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Pregnancy establishment and maintenance in cattle[1]

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ABSTRACT: A single ovulation, reciprocal embryo transfer study was used to investigate effects of oocyte competence and maternal environment on pregnancy establishment and maintenance in beef cows. Estrous cycles were synchronized in suckled beef cows and embryo donors were inseminated on d 0 ($n = 810$). Cows were classified on d 0 as having a small (<12.5 mm) or large (≥12.5 mm) ovulatory follicle and randomly chosen as donors or recipients to remove confounding effects of ovulatory follicle size on fertility. Embryos ($n = 393$) or oocytes ($n = 44$) were recovered on d 7, and all viable embryos were transferred into recipients ($n = 354$). All statistical analyses were conducted using the GLM procedure of SAS. Path analysis (with significance set at $P < 0.10$) was used to examine potential cause–effect relationships among the measured variables. Greater donor cow BW, circulating estradiol concentration at insemination, postpartum interval, and ovulatory follicle size directly increased ($P < 0.10$) fertilization success. Greater donor cow age was the only factor that directly decreased ($P < 0.10$) fertilization success. Viability of d-7 embryos was directly inhibited ($P < 0.10$) by rapid follicular growth rate from d –2 to 0 and heavier BW. Direct beneficial effects to embryo viability were increased serum progesterone concentration on d –2 and ovulatory follicle size. Pregnancy maintenance from d 7 to 27 was enhanced ($P < 0.10$) by increased serum estradiol concentration on d 0 and progesterone concentration on d 7 in the recipient cow. Increased follicular diameter in the recipient cow on d 0 was detrimental to pregnancy maintenance from d 7 to 27. This manuscript defines the complex interplay and relative contributions of endocrine and physical factors both prior and subsequent to fertilization that influence both oocyte competence and maternal environment and their roles in establishment and maintenance of pregnancy.

Key words: beef cow, embryo survival, fertilization, pregnancy

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INTRODUCTION

Successful fertilization, establishment, and maintenance of pregnancy are evolutionarily important in all mammalian species and agronomically and economically important in food producing animals. Numerous factors, including size or maturity of the ovulatory follicle (Lamb et al., 2001; Vasconcelos et al., 2001; Perry et al., 2005), endocrine sufficiency (Pelican et al., 2010; Wen et al., 2010; Var et al., 2011), and age, BW, and adiposity (Scheffer et al., 1999; Souter et al., 2011), impact successful fertilization and establishment and maintenance of pregnancy.

Control of the time of ovulation is important in various mammals. Gonadotropin-releasing hormone is

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commonly used to induce ovulation in cattle. Induced ovulation of small (presumably immature) follicles with GnRH decreased pregnancy rates and increased late-embryonic/fetal mortality in beef cows (Perry et al., 2005). The mechanisms by which GnRH induced ovulation of an immature dominant follicle and affected the establishment and maintenance of pregnancy are unknown but could involve ovulation of a less competent oocyte (Arlotto et al., 1996; Brevini and Gandolfi, 2001), inadequate corpus luteum (**CL**) function (Vasconcelos et al., 2001; Perry et al., 2005; Busch et al., 2008), altered oviductal environment, compromised uterine environment (Moore, 1985; Murdoch and van Kirk 1998; Bridges et al., 2010), or a combination thereof. To separate the usual confounding factors of oocyte competence and maternal environment sufficiency, a reciprocal embryo transfer (**ET**) experiment, classifying ovulatory follicle size as large or small, was conducted with suckled beef cows. Our goal was to study ovulatory follicle size and several other variables on measures of fertility in beef cows. Therefore, the primary objective of this experiment was to differentiate among determinants of oocyte competence and maternal environmental effects on establishment and maintenance of pregnancy.

MATERIALS AND METHODS

All protocols and procedures were approved by the Fort Keogh Livestock and Range Research Laboratory Animal Care and Use Committee (IACUC approval number 101106-3).

Animal Handling

This experiment occurred over 3 breeding seasons with 9, 11, and 5 groups of suckled postpartum beef cows (predominantly Hereford-Angus crossbred) in yr 1, 2, and 3, respectively. The timeline for data collection in each group is illustrated in Fig. 1. Suckled beef cows (n = 2550) were synchronized with the CO-Synch protocol [GnRH on d –9 (**GnRH1**) followed by $PGF_{2\alpha}$ on d –2 and GnRH on d 0 (**GnRH2**) with fixed-timed AI], but only cows that did not exhibit estrus before GnRH2 or 2 to 7 d after GnRH2 (n = 1164) were used. Cows were classified on d 0 as having a small (<12.5 mm) or large (≥12.5 mm) ovulatory follicle and randomly chosen as donors or recipients to remove confounding effects of ovulatory follicle size on fertility. Donor cows (n = 810) were inseminated by 1 of 3 AI technicians with semen from 1 sire (3 collections). Single embryos were recovered by uterine horn lavage 7 d after AI and all live embryos were transferred fresh into recipients (n = 354) the same day as recovery. Body weights and BCS (scale of

