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Effects of energy and protein nutrition in the dam on embryonic development

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Abstract

This review highlights the importance of energy and protein nutrition of the dam on embryo production and embryo development. Fertility is reduced by greater negative energy balance post-partum as manifest by reductions in fertility and embryo quality associated with lower body condition score (BCS) but particularly with greater postpartum loss of BCS. In addition, excessive energy intake, particularly from high carbohydrate diets can reduce fertilization and embryo quality in some but not all circumstances. High protein diets have been found to reduce embryo quality by day 7 after breeding, possibly due to greater blood urea nitrogen, however this negative effect is not observed in all studies. Sufficient circulating concentrations of amino acids, particularly rate-limiting amino acids such as methionine and lysine are critical for optimal milk production. The rate-limiting amino acids may also impact embryonic development, perhaps through improved amino acid profiles in the uterine lumen. Methionine may also have direct epigenetic effects in the embryo by methylation of DNA. Future studies are needed to replicate previously observed positive and negative effects of energy, excess protein, and amino acid supplementation in order to provide further insight into how embryonic development can be rationally manipulated using nutritional strategies.

Keywords: amino acids, embryo, energy, methionine, nutrition, protein.

Introduction

A number of reviews have highlighted the importance of nutrition in regulating bovine reproductive efficiency (Rabiee *et al.*, 2001; Vasconcelos *et al.*, 2003; Wu *et al.*, 2004; Wiltbank *et al.*, 2006; Grummer *et al.*, 2010; Santos *et al.*, 2010; Cardoso *et al.*, 2013). The effects of nutrition in the donor cow have been particularly emphasized (Santos *et al.*, 2008; Sartori *et al.*, 2010, 2013; Velazquez, 2011; Wu *et al.*, 2013). This review will specifically focus on the effects of energy and protein nutrition in the dam on bovine preimplantation embryo development.

The effects of inadequate or excessive energy, protein, or specific amino acids could be having effects at multiple stages of the reproductive process. First, effects during the early post-partum period have been postulated to alter the oocyte and subsequent embryo development after fertilization of this perturbed oocyte (Britt, 1992). Second, changes in circulating factors such as insulin, glucose, urea, or amino acids during the final stages of oocyte development, prior to ovulation, can profoundly impact fertilization or embryo development (Ocon and Hansen, 2003; Adamiak *et al.*, 2005, 2006; Bender *et al.*, 2014). A third obvious target of nutrition on the embryo, is during the first week of embryo development when changes in oviductal and uterine environment could alter development of the embryo to the blastocyst stage (Steeves and Gardner, 1999a, b; Steeves *et al.*, 1999). Finally, changes in circulating energy sources, such as glucose and propionate, and building blocks for cells, such as amino acids, could alter the uterine lumen and subsequently alter hatching and embryo elongation. The elongating embryo secretes the protein interferon-tau that is essential for rescue of the corpus luteum and can alter expression of specific proteins, such as amino acid transporters in endometrial epithelial cells, and thus alter the concentrations of many substances in the uterine lumen (Gao *et al.*, 2009c; Hugentobler *et al.*, 2010; Groebner *et al.*, 2011). Alternatively, select nutrients in the uterine lumen can also alter interferon-tau expression (Kim *et al.*, 2011). During all the time prior to embryo attachment to the uterine caruncles, the embryo is free-floating and is dependent upon uterine secretions into the uterine lumen, termed histotroph, for energy and the building blocks for development, including amino acids. Thus, deficiencies or excesses of energy, protein, or specific amino acids could have targeted impact on a specific stage of oocyte/embryo development or may have multiple, potentially additive effects, on reproductive processes. Complete characterization of all of these nutritional effects on reproduction is likely to be impossible but this manuscript attempts to review some of the key studies that have been designed to begin to unravel some of these specific effects. Due to space limitations, many specific nutritional effects will not be approached in this particular review article, including effects of fatty acid supplementation, as well as vitamin and mineral

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supplementation or deficiencies, however some of these aspects have been recently reviewed (Santos *et al.*, 2008; Velazquez, 2011; Leroy *et al.*, 2013, 2014).

Energy intake, carbohydrates, and insulin effects on embryo quality

Effects of BCS and changes in BCS on fertility and embryo quality

The relationships between energy intake, energy output, and form of dietary energy (fiber *vs*. non-fiber carbohydrate, NFC) have been shown to produce profound effects on metabolic status of the cow and, in some cases, reproductive performance of both dairy and beef cattle. Part of this effect is due to a delayed return to cyclicity. Negative energy balance decreases dominant follicle growth and estradiol (E2) production probably related to the decrease in LH pulses as well as the decrease in circulating insulin and IGF-1 (Canfield and Butler, 1990; Butler, 2003, 2005). The magnitude of BCS loss after calving can increase in the percentage of cows that are not cycling at the end of the voluntary waiting period (Gumen *et al.*, 2003; Santos *et al.*, 2004, 2009; Lopez *et al.*, 2005). An increase in percentage of anovular cows will lower reproductive efficiency in programs using detection of estrus or synchronized ovulation and timed AI (TAI; Gumen *et al.*, 2003; Santos *et al.*, 2009). Cows with lower BCS near the time of AI have decreased fertility (Moreira *et al.*, 2000; Souza *et al.*, 2008) and this may be related to increased anovulation as BCS decreases.

In a recent retrospective study (Carvalho *et al.*, 2014), we evaluated the effect of BCS near TAI on reproductive performance of lactating dairy cows treated with Double-Ovsynch (Souza *et al.*, 2008; Herlihy *et al.*, 2012) to induce cyclicity and synchronize ovulation. Cows with low BCS ($\leq$2.5) compared to higher BCS ($\geq$2.75) had greater incidence of anovulation (12.3% [21/171] *vs.* 4.9% [22/451]; P = 0.0006) and decreased pregnancies per AI (P/AI; 40.4% [105/260] *vs*. 49.2% [415/843]; P = 0.03). Thus, BCS near AI has a small but significant effect on fertility even when cows are induced into cyclicity using a GnRH-based protocol, such as Double-Ovsynch.

