

The effects of Transition Cow Nutrition on subsequent reproduction.

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Decreasing reproductive efficiency in dairy cattle has been documented not only in the USA but also in Ireland, United Kingdom, and Australia (Lucy, 2001). Butler (1998) showed a decline in first-service conception rate from approximately 65% in 1951 to 40% in 1996. Despite the “ideal” reproductive parameters for the lactating herd being well established, the goals are hard to reach and progress is slow. Between 1996 and 2007, the days to first breeding of the US Holstein herd were improved from 92 to 85 days, a trend in the right direction. However, the number of inseminations per lactation and calving interval increased from 2.1 to 2.5 and from 410 to 422 days respectively (Norman et al., 2009, Table 1). In order to have a calving interval of 12 to 13 months, cows need to be pregnant by 85 days in milk. Further, the cows need to maintain to term the embryo conceived at the first insemination. On average, in the US, Holstein cows are bred over 2.1 times per lactation (Norman et al., 2009). Santos et al., documented that cows lose 1 % of embryos during days 30 to 45 of pregnancy.

Table 1: Reproductive status of Holstein cows in the US

Year	DFB ¹	CR ¹ , %		NB ¹	CI ¹
		1st breeding	All breedings		
1996	92	34	33	2.1	410
1997	94	33	32	2.1	416
1998	94	32	30	2.2	422
1999	94	30	29	2.2	428
2000	91	30	28	2.2	426
2001	90	27	26	2.3	428
2002	90	30	29	2.3	426
2003	90	32	30	2.4	425
2004	87	33	31	2.5	422
2005	86	31	29	2.6	423
2006	86	31	30	2.5	422
2007	85	32	-	-	-
“Goals” ²	<80	>50	-	< 1.7	< 390

¹DFB; Days to first breeding, CR: Calvin interval, NB: Numbers of breeding per lactation, CI: Calving interval

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The Pregnancy Rate (PR) depends on both Heat Detection (HD) and Conception Rate. Improving the HD would improve the PR. Over the last several years, many insemination protocols have been designed to better synchronize heat, thus improving the odds of success to improve the PR. However, less than 50% of the cows become pregnant at first insemination, therefore cows need to be inseminated at least twice (Lucy, 2012). Lucy recommends a re-synchronization protocol prior to the second insemination to increase the efficiency of reducing the interval between inseminations. Despite the availability of many successful HD/synchronizing protocols, the ultimate goal must still be to create a healthy uterine environment to ensure the embryo reaches term. Success or failure depends on what happens during the transition period.

Reproduction and Nutrition

Nutrient demands for milk synthesis are increased in early lactation, and if no compensatory intake of nutrients is achieved to cope with milk production requirements, reproductive functions (i.e., synthesis and secretion of hormones, follicle ovulation, and embryo development) may be depressed. The incidence of diseases and disorders can be high during the periparturient period and have a negative impact on reproductive performance. The risk of pregnancy was reduced if cows lost more than one body condition score (BCS) unit (Butler, 2003 2005; Santos et al., 2008).

Milk production increases faster than energy intake in the first 4 to 6 weeks after calving. High yielding cows will experience negative energy balance (NEB) and blood concentrations of non-esterified fatty acids (NEFA) increase, and concentrations of insulin-like growth factor-I (IGF-I), glucose, and insulin are low. If extreme, these changes in blood metabolites and hormones may compromise ovarian function and fertility (Butler, 2005).

Different nutritional strategies have been proposed to improve reproduction of the dairy cow with no detrimental effect on lactation performance. Feeding high quality forages, increasing the concentrate:forage ratio, or adding supplemental fat to diets are some of the most common ways to improve energy intake in cows. Reproduction of dairy cattle may be benefited by maximizing DMI during the transition period, minimizing the incidence of peri-parturient problems.

Postpartum Cyclicity

The NEB experienced by dairy cows antagonizes the resumption of ovulatory cycles. During early postpartum, reproduction is deferred in favor of individual survival. In the modern dairy cow, lactation becomes the priority, in detriment to reproductive functions. During periods of energy restriction, oxidizable fuels consumed in the diet are prioritized toward essential processes such as cell maintenance, circulation, and neural activity (Wade and Jones, 2004). Homeostatic controls in early lactation assure that body tissue (adipose stores and muscle), will be mobilized in support of milk production (Kuhla et al. 2011). Delayed ovulation has been linked repeatedly with energy status (Butler, 2003). Energy shortage reduces the frequency of pulses of luteinizing hormone, therefore impairing follicle maturation and ovulation.

