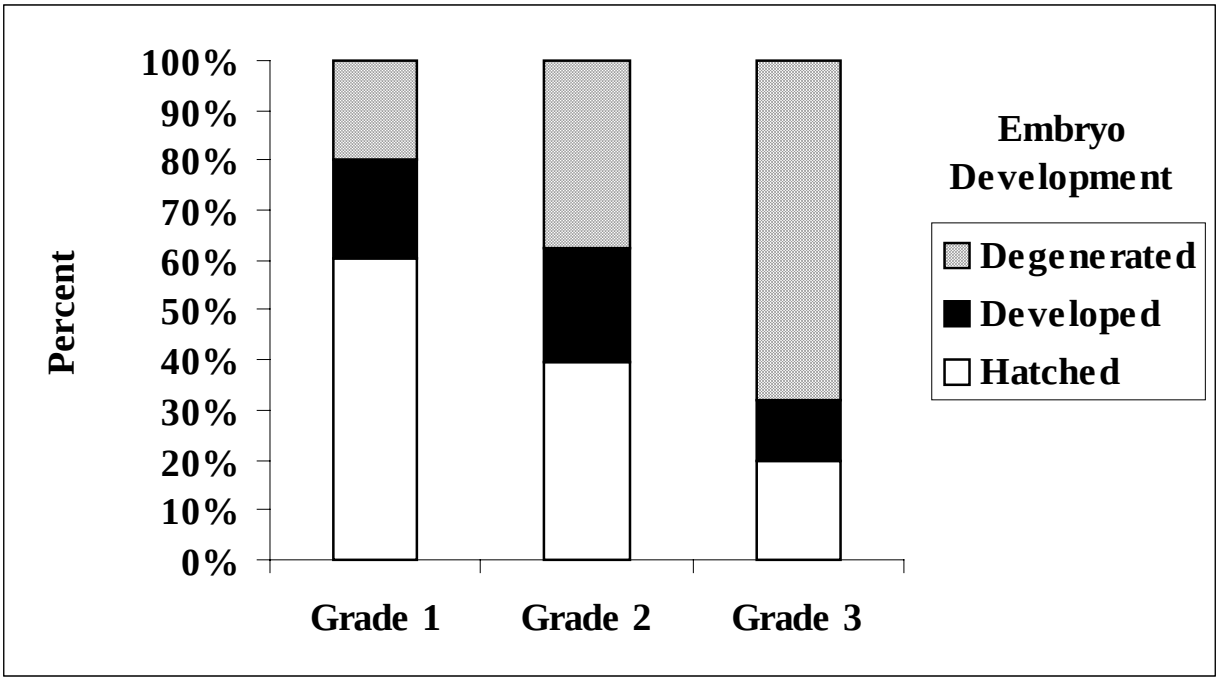
reported a significant decline in pregnancy rate in recipients when embryos were held more than 3 h after collection until freezing. Therefore, it appears that a shorter interval from collection to freezing is important in achieving a high pregnancy rate in cattle receiving a frozen-thawed embryo. If embryos are held longer than 4 to 6 h, pregnancy rates may be compromised. Furthermore, poor synchrony between the embryo stage of development and stage of the estrous cycle of the recipient or adverse environmental conditions could further exacerbate the potential for achieving low pregnancy rates.

During the period after embryo collection and prior to freezing (2, 6 or 12 h), embryos were refrigerated or maintained at room temperature. It was hypothesized that if embryos were refrigerated, especially those held for 12 h prior to freezing, it would improve post-thaw development rates when compared to that of embryos held at room temperature. If this were true, it might enable practitioners to freeze embryos once per day rather than at 2- to 3-h intervals, as is currently practiced. Unfortunately, results from this experiment failed to support this hypothesis. The development and hatching percentages of embryos that were held at room temperature or were refrigerated was similar at 24 and 72 h of culture. The percentage of degenerate embryos as well as embryo quality grade at each evaluation during *in vitro* culture was also comparable between embryos that were refrigerated or maintained at room temperature. These results are similar to those of Moore and Bilton (1973) who revealed that storage of sheep embryos at 5 °C prior to *in vitro* culture had no effect on subsequent development. In the current study, embryos were cryopreserved following storage at 5 °C whereas Moore and Bilton (1973) cultured the sheep embryos immediately following storage at 5°C. In both cases, holding embryos in a refrigerated environment prior to freezing or *in vitro* culture did not enhance embryonic development.

Morphological evaluations have been developed to predict embryo viability. These evaluations are non-invasive and include criteria such as embryo shape, number and compactness of cells and the number of extruded cells (Wright and Ellington, 1995). Lindner and Wright (1983) developed a four-category system to evaluate embryo quality, but the current study employed the three-category IETS (1998) guidelines for evaluation of embryo quality. In this experiment, embryo quality score was used to describe the “health” of the embryo. However, it should be noted that the embryo quality score recorded immediately post-thaw was a good indicator of the likelihood of an embryo to develop during *in vitro* culture. The percentage

of Grade 1, 2 or 3 embryos that developed or degenerated during *in vitro* culture is depicted in Figure 3-8. Grade 1 embryos had a greater hatching percentage during culture than Grade 2 or 3 embryos while the percentage of degenerate embryos was lower for Grade 1 embryos during culture than Grade 2 or 3 embryos.

Figure 3-8. Grade 1, 2 and 3 Embryos Evaluated Immediately Post-Thaw and the Percentage of these Embryos that Hatched, Developed or Degenerated at 72-h of In-Vitro Culture



The influence of embryo quality grade on embryo viability observed in the present study is similar to that described in previously reported studies. A retrospective analysis of data performed by Hasler (2001) revealed that the transfer of Grade 1 or 2 embryos resulted in a higher pregnancy rate in recipients (58 and 54%) than the transfer of Grade 3 embryos to recipients (38%). Furthermore, Wright (1981) reported that pregnancy rates of recipients were significantly different for cows that received a Grade 1, 2 or 3 embryo following transfer (64, 45 and 33%, respectively). Therefore, embryo quality grade can be used to predict embryo viability and the likelihood of the birth of offspring resulting from the transfer of embryos to synchronized recipients.

A bull contributes 50% of the genetic material to an embryo; thus, differences in embryonic development among the four bulls that supplied semen used for inseminating the donors was analyzed. Bull effects were not statistically significant for the percentage of embryos that hatched or degenerated at 72 h of *in vitro* culture, but differences among bulls were greater than expected. Specifically, the percentage of embryos collected that hatched at 72 h of culture was noticeably higher for cows bred to Bull 1 than for Bull 3 (62.0 and 37.1%, respectively). Meanwhile, hatching rate of embryos from cows that were inseminated with semen from Bulls 2 or 4 was comparable (47.2 and 48.3%, respectively). The percentage of embryos that degenerated during culture were similar for Bulls 1 and 2 (24.3 and 23.0%, respectively), and these percentages were somewhat lower than the percentage of degenerate embryos collected from cows bred to Bulls 3 or 4 (43.4 and 39.6%, respectively). Furthermore, embryo quality grade score was slightly lower (more desirable) for embryos produced by cows inseminated with semen from Bulls 1 or 2 (1.5 and 1.6, respectively) than Bulls 3 or 4 (1.9 and 2.0, respectively). In an effort to explain some differences in embryo development and quality grade, a semen analysis was performed using two straws from each bull. Semen was evaluated for motility and morphological characteristics (Table 3-4). Semen from each bull had a motility estimate of 60% or greater. Semen from Bulls 1 and 2 had 86 and 88% morphologically-normal sperm, respectively, whereas Bulls 3 and 4 had 69 and 74% morphologically-normal sperm, respectively. While these morphological estimates would be deemed acceptable by most bull studs, Bulls 3 and 4 had 22 and 18% abnormal sperm heads, respectively. This increase in the percentage of sperm head abnormalities could have resulted in a lowered embryo hatching percentage and a higher percentage of degenerate embryos following *in vitro* culture.

Sperm with subtly misshapen heads or normally shaped heads can gain access to the ovum following AI. However, sperm with abnormal heads have been shown to yield a higher frequency of low quality embryos and reduced fertilization rates (DeJarnette et al., 1992). While the zona pellucida provides a formidable barrier against the entry of sperm with misshapen heads, these normal or near-normal appearing sperm that gain access to the ovum may cause pregnancy wastage through early embryonic death (Saacke et al., 2000). This may explain the noticeable reduction in hatching percentage and increase in the percentage of embryos that degenerated during the 72 h of *in vitro* culture for Bull 3 in this experiment. Perhaps the sperm

from Bull 3 were able to penetrate the zona pellucida and fertilize the ovum, but due to some abnormality with the sperm, embryonic development was compromised.