Potentially even more important to fertility than the absolute BCS at the time of AI is the amount of BCS loss between parturition and first AI (López-Gatius *et al.*, 2003; Santos *et al.*, 2009). Consistent with this idea, in experiment 2 of our study (Carvalho *et al.*, 2014) we observed a much more dramatic effect on P/AI when we evaluated cows for BCS change between calving and 21 day after calving. The P/AI differed (P < 0.001) dramatically among BCS change categories and was greater for cows that gained BCS (83.5%; 353/423), intermediate for cows that maintained BCS (38.2%; 258/675), and least for cows that lost BCS (25.1%; 198/789). Thus, these results are consistent with the idea first introduced by Britt (1992), who postulated that energy status during the early post-partum period could alter follicular/oocyte quality resulting in negative effects on subsequent fertility in lactating dairy cows.

In experiment 3 (Carvalho *et al.*, 2014), we decided to directly test this hypothesis by evaluating the effect of early postpartum body weight loss on embryo quality from superovulated cows (Carvalho *et al.*, 2014). The body weight of lactating dairy cows (n = 71) was measured weekly from first to ninth week postpartum and then all cows had superovulation induced using a modified Double-Ovsynch protocol. Cows were divided into quartiles by percentage of body weight change (Q1 = least change; Q4 = most change) from calving until third week postpartum. There was no effect of quartile on number of ovulations, total embryos collected, or percentage of oocytes that were fertilized; however, the percentage of fertilized oocytes that were transferable embryos was greater for cows in Q1, Q2 and Q3 than Q4 (83.8, 75.2, 82.6, and 53.2%, respectively). In addition, percentage of degenerated embryos was least for cows in Q1, Q2, and Q3 and greatest for Q4 (9.6, 14.5, 12.6, and 35.2% respectively). Thus the effect of changes in BCS during the early post-partum period on subsequent fertility at first AI could be partially explained by the reduction in embryo quality and increase in degenerate embryos by day 7 after AI in cows that lost more body weight from first to third week postpartum. This result is obviously consistent with the hypothesis introduced by Britt (1992).

A retrospective study of 642 *Bos taurus* beef cattle donors (Table 1), predominantly Angus, evaluated the effect of BCS on superstimulation and embryo quality (Garcia Guerra *et al.*, 2007). Elevated but not lowered BCS was associated with decreased superovulatory response as measured by number of corpora lutea (P < 0.0001), total ova/embryo recovered (P = 0.0004), and number of fertilized ova (P < 0.0001). However, no differences in number of transferable embryos were detected, primarily because the percentage of fertilized ova that resulted in transferable embryos was greater (P < 0.0001) in donors with higher BCS.

Thus, BCS and particularly BCS change has been shown to have dramatic effects on fertility on early embryo development in dairy cattle. Results from this study in *Bos taurus* beef cattle were more consistent with a negative effect of elevated BCS on superovulatory response but not on embryo development.

Table 1. Effect of body condition score (BCS; on scale of 1 to 5 using 0.5 increments) on superovulatory responses.

Body condition score	2 to 2.5	3 to 3.5	4 to 4.5	5	P-value
Corpora Lutea	15.0 ± 1.4^{ab}	15.7 ± 0.5^{a}	13.9 ± 0.5^{b}	12.1 ± 0.9^{c}	<0.0001
Total ova/embryo	13.8 ± 1.6^{ab}	14.0 ± 0.5^{a}	12.3 ± 0.6^{b}	11.0 ± 1.0^{b}	0.0004
Fertilized ova	10.5 ± 1.5^{a}	9.6 ± 0.3^{a}	7.9 ± 0.4^{b}	6.9 ± 0.7^{b}	<0.0001
% Fertilized ova (Fertilized/Total)	74.0 ± 5.2	72.2 ± 1.5	71.6 ± 1.8	64.3 ± 3.3	0.1158
Transferable embryos	5.5 ± 0.8	5.1 ± 0.2	4.9 ± 0.2	4.6 ± 0.5	0.6196
% Transferable of fertilized (Transferableembryos/Fertilized)	59.7 ± 4.7^{bc}	57.6 ± 1.4^{c}	68.3 ± 1.7^{ab}	72.8 ± 2.7^{a}	<0.0001
% Transferable of total (Transferable embryos/Total)	40.3 ± 3.4^{ab}	40.3 ± 1.3^{b}	47.7 ± 1.8^{a}	45.4 ± 3.0^{ab}	0.0383

[a-c]Values with different superscripts within row differ (P<0.05); Mean ± SEM.

Effects of high energy diets or feed restriction on embryo quality

Another contrasting idea related to dietary energy intake and energy balance is reduction in embryo quality that has been observed in some studies when cows were fed excessive energy in the diet. Increases in feed intake or increased dietary NFC have been found to alter insulin (Adamiak *et al.*, 2005, 2006), progesterone (P4; Sangsritavong *et al.*, 2002; Vasconcelos *et al.*, 2003), and superovulatory success (Yaakub *et al.*, 1999). Superovulated beef heifers that were fed a high energy diet ad libitum (excessive energy) compared to 81% of ad libitum intake had reduced number of CL, reduced number of recovered structures, and dramatically reduced yield of transferrable embryos (Yaakub *et al.*, 1999). Thus, excessive energy consumption can alter embryo development, although the mechanism(s) for these effects and whether the effects are on the oocyte or directly on the early embryo are not yet fully described. An important idea that needs to still be adequately tested is that excessive energy could lead to overstimulation of the follicle and oocyte leading to subsequent reductions in embryo development (Webb and Campbell, 2007; Garnsworthy *et al.*, 2008a, b; Rooke *et al.*, 2009). Some evidence for negative effects of overfeeding on embryo development is provided by a study using superovulated ewes in which overfeeding (2.2 times maintenance) dramatically reduced embryo quality compared to underfed (0.5 times maintenance) ewes (Lozano *et al.*, 2003). This last study, as well as others in lactating cows (Sangsritavong *et al.*, 2002; Vasconcelos *et al.*, 2003), also observed that animals with higher feed intake had reduced circulating P4 concentrations. Previous studies have shown that increased circulating P4 concentrations during superovulation increased embryo quality and number of transferrable embryos (Nasser *et al.*, 2011; Rivera *et al.*, 2011). Lower circulating P4 may lead to increased LH pulses possibly leading to premature resumption of meiosis and ovulation of an oocyte of reduced fertility, as has been observed in persistent follicle models (Roberson *et al.*, 1989; Revah and Butler, 1996). In addition to effect of P4 during preovulatory follicle development, increasing circulating P4 concentrations after breeding, during early embryo development, can increase embryo development, particularly increasing length of the preimplantation embryo (Lonergan *et al.*, 2013; Lonergan and Forde, 2014; Maillo *et al.*, 2014; O'Hara *et al.*, 2014a, b; Wiltbank *et al.*, 2014).