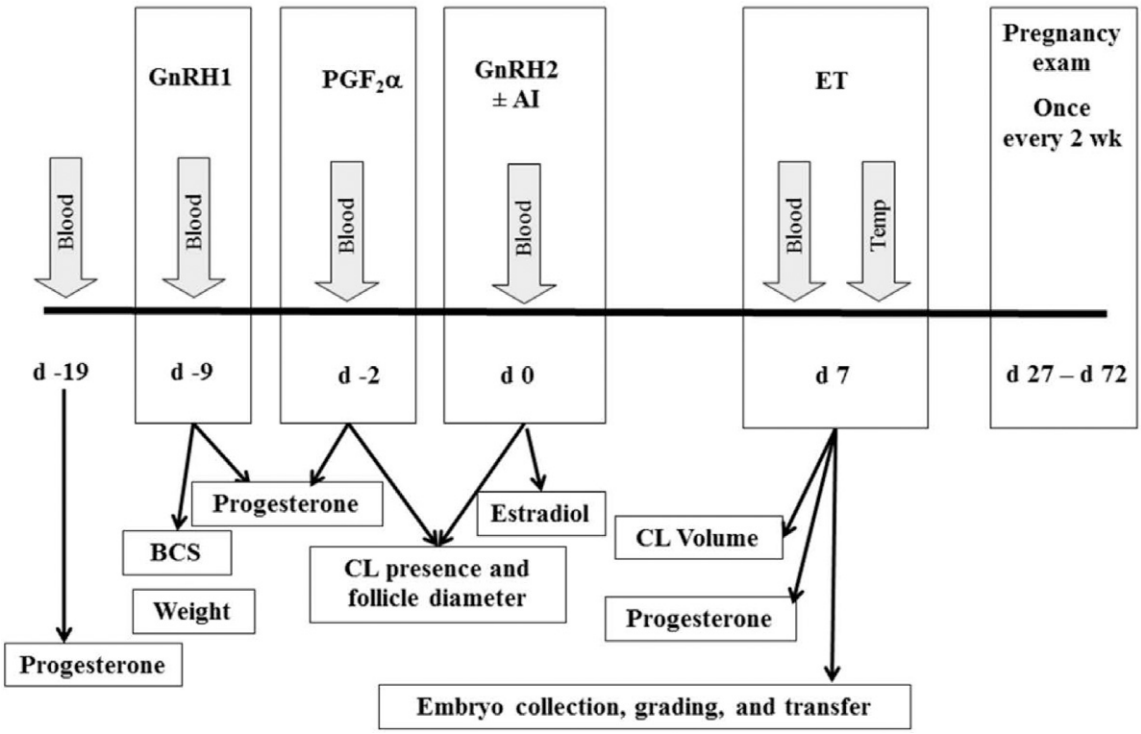

Figure 1. Experimental design. Treatments were based on ovulatory follicle size at GnRH2 of the donor and recipient cows. $PGF_{2\alpha}$ = prostaglandin $F_{2\alpha}$; ET = embryo transfer; Blood = blood collection for quantification of serum concentrations of progesterone and estradiol; Temp = rectal temperature; CL = corpus luteum.

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1 to 9 in which 1 = emaciated and 9 = obese; Whitman, 1975) were obtained on d –9. Rectal temperature was collected on d –9, 0, and 7 for donor and recipient cows.

Estrous Detection

Visual estrous detection occurred once daily from GnRH1 to $PGF_{2\alpha}$ and twice daily for 1 h from $PGF_{2\alpha}$ until GnRH2 in all groups and continued after GnRH2 in a subset of the groups (2 groups in yr 1 and all groups in yr 2 and 3). Estrotect (Western Point Inc., Apple Valley, MN) estrous detection patches were used on all cows to aid in estrous detection. Cows that were in estrus before GnRH2 or 2 to 7 d after GnRH2 were not included in the study.

Ovarian Structures

Ovaries were examined using an Aloka 500V ultrasound with a 7.5-MHz transducer (Aloka, Wallingford, CT). Diameter of the largest follicle on each ovary and the number of CL present were measured on d –2 ($PGF_{2\alpha}$) and d 0 (GnRH2). Follicle size was the average of the greatest diameter and the diameter perpendicular to it (Perry et al., 2005). The diameter and lumen of the CL were similarly measured in both donor and recipient cows on d 7.

Ovulatory Response

Ovulation after GnRH1 was estimated using estrous cyclicity status (described below) and number of CL present at $PGF_{2\alpha}$. Cows with 0 CL or cycling cows (concentrations of progesterone >1 ng/mL at GnRH1) with 1 CL were classified as not ovulating after GnRH1. Noncycling cows with 1 CL at $PGF_{2\alpha}$ and cycling cows with 2 CL were considered to have ovulated in response to GnRH1. A subset of cows were unable to be classified due to inconsistencies in the number of CL or cycling cows that had low progesterone at GnRH1 and a single CL at $PGF_{2\alpha}$ ($n = 217$). Among these cows, those with a CL at ET on the same ovary as the dominant follicle at GnRH2 were considered to have ovulated after GnRH2.

Embryo Recovery and Transfer

Embryos were recovered from donor cows by using a nonsurgical transcervical uterine catheterization technique on d 7. Catheters were placed in the uterine horn ipsilateral with the CL and a single horn was lavaged with ViGro Complete Flush Solution (Bioniche Animal Health, Athens, GA). Embryos were transferred fresh into the uterine horn ipsilateral to the CL on the same day as collected by 1 of 2 technicians. Without ET, there is a naturally occurring colinearity or confounding between ovulatory follicle size supporting embryogenesis and that supporting pregnancy. With ET, the effects of ovulatory follicle size on oocyte competence are separable from the effects of ovulatory follicle size on maternal recognition of pregnancy. Therefore, we chose to use the 12.5-mm threshold of follicle size as a convenient guide to break this confounding effect by transferring embryos that originated from large (≥12.5 mm) and small (<12.5 mm) follicles into recipients that had ovulated large and small follicles. Our decision to use 12.5 mm as the cutoff between large and small was based on the observation that optimal ovulatory follicle size varies across herds of cattle (Perry et al., 2005) and we wanted to be conservative in our approach. However, the statistical analysis of these data retains the continuous distribution of ovulatory follicle size. Therefore, ovulatory follicle size was not a treatment but a means of separating the confounding effects of this variable on pregnancy. An alternative conceptual model of this experiment is as a 2 × 2 factorial arrangement of treatments with follicle classifications divided by a threshold at 12.5 mm. However, this categorical arrangement denies the continuous nature of the distribution of follicle size and suggests that a follicle of 12.6 mm is somehow biologically distinct from one of 12.4 mm, which is very unlikely. Embryos collected from cows that ovulated a small follicle were transferred into cows that ovulated either a large ($n = 111$) or small ($n = 71$) follicle, and embryos collected from cows that ovulated a large follicle were transferred into cows that ovulated a large ($n = 50$) or small ($n = 122$) follicle.

Embryo Handling

Embryos and oocytes were washed 3 times in holding media (Biolife Holding Media; AgTech Inc., Manhattan, KS) and stored at 26°C until being graded and transferred. The interval between GnRH2 administration and grading was used as an estimation of embryo age. Embryo development (scale of 1 to 7 in which 1 = unfertilized oocyte and 7 = expanded blastocyst) and quality (scale of 1 to 4 in which 1 = excellent to good and 4 = degenerate or dead) were determined based on the classifications set by the International Embryo Transfer Society (**IETS**; Savoy, IL). All live embryos were randomly transferred into recipients except for embryos ($n = 8$) that were lost or damaged before transfer ($n = 6$) or for which a recipient was not available ($n = 2$). Additionally, some empty zona pellucidae ($n = 6$) were recovered but were not included in the analysis.

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Pregnancy Diagnosis

Pregnancy diagnosis began on d 27 to 29 after GnRH2 (or 20 to 22 d after transfer) and continued once every 2 wk until d 70 to 72 after GnRH2. Thus, pregnancy status of each recipient was determined 4 times. An Aloka 500V ultrasound was used with a 7.5-MHz or 5.0-MHz transducer (depending on the stage of pregnancy; Aloka) to determine presence of an embryo (d 27 to 29) or fetus (d 42 or later) and embryonic or fetal heartbeat.

