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Temporal Historical Observations, Rapidly Expanding Technological Tools, and Integration of Scientific Disciplines to Enhance Reproductive Performance of Lactating Dairy Cows: The Foster Mothers of The Human Race

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ABSTRACT

***Background*:** Lactating dairy cow of the 21st century is considered to be sub-fertile after intensive selection for milk yield. production of the dairy cows exemplifies the progress that can be made in the application of technology and holistic dairy cow management to optimize production. The hormonal and metabolic responses, associated with homeorrhetic and homeostatic regulatory responses to partition nutrients for lactation, coupled with intensive management contribute to the reduction in fertility. The numerous postpartum reproductive and metabolic disorders are associated with sub-optimal fertility in the breeding period. Continual advancements have been made in reproductive/lactation physiology, endocrinology, nutrition, herd health and management to improve reproductive herd fertility on commercial dairies. Objective of this presentation is to focus on current technologies and experimental approaches relative to their application in unraveling certain biological windows, which will further impact our ability to enhance reproductive efficiency coupled with increased food production and well-being of both animals and humans.

***Review*:** Feeding of omega 6- and omega 3- polyunsaturated fatty acids exert pro- and anti- inflammatory effects on the innate immune system that increases subsequent reproductive performance. Chronic exposure to a GnRH-agonist induced a marked atrophy of the postpartum uterus. Colostrum contains a pleothera of growth factors (lactocrine secretions) that influence uterine developmental programming in the pig, and neonatal exposure to estrogens/progesterone in pigs or cattle alters early programming of the uterus leading to dysfunctional reproductive tract consequences in the adult. Physiological systems to optimize ovarian and uterine function have led to timed insemination fertility programs that have enhanced herd pregnancy rates. Sequencing of the bovine genome has provided an array of new technological approaches to unravel the multi-factorial control systems to support conceptus-placental development throughout gestation and avoid pregnancy failure. A bovine microarray identified genes that were differentially expressed in conceptus and endometrial tissues at day 17 post-LH surge in cyclic and pregnant cows that were lactating or nonlactating. Expression of PAG genes within the conceptus and endometrium of pregnant cows and their association with other genes determined by standard partial correlation analyses infer a possible role of PAG in pregnancy maintenance and implantation by regulation of embryo development, trophoblast cell invasion, immune regulation, and prostaglandin metabolism. The associations detected are suggestive of potential pathways for investigation in early pregnancy at day 17 involving potential direct and indirect effects of PAG 11 produced by the conceptus. Development of microarrays of single nucleotide polymorphisms (SNP) across the bovine genome has led to Genomic Predicted Transmitting Abilities (GPTA) for various production traits including daughter pregnancy rates. More specific physiological reproductive traits have sufficient heritabilities that warrant consideration for selection. Furthermore, current technological advances are identifying candidate "fertility" genes for potential genetic selection. selection for production, health and reproductive traits will be the wave of the future as genomic and bioinformatic tools continue to be expanded and refined.

***Conclusion*:** This manuscript targets biological windows and technological advancements to improve reproductive performance of lactating dairy cows. Epidemiological analyses reveal that healthy postpartum lactating dairy cows are indeed fertile. Chronic exposure to a GnRH agonist induced postpartum uterine atrophy warranting additional research as to potential strategies to improve uterine health. Feeding of nutraceuticals such as polyunsaturated omega-6 and omega-3 fatty acids improves postpartum innate immune function and subsequent reproductive performance. Lactocrine secretions in colostrum and neonatal exposure to estrogens and progesterone influence uterine developmental programming related to subsequent reproductive competence. Reproductive management programs that optimize ovarian and uterine function permit a fertile single timed insemination to first and second inseminations. The sequencing of the bovine genome has led to thorough

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characterizations of the endometrium and conceptus transcriptones in response to key physiological periods such as pregnancy and lactation. Early expression of Pregnancy Associated Glycoprotein (PAG) genes within the conceptus and endometrium of pregnant cows and their association with other genes infer a possible role of PAG in pregnancy maintenance and implantation. The array of SNPs across the bovine genome and specific SNPs within candidate genes related to reproductive processes and fertility will enhance genetic selection for fertility along with production and health associated traits.

Keywords: genomics, timed insemination, immunosuppression, transcriptome analysis, neonate, colostrum, uterus.

I. INTRODUCTION

Modern dairy practices require considerably fewer resources than dairying in 1944 with 21% of animals, 23% of feedstuffs, 35% of the water, and only 10% of the land required to produce the same 1 billion kg of milk in the USA. Increased production of the dairy cows exemplifies the progress that can be made in the application of technology. Continual and expanding advances in technology across scientific disciplines hopefully will meet the majority of the global needs for food production to feed a continual expanding world population, since land use and water availability for agriculture is limited. Such efforts will require multi-interdisciplinary scientific efforts, political re-programming to internationalize agricultural production and distribution, and education of both producers and consumers as to the application of technology to produce safe foods.