In addition, excessive elevations in insulin may decrease oocyte quality and subsequent embryo development. Adamiak *et al.* (2005) conducted an elaborate experiment collecting oocytes via ultrasound-guided transvaginal follicular aspiration in beef x dairy crossbred heifers exposed to either maintenance (M) or two times maintenance (2M) feed levels over a period of three successive estrous cycles. The study found that the effect of feeding level on oocyte quality is dependent on body condition of the heifers; thus, the high feeding level had a positive impact on oocytes recovered from heifers in a low body condition score but had a negative impact on oocytes recovered from heifers of a moderately high body condition score. In addition, many of the moderately fat heifers were hyperinsulinemic, which also had a negative impact on oocyte quality. In a similar study, heifers exposed to a high starch diet had a corresponding increase in circulating insulin concentrations and a subsequent decrease in blastocyst production rate (Adamiak *et al.*, 2006). Thus, excessive energy intake may reduce embryo quality through elevations in LH pulses or through excessive insulin or other metabolic signal associated with consumption of a high carbohydrate diet or excess energy.

We tested these ideas in multiple experiments during the last few years. Nonlactating Nelore cows (n = 32) were offered maintenance (M, 1.2% of DM/kg of BW), 0.7 x M, or 1.5 x M diets for 42 days, in a cross-over design (Sartori *et al.*, 2009). Superstimulatory response was slightly lower in the low energy group compared to the maintenance group (14.6 ± 1.6^{a} *vs.* 12.6 ± 1.4^{b} *vs.* 13.6 ± 1.5^{ab} number of follicles > 6 mm; $P < 0.05$), whereas, there were no differences in superovulatory response (11.0 ± 1.4 *vs.* 9.8 ± 1.3 *vs.* 10.2 ± 1.3 corpora lutea; $P > 0.05$), fertilization rate ($P = 0.71$) or percentage of viable embryos ($P = 0.98$) among M, 0.7 x M, and 1.5 x M, respectively. In

another cross-over designed experiment by the Sartori laboratory, 14 Simmental x Nelore crossbred beef cows with high BCS, were fed either a maintenance diet or were overfed for 7 days prior to superovulation. Superstimulation and superovulation were similar, however, overfed cows had lower numbers of embryos/ova and viable embryos recovered as compared to the maintenance diet group (9.5 ± 1.8 *vs*. 14.1 ± 2.3 and 6.7 ± 1.5 *vs*. 10.7 ± 2.1, respectively (Sartori *et al.*, 2009). Thus in beef cattle, carefully-designed feeding trials (Sartori *et al.*, 2009) have found either no effect (*Bos indicus*) or a negative effect (cross-bred) of high energy diets on embryo production.

In later lactation Holstein dairy cows, energy intake generally exceeds energy output and therefore cows are in positive energy balance and circulating insulin is elevated. Acute restriction of feed intake reduced circulating insulin and increased circulating P4 in late lactation dairy cows (Ferraretto *et al.*, 2014). We used this model to test specific hypotheses related to feed intake (ad-libitum intake *vs*. 25% feed restricted) and LH (± additional LH) in superovulated Holstein cows in late lactation using a 2 x 2 Latin Square design (Bender *et al.*, 2014). Our first hypothesis was that acute feed restriction (25%) during a superovulation protocol would not alter ovulation rate but would increase embryo quality. Our second hypothesis was that increasing LH during the superovulation protocol would increase ovulation rate but might reduce embryo quality. Finally, we hypothesized that there would be important interactions between these two treatments with increasing LH in the FSH preparation, potentially leading to increased embryo yield in feed-restricted cows but potentially having a negative effect in cows fed ad libitum.

As expected, feed restriction had a substantial effect on circulating insulin concentrations without changing plasma glucose concentrations (Bender *et al.*, 2014). Large changes were not observed in numbers of large follicles on the final day of superstimulation, in the percentage of these follicles that ovulated, or in the number of CL on the day of flushing. Probably the most consistent and biologically-interesting result from this study was an interaction that was found between feed restriction and amount of LH during the superovulation protocol on the percentage of oocytes that were fertilized, and on the percentage of total structures that were Quality 1 and 2 embryos compared to degenerate embryos (Table 2). It appears that combining ad libitum feeding and high LH reduced percentage of oocytes that were fertilized and subsequent embryo quality of fertilized oocytes. This is consistent with the idea that high LH combined with high insulin can reduce embryo quality. Conversely, feed-restricted cows with low LH in the superovulation preparation also had reduced fertilization of oocytes, reduced percentage of Quality 1 and 2 embryos (of total structures), and increased degenerate embryos. However, increasing LH in feed-restricted cows increased embryo quality. Thus, there was an interaction between these two treatments on embryo quality that is consistent with the idea that optimizing superovulatory success requires consideration of both the hormonal and metabolic state of the superovulated cow with conditions that produce both high LH and high insulin (excess energy consumption) apparently being negative for fertilization and embryo quality (Bender *et al.*, 2014).

Table 2. Superovulatory response (LSM ± SEM) using data from 1st periods from experiment 1 and 2 (Bender *et al.*, 2014). Superovulated, lactating Holstein dairy cows were fed either ad libitum or were feed restricted and exposed to either low LH or high LH during superovulation.