Blood Collection and RIA

Blood was collected via tail venipuncture into 10 mL Vacutainer tubes (Fisher Scientific, Pittsburgh, PA) on d –19, –9, –2, 0, and 7. Blood was incubated at 4°C for 24 h and centrifuged at 1200 × *g* for 25 min at 4°C. Serum was harvested and stored at –20°C until RIA. Serum concentrations of progesterone were quantified by RIA with a Coat-a-Count RIA kit (Diagnostic Products Corporation, Los Angeles, CA; Bellows et al., 1991). Intra- and interassay CV were 1.8 and 13%, respectively, and the assay sensitivity was 0.08 ng/mL. Serum concentrations of progesterone at d –19 and d –9 were used to determine estrous cycling status of the cows (if both samples <1.0 ng/mL, then the cow was considered to be anestrus). Serum concentrations of estradiol on d 0 were quantified using RIA (Kirby et al., 1997). The intra- and interassay CV were 3.5 and 14%, respectively, and assay sensitivity was 0.5 pg/mL.

Statistical Analysis

Path analysis was used to examine potential cause–effect relationships among the measured variables (Wright, 1934). Path coefficients measure importance of a given path from cause to effect, defined as the ratio of the variability of the effect to be found when all causes are constant except the one in question, the variability of which is kept unchanged, to the total variability (Wright, 1920). Therefore, individual path coefficients are equivalent to standard partial regression coefficients and their squares measure the percentage of variation by each cause. Furthermore, the correlation between any 2 variables equals the sum of the products of the chains of path coefficients along all of the paths by which they are connected (Wright, 1920).

The overall sum of direct and indirect effects of independent variables on the primary dependent variables was measured using univariate standard linear regression. The primary dependent variables that were modeled included fertilization, embryo viability, quality, and developmental stage at d 7 and pregnancy at d 27 and d 72. Body condition score, days postpartum (**DPP**), BW, and age of both donor and recipient cows were considered strictly independent variables. Cycling status at start of treatment, ovulation after GnRH1, serum concentrations of progesterone at d –2 and d 7, serum concentrations of estradiol and follicle size at d 0, follicle growth from d –2 to d 0, rectal temperature at d 7, and embryo quality and stage (only donors with recovered embryos) were hypothesized to be independent with respect to the primary dependent variables but potentially themselves dependent on other independent variables, including those measured at a time before their being measured.

All statistical analyses were conducted using the GLM procedure (SAS Inst. Inc., Cary, NC). All data were first analyzed with a linear model that included fixed effects of year, group within year, and AI and ET technicians (when appropriate). Residuals (i.e., data adjusted for these nuisance effects) were subsequently calculated and standardized. These adjusted data values have mean = 0.0 and variance = 1.0 and were used in all subsequent analyses. Standard partial regression coefficients or path coefficients were estimated by multiple regression using the standardized data. For each dependent variable, an initial model was postulated that included as independent variables all variables that preceded the dependent variable in time of expression and that characterized the cows at the start of the experiment. These models were reduced by backward elimination until all variables remaining in the model had P-values ≤ 0.10 and path diagrams reflecting the final models were drawn.

RESULTS

Mean follicle diameter of all cows at GnRH2 was 12.5 ± 0.1 mm. Embryo recovery rate (including unfertilized oocytes) was 54% (439/810), of which, 90% (394/439) were fertilized embryos. Embryo quality and morphology were 1) excellent/good [63% (242/386)], 2) fair [22% (84/386)], 3) poor [9% (34/386)], and 4) dead [7% (26/386)] and 1) unfertilized (n = 45), 2) 2 to 12-cell (n = 19), 3) early morula (n = 54), 4) morula (n = 224), 5) early blastocyst (n = 79), 6) blastocyst (n = 9), and 7) expanded blastocyst (n = 1), respectively. Recipient pregnancy rates on approximately d 27, 42, 56, and 72 were 54 (190/354), 51 (182/354), 49 (175/354), and 49% (173/354), respectively. The mean (±SE) for each variable in this dataset was BCS (4.90 ± 0.02), DPP (86.6 ± 0.6 d), BW (517 ± 2 kg), age (3.93 ± 0.05 yr; range 2 to 11 yr), cyclicity (88.9 ± 0.7%), ovulation (59.5 ± 1.5%), follicle growth rate (1.99 ± 0.05 mm/d), d-0 serum estradiol concentration (8.50 ± 0.09 pg/mL), d –2 serum progesterone concentration (4.33 ± 0.07 ng/mL), live embryos (93.4 ± 1.2%), d-7 serum progesterone concentration (2.48 ± 0.04 ng/mL), and d-7 temperature (38.52 ± 0.03°C). A conservative level of significance (P < 0.10) was used to rule out variables that did not influence fertility measures.

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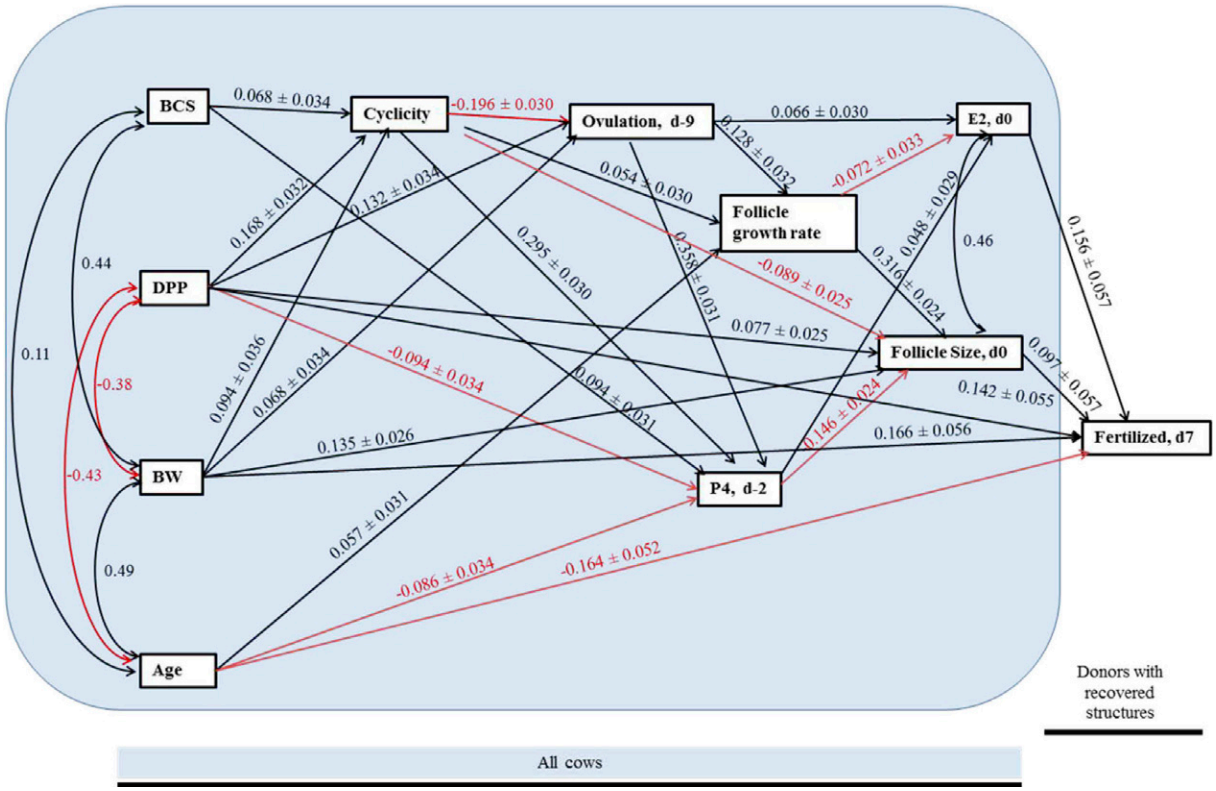