The lactating dairy cow of the 21st century is considered to be sub-fertile after intensive selection for milk yield. The hormonal and metabolic responses, associated with homeorrhetic and homeostatic regulatory responses to partition nutrients for lactation, coupled with management contribute to the reduction in fertility. Continual advancements have been made in reproductive/lactation physiology, endocrinology, nutrition, herd health and management to improve reproductive herd fertility on commercial dairies.

Current scientists and those in training have exciting repertoires of experimental models and technological tools to bring to bear in improving animal productivity with the use of the whole animal and various aspects of molecular and cell biology. However, an understanding of the scientific past is very insightful and can improve the efficiency of one's career and that of their cooperative multi-interdisciplinary efforts. To make this point, in particular to trainee's, a vivid example of the past that was extremely insightful to the author was the publication of Dr. Joseph Halbane's (Figure 1;[29]). "EmbryonoalImpulse" represents rapid growth of the fetal mammary gland in the 8thand 9thmonths of pregnancy. Neonatal swelling and regression of the mammary gland and uterus were due to active substances from the placenta and their withdrawal. There is a "Puberty Impulse" on the mammary gland and uterus due to activity of the ovaries, and this was further characterized by ovariectomy and re-transplantation experiments. After puberty, periodic swelling of the mammary gland and uterus occurs with re-occurring estrous cycles (i.e., in humans the "MenstrualImpulse"). There is the "Pregnancy Impulse" of rapid proliferation with hyperplasia of the glandular tissue but at a much greater rate than post-pubertal changes leading to the inference that placenta produces more regulatory substances than does the ovary. Pregnancy changes were not due to fetus but due to the placenta since growth proceeds after death of the fetus and subsides with loss of placenta. Secretions produced by epithelium of the placenta (i.e., tropholast and chorionic epithelium) and not stromal tissue were inferred. The CL persists during pregnancy under influence of the placenta. Changes in the maternal and fetal uterus were correlated due to the fact that both regress in the postpartum or puerperium period. Puerperal involution of the maternal uterus occurs only after delivery of the placenta and was considered a true atrophy. Emptying of the uterus was considered critical to onset of milk secretion (i.e., 3-4 days after birth). Secretion before placental delivery was characterized as colostrum not milk. Suckling did not induce milk secretion and only maintained secretion after the uterus is emptied and suckling was associated with quiescence of the ovary.

Insightful components in Figure 1 relate to several physiological periods such as the periparturient period in which distinct changes are being programmed on both the newborn relative to potential function of the uterus, mammary gland and well-being of the newborn, and the maternal processes of mammary gland lactogenesis, regression of the uterus, homeorethetic/homeostatsis responses to support

lactation, and immune status. All of these processes influence efficiency of production and reproduction. Also note that the distinct differences Halbane [29] deduced following pregnancies in the non-suckled and suckled states. Non-suckled or non-lactation resulted in a coupled decrease in both the uterus and mammary gland and an earlier recrudescence of ovarian cycles. In contrast, the suckled or lactating state resulted in a greater rate of uterine regression and a marked delay in recrudescence of ovarian cycles. Presently, following 105 years after these initial observations in a human clinical practice and a pleothera of animal husbandry publications to improve animal management and production, major new findings and experimental approaches are being applied to further optimize the productivity and well-being of animals and humans.

Objective of this presentation is to focus on current technologies and experimental approaches relative to their application in unraveling certain biological windows described by Halbane [29], which will further impact our ability to increase food production and well-being of both animals and humans.

II. PERIPARTUM AND POSTPARTUM EFFECTS ON MATERNAL AND NEONATAL REPRODUCTIVE FUNCTION

Genetic and environmental differences among cows in the peripartum period are associated with various endocrine and biochemical systems regulating postpartum milk secretion, ovarian recrudescence, uterine regression, and health of the