Table 3-4. Semen Analysis for the Four Bulls used to Inseminate Donors

Bull	Motility (%)	Morphology (%)			
		Normal	Head Abnormalities	Droplets	Tail Abnormalities
1	60	86	6	4	4
2	70	88	7	2	3
3*	60	69	22	4	5
4^	60	74	18	3	5

* Head abnormalities for Bull 3 = 15% pyriformed, tapered and asymmetrical sperm heads
^ Head abnormalities for Bull 4 = 12% long sperm heads

The effect of dietary intake of the donors during the 45 d prior to embryo recovery was analyzed to determine if diet influenced embryonic development. Results from Exp. 1 revealed that the three diets (CON, SBM and LITTER) failed to influence the number or quality of embryos recovered from superovulated donors. Differences in the source and amount of dietary protein fed to donors also did not cause statistically significant changes in the development or quality of embryos cultured after freezing and thawing. However, close inspection of the results from Exp. 2 reveal that embryos collected from donors fed the CON or SBM diet had a notably greater percentage of embryos that hatched at 72 h of *in vitro* culture (55.4 and 52.6%, respectively) than embryos recovered from cows fed the LITTER diet (31.1%). Conversely, the percentage of embryos that degenerated from donors fed the CON or SBM diet was lower (23.9 and 29.6%, respectively) than those from cows fed the LITTER diet (44.2%). In addition, embryo quality grade at 72 h of culture was slightly better for cows fed the CON diet (1.5) than for cows fed the SBM or LITTER diet (1.7 and 2.1, respectively). Although the effects of the diet fed to donors on embryo development were not statistically significant, the unexpectedly lower rate of development in embryos from donors fed the LITTER diet and the subtle reduction in quality of embryos collected from donors on the high-protein diets (LITTER, SBM) are concerning. Feeding excess amounts of RDP may result in fertilization failure or poor embryonic development, possibly due to an altered uterine environment (Elrod and Butler, 1993;

Blanchard et al., 1990). Thus, the possibility that differences in the solubility or degradability of the CP may lead to poor embryonic development should not be dismissed completely.

The principal objective of Exp. 2 was to characterize the effects of varying the interval from embryo collection to freezing on post-thaw viability and embryo development when embryos were held at room temperature or in a refrigerated environment from the time of collection to freezing. Embryos held for 2 h from collection to freezing had a greater hatching percentage than embryos held for 12 h prior to freezing. During the interval from embryo collection to freezing, holding embryos in a refrigerated environment or at room temperature did not enhance post-thaw embryonic development. In addition, it was determined that the diet of the donor and the four bulls that supplied semen to breed donors did not have a statistically significant influence on the development or quality of embryos collected from the donors. Nonetheless, differences among bulls and diets were larger than expected and may require further investigation.

E. Implications

Increasing the interval between embryo collection and freezing compromises embryonic development after thawing. The percentage of embryos that hatched following 72 h of *in vitro* culture was not different for embryos held for 2 or 6 h from collection to freezing. However, embryos held for 2 h prior to freezing had an 11% higher hatching rate than those embryos held for 6 h until freezing. Thus, ET practitioners should freeze embryos 2 to 3 h following embryo recovery in order to maximize pregnancy rates of recipients receiving a frozen-thawed embryo. Holding embryos for 2, 6 or 12 h in a refrigerated environment or at room temperature did not influence embryonic development after thawing, thus allowing practitioners to hold embryos at either temperature without impairing embryonic development.

Although the semen from the four bulls used for inseminating donors for this experiment would meet the standards for most bull studs, cows bred to the two bulls with a greater percentage of misshapen sperm heads had a slightly lower embryo hatching percentage and a higher percentage of degenerate embryos at the end of *in vitro* culture. Additionally, this experiment provided additional evidence indicating that the diet of the donor may influence embryonic development. While no statistically significant differences were detected in the viability of embryos collected from cows receiving the CON, SBM or LITTER diet, the hatching rate was greater for embryos collected from donors fed the CON or SBM diet than for embryos collected from donors receiving the LITTER diet. The differences among bulls and diets were larger than anticipated, and further research needs to be performed to investigate these differences and their impact on embryonic development.

CHAPTER IV. GENERAL DISCUSSION

The goals of the current study were two-fold: 1) To determine the effects of differences in dietary protein on the production and quality of embryos collected from superovulated Angus cows; and 2) To determine the effects of the interval from embryo collection to freezing on post-thaw viability when embryos were held at room temperature or in a refrigerated environment from the time of collection to freezing. The underlying goal of Exp. 1 was to determine if deep stacked poultry litter could be utilized as an inexpensive source of protein for superovulated donors without negatively influencing the number or quality of embryos collected or their subsequent development during *in vitro* culture. For Exp. 2, it was hypothesized that holding embryos in a refrigerated environment from collection to freezing would better sustain embryonic development after thawing than those embryos maintained at room temperature, especially for those embryos that were held for 12 h until freezing.

For Exp. 1, donors were randomly assigned to receive a diet consisting of peanut-hulls (CON), SBM or poultry litter (LITTER) as the main source of dietary protein. Donors were given 30 d on the diets prior to estrus synchronization and the initiation of superovulatory treatment. When embryos were collected, no differences were detected in the numbers or quality of embryos recovered from donors that received the CON, SBM or LITTER diet. These results demonstrated that poultry litter can be fed to donors without compromising the response to superovulatory treatment or embryo production.

Embryos collected in Exp. 1 were then cultured for 72 h to assess embryonic development. No statistically significant differences were detected for embryonic development after thawing for cows fed the CON, SBM or LITTER diet. However, after 72 h of culture, embryos collected from donors that were fed the CON or SBM diet had a higher percentage that developed to the expanded blastocyst stage and hatched from the zona pellucida (24.3 and 21.5%) or developed past the early blastocyst stage (20.3 and 14.7%) than embryos collected from donors assigned to the LITTER diet. Furthermore, embryos recovered from donors that received the CON or SBM diet had a lower chance of being classified as degenerate (-20.3 and -14.6%) as well as a more desirable average quality grade (1.5 and 1.7) than embryos from cows fed the LITTER diet (2.1). Although the magnitude of these differences was not statistically significant, they raise serious questions about the ability of embryos collected from donors fed poultry litter to develop normally after thawing. Therefore, cattle producers should be aware of

the potential detrimental effects on embryonic development when feeding poultry litter to donors.

Metabolic differences were detected among donors that received the CON, SBM or LITTER diet. It is important to note that blood samples collected from donors fed the SBM diet had a significantly higher concentration of PUN before and after feeding than donors assigned to the CON or LITTER diet. While the concentration of PUN increased significantly following feeding in blood samples collected from donors fed the LITTER diet, the concentration of PUN did not approach that measured in blood samples collected from donors assigned to the SBM diet. If elevated PUN concentrations are the potential cause of compromised embryonic development, then donors fed the SBM diet might have been expected to produce embryos that had reduced embryonic growth. However, donors fed the SBM diet had a comparable percentage of embryos that developed or hatched as those embryos recovered from donors that received the CON diet, while embryos collected from donors assigned to the LITTER diet had the lowest hatching percentage and developmental rate. Perhaps some other component in poultry litter, yet to be determined, negatively impacted the developmental capacity of embryos collected from superovulated donors. Although poultry litter is relatively inexpensive and can adequately supply protein in the diets of cattle, interpretation of data from this experiment raise the question of whether embryonic development may be impaired in cows fed poultry litter.

While bull effects were greater than expected, no significant differences in embryonic development were detected among bulls. Donors inseminated with semen from bulls that had a greater percentage of sperm head abnormalities produced more embryos that degenerated and more embryos that had a less desirable quality grade following culture than those donors inseminated with semen from bulls with a greater percentage of morphologically-normal sperm. A possible reason for this failure in embryonic development could be attributed to the sperm with subtle abnormalities from Bulls 3 and 4 penetrating the zona pellucida of the ovum, achieving successful fertilization, but ultimately causing early embryonic death.

Using the most advanced cryoprotective techniques for freezing bovine embryos, Exp. 2 was designed to determine the effects of the interval from embryo collection to freezing when embryos were maintained in a refrigerated environment or at room temperature from embryo collection to freezing. It was evident that holding embryos longer than 6 h until freezing resulted in significantly lower developmental rates and a higher percentage of embryos that degenerated

following culture. Embryos that were held for 2 h prior to freezing resulted in a higher hatching percentage (+11%) as well as a higher development rate (+6%) than embryos held for 6 h until freezing. While the magnitude of these differences was not statistically significant, practitioners should still be advised to freeze embryos within 2 to 3 h after collection to maximize their developmental potential when transferred to a synchronized recipient following thawing.

It was hypothesized that holding embryos in a refrigerated environment between the time of collection and freezing may improve their potential for development after thawing. This could enable ET practitioners to increase the interval from embryo collection to freezing. However, this hypothesis was rejected, as embryos that were held in a refrigerated environment prior to freezing or at room temperature had similar developmental rates during *in vitro* culture. Thus, embryos can be held at either temperature from collection to freezing without influencing embryonic growth after thawing.