Parameter	Feed				Feed	LH	Feed*LH Interaction
	Ad-libitum High LH	Ad-libitum Low LH	Feed restricted High LH	Feed restricted Low LH			
CL Number (n)	22.1 ± 3.9	16.6 ± 3.9	17.0 ± 3.9	19.4 ± 3.9	0.76	0.69	0.32
Fertilization Rate (%)	47.9^{b} ± 10.0	89.4^{a} ± 10.8	80.1^{a} ± 10.8	59.9^{b} ± 10.0	0.90	0.32	<0.01
Quality 1 & 2 Embryos							
% of fertilized	59.5^{B} ± 12.0	76.7^{AB} ± 12.0	88.3^{A} ± 12.0	70.3^{AB} ± 11.1	0.35	0.97	0.15
% of total structures	$35.6^{b,B}$ ± 11.6	$76.7^{a,A}$ ± 11.6	$73.4^{a,A}$ ± 11.6	$47.3^{ab,B}$ ± 10.8	0.72	0.52	<0.01
Degenerate embryos							
% of fertilized	37.8^{A} ± 12.0	22.8^{AB} ± 12.0	9.1^{B} ± 12.0	29.8^{AB} ± 11.1	0.37	0.81	0.14

^{a,b}Within response variable, means with different superscript significantly differ ($P < 0.05$). ^{A,B}Within response variable, means with different superscript tend to differ ($P < 0.15$).

An inverse way to test this hypothesis is to increase insulin and/or LH in cows on a maintenance diet. In a preliminary experiment, 8 lactating, pregnant Holstein cows were utilized to determine an effective propylene glycol (PROP) dose to produce an increase in circulating insulin (Hackbart *et al.*, 2011). In the main experiment (Hackbart and Wiltbank, 2014, Department of Dairy Science, University of Wisconsin-Madison, WI, USA, unpublished), seventeen non-lactating Holstein cows were superovulated in an experiment that used a Latin-square design to determine the effects of increased insulin (PRO) and/or LH during antral follicle development (superstimulation) on oocyte quality as determined by fertilization and early embryo quality.

Cows were orally drenched with PROP every 4 h from the start of the ovulatory follicle wave until ovulation (8 h after follicular aspiration until 24 h after GnRH to induce superovulation; ~7 day) to induce hyperinsulinemia. The LH concentrations were altered by increasing LH (3-fold) in the FSH preparation during the last 2 days of superovulation. Treatment groups were: Control-oral drenching with water and administration of a low-LH preparation; High LH-drenching with water and use of a high-LH preparation; High Insulin-drenching with PROP and administration of a low-LH preparation; High Insulin & High LH - drenching with PROP and administration of a high-LH preparation. PROP was effective at increasing glucose ($P < 0.05$) and insulin ($P < 0.02$) concentrations at all times that were analyzed and there was evidence that insulin resistance was produced by day 5 of treatment with PROP. Neither insulin nor LH affected numbers of follicles >9 mm at the time of the GnRH-induced LH surge, although the percentage of these follicles that ovulated was decreased by both insulin ($P = 0.002$) and LH ($P = 0.048$). In addition, insulin tended ($P = 0.056$) to decrease the total number of ovulations. Insulin reduced ($P = 0.028$) the fertilization rate, while LH tended ($P = 0.092$) to increase fertilization rate. The negative effect of insulin on fertilization was also reflected in the negative correlation between fertilization rate and insulin concentrations on day 5 when data from all cows were analyzed ($r = -0.23$; $P < 0.1$). There was no effect of either insulin or LH on any measure of embryo quality including percentage of embryos that were degenerate, Quality 1 or Quality 1 and 2 embryos of total recovered structures or of fertilized structures (Table 3). Consistent with our results, a previous study of cultured bovine follicles found that addition of insulin reduced cleavage rates after *in vitro* fertilization but did not reduce the percentage of cleaved embryos that developed to blastocysts (Fouladi-Nashta and Campbell, 2006). In contrast, other studies report no reduction in cleavage rates in hyperinsulinemic cows, although the percentage of cleaved embryos that develop to blastocysts is altered (Adamiak *et al.*, 2005, 2006). Thus, all of these results are consistent with the concept that an acute elevation in insulin during the preovulatory follicular wave can alter follicular function resulting in lower percentage of large follicles that ovulate, particularly when combined with increased LH, and, in some cases, reduced fertilization of ovulated oocytes.

In conclusion, it seems clear that negative energy balance during the first three weeks after calving can have a negative impact on fertility at the first AI, even though the AI occurred more than five weeks after the original negative energy balance. The harmful effect of negative energy balance during the transition period is manifest in reduced embryo development during the first week after AI, suggesting a lingering effect of the transition problems on oocyte competence. In addition, excessive energy intake during the final stages of follicular development did not appear to have a negative effect in Nelore cows but was somewhat negative for embryo quality in over conditioned cross-bred beef cattle. In late lactation Holstein cows, feed restriction had a positive effect on embryo quality in cows that had supplemental LH but was negative in cows without additional LH. In dry Holstein cows on a maintenance diet, elevations in insulin reduced fertilization, suggesting a negative effect of insulin on oocyte quality, but did not alter subsequent embryo development or quality. Thus, the breed, BCS, and current metabolic status of the cow need to be considered when deciding the optimal nutritional and hormonal programs to use during embryo production.

Table 3. Ovarian and hormonal parameters in non-lactating Holstein cows that were orally drenched with water and superovulated with low concentrations of LH (Control) or high concentrations of LH (High LH) or orally drenched with propylene glycol every 4 h for 7 day and superovulated with low concentrations of LH (High Insulin) or with high concentrations of LH (High Insulin and High LH).

Parameter	Mean (± SEM)				Effects		
	Control	High LH	High Insulin	High Insulin&LH	Insulin	LH	Insulin*LH
CL Number	15.8 ± 2.5	15.4 ± 2.1	13.9 ± 1.8	12.6 ± 1.6	0.0560	0.6185	0.9658
Fertilization (%)	71.1 ± 8.7^{ab,A}	76.3 ± 8.5^{a,A}	51.5 ± 8.0^{b,B}	75.4 ± 4.5^{ab,AB}	0.0281	0.0917	0.2627
Quality 1 & 2 embryos							
% of fertilized	63.1 ± 10.0	61.6 ± 10.8	53.6 ± 10.6	75.4 ± 6.1	0.9192	0.3912	0.2496
% of total structures	52.5 ± 10.1	51.3 ± 10.7	34.1 ± 8.0	58.1 ± 6.7	0.4538	0.1834	0.1585
Degenerate embryos							
% of fertilized	29.0 ± 10.0	36.6 ± 10.8	34.6 ± 10.9	19.4 ± 6.6	0.3255	0.8675	0.1903

^{a,b}Within response variable, means with different superscript significantly differ ($P < 0.05$). ^{A,B}Within response variable, means with different superscript tend to differ ($P < 0.15$).