Figure 2. Path analysis of factors leading to successful fertilization. All cows (donor and recipients; $n = 1164$) were used to calculate the standardized partial regression coefficients in the shaded area but only donor cows ($n = 437$) from which an embryo or unfertilized oocyte was recovered were used to determine the partial regression coefficients leading to fertilization. Red arrows correspond to a negative association and black correspond to a positive association. All arrows indicate significant effects ($P < 0.10$) and the absence of an arrow indicates no significant effect ($P \geq 0.10$). DPP = days postpartum; P4 = serum concentrations of progesterone; E2 = serum concentrations of estradiol.

Observations Leading to Fertilization

At the beginning of treatment, older cows were heavier, had fewer DPP, and had slightly greater BCS (i.e., were fatter) than younger cows ($P < 0.10$; Fig. 2). Cows that were heavier also had greater BCS and shorter DPP. Collectively, DPP, BW, and BCS explained 3.5% of the variation in whether or not the cows were cycling before d –9 (GnRH1). Days postpartum was approximately 1.8 times more important than BW in explaining variation in cycling status, with BW being nearly 1.4 times more important than BCS. Whether or not a cow ovulated after GnRH1 on d –9 was directly affected by whether or not she was cycling, DPP, and BW, with these 3 variables explaining 4.9% of the variation. Cows that were determined to be cycling before d –9 were less likely to ovulate at the beginning of the synchronization protocol (GnRH1) and this effect was 1.5 to 2.9 times more important than the effect of DPP or BW, respectively.

Follicular growth rate from d –2 to 0 was greater ($P < 0.10$) in cows that ovulated in response to GnRH1 compared with cows that did not ovulate. Follicular growth leading up to induced ovulation (d 0) was more rapid in cycling and older cows with these effects being of approximately equal magnitude but of substantially less (0.43×) importance than ovulation at GnRH1. Collectively, the direct effects explained 2% of the variation in follicular growth rate. Twenty-two percent of the variation in follicular diameter on d 0 was explained by (magnitude of effects relative to that of follicular growth rate shown parenthetically) follicular growth rate, serum concentration of progesterone on d –2 (0.46), BW (0.43), cyclicity (0.28), and DPP (0.24). Other things being equal, return to estrous cyclicity and increased concentration of serum progesterone on d –2 compromised ovulatory follicle size, and greater follicular growth rate from d –2 to 0, BW, and DPP increased ovulatory follicle size.

Endocrine status was characterized by serum concentrations of progesterone at d –2 and estradiol at d 0. Collectively, the direct effects explained 18% of the variation in progesterone but only 3% of the variation in estradiol. Estrous cyclicity and ovulation response to GnRH1 had large positive effects ($P < 0.10$) on serum progesterone concentrations on d –2, with a relatively minor additional positive contribution from increased BCS. Variables having negative effects ($P < 0.10$) on serum progesterone concentration on d –2 were increased cow age and increased DPP. Rapid follicular growth rate from d –2 to d 0 reduced ($P < 0.10$) estradiol concentration in serum on d 0 whereas ovulation in response to

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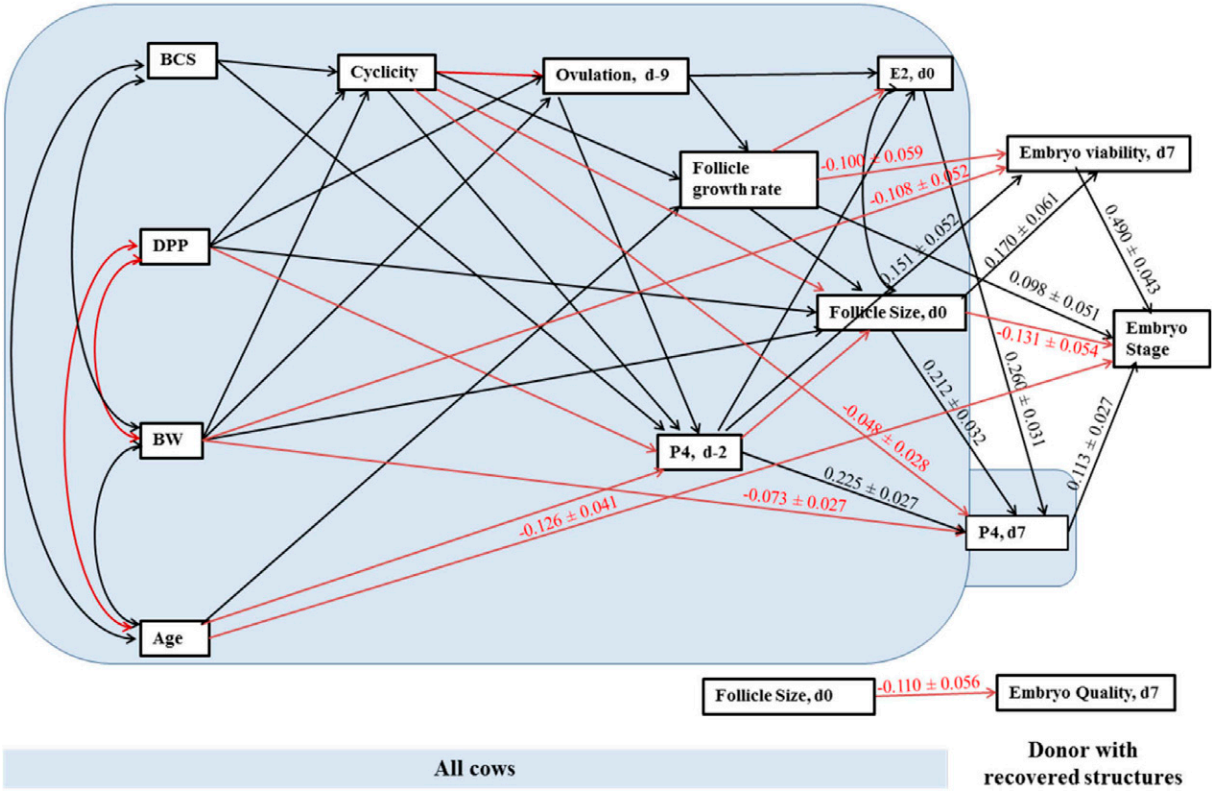