In conclusion, cattle producers that feed poultry litter to donors should be concerned about the reduction in embryonic development detected in this study. Additional studies need to be conducted to characterize the developmental capacity of embryos collected from donors fed poultry litter. Based on the results of this study, ET practitioners should be advised to freeze embryos within 6 h following collection, but embryo hatching percentage and developmental rates are maximized when embryos are frozen 2 to 3 h after collection. If practitioners hold embryos prior to freezing, the embryos may be stored at room temperature or in a refrigerated environment without affecting embryonic development.

Literature Cited

Arreseigor, C.J., A. Sisul, A.E. Arreseigor and R.C. Stahringer. 1998. Effect of cryoprotectant, thawing method, embryo grade and breed on pregnancy rates of cryopreserved bovine embryos. Theriogenology. 49:160 (Abstr.).

Bakshi, M.P.S. and J.P. Fontenot. 1998. Processing and nutritive evaluation of broiler litter as livestock feed. Anim. Feed Sci. Technol. 74:337-345.

Baracaldo, M.I., M.F. Martinez, G.P. Adams and R.J. Mapletoft. 2000. Superovulatory response following transvaginal follicle ablation in cattle. Theriogenology. 53:1239-1250.

Bavister, B.D. 1995. Culture of preimplantation embryos: facts and artifacts. Human Reprod. Update. 1:91-148.

Bavister, B.D., T.A. Rose-Hellekant and T. Pinyopummintr. 1992. Development of in vitro matured/in vitro fertilized bovine embryos into morulae and blastocysts in defined culture media. Theriogenology. 37:127-146.

Beckman Instruments, Inc. 1995. Beckman SYNCHRON CX Clinical Systems Operating Instructions manual. Glucose Chemistry Information, #442640. Brea, CA.

Bhattacharya, A.N. and J.P. Fontenot. 1966. Protein and energy value of peanut hull and wood shaving poultry litter. J. Anim. Sci. 25:367-372.

Bhattacharya, A.N. and J.C. Taylor. 1975. Recycling animal waste as a feedstuff: a review. J. Anim. Sci. 41:1438-1457.

Blanchard, T., J. Ferguson, L. Love, T. Takeda, B. Henderson, J. Hasler and W. Chalupa. 1990. Effect of dietary crude-protein type on fertilization and embryo quality in dairy cattle. Am. J. Vet. Res. 51:905-908.

Boland, M.P., P. Lonergan and D. O'Callaghan. 2001. Effect of nutrition on endocrine parameters, ovarian physiology, and oocyte and embryo development. Theriogenology. 55:1323-1340.

Butler, W.R. 1998. Review: Effect of protein nutrition on ovarian and uterine physiology in dairy cattle. J. Dairy Sci. 81:2533-2539.

Canseco, R.S., F.C. Gwazdauskas, R. Rajamahendran and W.E. Vinson. 1988. Culture of bovine morulae in media supplemented with immunoglobulins. J. Dairy Sci. 71:2767-2771.

Canseco, R.S., A.E.T. Sparks, R.E. Pearson and F.C. Gwazdauskas. 1992. Embryo density and medium volume effects on early murine embryo development. J. Assis. Reprod. Genet. 9:454-457.

Chalupa, W. 1984. Discussion of protein symposium. J. Dairy Sci. 67:1134-1146.

DeJarnette, J.M., R.G. Saacke, J. Bame and C.J. Vogler. 1992. Accessory sperm: their importance to fertility and embryo quality, and attempts to alter their numbers in artificially inseminated cattle. J. Anim. Sci. 70:484-491.

D'Occhio, M.J., D. Jillella and B.R. Lindsey. 1999. Factors that influence follicle recruitment, growth and ovulation during ovarian superstimulation in heifers: opportunities to increase ovulation rate and embryo recovery by delaying the exposure of follicles to LH. Theriogenology. 51:9-35.

Dochi, O., Y. Yamamoto, H. Saga, N. Yoshiba, N. Kano, J. Maeda, K. Miyata, A. Yamauchi, K. Tominaga, Y. Oda, T. Nakashima and S. Inohae. 1998. Direct transfer of bovine embryos frozen-thawed in the presence of propylene glycol or ethylene glycol under on-farm conditions in an integrated embryo transfer program. Theriogenology. 49:1051-1058.

Driancourt, M.A. 2001. Regulation of ovarian follicular dynamics in farm animals. Implications for manipulation of reproduction. Theriogenology. 55:1211-1239.

Elrod, C.C. and W. R. Butler. 1993. Reduction of fertility and alteration of uterine pH in heifers fed excess ruminally degradable protein. J. Anim. Sci. 71:694-701.

Evers, G.W., L. W. Greene, J.B. Carey and D.S. Doctorian. 1996. Feeding broiler litter to beef cattle. Texas Agricultural Exp. Station: MP-1773. Texas A&M Univ., College Station.

Fontenot, J.P., L.W. Smith and A.L. Sutton. 1983. Alternative utilization of animal wastes. J. Anim. Sci. 57(Suppl. 2):221-233.

Fontenot, J.P. 1991. Recycling animal wastes by feeding to enhance environmental quality. Prof. Anim. Sci. 7:1-8.

Fontenot, J.P. 1996. Feeding poultry wastes to cattle. Paper presented at the Virginia Cattle and Poultry Litter Conference, November 6, Harrisonburg, VA.

Fontenot, J.P. 1999. Nutrient recycling: the North American experience – review. Asian-Aus. J. Anim. Sci. 12:642-650.

Garcia-Bojalil, C.M., C.R. Staples, W.W. Thatcher and M. Drost. 1994. Protein intake and development of ovarian follicles and embryos of superovulated nonlactating dairy cows. J. Dairy Sci. 77:2537-2548.

Ginther, O.J., K. Kot, L.J. Kulick and M.C. Wiltbank. 1997. Emergence and deviation of follicles during the development of follicular waves in cattle. Theriogenology. 48:75-87.

Ginther, O.J., M.C. Wiltbank, P.M. Fricke, J.R. Gibbons and K. Kot. 1996. Selection of the dominant follicle in cattle. Biol. Reprod. 55:1187-1194.

Hasler, J.F. 1992. Current status and potential of embryo transfer and reproductive technology in dairy cattle. J. Dairy Sci. 75:2857-2879.

Hasler, J.F. 2001. Factors affecting frozen and fresh embryo transfer pregnancy rates in cattle. Theriogenology. 56:1401-1415.

Heidel, R.D. 1996. Litter handling and feeding ideas. Paper presented at the Virginia Cattle and Poultry Litter Conference, November 6, Harrisonburg, VA.

Hopkins, B.A. and M.H. Poore. 2001. Deep-stacked broiler litter as a protein source for dairy replacement heifers. J. Dairy Sci. 84:299-305.

Hiller, A., J. Plazin and D.D. Van Slyke. 1948. Study of conditions for the Kjeldahl determination of nitrogen in proteins. J. Biol. Chem. 176:1401-1420.

IETS. 1998. Manual of the International Embryo Transfer Society. 3rd ed. Savoy, IL.

Jacob, J.P., W.E. Kunkle, R.S. Tervola, R.D. Miles and F.B. Mather. 1997. Broiler litter, part 1: a feed ingredient for ruminants. Florida Cooperative Extension Service: Factsheet PS-13. Univ. of Florida, Gainesville.

Jordan, E.R., T.E. Chapman, D.W. Holtan and L.V. Swanson. 1983. Relationship of dietary crude protein to composition of uterine secretions and blood in high-producing postpartum dairy cows. J. Dairy Sci. 66:1854-1862.

Jurgens, M.H. 1997. Beef cattle feeding guides. Page 357 in Animal Feeding and Nutrition. 8th ed. Kendall/Hunt Publishing Company. Dubuque, IA.

Kim, I.H., D.S. Son, S.H. Yeon, S.H. Choi, S.B. Park, I.S. Ryu, G.H. Suh, D.W. Lee, C.S. Lee, H.J. Lee and J.T. Yoon. 2001. Effect of dominant follicle removal before superstimulation on follicular growth, ovulation and embryo production in Holstein cows. Theriogenology. 55:937-945.

Kunkle, W.E., J.P. Jacob, R.S. Tervola, R.D. Miles and F.B. Mather. 1997. Broiler litter, part 2: feeding to ruminants. Florida Cooperative Extension Service: Factsheet PS-14. Univ. of Florida, Gainesville.

Leech, R. 1996. Litter storage ideas. Paper presented at the Virginia Cattle and Poultry Litter Conference, November 6, Harrisonburg, VA.

Leibo, S.P. 1986. Cryobiology: preservation of mammalian embryos. Basic Life Sci. 37:251-272.