Effects of protein and amino acid nutrition on embryo quality

Effects of high protein diets and elevated blood urea nitrogen on embryos

Protein nutrition has also been investigated in relation to reproductive efficiency and embryonic development in many types of studies (Butler, 1998; Santos *et al.*, 2008; Velazquez, 2011). One major idea is that elevated crude protein or protein degradability in the diet leads to elevated urea nitrogen. This high urea nitrogen, measured in blood or milk is associated with, and may be the cause of, reduced fertility in lactating dairy cows. A recent meta-analysis evaluated the results from 32 treatment comparisons published in 21 studies (Lean *et al.*, 2012). In these studies, increased dietary protein or increased degradability of dietary protein decreased risk of pregnancy by 9%. However there was no association between blood urea nitrogen (BUN) and risk of pregnancy, possibly due to technical aspects of BUN quantification or a relatively minor role of this metabolite in the reduced fertility. Nevertheless, there are many reasons to reduce crude protein in dairy cow diets including costs, environmental impacts, and efficiency of nitrogen utilization (Sinclair *et al.*, 2014).

Reduced development of early embryos has been demonstrated when cows are fed excess rumen degradable protein (Blanchard *et al.*, 1990) or excess dietary urea (Dawuda *et al.*, 2002). In sheep, excess dietary urea reduced fertility due primarily to effects that occurred before day 4 of pregnancy (McEvoy *et al.*, 1997; Fahey *et al.*, 2001), with some probable effects on fetal growth in surviving embryos (McEvoy *et al.*, 1997). In addition, cows with high serum urea nitrogen concentrations had reduced fertilization and embryo development (Leroy *et al.*, 2008; Lee *et al.*, 2012). For example in a retrospective study of 180 lactating, superovulated dairy cows (Lee *et al.*, 2012), lactating cows with medium milk urea nitrogen (MUN; $12 \leq$ MUN < 18 mg/dL) had greater number of transferable embryos (7.4 ± 0.6 *vs*. 4.6 ± 0.6; $P < 0.05$) compared to cows with high MUN ($\geq$18 mg/dL). In an elegant study using beef heifers supplemented with urea with either high energy or low energy diets, there was no effect of urea on fertility following transfer of an *in vitro* produced embryo on day 7 after estrus (Gath *et al.*, 2012). This indicated that high blood urea nitrogen did not seem to disrupt pregnancy between day 7 to 35 after breeding, at least in this animal model. Heifers were also superovulated on these three diets (Control = high energy; High energy with high urea; Low energy with high urea) and embryos were flushed from the oviduct at 3 day after AI. There was no effect of diet on number of CL or number of structures recovered, however heifers supplemented with urea (both groups) had greater fertilization rate compared to controls (61.3 *vs*. 92.0 and 86.8%; $P < 0.05$). Thus, no negative effects of dramatic increases in blood urea nitrogen were observed in this experiment. Perhaps the negative effects of urea are at a later stage than 3 days after AI. Indeed, an *in vitro* study provides some evidence for this concept (Ocon and Hansen, 2003). When oocytes were matured in media containing higher concentrations of urea, the subsequent fertilization rate was similar but the percentage that developed to the blastocyst stage was dramatically reduced. Surprisingly, unlike the negative effects on embryo development observed with increased urea during oocyte maturation, there was no effect on embryo development when fertilized embryos were subsequently cultured in high urea concentrations (Ocon and Hansen, 2003). High blood urea nitrogen is thought to affect embryos by decreasing pH in the uterine lumen. In this experiment (Ocon and Hansen, 2003), decreasing media pH during incubation of fertilized zygotes was found to inhibit progression to blastocyst. Thus, high urea can have direct effects on the oocyte during the final stages of follicular development producing an embryo with reduced embryonic growth potential, as well as indirect effects by reducing uterine pH in embryos after fertilization.

In contrast, an *in vivo* study using Nelore cows ($n = 68$) treated with elevated urea for the 5 days before or 5 days after AI provided evidence for a primary effect of urea during the fertilization and early embryo development period (Alves *et al.*, 2010). Fertilization was similar in control and cows treated with urea before AI but tended to be reduced ($P = 0.07$) in cows treated with urea after AI (82.2 and 75.3 *vs*. 50.7%). Surprisingly, embryos from cows treated with urea after AI, although fewer in number, developed more rapidly as shown by percentage of embryos that had only attained the compact morula stage when collected on day 7 after AI (Control = 76.9%; Urea before = 68.8%; Urea after = 38.6%; $P = 0.02$). Thus, increases in blood urea nitrogen due to feeding of diets with urea or excess rumen-degradable protein can reduce fertility potentially due to effects on the oocyte, fertilization, and changes in uterine pH. However, under certain circumstances, dramatic increases in blood urea nitrogen have no effect on the embryo or may even show some positive effects, although this needs to be confirmed in studies specifically directed at testing this hypothesis.

Effects of supplementation of specific amino acids on embryos

Less emphasis has been placed on potential positive effects that amino acid supplementation can have on reproduction in dairy cattle. Some amino acids are limiting for optimal milk production as evidenced by an increase in milk yield, percentage of milk protein, and milk protein yield after supplementation with specific, rumen-protected amino acids (Socha *et al.*, 2005; Cho *et al.*, 2007; Patton, 2010). Generally the first

three rate-limiting amino acids for milk production are considered to be Methionine, Lysine, and Histidine. In addition, many amino acids can have positive effects on physiological processes that are independent of their effects on synthesis of proteins. This has been termed "functional effects" of amino acids and methionine and arginine effects are the best studied "functional amino acids" that have been linked to reproduction (Bazer *et al.*, 2010; Penagaricano *et al.*, 2013). This part of the review will focus on concentrations of amino acids in oviduct and uterus, followed by a discussion of reproductive stages that may be altered by amino acids.