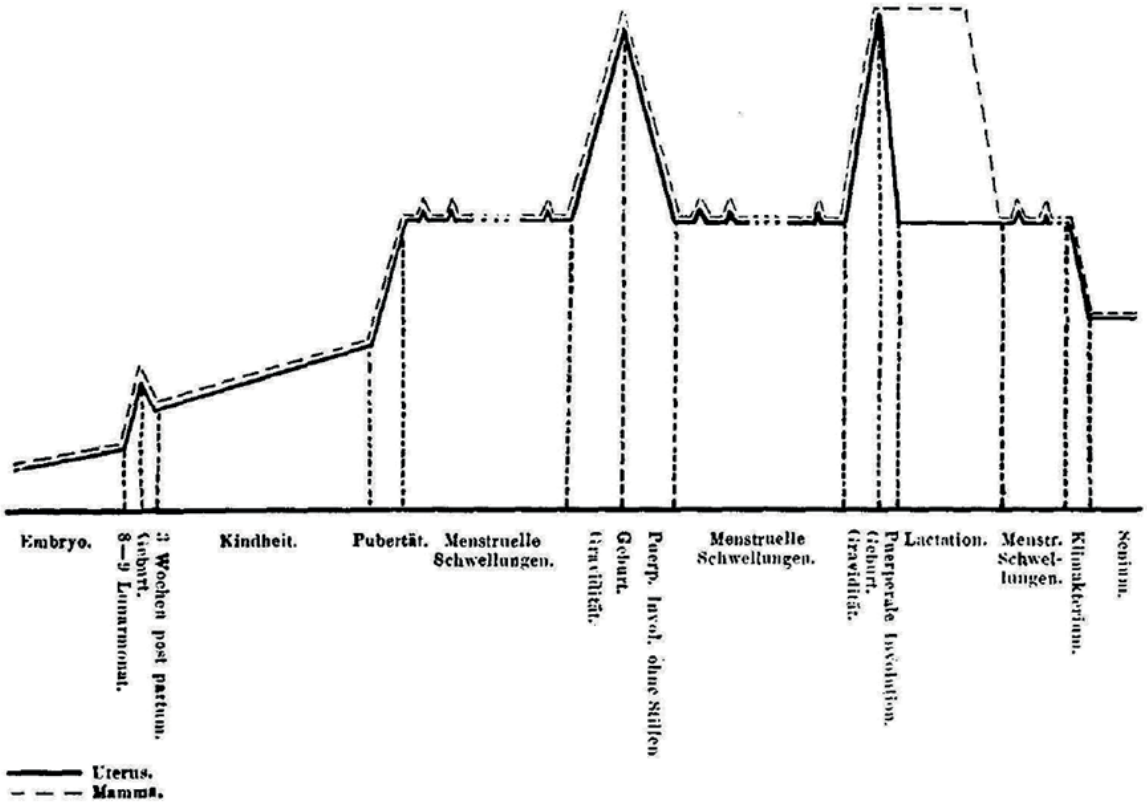

Figure 1. Original figure translated from German entitled : "The Inner secretion of the Ovary and Placenta and its Importance for Function of the Mammary Gland" (J. Halban 1905 Archiv fur Gynakologie 75:353; in: Obstet Gyn 1955 6(5):559-565); Translations:Mamma (Mammary Gland), Lunarmonat (lunar month), Geburt (birth), Wochen postpartum (weeks postpartum), Kindheit (childhood), Pubertat (puberty), Menstruelle Schwellungen (menstrual swellings), Puerperale Invol. Ohne Stillen (postpartum involution without breast feeding), Klimakterium (menopause), Senium (senescence).

maternal unit as well as the newborn. Furthermore, the influence of the peripartum period on subsequent reproductive processes has been somewhat of a myoptic view that has not integrated the collated physiological endocrine, immune and nutritional physiology on reproductive processes. The conceptus (i.e., fetus and placenta) may be central to regulation of these various processes [59]. A series of experiments demonstrated quantitative hormonal and physiological differences among cows: in the peripartum period attributable to cows selected for milk yield versus a non-selected control line [17,18]; first calf Holstein cows bearing either Holstein, Holstein*Angus or Holstein*Brahman conceptuses [27, 28]; and cows managed during the prepartum period under a shade heat abatement system versus a control no-shade environment [13,38]. Distinct differences in free and sulfated estrogens, progesterone, prolactin, 13-14 dihydroxy- 15 keto $PGF_{2\alpha}$, thyroxine and triiodothyronine, as well as physiological differences in conceptus birth weights, postpartum milk yield, and uterine regression were detected in response to these distinct experimental differences.