Figure 3. Path analysis of factors leading to embryo characteristics. All cows (donors and recipients; $n = 1164$) were used to calculate the standardized partial regression coefficients in the shaded area but only donor cows from which an embryo was recovered ($n = 393$) were used to determine the partial regression coefficients for embryo measurements. Red arrows correspond to a negative association and black correspond to a positive association. All arrows indicate significant effects ($P < 0.10$) and the absence of an arrow indicates no significant effect ($P \geq 0.10$). Values for arrows without coefficients may be found in Fig. 2. DPP = days postpartum; P4 = serum concentrations of progesterone; E2 = serum concentrations of estradiol; embryo quality = 1 (excellent/good), 2 (fair), 3 (poor), and 4 (dead); embryo stage = 1 (unfertilized), 2 (2 to 12-cell), 3 (early morula), 4 (morula), 5 (early blastocyst), 6 (blastocyst), and 7 (expanded blastocyst).

GnRH1 of the CO-Synch protocol and increased progesterone on d –2 enhanced ($P < 0.10$) the concentration of estradiol. It is noteworthy that despite efforts to mechanistically describe the relationship between follicle size and estradiol on d 0, they remained correlated (0.46; $P < 0.10$) after accounting for the modeled relationships.

Establishment and Maintenance of Pregnancy

The total recovery rate of both oocytes and embryos was 54% (439/810), and of these, 10.3% ($n = 45$) were unfertilized. Successful fertilization was enhanced ($P < 0.10$) in heavier cows, in younger cows, in cows with greater serum concentration of estradiol on d 0, in cows with a longer post partum period, and by increased size of the ovulatory follicle (Fig. 2). These variables accounted for a total of 9% of the variation in fertilization success. The relative magnitudes of effects of all factors affecting fertilization were similar. Mean (±SEM) ovulatory follicle size of cows from which a fertilized embryo was recovered was 12.6 ± 0.1 compared with 11.7 ± 0.4 for unfertilized oocytes ($P < 0.005$).

The probability that the embryo collected on d 7 was alive was enhanced by a larger ovulatory follicle, greater concentration of progesterone in serum on d –2, lighter BW, and a follicle that grew more slowly from d –2 to d 0 (Fig. 3). Collectively, however, these factors accounted for only approximately 5% of the variation in embryo survival with all effects being of similar magnitude.

The concentration of serum progesterone in both donor and recipient cows at ET (d 7) was enhanced ($P < 0.10$) by increased concentrations of estradiol on d 0 and progesterone on d –2 and by increased follicle size on d 0. The magnitudes of these effects were similar. Increased BW and cyclicity had relatively minor negative effects on serum progesterone on d 7. Taken together, these effects explained 21% of the variation in serum progesterone on d 7. Embryonic development was obviously enhanced by embryo viability and to a lesser degree by growth rate of the ovulatory follicle and the serum concentration of progesterone on d 7. Increased follicle size and advancing cow age compromised embryonic development. Collectively these factors explained 29% of the variation in the stage of embryonic development. Ovulatory follicle size directly improved embryo quality (Fig. 3) and serum estradiol concentration had an equal but indirect positive effect on embryo quality (Table 1).

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Table 1. Standardized linear regression coefficients (sum of all direct and indirect effects) for traits hypothesized as potentially affecting variation in the dependent variables fertilization, embryo viability, quality, and stage on d 7 and pregnancy on d 27 and 72 in beef cows

Independent variable[1]		Fertilization	Embryo viability	Embryo quality	Embryo stage	Pregnant, d 27	Pregnant, d 72
Age	Donor	–0.127 ± 0.044*	–0.048 ± 0.048	0.054 ± 0.048	–0.169 ± 0.048*	0.012 ± 0.052	0.025 ± 0.063
	Recipient	–	–	–	–	0.071 ± 0.057	0.127 ± 0.067*
DPP	Donor	0.140 ± 0.051*	0.033 ± 0.053	0.047 ± 0.053	0.071 ± 0.053	–0.009 ± 0.056	0.145 ± 0.070*
	Recipient	–	–	–	–	–0.069 ± 0.048	–0.021 ± 0.062
BW	Donor	0.060 ± 0.049	–0.097 ± 0.051*	0.009 ± 0.052	0.074 ± 0.052	0.011 ± 0.056	0.097 ± 0.068
	Recipient	–	–	–	–	–0.009 ± 0.053	0.025 ± 0.071
BCS	Donor	0.005 ± 0.049	–0.023 ± 0.051	–0.042 ± 0.051	0.004 ± 0.051	–0.058 ± 0.056	0.082 ± 0.071
	Recipient	–	–	–	–	–0.021 ± 0.053	–0.017 ± 0.071
Cyclicity	Donor	–0.006 ± 0.043	–0.010 ± 0.029	0.019 ± 0.029	–0.033 ± 0.029	0.025 ± 0.031	0.043 ± 0.041
	Recipient	–	–	–	–	0.001 ± 0.031	0.007 ± 0.042
Ovulation, d –9	Donor	0.064 ± 0.051	0.109 ± 0.053*	–0.055 ± 0.053	0.086 ± 0.053	–0.059 ± 0.057	–0.009 ± 0.078
	Recipient	–	–	–	–	0.145 ± 0.059*	–0.100 ± 0.077
P4, d –2	Donor	–0.011 ± 0.048	0.123 ± 0.050*	–0.064 ± 0.051	0.160 ± 0.050*	–0.015 ± 0.054	0.058 ± 0.065
	Recipient	–	–	–	–	0.111 ± 0.050*	–0.043 ± 0.062
E2, d 0	Donor	0.211 ± 0.051*	0.094 ± 0.056*	–0.110 ± 0.056*	0.010 ± 0.057	–0.028 ± 0.061	0.023 ± 0.077
	Recipient	–	–	–	–	0.216 ± 0.052*	–0.020 ± 0.073
Follicle size, d 0	Donor	0.178 ± 0.050*	0.094 ± 0.056	–0.110 ± 0.056*	–0.038 ± 0.056	–0.007 ± 0.062	0.005 ± 0.080
	Recipient	–	–	–	–	0.003 ± 0.057	–0.004 ± 0.078
Follicle growth	Donor	0.084 ± 0.051*	–0.043 ± 0.058	–0.006 ± 0.058	0.033 ± 0.057	0.057 ± 0.061	–0.056 ± 0.075
	Recipient	–	–	–	–	0.025 ± 0.055	–0.094 ± 0.078
Temp, d 0	Donor	0.123 ± 0.083	0.045 ± 0.050	–0.074 ± 0.051	–0.016 ± 0.051	–	–
	Recipient	–	–	–	–	–	–
P4, d 7	Donor	–	0.153 ± 0.058*	–0.122 ± 0.059*	0.156 ± 0.059*	–0.016 ± 0.064	0.003 ± 0.086
	Recipient	–	–	–	–	0.231 ± 0.049*	0.006 ± 0.066
Temp, d 7	Donor	–	0.004 ± 0.050	–0.069 ± 0.051	0.026 ± 0.051	0.004 ± 0.055	0.007 ± 0.076
	Recipient	–	–	–	–	0.139 ± 0.057*	–0.018 ± 0.072
Embryo age	Donor	–	–	0.011 ± 0.051	0.038 ± 0.051	–	–
Embryo viability	Donor	–	–	–0.685 ± 0.037*	0.499 ± 0.044*	–	–
Embryo stage	Recipient	–	–	–	–	0.064 ± 0.065	0.064 ± 0.087
Embryo quality	Recipient	–	–	–	–	0.045 ± 0.072	–0.173 ± 0.089*