Leibo, S.P., A. Martino, S. Kobayashi and J.W. Pollard. 1996. Stage-dependent sensitivity of oocytes and embryos to low temperatures. Anim. Reprod. Sci. 42:45-53.

Lerner, S.P, W.V. Thayne, R.D. Baker, T. Henschen, S. Meredith, E.K. Inskeep, R.A. Dailey, P.E. Lewis and R.L. Butcher. 1986. Age, dose of FSH and other factors affecting superovulation in Holstein cows. J. Anim. Sci. 63:176-183.

Lester, T.D., R.W. McNew and R.W. Rorie. 1999. Use of electronic estrous detection to evaluate the effect of embryo-recipient synchrony on pregnancy rate in cattle. Theriogenology. 51:265 (Abstr.).

Lindner, G.M. and R.W. Wright, Jr. 1983. Bovine embryo morphology and evaluation. Theriogenology. 20:407-416.

Mazur, P. 1980. Fundamental aspects of the freezing of cells, with emphasis on mammalian ova and embryos. Proc. of a Plenary Session on Artificial Insemination at the IX International Congress on Animal Reproduction and Artificial Insemination. Madrid, Spain. 99-114.

McCaskey, T.A. and J.B. Martin. 1988. Evaluation of a process for improved quality and microbial safety of broiler litter. Biol. Wastes. 25:209-218.

McKinnon, B.R. 1996. The nutritional and economic impact litter can make with cows. Paper presented at the Virginia Cattle and Poultry Litter Conference, November 6, Harrisonburg, VA.

Moore, N.W. and R.J. Bilton. 1973. The storage of fertilized sheep ova at 5 °C. Aust. J. Biol. Sci. 26:1421-1427.

Nibart, M. and P. Humblot. 1997. Pregnancy rates following direct transfer of glycerol sucrose or ethylene glycol cryopreserved bovine embryos. Theriogenology. 47:371 (Abstr.).

Nolan, R., D. O'Callaghan, R.T. Duby, P. Lonergan and M.P. Boland. 1998. The influence of short-term nutrient changes on follicle growth and embryo production following superovulation in beef heifers. 50:1263-1274.

O'Doherty, E.M., M.G. Wade, J.L. Hill and M.P. Boland. 1997. Effects of culturing bovine oocytes either singly or in groups on development to blastocysts. Theriogenology. 48:161-169.

Pettit, W.H., Jr. 1985. Commercial freezing of bovine embryos in glass ampules. Theriogenology. 23:13-16.

Pugh, D.G., D.L. Rankins, T. Powe and G. D'Andrea. 1994. Feeding broiler litter to beef cattle. Vet. Med. July. p 665.

Rajamahendran, R., R.S. Canseco, F.C. Gwazdauskas and W.E. Vinton. 1985. Observations on in vitro development of bovine morulae in Ham's F-10 and Dulbecco's phosphate buffered saline supplemented with normal steer serum. Theriogenology. 24:369-374.

Robertson, E. 2000. Non-surgical embryo transfer. Harrogate Genetics International, Inc. 6th ed. Harrogate, TN.

Rouillier, P., L.A. Guibault, J.G. Lussier and P. Matton. 1996. Changes in morphological appearance and functional capacity of recruited follicles in cows treated with FSH in the presence or absence of a dominant follicle. Theriogenology. 46:1053-1061.

Saacke, R.G., J.C. Dalton, S. Nadir, R.L. Nebel and J.H. Bame. 2000. Relationship of seminal traits and insemination time to fertilization rate and embryo quality. Anim. Reprod. Sci. 60-61:663-677.

SAS. 2001. Statistical Analysis System: A User's Guide. Version 8.02, 4th ed. Statistical Analysis System Institute, Inc., Cary, NC.

Shaw, D.W. and T.E. Good. 2000. Recovery rates and embryo quality following dominant follicle ablation in superovulated cattle. Theriogenology. 53:1521-1528.

Sherwood, L. 1997. The peripheral endocrine glands. 3rd ed. Human Physiology: From Cells to Systems. p 677. Wadsworth Publishing Company, Belmont, CA.

Singh, J. and G.P. Adams. 1998. Immunohistochemical distribution of follistatin in dominant and subordinate follicles and the corpus luteum of cattle. Biol. Reprod. 59:561-570.

Smith, L.W. 1973. Recycling animal wastes as protein sources. In: Alternative sources of protein for animal production. p 153. National Academy of Sciences. Washington, D.C.

Spell, A.R., W.E. Beal, L.R. Corah and G.C. Lamb. 2001. Evaluating recipient and embryo factors that affect pregnancy rates of embryo transfer in beef cattle. Theriogenology. 56:287-297.

Stephenson, A.H., T.A. McCaskey and B.G. Ruffin. 1990. A survey of broiler litter composition and potential value as a nutrient source. Biol. Wastes. 34:1-9.

Starkey, J. 2002. CAFO revisions: regulation without purpose? Poultry USA. January. p 98-102.

Talke, H. and G.E. Schubert. 1965. Enzymic determination of urea in blood serum, by the Warburg optical test. Klin. Wochschr. 43(3):174-175.

Tiffany, T.O., J. M. Jansen, C.A. Burtis, J.B. Overton and C.D. Scott. 1972. Enzymic kinetic rate and end-point analyses of substrate, by use of a GeMSAEC Fast Analyzer. Clin. Chem. 18(8):829-840.

U.S. Poultry and Egg Association. 2002. Frequently asked questions. Available at: http://www.poultryegg.org/Industry_Info/FAQ/faq.html. Accessed April 11, 2002.

USDA/ERS (U.S. Department of Agriculture, Economic Research Service). 1999. Food consumption, prices, and expenditures, 1970-97. SB965. Washington, DC: U.S. Department of Agriculture, Economic Research Service. April. http://www.ers.usda.gon/publications/sb965/

USEPA (U.S. Environmental Protection Agency). 2001. Development document for the proposed revisions to the national pollutant discharge elimination system regulation and the effluent guidelines for concentrated animal feeding operations. EPA-821-R-01-003.

Webb, K.E. and J.P. Fontenot. 1975. Medicinal drug residues in broiler litter and tissues from cattle fed litter. J. Anim. Sci. 41:1212-1217.

Wright, J.M. 1981. Non-surgical embryo transfer in cattle. Theriogenology. 15:43-56.

Wright, J.M. 1985. Commercial freezing of bovine embryos in straws. Theriogenology. 23:17-29.

Wright, P.A. 1995. Review: Nitrogen excretion: three end products, many physiological roles. J. Exp. Physiol. 198:273-281.

Wright, R.W. Jr. and J. Ellington. 1995. Morphological and physiological differences between in vivo- and in vitro-produced preimplantation embryos from livestock species. Theriogenology. 44:1167-1189.

Yaakub, H., D. O'Callaghan and M.P. Boland. 1999. Effect of type and quantity of concentrates on superovulation and embryo yield in beef heifers. Theriogenology. 51:1259-1266.

Appendices

ANOVA Tables from MIXED Model of SAS (2001) for Experiment 1

Appendix A. Table 1. Type 3 tests for fixed effects for differences in weight taken at the beginning of feeding (Wt1) for cows fed one of the three diets.

Effect	Numerator DF	Denominator DF	F-Value	Pr > F
Diet	2	43	0.55	0.5837

Appendix A. Table 2. Least square means for differences in weight taken at the beginning of feeding (Wt1) for cows fed one of the three diets.

Diet	Estimate	Standard Error
CON	595.8	20.6
SBM	600.6	23.1
LITTER	569.6	23.1

Appendix A. Table 3. Type 3 tests for fixed effects for differences in weight taken at estrus synchronization (Wt2) for cows fed one of the three diets.

Effect	Numerator DF	Denominator DF	F-Value	Pr > F
Diet	2	43	0.53	0.5943

Appendix A. Table 4. Least square means for differences in weight taken at estrus synchronization (Wt2) for cows fed one of the three diets.

Diet	Estimate	Standard Error
CON	600.5	21.3
SBM	598.0	23.8
LITTER	570.6	23.8

Appendix A. Table 5. Type 3 tests for fixed effects for differences in weight taken prior to embryo recovery (Wt3) for cows fed one of the three diets.

Effect	Numerator DF	Denominator DF	F-Value	Pr > F
Diet	2	43	0.18	0.8370

Appendix A. Table 6. Least square means for differences in weight taken prior to embryo recovery (Wt3) for cows fed one of the three diets.

Diet	Estimate	Standard Error
CON	597.2	20.7
SBM	599.5	23.3
LITTER	581.4	23.3

Appendix A. Table 7. Type 3 tests for fixed effects for differences in ova/embryos recovered from donors at embryo collection.

Effect	Numerator DF	Denominator DF	F-Value	Pr > F
Diet	2	40	1.37	0.2647
Date	3	40	2.64	0.0626

Appendix A. Table 8. Least square means for differences in ova/embryos recovered from cows that received one of the three diets.