Table 4 summarizes the concentrations of amino acids in plasma, in the oviduct (average of days 0, 2, 3, 4, and 6 of estrous cycle), and in the uterus (average days 6, 8, and 14 of estrous cycle). The data are from an elegant study done in crossbred beef heifers (Hugentobler *et al.*, 2007). There was no effect of day of the cycle on oviductal concentrations of amino acids so the average of all measured days is shown. The plasma concentrations are the average of the same days as oviductal measurements. Nine of the 20 amino acids were present at significantly greater concentrations in the oviduct than plasma indicating that mechanisms are present in the cells of the oviduct that allow concentration of amino acids. The uterus also had greater concentrations of many amino acids than found in plasma from cows on the same days of the estrous cycle. The amino acids that were most dramatically elevated in uterus, Asp, Asn, Glu, were mostly similar to the oviduct. One major difference is that the concentration of Tau is much greater in uterus compared to oviduct, where Tau was not concentrated compared to plasma (Table 4).

Table 4. Concentrations of 19 amino acids in plasma, oviduct, and uterus based on results from Hugentobler *et al.*, 2006. In addition, the last column compares amino acid concentrations in pregnant *vs*. non-pregnant uterus near embryo elongation (day 15 in sheep (Gao *et al.*, 2009c); day 18 in cattle (Groebner *et al.*, 2011).

Amino Acid	Oviductal [μM]	Plasma [μM]	Uterine [μM]	Oviduct /Plasma, %	Uterus /Plasma %	Fold Increase in pregnant uterus
Ala	592.2	252.52	353.07	235%	156%	2.87X
Arg	133.3	94.50	193.87	141%	196%	7.58X
Asn	41.0	19.60	72.17	209%	357%	5.5X
Asp	135.5	6.72	120.80	2016%	2059%	4.93X
Gln	194.7	236.80	208.57	82%	89%	4.06X
Glu	346.3	62.12	217.63	558%	341%	3.45X
Gly	1557.6	680.88	1215.73	229%	183%	1.24X
His	68.8	57.04	109.23	121%	195%	11.48X
Ile	87.6	86.10	94.10	102%	103%	7.06X
Leu	192.2	154.72	201.03	124%	121%	4.41X
Lys	223.7	105.34	209.23	212%	176%	14.39X
Met	39.8	24.88	40.40	160%	201%	12.39X
Phe	68.1	38.42	75.50	177%	175%	7.31X
Ser	172.7	85.54	252.73	202%	301%	2.52X
Tau	49.4	47.34	440.03	104%	783%	1.09X
Thr	162.6	133.60	144.60	122%	96%	3.29X
Trp	36.1	27.52	38.40	131%	134%	4.99X
Tyr	54.4	25.62	63.73	212%	227%	5.3X
Val	181.4	170.04	192.47	107%	106%	4.63X

In addition to the mechanisms that concentrate amino acids in the uterus in non-pregnant ruminants, there are additional mechanisms that result in further increases in concentrations of amino acids in the uterine lumen in pregnant ruminants near the time of embryo elongation (day 14-18). Three studies have provided amino acid concentrations near the time of embryo elongation; two in sheep (Gao *et al.*, 2009c) and one in cattle (Groebner *et al.*, 2011). Although there seems to be very little change in amino acid concentrations between day 10 and 16 in non-pregnant sheep, there are dramatic increases from 3 to 23-fold in specific amino acids in the uterine lumen of pregnant sheep (Gao *et al.*, 2009c). In order to provide some idea of changes in uterine amino acids during early pregnancy, we have combined the results from these 3 studies into a fold increase in amino acids during the time of embryo elongation. As shown in Table 1, there is an increase in almost all amino acids at the time of embryo elongation. Of particular interest for dairy cattle, the three amino acids that are considered rate-limiting for milk production, Met, His, and Lys, are the amino acids with the greatest increase in concentrations in the uterine lumen during embryo elongation (>10-fold increase on average from these three studies). Arginine is another amino acid that has been studied extensively in relation to reproduction (Lassala *et al.*, 2011; Wu *et al.*, 2013; Li *et al.*, 2014) and it is also highly concentrated in the

pregnant uterus. No study has evaluated these increases in lactating dairy cows, particularly in dairy cows that are deficient *vs*. sufficient in particular amino acids. In a sheep model, maternal nutrient restriction can dramatically reduce plasma, uterine, and fetal fluid concentrations of amino acids (Kwon *et al.*, 2004) and cause fetal growth restriction. This growth restriction can be overcome by provision of arginine or sildenafil citrate (Viagra; phosphodiesterase-5 inhibitor) that both increase uterine blood flow and amino acid concentrations in the uterine fluid (Lassala *et al.*, 2010; Satterfield *et al.*, 2010). Thus, Arg, although not considered rate-limiting for milk production under most circumstances, could be limiting for uterine blood flow and thereby limit reproductive efficiency of dairy cattle. Inadequate supply of other amino acids, particularly the rate-limiting amino acids, Met, His, and Lys, could hinder the rapid growth of the embryo that occurs between day 14 and 19 in the pregnant cow or subsequent growth of embryonic, fetal, and placental tissues.

The increase in specific amino acids in the uterus near the time of embryo elongation appears to be due to an induction of specific amino acid transporters in the uterine endometrial cells (Gao *et al.*, 2009a, b; Groebner *et al.*, 2011). The induction of these amino acid transporters is most likely induced by the protein interferon-tau that is secreted by the elongating embryo. For example, interferon-tau treatment dramatically increased one specific amino acid transporter, SLC15A3, in both glandular epithelial (36-fold) and stromal epithelial (177-fold) uterine cells (Groebner *et al.*, 2011). Thus, there is likely a positive feedback system occurring during this critical time of embryo elongation with uterine amino acids being essential for rapid embryo growth and embryonic interferon-tau production; whereas, interferon-tau stimulates active amino acid transport through the uterine epithelial cells to increase amino acid supply to the elongating embryo. Disturbances in the temporal relationship between uterine blood flow, induction of uterine amino acid transport, uterine amino acid concentrations, embryonic growth, embryonic interferon-tau production, and rescue/regression of the corpus luteum may reduce fertility and increase pregnancy losses.