*$P < 0.10$

[1]DPP = days postpartum; P4 = serum concentrations of progesterone; E2 = serum concentrations of estradiol; Temp = rectal temperature; embryo age = time from GnRH2 to transfer of embryo; embryo quality = 1 (excellent/good), 2 (fair), 3 (poor), and 4 (dead); embryo stage = 1 (unfertilized), 2 (2 to 12-cell), 3 (early morula), 4 (morula), 5 (early blastocyst), 6 (blastocyst), and 7 (expanded blastocyst).

Serum progesterone concentration on d 7 had a similar positive but indirect effect on embryo quality (Table 1).

Factors directly related to successful maintenance of pregnancy to d 27 were all characteristics of the recipient cows receiving a live embryo on d 7 (Fig. 4). Increased serum concentrations of progesterone on d 7 and estradiol on d 0 enhanced ($P < 0.10$) successful maintenance of pregnancy to d 27, as did advancing age and increased body temperature of the recipient. Increased size of the ovulatory follicle in the recipient female was detrimental to maintenance of pregnancy to d 27 when all other variables were held constant, including serum estradiol on d 0 and serum progesterone on d 7. However, if differences in serum progesterone concentration are allowed, then the effect of follicle size on pregnancy is positive ($P < 0.10$). Collectively these factors explained 12% of the variation in the probability that pregnancy was successfully maintained to d 27. For those pregnancies that were successful at d 27, greater-quality embryos (lower IETS quality score) and older recipients were indicative of a greater probability that pregnancy would be maintained through d 72.

DISCUSSION

Conclusions from inspection of Figs. 2 through 4 are 1) relationships among factors affecting measures of fertility are numerous and are both direct and mediated through other intermediate factors, 2) no single variable controlled a preponderance of the variation in any of the

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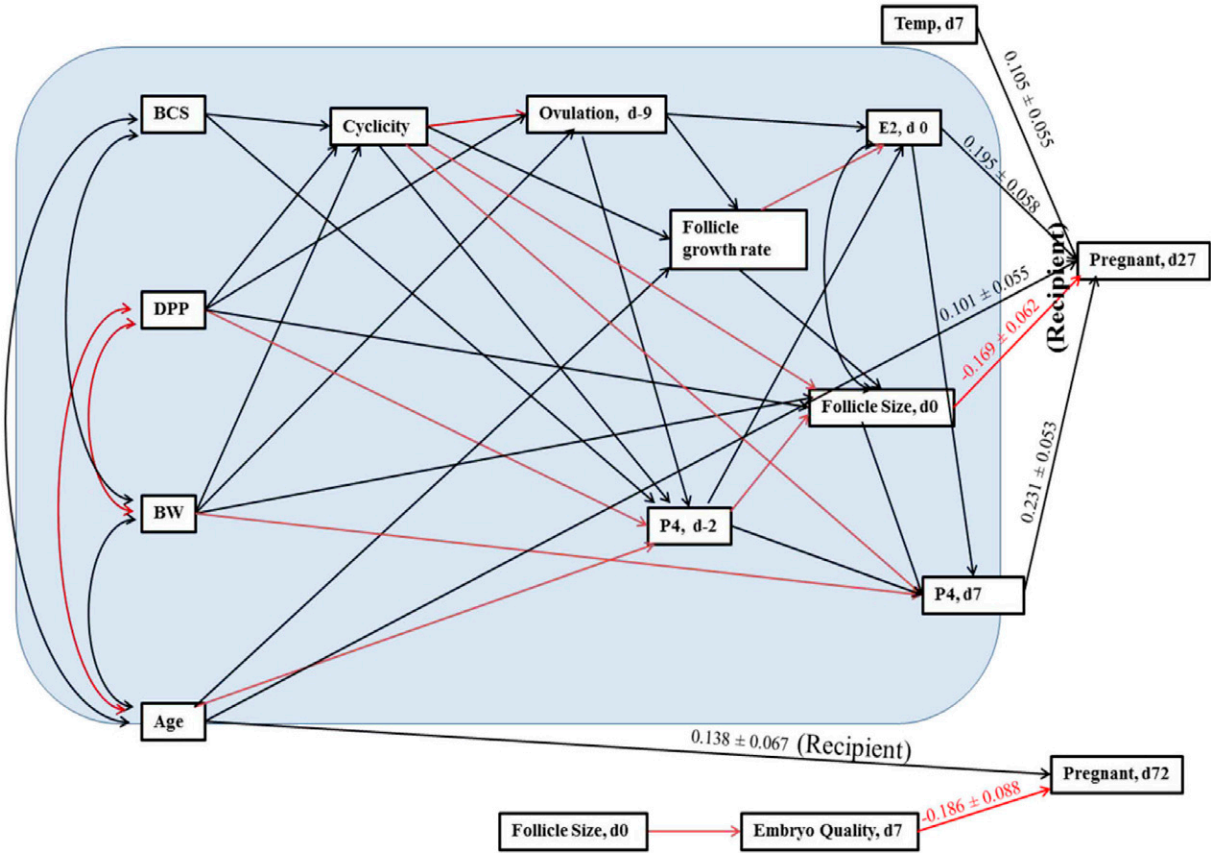