The transition period, typically considered from the time 3 weeks prepartum until 3 weeks postpartum, is marked by declining dry matter intake, negative energy status once lactation is initiated, and inadequate innate immunity that increases the risk of uterine diseases. A major issue facing dairy cows under intensive management systems is the high incidence of health problems, particularly those that affect the reproductive tract and those of metabolic origin that affect subsequent reproductive performance. Data from 5,719 postpartum dairy cows evaluated daily for health disorders from seven dairy farms were compiled [50]. Cows were subjected to presynchronized timed AI programs. Only 55.8% of them were considered healthy and did not develop any disease event in the first 60 days postpartum. Incidence of clinical diseases (calving related problems, 14.6%; metritis, 16.1%; clinical endometritis, 20.8%; fever, 21.0%; mastitis, 12.2%; ketosis, 10.4%; lameness, 6.8%; digestive problems, 2.8%; pneumonia, 2.0%) was high and 27.0% of the cows were diagnosed with a single disease event, whereas 17.2% had at least 2 disease events in the first 2 months of lactation. In spite of similar milk yield, cows diagnosed with health problems were less likely to be cyclic at 65 days postpartum. Calving related disorders and those that affect the reproductive tract were the major contributors for the depression in cyclicity (cyclic: healthy 84.1 % versus 70.7% > 1 disease). Diagnosis of health disorders in early lactation markedly depressed the risk of cows to become pregnant at the first postpartum AI (pregnancy per TAI: healthy 51.4% versus 34.7% > 1 disease), and increased the risk of pregnancy loss in the first 60 days of gestation (pregnancy loss %: healthy 8.9% versus 15.8% > 1 disease). These responses indicate that reduction in morbidity by prevention of periparturient diseases has the potential to enhance fertility of dairy cows by improving resumption of postpartum ovulation, increasing pregnancy per AI, and minimizing the risk of pregnancy loss. A major focus point to be gleaned is that restoration of uterine/ovarian function and optimization of immune function are considered important researchable areas to sustain reproduction in lactating dairy cows. Furthermore, epidemiological data analyses are a powerful tool to identify reproductive inefficiencies and potential causative associations but do not prove cause and effect.

2.1 Restoration of postpartum uterine function

Silvestre *et al*. [52] documented clearly that at 2 + 1 days postpartum chronic treatment subcutaneously with a non-degradable implant containing 5 mg of the GnRH-Agonist Deslorelin had a profound effect on uterine involution. The Deslorelin implant suppressed follicular development, enhanced physical involution of the uterus and cervix, increased tonicity of the uterine wall, reduced frequency of abnormal cervical discharges, and decreased inflammatory processes of the reproductive tract. Likewise, a degradable Deslorelin implant enhanced uterine involutionary processes but also delayed onset of ovulations, which offset the potential benefit of improved uterine regression and health [55]. Future research should focus on GnRH agonist delivery systems in which initiation and termination of treatments could be manipulated practically into a restricted time frame through 22 days postpartum to optimize uterine involution. Also the mechanism of Deslorelin action needs further investigation as to either direct or indirect

effects on the uterus. This may avoid a prolonged period of anovulation. If the agonist is directly effective, can a molecule be developed that exerts a uterine effect without desensitizing gonadotrophs that secrete LH and FSH Perhaps the decreased secretion of LH and/ or FSH has direct effects on the uterus since clearly follicle development and turnover is reduced. This is the same endocrine status of suckled beef cows that have a greater rate of uterine regression and less uterine health problems than lactating dairy cows. Induced atrophy of the uterus via suppression of gonadotrophin secretion, possibly independent of oxytocin secretion, is an intriguing hypothesis.

2.2 Nutritional modulation of postpartum innate immunity and subsequent fertility

Nutritional management to alter innate immunity in the transition period followed by a dietary change that enhances subsequent pregnancy rates in high producing dairy cows are strategies that are complementary to reproductive management programs and potentially more acceptable to both producers and consumers. Silvestre *et al*. [53,54] designed an experiment to evaluate the effects of differential temporal supplementation of various calcium (Ca) salts of fatty acids on reproduction, production and innate immune responses. Holstein cows (n = 1,380) were assigned randomly to be fed either transition diets supplemented with 1.5% of the DM as Ca salts of either palm oil (PO; mostly saturated and monounsaturated fatty acids) or safflower oil (SO; mostly linoleic acid). At 31days postpartum, cows within each transition diet were randomized to receive either PO or Ca salts containing fish oil (FO; enriched in :5$_{n-3}$ that leads to suppression in the biosynthesis of inflammatory molecules) until 160 days postpartum.

Feeding SO during the transition period improved early postpartum neutrophil bactericidal function, abundance of the L-selectin adhesion molecule, and neutrophil production of pro-inflammatory cytokines [53]. The SO dietary supplement elevated plasma concentrations of haptoglobin and fibrinogen. Conversely, feeding FO during the breeding period attenuated cytokine secretion from neutrophils.