Usually, the first ovulation after calving in dairy cattle occurs 10 to 14 d after the nadir of NEB (Butler, 2003). Drastic weight and BCS losses caused by inadequate feeding or disease are associated with anovulation and anestrus in dairy cattle. Cows with low BCS at 65 d postpartum are more likely to be anovular (Santos et al., 2008, Carvalho et al., 2014), which compromises reproductive success at first postpartum insemination. Extended postpartum anovulation or anestrus extends the period from calving to first artificial insemination (AI) and reduces fertility during the first postpartum service (Santos et al., 2008). Anovular cows not only have reduced estrus detection and conception rates, but also have lower % pregnancies per AI (Carvalho et al., 2014). The timing of the first postpartum ovulation determines and limits the number of estrous cycles occurring before the beginning of the insemination period. In most dairy herds, fewer than 20 % of cows should be anovulatory by 60 d after calving (Santos et al., 2008).

Feeding management that minimizes loss of BCS during the early postpartum period and incidence of metabolic diseases during early lactation should increase the number of cows experiencing first ovulation during the first 4 to 6 weeks after calving.

Energy

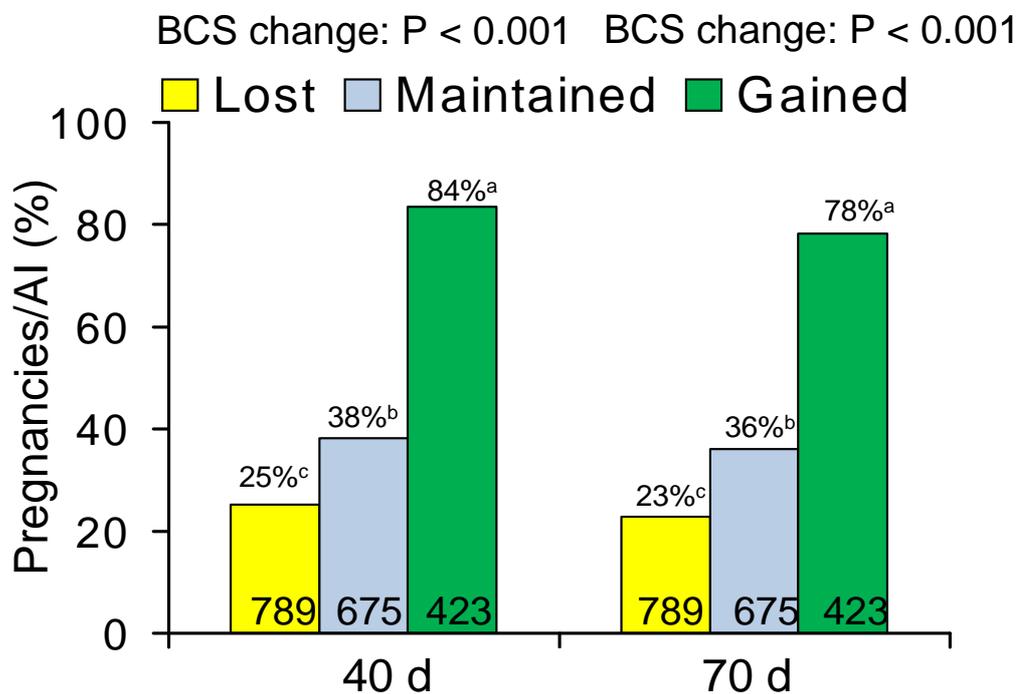
The early-lactation NEB and a subsequent failure to replenish body energy stores have been associated with reduced reproductive performance (Butler and Smith, 1989; Buckley et al., 2003; Roche et al., 2007). Drackley (2006) reported that variation in energy balance in postpartum Holstein cows was influenced most strongly by DMI ($r = 0.73$) and less by milk yield ($r = -0.25$). Therefore, differences in NEB between cows are more related to how much energy they consume than with how much milk they produce. Plasma progesterone concentrations are affected by the energy balance of dairy cows. Glucose, insulin, and IGF-I, which are low during periods of NEB, together with progesterone have been shown to affect folliculogenesis, ovulation, and steroid production in vitro and in vivo (Thatcher et al., 2011). Even though the exact mechanism by which energy affects secretion of reproductive hormones is not well defined, it is clear that lower levels of blood glucose, IGF-I, and insulin may mediate this process. Post calving NEB three weeks after calving was not related with milk yield, or solid corrected milk yield, but it was highly correlated with early post-partum DMI (Drackley, 2006). Therefore, improving DMI during early lactation may improve reproductive performance.

What happens during transition affects the cow's reproduction.