Figure 4. Path analysis of factors leading to pregnancy success in beef cows. Only data of recipients and donors from which an embryo was transferred (n = 354) were used to determine factors affecting pregnancy establishment (d 27) or maintenance (d 72). Red arrows reflect a negative association and black correspond to a positive association. All arrows indicate significant effects ($P < 0.10$) and the absence of an arrow indicates no significant effect ($P \geq 0.10$). Values for arrows without coefficients may be found in Fig. 2 or 3. The shaded region of the figure represents all cows in the study whereas the unshaded portion represents direct effects on cows that received an embryo. DPP = days postpartum; P4 = serum concentrations of progesterone; E2 = serum concentrations of estradiol; Temp = rectal temperature.

primary response variables, 3) understanding factors affecting the primary response variables requires simultaneous consideration of several causative variables, and 4) much of the biology underlying establishment and maintenance of pregnancy remains to be elucidated. The decision to use $P < 0.10$ over $P < 0.05$ in the path analysis was the result of a compromise between the power of the test and level of significance. It could be viewed that we chose the more conservative approach in that we wanted to rule out only those factors that truly were not relevant.

State of the Female before Insemination

Several of the variables measured in this study (including BCS, DPP, BW, and age) have been reported to affect pregnancy rate in cattle, but the mechanisms by which these variables influence pregnancy have not been critically evaluated (Short et al., 1990). Some of the earlier literature included studies designed to create diverse ranges in these variables, but the current study was designed to ensure adequate BCS and DPP to minimize influences on dependent variables. Nonetheless, although these variables were strongly correlated with each other and affected several of the intermediate variables in the path analyses, their overall effects on fertilization, recovery of a live embryo, embryo developmental stage, embryo quality, and pregnancy establishment and maintenance were minimal and are not the focus of this discussion. Based on the current study, it appears that the positive influences of BCS, DPP, and BW on reproduction are mediated through return to estrous cyclicity and small improvements in fertilization success. The negative influences of cow age on fertilization success and embryo quality were countered by positive influences of age on embryo developmental stage and pregnancy establishment and maintenance.

Increased serum concentrations of progesterone during the cycle before conception have been associated with increased conception rates (Folman et al., 1973; Corah et al., 1974; Fonseca et al., 1983; Bello et al., 2006). Serum concentrations of progesterone at ET (d 7) and $PGF_{2\alpha}$ (d –2) directly and indirectly advanced embryo stage of development in donor cows at ET. Although it is not known how increased circulating concentrations of progesterone from the previous cycle affect conception rate, it is possible that oocyte competence is com-

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promised by reduced concentrations of progesterone (Mihm et al., 1994).

Ovulation in response to GnRH1 on d –9 increased the growth rate and diameter of the ovulatory follicle at GnRH2 in the present study and others (Atkins et al., 2010a,b) and reduced the variation in ovulatory follicle size at GnRH2 (Bello et al., 2006). Ovulation response after GnRH1 would also affect the age of the follicle induced to ovulate after GnRH2 and may have resulted in ovulation of an older follicle because cows that exhibited estrus before GnRH2 were removed from the study. Although the increased follicular growth rate from d –2 to 0 greatly increased size of the ovulatory follicle, it also decreased serum estradiol concentrations slightly, which would not be expected.

State of the Female at Insemination

The strong correlation between serum estradiol concentration and ovulatory follicle size on d 0 demonstrates that examining effects of either variable without concurrent consideration of the other would overstate the importance of either one. Even with the strong correlation between these traits, each still had an independent direct effect on fertilization success. Induced ovulation of a large follicle was associated with increased serum concentrations of estradiol at ovulation (Vasconcelos et al., 2001; Perry et al., 2005, 2007; Bello et al., 2006; Busch et al., 2008). In the present study, successful fertilization increased with increasing serum estradiol and diameter of the ovulatory follicle. Therefore, prematurely ovulated follicles of smaller size that result in lesser serum estradiol likely provide a lower quality oocyte. Bovine oocytes and surrounding cumulus cells have nuclear estradiol receptors (Beker-Van Woudenberg et al., 2004); therefore, a direct or indirect effect of estradiol is feasible. Competence of bovine oocytes increased with follicular diameter (Arlotto et al., 1996) and administration of an aromatase inhibitor reduced the ability of primate oocytes to mature to metaphase II (Zelinski Wooten et al., 1993). The oocyte continues cytoplasmic, nuclear, and molecular maturation within the growing follicle (Sirard et al., 2006) and oocyte growth is intimately associated with follicular maturation (Fair, 2003). Induced ovulation of a premature follicle may result in the release of an oocyte that is less capable of being fertilized, less able to activate the embryonic genome, or possibly result in reduced development potential of the embryo/fetus/offspring.

Increased serum estradiol (d 0) concentrations and ovulatory follicle size among donor cows were associated with improved embryo quality. The ovum, zygote, and early embryo are dependent on mRNA and proteins synthesized before the LH surge. Protein and mRNA synthesis continued to occur in oocytes from bovine follicles up to 15 mm in diameter (Arlotto et al., 1996). In bovine oocytes, transcription increased until just before germinal vesicle breakdown (Rodriguez and Farin, 2004), after which the oocyte was unable to continue mRNA synthesis. The bovine embryo is unable to transcribe mRNA until the maternal to embryonic transition, which occurs around the 8- to 16-cell stage (Brevini and Gandolfi, 2001). Therefore, premature exposure of an oocyte to an LH surge could reduce the maternally derived mRNA that might be important for development before activation of the embryonic genome.

Another emerging area of research that is relevant to oocyte effects on the establishment and maintenance of pregnancy is the epigenetic control and imprinted gene contribution to embryo development. Several genes are reported to be imprinted for specific expression by either the paternal or maternal allele (Tycko and Morison, 2002; Miyoshi et al., 2006) and aberrant imprinting has been implicated in abnormalities during embryonic, fetal, and neonatal development (Moore and Reik, 1996; Farin et al., 2006). It is possible that the follicular environment may alter imprinting of specific genes as oocytes from superstimulated mice and women had a differential imprinting pattern compared with controls (Sato et al., 2007) and concentrations of steroids and gonadotropins were reported to change the global methylation pattern of mouse oocytes in vivo (Murray et al., 2008).