Effect of supplementing specific rumen-protected amino acids on fertility

Numerous studies have evaluated the effects of rumen-protected amino acids, particularly methionine, on milk production. For example, a recent meta-analysis (Vyas and Erdman, 2009) evaluated the results from 35 experiments on production effects of postruminal supplementation with methionine. At low methionine intakes (25 g per cow per day) there were dramatic increases in milk protein (16 g of milk protein per gram of metabolizable methionine intake); whereas, the production response was more muted at high methionine intake (70 g per cow per day; increase of 4 g of milk protein per g of metabolizable methionine intake). Unfortunately, we have been unable to find studies in the scientific literature, which were specifically designed and adequately powered to evaluate the effects of specific amino acids on reproductive efficiency of lactating dairy cows. The largest study (Polan *et al.*, 1991) combined results from 259 cows at 6 Universities evaluating rumen-protected methionine and lysine supplementation. They detected no significant effect on days to first service, services per conception, or calving interval, although no details were provided on reproductive measures in each specific treatment group. It is obvious that large studies are needed to validly evaluate the effects of supplementing amino acids on measures of reproductive efficiency in lactating dairy cows.

One of the reasons for the poor definition of the role of specific amino acids in reproduction has been the use of experimental designs that generally are not optimal for making firm conclusions about reproductive traits. Some nutrition-reproduction studies use individually fed cattle, generally at university facilities, providing data that are valid for quantitative variables, such as milk production and hormonal concentrations, but are underpowered (too few cows per treatment) for evaluating binomial variables such as fertility (Tempelman, 2009). Alternatively, researchers use sufficient numbers of cows on commercial operations but nutritional strategies are applied to too few pens to allow valid statistical analyses, as previously discussed (Tempelman, 2009). To detect a 10% difference in pregnancies per AI (P/AI) there would need to be at least 180 cows per treatment group. Detection of smaller differences would require much greater numbers of cows in the experiment. In one manuscript the authors state “As nutritional scientists, we tend to put production responses above all other responses. However, maintaining the health of the cow also has its economic benefits and we must consider health responses when evaluating the effects and benefits of using supplemental Met sources during the periparturient period” (Ordway *et al.*, 2009). In other species, fecundity and embryo development are dependent upon optimal methionine balance (Coelho *et al.*, 1989; Rosenkrans *et al.*, 1989; Coelho and Klein, 1990; Grandison *et al.*, 2009). For example, supplementation of culture media with methionine increased percentage of porcine embryos that initiated hatching (measure of normal embryo development) from 56 to 89% (Rosenkrans *et al.*, 1989).

Effect of methionine on embryo development

One particularly interesting study (Coelho *et al.*, 1989) used serum from lactating dairy cows in the media to grow head-fold stage rat embryos (day 9.5

after breeding). Complete development of these embryos requires serum and development is normal in rat serum. When embryos are grown in serum from dairy cows embryonic development is abnormal (Table 5, Line 1) when measured as total embryo protein, somite pairs, or percentage of the embryos that are abnormal (no neural tube closure, abnormal shape, no development of eyes and branchial arches). Supplementation of bovine serum with amino acids and vitamins produced normal development (Line 2). Amino acid supplementation alone but not vitamin supplementation produced normal development. Supplementation of methionine alone was sufficient to produce normal development of the rat embryos in cow serum (Next to last line of Table 5). In a separate experiment, use of serum from cows that were supplemented with rumen-protected methionine (110 g/day) also produced normal embryo development. Thus, bovine serum has such low methionine concentrations that normal development of rat embryos is retarded.

Table 5. Effect of supplementation of various components on development of head-fold stage rat embryos in bovine serum. Data from Coelho *et al.*, 1989.

Cow serum with	Embryo protein	Somite pairs	% Abnormal
None	73.7 ± 8.6^a	12.5 ± 1.3^a	100%
Amino acids + vitamins	130.0 ± 7.7^b	21.5 ± 0.6^b	0%
Amino acids	117.1 ± 8.5^b	21.3 ± 0.2^b	0%
Vitamins	56.6 ± 5.76^a	9.3 ± 0.8^a	100%
Amino acids w/o methionine	82.9 ± 8.7^a	11.0 ± 0.7^a	100%
Methionine	133.7 ± 5.5^b	22.3 ± 0.4^b	0%
Serum from cow supplemented with 110 g/d rumen-protected Met	135.2 + 9.1 (Separate study)	Not measured	0%

The requirements for complete development of bovine embryos have not yet been determined. Current culture conditions allow production of bovine embryos to the blastocyst stage (day 7-8) and even allow hatching of a percentage of embryos (day 9), however conditions have not been developed that allow elongation of embryos *in vitro*, and definitely do not allow culture of bovine embryos to the head-fold stage that was analyzed in the rat embryo experiments. The methionine requirements for cultured preimplantation bovine embryos (day 7-8) was recently determined in studies from University of Florida (Bonilla *et al.*, 2010). There was a surprisingly low methionine requirement (7 µM) for development of embryos to the blastocyst stage by day 7, however development to the advanced blastocyst stage by day 7 appeared to be optimized at about 21 µM (Bonilla *et al.*, 2010). Thus, the results of this study indicated that development of morphologically normal bovine embryos did not require elevated methionine concentrations (>21 µM), at least during the first week after fertilization.

A recent study (Ikeda *et al.*, 2012) evaluated whether methionine metabolism was required for normal development of bovine embryos. The researchers added ethionine or additional methionine to cultures of bovine embryos. Ethionine blocks metabolism of methionine into the one-carbon pathway (termed antimetabolite of methionine). Ethionine did not block development to the morula stage but blocked development to the blastocyst stage (Control = 38.5%; Ethionine = 1.5%). Development to the blastocyst stage in the presence of ethionine was partially restored by adding S-adenosylmethionine (SAM) which would restore the methylation pathway but not restore protein synthesis. Thus, methionine has an essential role in the development of the bovine embryo from morula to blastocyst, that is probably partially mediated by hypomethylation in the absence of sufficient methionine.