Diet	Estimate	Standard Error
CON	17.7	1.9
SBM	17.9	2.1
LITTER	21.9	2.2

Appendix A. Table 9. Type 3 tests for fixed effects for differences in Stage 4 embryos recovered from donors at embryo collection.

Effect	Numerator DF	Denominator DF	F-Value	Pr > F
Diet	2	40	2.50	0.0945
Date	3	40	1.03	0.3893

Appendix A. Table 10. Least square means for differences in Stage 4 embryos recovered from cows that received one of the three diets.

Diet	Estimate	Standard Error
CON	4.1	1.0
SBM	7.0	1.1
LITTER	4.7	1.1

Appendix A. Table 11. Type 3 tests for fixed effects for differences in Stage 5 embryos recovered from donors at embryo collection.

Effect	Numerator DF	Denominator DF	F-Value	Pr > F
Diet	2	40	1.71	0.1933
Date	3	40	0.06	0.9798

Appendix A. Table 12. Least square means for differences in Stage 5 embryos recovered from cows that received one of the three diets.

Diet	Estimate	Standard Error
CON	4.9	1.1
SBM	1.9	1.2
LITTER	3.8	1.3

Appendix A. Table 13. Type 3 tests for fixed effects for differences in Stage 6 embryos recovered from donors at embryo collection.

Effect	Numerator DF	Denominator DF	F-Value	Pr > F
Diet	2	40	2.62	0.0851
Date	3	40	0.22	0.8809

Appendix A. Table 14. Least square means for differences in Stage 6 embryos recovered from cows that received one of the three diets.

Diet	Estimate	Standard Error
CON	0.1	0.1
SBM	0.0	0.1
LITTER	0.4	0.2

Appendix A. Table 15. Type 3 tests for fixed effects for differences in Grade 1 embryos recovered from donors at embryo collection.

Effect	Numerator DF	Denominator DF	F-Value	Pr > F
Diet	2	40	0.51	0.6064
Date	3	40	0.21	0.8877

Appendix A. Table 16. Least square means for differences in Grade 1 embryos recovered from cows that received one of the three diets.

Diet	Estimate	Standard Error
CON	5.8	1.0
SBM	4.4	1.1
LITTER	5.6	1.2

Appendix A. Table 17. Type 3 tests for fixed effects for differences in Grade 2 embryos recovered from donors at embryo collection.

Effect	Numerator DF	Denominator DF	F-Value	Pr > F
Diet	2	40	0.99	0.3812
Date	3	40	1.58	0.2092

Appendix A. Table 18. Least square means for differences in Grade 2 embryos recovered from donors that received one of the three diets.

Diet	Estimate	Standard Error
CON	2.9	0.9
SBM	4.1	0.9
LITTER	2.9	1.0

Appendix A. Table 19. Type 3 tests for fixed effects for differences in Grade 3 embryos recovered from donors at embryo collection.

Effect	Numerator DF	Denominator DF	F-Value	Pr > F
Diet	2	40	0.55	0.5826
Date	3	40	0.86	0.4684

Appendix A. Table 20. Least square means for differences in Grade 3 embryos recovered from donors that received one of the three diets.

Diet	Estimate	Standard Error
CON	0.5	0.2
SBM	0.8	0.2
LITTER	0.5	0.2

Appendix A. Table 21. Type 3 tests for fixed effects for differences in average quality score for embryos recovered from donors at embryo collection.

Effect	Numerator DF	Denominator DF	F-Value	Pr > F
Diet	2	40	0.95	0.3947
Date	3	40	0.79	0.5081

Appendix A. Table 22. Least square means for differences in average quality score for embryos recovered from cows that received one of the three diets.

Diet	Estimate	Standard Error
CON	1.4	0.1
SBM	1.6	0.2
LITTER	1.4	0.2

Appendix A. Table 23. Type 3 tests for fixed effects for differences in the number of degenerate embryos recovered from donors at embryo collection.

Effect	Numerator DF	Denominator DF	F-Value	Pr > F
Diet	2	40	0.60	0.5543
Date	3	40	0.33	0.8018

Appendix A. Table 24. Least square means for differences in the number of degenerate embryos recovered from cows that received one of the three diets.

Diet	Estimate	Standard Error
CON	1.4	0.7
SBM	2.2	0.7
LITTER	2.2	0.7

Appendix A. Table 25. Type 3 tests for fixed effects for differences in the number of unfertilized ova recovered from donors at embryo collection.

Effect	Numerator DF	Denominator DF	F-Value	Pr > F
Diet	2	40	1.39	0.2620
Date	3	40	1.34	0.2739

Appendix A. Table 26. Least square means for differences in the number of unfertilized ova recovered from cows that received one of the three diets.

Diet	Estimate	Standard Error
CON	7.2	1.8
SBM	6.8	2.0
LITTER	10.8	2.1

Appendix A. Table 27. Type 3 tests for fixed effects for differences in transferable embryos (Grade 1 + Grade 2 + Grade 3) recovered from donors at embryo collection.

Effect	Numerator DF	Denominator DF	F-Value	Pr > F
Diet	2	40	0.01	0.9928
Date	3	40	0.46	0.7123

Appendix A. Table 28. Least square means for differences in transferable embryos (Grade 1 + Grade 2 + Grade 3) recovered from cows that received one of the three diets.

Diet	Estimate	Standard Error
CON	9.1	1.7
SBM	9.3	1.8
LITTER	9.0	1.9

Appendix A. Table 29. Type 3 tests for fixed effects for differences in non-transferable embryos (degenerate embryos + unfertilized ova) recovered from donors at embryo collection.

Effect	Numerator DF	Denominator DF	F-Value	Pr > F
Diet	2	40	1.68	0.1991
Date	3	40	1.42	0.2506

Appendix A. Table 30. Least square means for differences in non-transferable (degenerate embryos + unfertilized ova) recovered from cows that received one of three diets.

Diet	Estimate	Standard Error
CON	8.6	1.8
SBM	8.9	2.0
LITTER	13.1	2.1

Appendix A. Table 31. Type 3 tests for fixed effects for differences in freezable embryos (Grade 1 + Grade 2) recovered from donors at embryo collection.

Effect	Numerator DF	Denominator DF	F-Value	Pr > F
Diet	2	40	0.00	0.9982
Date	3	40	0.46	0.7134

Appendix A. Table 32. Least square means for differences in freezable embryos (Grade 1 + Grade 2) recovered from cows that received one of three diets.

Diet	Estimate	Standard Error
CON	8.6	1.7
SBM	8.5	1.8
LITTER	8.5	1.9

Appendix A. Table 33. Type 3 tests for fixed effects for differences in the concentration of plasma urea nitrogen (PUN) pre-feeding (PUN 1) at estrus synchronization.

Effect	Numerator DF	Denominator DF	F-Value	Pr > F
Diet	2	43	211.54	< 0.0001

Appendix A. Table 34. Least square means for differences in the concentration of PUN pre-feeding (PUN 1), measured in mg/dL, for dietary treatments at estrus synchronization.

Diet	Estimate	Standard Error
CON	9.3	0.5
SBM	22.6	0.5
LITTER	10.4	0.5

Appendix A. Table 35. Type 3 tests for fixed effects for differences in the concentration of PUN post-feeding (PUN 2) at estrus synchronization.

Effect	Numerator DF	Denominator DF	F-Value	Pr > F
Diet	2	43	171.60	< 0.0001

Appendix A. Table 36. Least square means for differences in the concentration of PUN post-feeding (PUN 2), measured in mg/dL, for dietary treatments at estrus synchronization.

Diet	Estimate	Standard Error
CON	10.3	0.5
SBM	23.1	0.5
LITTER	16.4	0.5

Appendix A. Table 37. Type 3 tests for fixed effects for changes in the concentration of PUN (PUN 2 – PUN 1) at estrus synchronization.

Effect	Numerator DF	Denominator DF	F-Value	Pr > F
Diet	2	43	230.24	< 0.0001

Appendix A. Table 38. Least square means for changes in the concentration of PUN (PUN 2 – PUN 1), measured in mg/dL, for dietary treatments at estrus synchronization.

Diet	Estimate	Standard Error
CON	1.1	0.2
SBM	0.5	0.2
LITTER	5.9	0.2

Appendix A. Table 39. Type 3 tests for fixed effects for differences in the concentration of PUN pre-feeding (PUN 3) 1 d prior to embryo collection.

Effect	Numerator DF	Denominator DF	F-Value	Pr > F
Diet	2	43	106.31	< 0.0001

Appendix A. Table 40. Least square means for differences in the concentration of PUN pre-feeding (PUN 3), measured in mg/dL, for dietary treatments 1 d prior to embryo recovery.