Increased subsequent serum concentrations of progesterone have been associated with increased ovulatory follicle size and serum estradiol concentrations (Vasconcelos et al., 2001; Perry et al., 2005; Busch et al., 2008; Stevenson et al., 2008). Either or both of the preceding alterations in ovarian steroid secretion could alter the oviductal and uterine environments to impact fertilization success. Estradiol may affect the oviductal or uterine environment by changing the pH of the uterus (Elrod and Butler, 1993; Perry and Perry, 2008a,b), altering sperm transport and longevity (Allison and Robinson, 1972; Hawk, 1983) to improve fertilization success and oviduct secretions (e.g., oviductal glycoprotein; Buhi, 2002) and indirectly stimulate progesterone activity through induction of progesterone receptors in the uterus (Stone et al., 1978; Zelinski et al., 1982; Ing and Tornesi, 1997). Ovarian steroids have been implicated in oviductal gene expression and deficiencies in estradiol and or progesterone would likely result in nutrient insufficiency for optimal embryo development (Bauersachs et al., 2004).

Embryo Viability, Developmental Stage, and Quality

The negative direct effect of ovulatory follicle size on embryo development is overridden by the sum

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of positive indirect effects of ovulatory follicle size through increased d-7 serum progesterone concentration, increased d-0 estradiol concentration (via increased d-7 serum progesterone concentration), and increased incidence of a live embryo. The negative effect of ovulatory follicle size on embryo quality score actually denotes improved embryo quality because a lower score reflects better quality. Besides embryo viability, the largest factors improving embryo quality and developmental stage were donor serum progesterone concentrations on d 7 and d –2 and follicle size and serum estradiol concentrations on d 0. Advanced embryo development in donor cows with increased serum progesterone on d –2 may have been partially due to oviductal or uterine priming effects. Positive effects of improved embryo developmental stage and quality on pregnancy establishment have been reported previously in cattle (Donaldson, 1985; Hasler, 2001) and other species (Balaban et al., 2000). A beneficial effect of progesterone as early as 2 to 3 d after estrus on embryo development has been reported previously (Maurer and Echternkamp, 1982) and is obvious from the results presented here.

Pregnancy at Day 27 and Day 72

Clearly progesterone is important to the establishment and maintenance of pregnancy. Progesterone affects uterine environment by inducing histotrophe secretions from the uterine glands and decreasing myometrial contractions (Geisert et al., 1992; Spencer et al., 2004). It is possible that progesterone has a direct effect on the embryo as bovine embryos were reported recently to have progesterone receptors (Clemente et al., 2009). However, addition of progesterone to cultured embryos did not change embryo quality or development; therefore, a direct progesterone action on the embryo is still unclear (Fukui and Ono, 1989). Serum estradiol concentration at the time of GnRH2-induced ovulation was the second most important factor affecting pregnancy establishment and is likely acting through increasing progesterone receptors in the uterine endometrium (Miller et al., 1977; Bridges et al., 2007). From the combination of indirect effects, ovulatory response to GnRH1 in recipient cows was the next most important effect on pregnancy establishment and may be related to formation of a healthier CL from a more optimally developed follicle. Luteal cells are derived from follicular cells, so factors affecting follicular differentiation and maturation may also affect subsequent luteinization. Follicular secretion of estradiol may affect luteal production of progesterone, as ewes treated with an aromatase inhibitor before induced ovulation had a delayed increase in serum concentrations of progesterone (Benoit et al., 1992). Progesterone production by human luteinized granulosa cells was affected by the follicular environment (McNatty and Sawers, 1975) or gonadotropin exposure before ovulation (Lobb and Younglai, 2001). Furthermore, vascular development of the follicle could affect the blood supply to the subsequent CL (Tamanini and De Ambrogi, 2004). Therefore, ovulatory response to GnRH1 may be an important variable driving success of timed AI programs in beef cattle.

Younger recipient cows experienced greater embryonic mortality between d 7 and pregnancy diagnosis on d 27 and 72, even though younger donor cows experienced greater fertilization success. In dairy cattle, younger cows (≤4 yr old) tended to have decreased embryonic mortality compared with older cows (≥5 yr old) between wk 5 to 9 of gestation (Starbuck et al., 2004). This is the first demonstration of greater embryonic mortality in younger cows that have been observed previously to have decreased pregnancy rates (Short et al., 1990). The younger cows used in the present study had greater days postpartum but decreased energy reserves (BCS) than older cows. Taken together, it would appear that pregnancy maintenance (at least among young cows) ranks lower in nutritional partitioning than pregnancy establishment, contrary to earlier reports (Short et al., 1990). Increased recipient cow temperature at time of ET translated to greater pregnancy establishment (d 27) contrary to earlier reports but the range in body temperature in the present study was less than reported previously (Vasconcelos et al., 2006). The negative effect of ovulatory follicle size on pregnancy establishment (d 27) is eliminated through positive indirect effects of follicle size on d-27 pregnancy. Improved embryo quality had the greatest impact on pregnancy maintenance followed by donor cow postpartum interval and recipient cow age.

Given the numerous variables measured in this study, they still accounted for only a minor percentage of the variation in each fertility trait. The independent variables accounted for only 9.4% of the variation in fertilization success, 4.6% of the variation in embryo viability, 47.9% of the variation in embryo quality (largely due to effects of embryo viability on embryo quality), and 29.2% of the variation in embryo developmental stage (again largely due to effects of embryo viability). Only 11.9 and 4.1% of the variation in pregnancy at d 27 and 72, respectively, were accounted for by the independent traits measured in this study. Therefore, there is still much progress to be made in understanding all of the variables affecting fertility in beef cows.

In summary, the follicular environment of the donor cow affected fertilization rate and embryonic survival before d 7 but maintenance of pregnancy subsequent to d 7 was dependent on ovulatory follicle size and estradiol production and subsequent progesterone production

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in the recipient cows (independent of the ovulatory follicle size of the donor cow) after single embryo recovery and transfer. This result indicates a potential role of oocyte competence in fertilization and early embryo survival (although the oviductal and uterine environment may play a role) 7 d after breeding. The maternal environment (as influenced primarily by estradiol and progesterone) affected the maintenance of pregnancy after ET. The amount of variation in the primary dependent variables measured (fertilization on d 7 and pregnancy maintenance to d 28 and d 72) accounted for by the multiple variables measured in this study was very small (9.4, 11.9, and 4.1%, respectively).

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