We recently evaluated the effect of supplementation with rumen-protected methionine on early embryo development in superovulated cows (Souza *et al.*, 2012a, b). We used superovulated animals so that we would have sufficient statistical power by evaluating numerous embryos in order to validly test the *in vivo* effects of methionine supplementation on early embryo development in lactating dairy cows. In this experiment, animals were blocked by parity and calving date and randomly assigned to two treatments differing in level of dietary methionine supplementation: 1) Methionine (MET); diet composed of (%DM) corn silage (39.7), alfalfa silage (21.8), HMSC (17.2), roasted soybeans (8.6), grass hay (4.6), canola meal (4.0), mineral-vitamin mix (2.7) and ProVAAL Ultra (w/Smartamine®, 1.4), formulated to deliver 2875 g MP with 6.8 Lys %MP and 2.43 Met %MP; 2) Control (CON); cows fed the same basal diet but replacing ProVAAl Ultra by ProVAAL Advantage (no added Smartamine®), formulated to deliver 2875 gr MP with 6.8 Lys %MP and 1.89 Met %MP. There was an increase in both kg of milk protein produced and percentage of protein in the milk (Souza *et al.*, 2012b). Thus, from a protein production standpoint, methionine appeared to be rate-limiting. We measured plasma methionine concentrations in this study and found a large effect of feeding rumen-protected methionine on circulating methionine concentrations (Control = 16.8 µM *vs*. Met-supplemented = 22.9 µM).

Our primary interest was the effect of supplemental Met on embryo quality (Souza *et al.*, 2012a). We evaluated a total of 570 embryos in this

experiment and found no differences in fertilization or embryo quality (Table 6). Thus, methionine supplementation did not alter early embryo development, at least from a gross morphological standpoint.

Even though methionine supplementation during the later stages of follicle development and early embryo development may not have produced morphological changes in the early embryo, it is well known that methionine during this time can have dramatic effects on the epigenome of the embryo (Sinclair *et al.*, 2007). This means that the genes can be changed in such a way that they are not expressed in the same way due to addition of groups, generally methyl groups to the DNA of the cells. For example, a previous study in sheep restricted methyl donors by restricting methionine, vitamin B12, and folate before and for the first 6 days after breeding (Sinclair *et al.*, 2007). They then transferred normally-appearing embryos into control sheep and then evaluated the lambs after parturition. The embryos that were produced in low methionine produced lambs that had substantial differences in blood pressure and immune function. To test this idea in cattle, we evaluated whether the embryos that were recovered from cows that had been supplemented or not supplemented with methionine had differences in gene expression (Penagaricano *et al.*, 2013).

The objective of this part of the study was to evaluate the effect of maternal methionine supplementation on the transcriptome of bovine preimplantation embryos (Penagaricano *et al.*, 2013). Only high quality embryos from individual cows were pooled and then analyzed by a powerful technique that allows evaluation of all genes that are expressed in these embryos, called RNA sequencing. Remarkably, the small difference that we produced in circulating methionine produced a substantial difference in expression of genes in the embryo. A total of 10,662 genes were significantly expressed in the bovine embryos. A total of 276 genes were expressed differently, statistically, in embryos from cows supplemented or not supplemented with methionine. Most of these genes were turned off in embryos from cows that were supplemented with methionine. This would be expected since methionine supplementation leads to methylation of the DNA and this can inhibit expression of some specific genes until cells differentiate to the appropriate stage when gene expression should commence (Wolff *et al.*, 1998; Burdge *et al.*, 2007). Thus methionine supplementation seemed to change gene expression in a way that may lead to improved pregnancy outcomes and improved physiology of the offspring. Many of the genes are involved in immune function and later stages of embryo development that may be critical for pregnancy progression and normal immune function after birth. Further studies are needed to determine if these changes in gene expression lead to changes in embryo development, reduced pregnancy loss, and altered physiology of the offspring.

Thus, supplementation of rate-limiting amino acids can have substantial effects on milk protein content and yield, however, effects on reproduction have not yet been adequately evaluated. The dramatic induction of the rate-limiting amino acids, Met, His, and Lys, in the uterine fluid of pregnant cows near the time of embryo elongation suggests that elevated amounts of these amino acids may be critical for this important stage of embryo development. Supplementation of cows with methionine during the final stages of follicular development and early embryo development, until day 7 after breeding, did not lead to gross morphological changes in the embryos but did result in dramatic differences in gene expression in the embryo. Further studies are needed to evaluate whether supplementation with these essential amino acids to lactating cows would have a beneficial impact on embryo survival and if these changes in the early embryo translate into changes in pregnancy outcomes or physiology of the resulting calf.

Table 6. Effect of methionine supplementation with Smartamine®, 1.4 on reproductive parameters in superovulated lactating dairy cows.

Number of cows	MET	CON	
	35	37	P-value
CL number	17.0 ± 1.3	17.7 ± 1.5	0.90
Total ova/embryos recovered	9.1 ± 1.4	6.8 ± 1.0	0.18
Number of fertilized ova	6.5± 1.1	5.5 ± 0.9	0.56
% Fertilized ova	74.7 ± 5.6	82.2 ± 3.8	0.27
Number of transferable embryos	5.0 ± 0.9	4.3 ± 0.1	0.57
% Transferable embryos	56.3 ± 6.5	62.5 ± 6.0	0.49
Number of degenerate embryos	1.5 ± 0.4	1.3 ± 0.4	0.75
% Degenerate embryos	18.5 ± 4.6	19.7 ± 4.7	0.83
% Degenerate of fertilized ova	25.1 ± 5.8	27.5 ± 6.0	0.74

Final conclusions

Thus, preimplantation embryo development can be affected in a positive or negative way by deficiencies or excesses of energy/carbohydrates and protein/amino acids. Some of the effects on embryo development may be occurring during the final stages of oocyte development within the preovulatory follicle but are only manifest by the blastocyst stage. For example, the effects discussed above using feed restriction and LH supplementation during follicle development can alter subsequent embryo development (Bender *et al.*, 2014). In addition, some of the effects on embryo function may not be manifest in gross morphological appearance of the embryos but result in dramatic differences in gene expression as observed in the study that evaluated embryonic gene expression using RNASeq in embryos produced in dams that were supplemented or not supplemented with methionine (Penagaricano *et al.*, 2013). There is still a great deal more fundamental biology that needs to be done to fully understand how embryo development can be most practically manipulated using nutritional strategies.

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