In a recent retrospective study, Carvalho et al., 2014) evaluated the effect of BCS near TimedAI (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 (≤ 2.5) compared to higher BCS (≥ 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]; $P = 0.03$). Consistent with this idea, in a second experiment Carvalho et al., 2014 observed a

significant effect on P/AI from cows that experienced a change in BCS between calving and 21 d after calving. The P/AI differed ($P < 0.001$) 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.

Figure 1: Impact of body condition losses on pregnancies per AI (P/AI)



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. Further, these results highlight the importance of providing the right feeding regime to the early lactation cows to minimize body weight losses.

The importance of amino acids

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. The first three limiting amino acids for milk

production are considered to be Methionine, Lysine (NRC, 2001), and Histidine (Hutannen, 2002). In addition, many amino acids can have positive effects on physiological processes that are independent of their effects on synthesis of proteins (Wu, 2013).

Fertilization and the first few days of embryo development occur in the oviduct. By about 5 days after estrus the embryo arrives in the uterine horn. The embryo reaches the blastocyst stage by 6 to 7 days after estrus. The embryo hatches from the zona pellucida by about Day 9 after estrus and then elongates on Days 14-19. The elongating embryo secretes the protein interferon-tau that is essential for rescue of the corpus luteum and continuation of the pregnancy. By Day 25-28 the embryo attaches to the caruncles of the uterus and begins to establish a vascular relationship with the dam through the placenta. During all the time prior to embryo attachment, the embryo is free-floating and is dependent upon uterine secretions for energy and the building blocks for development, including amino acids. Thus, it is critical to understand the changes in amino acid concentrations in the uterus that accompany these different stages of embryo development.

The lipid profile of oocytes and early embryo can be influenced by the environment of the cow. Acosta et al., (2014) ran a trial with the objective to determine the effect of supplementing rumen-protected methionine on DNA methylation and lipid accumulation in preimplantation embryos of dairy cows. Lactating Holsteins entering their 2nd or greater lactation were randomly assigned to two treatments from 30 ± 2 DIM to 72 ± 2 DIM; Control (CON; n=5, fed a basal diet with a 3.4:1 Lys:Met) and Methionine (MET; n=5, fed the basal diet plus Smartamine M to a 2.9:1 Lys:Met). Embryos were flushed 6.5 d after artificial insemination. Embryos with stage of development 4 or greater were used for analysis. For lipids, fluorescence intensity of Nile Red staining was compared against a negative control embryo (subtraction of background). A total of 37 embryos were harvested from cows (MET= 16; CON=21). Cows receiving MET had greater (P=0.021) lipid accumulation when compared with cows receiving CON. There were no treatment effects (P> 0.511) on number of cells or stage of development. In conclusion, cows supplemented with methionine produced embryos with higher lipid concentration when compared to CON which could potentially serve as an important source of energy for the early developing embryo.

Hugentobler, et al. (2007) summarized the concentrations of amino acids in plasma (average of days 0, 2, 3, 4 and 6 of estrous cycle), in the oviduct of crossbred beef heifers, and in the uterus (average days 6, 8, and 14 of estrous cycle). There was no effect of day of the cycle on oviductal concentrations of amino acids. 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 elevated in uterus, Asp, Asn, Glu, were mostly similar to the oviduct. 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 large 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, Wiltbank et al., (2014) combined the results from these 3 studies into a fold increase in amino acids during the time of embryo elongation. 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 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

Thus, Arg, although not considered 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 limiting amino acids, Met, His, and Lys, could hinder the rapid development of the embryo that occurs between Day 14 and 19 in the pregnant cow or subsequent growth of embryonic, fetal, and placental tissues.

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 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 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. Amino acid supplementation alone but not vitamin supplementation produced normal development. Use of serum from cows that were supplemented with rumen-protected methionine also produced normal embryo development. Thus, bovine serum has such low methionine concentrations that normal development of rat embryos is retarded.

The requirements for complete development of bovine embryos have not yet been determined. Current culture conditions allow development 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 *in vitro* that allow elongation of embryos . The methionine requirements for cultured pre-implantation bovine embryos (day 7-8) was 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 around 21 μM (Bonilla et al., 2010). Thus, the results of these studies 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.

Souza et al., (2012a, b) recently evaluated the effect of supplementation with rumen-protected methionine on early embryo development in super-ovulated cows Super ovulation increased the number of embryos available and thus the statistical power to 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 milk protein synthesis standpoint, methionine was concluded to be the first limiting amino acid. A large significant effect ($P < 0.01$) of feeding the rumen-protected methionine on circulating methionine concentrations (Control=16.8 μM vs. Met-supplemented=22.9 μM) was observed.