Diet	Estimate	Standard Error
CON	7.6	0.7
SBM	20.9	0.8
LITTER	10.1	0.8

Appendix A. Table 41. Type 3 tests for fixed effects for differences in the concentration of PUN post-feeding (PUN 4) 1 d prior to embryo recovery.

Effect	Numerator DF	Denominator DF	F-Value	Pr > F
Diet	2	43	100.43	< 0.0001

Appendix A. Table 42. Least square means for differences in the concentration of PUN post-feeding (PUN 4), measured in mg/dL, for dietary treatments 1 d prior to embryo recovery.

Diet	Estimate	Standard Error
CON	9.2	0.7
SBM	21.4	0.7
LITTER	16.1	0.7

Appendix A. Table 43. Type 3 tests for fixed effects for changes in the concentration of PUN (PUN 4 – PUN 3) 1 d prior to embryo recovery.

Effect	Numerator DF	Denominator DF	F-Value	Pr > F
Diet	2	43	120.84	< 0.0001

Appendix A. Table 44. Least square means for changes in the concentration of PUN (PUN 4 – PUN 3), measured in mg/dL, for dietary treatments 1 d prior to embryo recovery.

Diet	Estimate	Standard Error
CON	1.5	0.3
SBM	0.5	0.3
LITTER	6.0	0.3

Appendix A. Table 45. Type 3 tests for fixed effects for differences in the concentration of total plasma protein (TPP) pre-feeding (TPP 1) at estrus synchronization.

Effect	Numerator DF	Denominator DF	F-Value	Pr > F
Diet	2	43	14.33	< 0.0001

Appendix A. Table 46. Least square means for differences in the concentration of TPP pre-feeding (TPP 1), measured in g/dL, for dietary treatments at estrus synchronization.

Diet	Estimate	Standard Error
CON	6.8	0.1
SBM	7.2	0.1
LITTER	6.6	0.1

Appendix A. Table 47. Type 3 tests for fixed effects for differences in the concentration of TPP post-feeding (TPP 2) at estrus synchronization.

Effect	Numerator DF	Denominator DF	F-Value	Pr > F
Diet	2	43	6.31	0.0040

Appendix A. Table 48. Least square means for differences in the concentration of TPP post-feeding (TPP 2), measured in g/dL, for dietary treatments at estrus synchronization.

Diet	Estimate	Standard Error
CON	6.8	0.1
SBM	7.1	0.1
LITTER	6.8	0.1

Appendix A. Table 49. Type 3 tests for fixed effects for changes in the concentration of TPP (TPP 2 – TPP 1) at estrus synchronization.

Effect	Numerator DF	Denominator DF	F-Value	Pr > F
Diet	2	43	5.30	0.0419

Appendix A. Table 50. Least square means for changes in the concentration of TPP (TPP 2 – TPP 1), measured in g/dL, for dietary treatments at estrus synchronization.

Diet	Estimate	Standard Error
CON	0.0	0.1
SBM	-0.1	0.1
LITTER	0.1	0.1

Appendix A. Table 51. Type 3 tests for fixed effects for differences in the concentration of TPP pre-feeding (TPP 3) prior to embryo collection.

Effect	Numerator DF	Denominator DF	F-Value	Pr > F
Diet	2	43	12.38	< 0.0001

Appendix A. Table 52. Least square means for differences in the concentration of TPP pre-feeding (TPP 3), measured in g/dL, for dietary treatments prior to embryo recovery.

Diet	Estimate	Standard Error
CON	6.7	0.1
SBM	7.2	0.1
LITTER	6.6	0.1

Appendix A. Table 53. Type 3 tests for fixed effects for differences in the concentration of TPP post-feeding (TPP 4) prior to embryo recovery.

Effect	Numerator DF	Denominator DF	F-Value	Pr > F
Diet	2	43	11.00	0.0001

Appendix A. Table 54. Least square means for differences in the concentration of TPP post-feeding (TPP 4), measured in g/dL, for dietary treatments prior to embryo recovery.

Diet	Estimate	Standard Error
CON	6.9	0.1
SBM	7.3	0.1
LITTER	6.7	0.1

Appendix A. Table 55. Type 3 tests for fixed effects for changes in the concentration of TPP (TPP 4 – TPP 3) prior to embryo recovery.

Effect	Numerator DF	Denominator DF	F-Value	Pr > F
Diet	2	43	0.23	0.7929

Appendix A. Table 56. Least square means for changes in the concentration of TPP (TPP 4 – TPP 3), measured in g/dL, for dietary treatments prior to embryo recovery.

Diet	Estimate	Standard Error
CON	0.2	0.1
SBM	0.1	0.1
LITTER	0.1	0.1

Appendix A. Table 57. Type 3 tests for fixed effects for differences in the concentration of plasma glucose (PGL) pre-feeding (PGL 1) at estrus synchronization.

Effect	Numerator DF	Denominator DF	F-Value	Pr > F
Diet	2	43	5.69	0.0064

Appendix A. Table 58. Least square means for differences in the concentration of PGL pre-feeding (PGL 1), measured in mg/dL, for dietary treatments at estrus synchronization.

Diet	Estimate	Standard Error
CON	62.3	0.8
SBM	65.9	0.9
LITTER	62.5	0.9

Appendix A. Table 59. Type 3 tests for fixed effects for differences in the concentration of PGL post-feeding (PGL 2) at estrus synchronization.

Effect	Numerator DF	Denominator DF	F-Value	Pr > F
Diet	2	43	1.38	0.2627

Appendix A. Table 60. Least square means for differences in the concentration of PGL post-feeding (PGL 2), measured in mg/dL, for dietary treatments at estrus synchronization.

Diet	Estimate	Standard Error
CON	61.2	1.3
SBM	61.7	1.5
LITTER	64.2	1.5

Appendix A. Table 61. Type 3 tests for fixed effects for changes in the concentration of PGL (PGL 2 – PGL 1) at estrus synchronization.

Effect	Numerator DF	Denominator DF	F-Value	Pr > F
Diet	2	43	5.15	0.0098

Appendix A. Table 62. Least square means for changes in the concentration of PGL (PGL 2 – PGL 1), measured in mg/dL, for dietary treatments at estrus synchronization.

Diet	Estimate	Standard Error
CON	-1.1	1.2
SBM	-4.2	1.3
LITTER	1.7	1.3

Appendix A. Table 63. Type 3 tests for fixed effects for differences in the concentration of PGL pre-feeding (PGL 3) prior to embryo collection.

Effect	Numerator DF	Denominator DF	F-Value	Pr > F
Diet	2	43	3.86	0.0288

Appendix A. Table 64. Least square means for differences in the concentration of PGL pre-feeding (PGL 3), measured in mg/dL, for dietary treatments prior to embryo recovery.

Diet	Estimate	Standard Error
CON	62.9	1.0
SBM	66.3	1.1
LITTER	65.0	1.1

Appendix A. Table 65. Type 3 tests for fixed effects for differences in the concentration of PGL post-feeding (PGL 4) prior to embryo recovery.

Effect	Numerator DF	Denominator DF	F-Value	Pr > F
Diet	2	43	1.50	0.2343

Appendix A. Table 66. Least square means for differences in the concentration of PGL post-feeding (PGL 4), measured in mg/dL, for dietary treatments prior to embryo recovery.

Diet	Estimate	Standard Error
CON	61.8	0.9
SBM	63.8	1.0
LITTER	63.8	1.0

Appendix A. Table 67. Type 3 tests for fixed effects for changes in the concentration of PGL (PGL 4 – PGL 3) prior to embryo recovery.

Effect	Numerator DF	Denominator DF	F-Value	Pr > F
Diet	2	43	0.55	0.5818

Appendix A. Table 68. Least square means for changes in the concentration of PGL (PGL 4 – PGL 3), measured in mg/dL, for dietary treatments prior to embryo recovery.

Diet	Estimate	Standard Error
CON	-1.1	1.1
SBM	-2.5	1.2
LITTER	-1.2	1.2

ANOVA Tables from GLM Procedure of SAS (2001) for Experiment 2

Appendix B. Table 1. Test for differences in the percentage of embryos that hatched for main effects at 24 h of *in vitro* culture (Type III MS).

Source	DF	Mean Square	F-Value	Pr > F
Diet	2	0.0130	0.09	0.9113
Bull	3	0.2604	1.86	0.1365
Cow (Diet•Bull)	41	0.2037	1.45	0.0408
Time	2	0.8833	6.30	0.0020
Temp	1	0.0017	0.01	0.9120
Error	356	0.1402		

Appendix B. Table 2. Test for differences in the percentage of embryos that hatched for bull and diet effects using the Type III MS for cow nested within diet by bull as the error term at 24 h of *in vitro* culture.