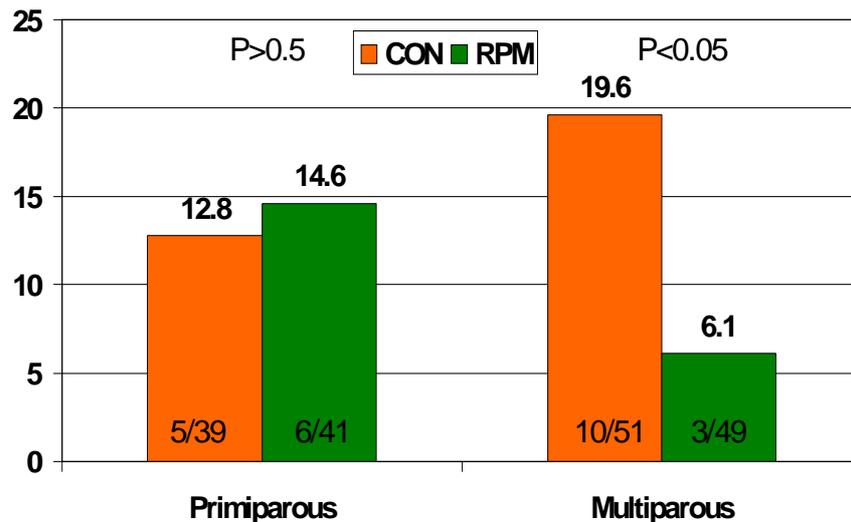
The researchers primary interest was the effect of supplemental Met on embryo quality (Souza et al., 2012a). They evaluated a total of 570 embryos in this experiment and found no differences in fertilization or embryo quality. Thus, methionine supplementation did not alter early embryo development, at 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 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. To test this hypothesis, Penagaricano et al., (2013), evaluated whether the embryos that were recovered from cows that had been supplemented or not supplemented with methionine had differences in gene expression. The objective was to evaluate the effect of maternal methionine supplementation on the transcriptome of bovine pre-implantation embryos. 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 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. 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.

As a follow-up to the Souza et al. trial, researchers from the same laboratory at the Univ. of Wisconsin conducted a trial in a commercial dairy to test the hypothesis that cows fed a methionine enriched diet would: 1) increase milk protein production, 2) Increase the number of cows that are pregnant on days 28, 32 and 61 after TAI at day 63±3 and 3) Reduce pregnancy losses (Toledo et al., unpublished). A total of 309 cows (138 primiparous and 171 multiparous) were blocked by parity and randomly assigned to two treatments; 1) CON: Cows fed a ration formulated to deliver 2500 g of MP with 6.9% Lys (% MP) and 1.9 Met (% MP) and 2) RPM: Cows fed a ration formulated to deliver 2500 g of MP with 6.9% Lys % MP) and 2.3 & Met (% MP). Cows were randomly assigned to three pens with head-locks and fed a single basal TMR twice daily. From 28 to 128 DIM, after the AM milking, cows were head-locked for 30 minutes and the TMR of CON and RPM cows were individually top dressed with 50 g of DDG or 50 g of a mix of DDG (29 g) and Smartamine (21 g) respectively. Following a double ovsynch protocol, cows were inseminated and pregnancy checked at 28 (plasma Pregnancy Specific Protein-B concentration), and at 32, 47 and 61 d (ultrasound). Individual milk samples were taken once a month and analyzed for composition. There were no statistical differences in milk production or ECM, but RPM cows had a higher milk protein concentration ($P < 0.01$). Cow fed the methionine enriched diet had a lower pregnancy loss from 21 to 61 after AI (16.7 % RPM cows vs. 10.0% from CON cows; $P = 136$). Pregnancy losses between days 28 and 61 were not different in the primiparous cows (12/8% CON and 14.6% RPM; $P = 0.54$), however, pregnancy losses between

treatments were significant for the multiparous cows (19.6% CON vs. 6.1% RPM; P=0.04, Figure 2).

Figure 2: Pregnancy losses between days 21 and 61 after timed AI of primiparous and multiparous cows fed a control diet (CON) or a methionine enriched diet (RPM)



Supplementation with limiting amino acids have shown positive benefits on milk protein content and yield. The elevated concentration of the 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. The lower pregnancy losses from cows fed a methionine enriched diets suggest that methionine favors the embryo survival, at least in multiparous cows. Further studies are needed to corroborate whether supplementation with methionine 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.

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