Source	DF	Mean Square	F-Value	Pr > F
Diet	2	0.0130	0.06	0.9382
Bull	3	0.2604	1.28	0.2947

Appendix B. Table 3. Least square means for differences in the percentage of embryos that hatched due to the interval from embryo collection to freezing at 24 h of *in vitro* culture.

Time (h)	Estimate	Standard Error
2	0.257	0.041
6	0.212	0.041
12	0.096	0.042

Appendix B. Table 4. Test for differences in the percentage of embryos that hatched for main effects at 48 h of *in vitro* culture (Type III MS).

Source	DF	Mean Square	F-Value	Pr > F
Diet	2	0.3987	1.99	0.1381
Bull	3	0.3543	1.77	0.1528
Cow (Diet•Bull)	41	0.4260	2.13	0.0001
Time	2	2.8030	13.99	<0.0001
Temp	1	0.7684	3.84	0.0509
Error	356	0.2003		

Appendix B. Table 5. Test for differences in the percentage of embryos that hatched for bull and diet effects using the Type III MS for cow nested within diet by bull as the error term at 48 h of *in vitro* culture.

Source	DF	Mean Square	F-Value	Pr > F
Diet	2	0.3987	0.94	0.4005
Bull	3	0.3543	0.83	0.4843

Appendix B. Table 6. Least square means for differences in the percentage of embryos that hatched due to the interval from embryo collection to freezing at 48 h of *in vitro* culture.

Time (h)	Estimate	Standard Error
2	0.535	0.049
6	0.416	0.049
12	0.240	0.051

Appendix B. Table 7. Least square means for differences in the percentage of embryos that hatched due to the holding temperature during the interval from embryo collection to freezing at 48 h of *in vitro* culture.

Temp	Estimate	Standard Error
Room	0.442	0.044
Refrigerated	0.352	0.045

Appendix B. Table 8. Test for differences in the percentage of embryos that hatched for main effects at 72 h of *in vitro* culture (Type III MS).

Source	DF	Mean Square	F-Value	Pr > F
Diet	2	0.5384	2.61	0.0747
Bull	3	0.4583	2.22	0.0850
Cow (Diet•Bull)	41	0.4172	2.02	0.0004
Time	2	3.3261	16.14	<0.0001
Temp	1	0.1880	0.91	0.3402
Error	356	0.2060		

Appendix B. Table 9. Test for differences in the percentage of embryos that hatched for bull and diet effects using the Type III MS for cow nested within diet by bull as the error term at 72 h of *in vitro* culture.

Source	DF	Mean Square	F-Value	Pr > F
Diet	2	0.5384	1.29	0.2860
Bull	3	0.4583	1.10	0.3607

Appendix B. Table 10. Least square means for differences in the percentage of embryos that hatched due to the interval from embryo collection to freezing at 72 h of *in vitro* culture.

Time (h)	Estimate	Standard Error
2	0.629	0.050
6	0.520	0.050
12	0.311	0.051

Appendix B. Table 11. Least square means for differences in the percentage of embryos that hatched due to diet effects at 72 h of *in vitro* culture.

Diet	Estimate	Standard Error
CON	0.554	0.056
SBM	0.526	0.050
LITTER	0.380	0.088

Appendix B. Table 12. Least square means for differences in the percentage of embryos that hatched due to bull effects 72 h of *in vitro* culture.

Bull	Estimate	Standard Error
1	0.620	0.062
2	0.472	0.061
3	0.371	0.060
4	0.483	0.113

Appendix B. Table 13. Test for differences in the percentage of embryos that developed for main effects at 24 h of *in vitro* culture (Type III MS).

Source	DF	Mean Square	F-Value	Pr > F
Diet	2	0.4562	2.46	0.0869
Bull	3	0.4811	2.59	0.0524
Cow (Diet•Bull)	41	0.3266	1.76	0.0038
Time	2	4.3288	23.34	<0.0001
Temp	1	0.2364	1.27	0.2596
Error	356	0.1854		

Appendix B. Table 14. Test for differences in the percentage of embryos that developed for bull and diet effects using the Type III MS for cow nested within diet by bull as the error term at 24 h of *in vitro* culture.

Source	DF	Mean Square	F-Value	Pr > F
Diet	2	0.4562	1.40	0.2589
Bull	3	0.4811	1.47	0.2360

Appendix B. Table 15. Test for differences in the percentage of embryos that developed for main effects at 48 h of *in vitro* culture (Type III MS).

Source	DF	Mean Square	F-Value	Pr > F
Diet	2	0.4415	2.49	0.0844
Bull	3	0.4475	2.52	0.0575
Cow (Diet•Bull)	41	0.2768	1.56	0.0186
Time	2	3.2483	18.32	<0.0001
Temp	1	0.1625	0.92	0.3391
Error	356	0.1773		

Appendix B. Table 16. Test for differences in the percentage of embryos that developed for bull and diet effects using the Type III MS for cow nested within diet by bull as the error term at 48 h of *in vitro* culture.

Source	DF	Mean Square	F-Value	Pr > F
Diet	2	0.4415	1.59	0.2153
Bull	3	0.4475	1.62	0.2003

Appendix B. Table 17. Test for differences in the percentage of embryos that developed for main effects at 72 h of *in vitro* culture (Type III MS).

Source	DF	Mean Square	F-Value	Pr > F
Diet	2	0.4450	2.51	0.0829
Bull	3	0.4269	2.41	0.0672
Cow (Diet•Bull)	41	0.2605	1.47	0.0367
Time	2	3.3082	18.64	<0.0001
Temp	1	0.2216	1.25	0.2646
Error	356	0.1775		

Appendix B. Table 18. Test for differences in the percentage of embryos that developed for bull and diet effects using the Type III MS for cow nested within diet by bull as the error term at 72 h of *in vitro* culture.

Source	DF	Mean Square	F-Value	Pr > F
Diet	2	0.4450	1.71	0.1938
Bull	3	0.4269	1.64	0.1953

Appendix B. Table 19. Least square means for differences in the percentage of embryos that developed due to the interval from embryo collection to freezing at 72 h of *in vitro* culture.

Time (h)	Estimate	Standard Error
2	0.796	0.046
6	0.735	0.046
12	0.492	0.048

Appendix B. Table 20. Least square means for differences in the percentage of embryos that developed due to diet effects at 72 h of *in vitro* culture.

Diet	Estimate	Standard Error
CON	0.761	0.052
SBM	0.704	0.046
LITTER	0.558	0.082

Appendix B. Table 21. Test for differences in the percentage of embryos that degenerated for main effects at 24 h of *in vitro* culture (Type III MS).

Source	DF	Mean Square	F-Value	Pr > F
Diet	2	0.4561	2.46	0.0869
Bull	3	0.4811	2.59	0.0524
Cow (Diet•Bull)	41	0.3266	1.76	0.0038
Time	2	4.3288	23.34	<0.0001
Temp	1	0.2364	1.27	0.2596
Error	356	0.1854		

Appendix B. Table 22. Test for differences in the percentage of embryos that degenerated for bull and diet effects using the Type III MS for cow nested within diet by bull as the error term at 24 h of *in vitro* culture.

Source	DF	Mean Square	F-Value	Pr > F
Diet	2	0.4561	1.40	0.2589
Bull	3	0.4811	1.47	0.2360

Appendix B. Table 23. Least square means for differences in the percentage of embryos that degenerated due to the interval from embryo collection to freezing at 24 h of *in vitro* culture.

Time (h)	Estimate	Standard Error
2	0.217	0.047
6	0.311	0.047
12	0.573	0.049

Appendix B. Table 24. Least square means for differences in the percentage of embryos that degenerated due to diet effects at 24 h of *in vitro* culture.

Diet	Estimate	Standard Error
CON	0.267	0.053
SBM	0.367	0.047
LITTER	0.467	0.084

Appendix B. Table 25. Least square means for differences in the percentage of embryos that degenerated due to bull effects 24 h of *in vitro* culture.

Bull	Estimate	Standard Error
1	0.251	0.059
2	0.294	0.058
3	0.472	0.056
4	0.452	0.107

Appendix B. Table 26. Test for differences in the percentage of embryos that degenerated for main effects at 48 h of *in vitro* culture (Type III MS).

Source	DF	Mean Square	F-Value	Pr > F
Diet	2	0.4415	2.49	0.0844
Bull	3	0.4475	2.52	0.0575
Cow (Diet•Bull)	41	0.2768	1.56	0.0186
Time	2	3.2483	18.32	<0.0001
Temp	1	0.1625	0.92	0.3391
Error	356	0.1773		

Appendix B. Table 27. Test for differences in the percentage of embryos that degenerated for bull and diet effects using the Type III MS for cow nested within diet by bull as the error term at 48 h of *in vitro* culture.

Source	DF	Mean Square	F-Value	Pr > F
Diet	2	0.4415	1.59	0.2153
Bull	3	0.4475	1.62	0.2003

Appendix B. Table 28. Least square means for differences in the percentage of embryos that degenerated due to the interval from embryo collection to freezing at 48 h of *in vitro* culture.

Time (h)	Estimate	Standard Error
2	0.204	0.046
6	0.273	0.046
12	0.508	0.048

Appendix B. Table 29. Least square means for differences in the percentage of embryos that degenerated due to diet effects at 48 h of *in vitro* culture.

Diet	Estimate	Standard Error
CON	0.239	0.052
SBM	0.304	0.046
LITTER	0.441	0.082

Appendix B. Table 30. Least square means for differences in the percentage of embryos that degenerated due to bull effects 48 h of *in vitro* culture.

Bull	Estimate	Standard Error
1	0.243	0.057
2	0.230	0.057
3	0.435	0.055
4	0.404	0.105

Appendix B. Table 31. Test for differences in the percentage of embryos that degenerated for main effects at 72 h of *in vitro* culture (Type III MS).

Source	DF	Mean Square	F-Value	Pr > F
Diet	2	0.4450	2.51	0.0829
Bull	3	0.4269	2.41	0.0672
Cow (Diet•Bull)	41	0.2605	1.47	0.0367
Time	2	3.3082	18.64	<0.0001
Temp	1	0.2216	1.25	0.2646
Error	356	0.1775		

Appendix B. Table 32. Test for differences in the percentage of embryos that degenerated for bull and diet effects using the Type III MS for cow nested within diet by bull as the error term at 72 h of *in vitro* culture.

Source	DF	Mean Square	F-Value	Pr > F
Diet	2	0.4450	1.71	0.1938
Bull	3	0.4269	1.64	0.1953

Appendix B. Table 33. Least square means for differences in the percentage of embryos that degenerated due to the interval from embryo collection to freezing at 72 h of *in vitro* culture.

Time (h)	Estimate	Standard Error
2	0.204	0.046
6	0.266	0.046
12	0.508	0.048

Appendix B. Table 34. Least square means for differences in the percentage of embryos that degenerated due to diet effects at 72 h of *in vitro* culture.

Diet	Estimate	Standard Error
CON	0.239	0.052
SBM	0.296	0.046
LITTER	0.442	0.082

Appendix B. Table 35. Least square means for differences in the percentage of embryos that degenerated due to bull effects 72 h of *in vitro* culture.

Bull	Estimate	Standard Error
1	0.243	0.057
2	0.230	0.057
3	0.434	0.055
4	0.396	0.105

Appendix B. Table 36. Test for differences in embryo quality grade for main effects at 24 h of *in vitro* culture (Type III MS).

Source	DF	Mean Square	F-Value	Pr > F
Diet	2	0.9787	2.79	0.0625
Bull	3	0.7649	2.18	0.0896
Cow (Diet•Bull)	41	0.7755	2.21	< 0.0001
Time	2	7.9582	22.72	<0.0001
Temp	1	1.2879	3.68	0.0560
Error	356	0.3503		

Appendix B. Table 37. Test for differences in embryo quality grade for bull and diet effects using the Type III MS for cow nested within diet by bull as the error term at 24 h of *in vitro* culture.

Source	DF	Mean Square	F-Value	Pr > F
Diet	2	0.9787	1.26	0.2938
Bull	3	0.7649	0.99	0.4086

Appendix B. Table 38. Least square means for differences in embryo quality grade due to the interval from embryo collection to freezing at 24 h of *in vitro* culture.

Time (h)	Estimate	Standard Error
2	1.3	0.1
6	1.4	0.1
12	1.8	0.1

Appendix B. Table 39. Least square means for differences in embryo quality grade due to the temperature during the interval from embryo collection to freezing at 24 h of *in vitro* culture.

Temp	Estimate	Standard Error
Room	1.4	0.1
Refrigerated	1.6	0.1

Appendix B. Table 40. Least square means for differences in embryo quality grade due to diet effects at 24 h of *in vitro* culture.

Diet	Estimate	Standard Error
CON	1.4	0.1
SBM	1.6	0.1
LITTER	1.6	0.1

Appendix B. Table 41. Least square means for differences in embryo quality grade due to bull effects at 24 h of *in vitro* culture.

Bull	Estimate	Standard Error
1	1.4	0.1
2	1.4	0.1
3	1.6	0.1
4	1.6	0.2

Appendix B. Table 42. Test for differences in embryo quality grade for main effects at 48 h of *in vitro* culture (Type III MS).

Source	DF	Mean Square	F-Value	Pr > F
Diet	2	1.1071	2.20	0.1128
Bull	3	1.2264	2.43	0.0648
Cow (Diet•Bull)	41	1.0276	2.04	0.0003
Time	2	9.2687	18.38	<0.0001
Temp	1	0.4607	0.91	0.3398
Error	356	0.5042		

Appendix B. Table 43. Test for differences in embryo quality grade for bull and diet effects using the Type III MS for cow nested within diet by bull as the error term at 48 h of *in vitro* culture.

Source	DF	Mean Square	F-Value	Pr > F
Diet	2	1.1071	1.08	0.3499
Bull	3	1.2264	1.19	0.3242

Appendix B. Table 44. Least square means for differences in embryo quality grade due to the interval from embryo collection to freezing at 48 h of *in vitro* culture.

Time (h)	Estimate	Standard Error
2	1.4	0.1
6	1.5	0.1
12	1.9	0.1

Appendix B. Table 45. Least square means for differences in embryo quality grade due to bull effects at 48 h of *in vitro* culture.

Bull	Estimate	Standard Error
1	1.4	0.1
2	1.5	0.1
3	1.8	0.1
4	1.6	0.2

Appendix B. Table 46. Test for differences in embryo quality grade for main effects at 72 h of *in vitro* culture (Type III MS).

Source	DF	Mean Square	F-Value	Pr > F
Diet	2	3.6183	5.51	0.0044
Bull	3	1.8524	2.82	0.0388
Cow (Diet•Bull)	41	1.1713	1.78	0.0031
Time	2	10.6568	16.23	<0.0001
Temp	1	0.0294	0.04	0.8325
Error	356	0.6566		

Appendix B. Table 47. Test for differences in embryo quality grade for bull and diet effects using the Type III MS for cow nested within diet by bull as the error term at 72 h of *in vitro* culture.

Source	DF	Mean Square	F-Value	Pr > F
Diet	2	3.6183	3.09	0.0563
Bull	3	1.8524	1.58	0.2085

Appendix B. Table 48. Least square means for differences in embryo quality grade due to the interval from embryo collection to freezing at 72 h of *in vitro* culture.

Time (h)	Estimate	Standard Error
2	1.5	0.1
6	1.7	0.1
12	2.1	0.1

Appendix B. Table 49. Least square means for differences in embryo quality grade due to diet effects at 72 h of *in vitro* culture.

Diet	Estimate	Standard Error
CON	1.5	0.1
SBM	1.7	0.1
LITTER	2.1	0.2

Appendix B. Table 50. Least square means for differences in embryo quality grade due to bull effects at 72 h of *in vitro* culture.

Bull	Estimate	Standard Error
1	1.5	0.1
2	1.6	0.1
3	1.9	0.1
4	2.0	0.2

VITA

Frank Dean Jousan, son of Frank and Beverly Jousan, Jr., was born on September 26, 1977. He graduated from Joaquin High School in Joaquin, TX, in 1996. During high school, he was interested in beef cattle reproduction and exhibited Simmental and Simbrah cattle across the country. In addition, he served on the Texas Junior Simmental/Simbrah Association Board of Directors, including one term as President. Furthermore, he served on the American Junior Simmental Association Board of Directors for two years, including one year as 1st Vice-President as well as Chairman for the South-Central Region for the AJSA.

In September 1996, he enrolled at Texas A&M University to pursue a Bachelor of Science degree in Animal Science with a Science Option. While at Texas A&M, he was involved in an undergraduate research project under the direction of Dr. Ted Friend. Following the completion of the project, he was a co-author for the paper, along with M.N. Collins, T.H. Friend and S.C. Chen, titled, *Effects of density on displacement, falls, injuries, and orientation during horse transportation,* that was published in Applied Animal Behavior Science in 2000 (67(3):169-179). In May 2000, he received his degree from Texas A&M University.

In August 2000, he pursued graduate studies in Physiology of Reproduction in cattle in the Department of Animal and Poultry Sciences at Virginia Polytechnic Institute and State University. During his time at VPI&SU, he received a Graduate Teaching Assistantship and taught Introduction to Animal and Poultry Science and Physiology of Reproduction laboratories for two years. He is an active member of the American Society for Animal Science and the Society for the Study of Reproduction. He recently accepted a graduate position to pursue his Ph.D. in Animal Sciences, with an emphasis in Animal Cell and Molecular Biology, under the direction of Dr. Peter Hansen at the University of Florida.

F. Dean Jousan