Transition and breeding diets did not affect the proportion of cows cycling at 74 days postpartum (80.0%; [54]). Overall first service pregnancy per AI at 30 and 60 days after insemination were 39.3% and 33.3%, respectively, and there were no effects of diets. However, fewer cows fed FO diets lost their pregnancy (6.3 versus 13.6%). Perhaps the anti-inflammatory effects of the omega-3 FA in FO [53] fed during the breeding period reduced pregnancy losses. Furthermore, cows fed FO had an increased pregnancy per AI to the second service at both 30 days (36.2 vs 27.2%) and 60 days (34.5 vs 23.7%) after insemination. At second service, cows fed SO in the transition period followed by FO during the breeding period had the highest proportion pregnant (i.e., 43.3% at 30 days and 43.1% at 60days). The combination of reduced pregnancy loss at first AI and increased pregnancy at the second AI resulted in a greater proportion of cows fed FO that were pregnant after two postpartum inseminations. The mechanisms regarding the positive effects of FO supplementation are speculative, but could be associated with an improved ratio of IGF-2 to IGF-1 gene expression, and/or anti-inflammatory effects within the endometrium that are complementary to the immunosuppressive and anti-luteolytic effects of the conceptus that maintain pregnancy.

Collectively, strategic supplementation of fatty acids benefitted immune function early postpartum and exerted immunosuppressive effects during the breeding period, which could explain some of the improvement observed in fertility [53,54]. Clearly feeding nutriceutical components in the diet that improve reproductive performance will be perceived by the consumer as a more acceptable management strategy compared to treatment with safe and improved hormones.

2.3 Maternal regulation of neonatal passive immunity and lactocrine programming of uterine development

Parturition marks the cessation of intrauterine life but does not end maternal effects in the sense that the newborn calf suckles colostrum (e.g., beef calf) or receives via supplementation fresh or stored frozen/thawed colostrum, during the first 24 h after birth (e.g., intensive management of a commercial dairy) to acquire passive immunity. Whether the mean transfer of 248 g of IgG or 7 kg of colostrum to the newborn [41] contributes to the period of postpartum immunosuppression of the mother and is related to peripartum reproductive disorders (e.g. puberalmetritis, endometritis, subclinical endometritis) warrants investigation [30]. Perhaps peripar-

turient cows need to be acutely supplemented with immunoglobulins to compensate for major losses in the colostrum during the first 24 h of lactation.

Colostrum contains a plethora of growth factors (e.g., EGF, IGF-1, IGF-2, and other unidentified factors) that target the neonate to affect differentiation of anterior pituitary mammotropes, gastrointestinal tract development, and maturation of the immune system [11, 44]. The term lactocrine was coined to describe a mechanism through which a bioactive factor or hormone reaches the neonatal circulation shortly after birth, as a specific consequence of nursing [68]. Lactocrine-acting factors, whether natural or man-made, may affect developmental events associated with programming of female reproductive tract tissues. This has been demonstrated vividly in the pig based upon the following lines of evidence[4]: (1) colostrum is a source of bioactive relaxin; (2) relaxin can be detected in the circulation of newborn pigs only if they are allowed to nurse; (3) the neonatal uterus is RLX receptor (RXFP1) positive at birth and prior to onset of endometrial estrogen receptor (ESR1) expression; (4) administration of exogenous relaxin to newborn gilts stimulates both uterine ESR1 and vascular endothelial growth factor (VEGFA) expression by postnatal day 2; (5) such effects of relaxin administered from birth can be attenuated by pretreatment of gilts with the ESR1 antagonist ICI 182,780 indicative that relaxin may act, in part, via cross talk with the estrogen signaling system.

Other mammary born bioactive factors or cooperative lactocrine mechanisms affect the neonatal uterine developmental program based upon comparisons between newborn pigs that nursed ad libitum or received a milk replacer with or without exogenous relaxin for 48 h after birth [11]. Colostrum consumption was required for normal uterine protein and/or transcript expression on postnatal day 2 of RXFP1, ESR1, VEGFA, and matrix metalloproteinase 9 (MMP9). In contrast, uterine ESR1, VEGFA, and MMP9 protein levels were below detection limits in replacer-fed gilts. Supplemental relaxin increased uterine ESR1 protein and mRNA in nursed gilts, as well as VEGFA protein in nursed and VEGFA mRNA in both nursed and replacer-fed gilts. Relaxin treatment did not affect uterine MMP9 mRNA levels. When compared with replacer-fed gilts on postnatal day 2, uterine RXFP1 mRNA was reduced in nursed gilts and in relaxin-supplemented replacer-fed gilts. The authors concluded that establishment of the neonatal porcine uterine developmental program requires maternal lactocrine support beyond just the presence of relaxin.

In porcine neonates, estradiol valerate exposure from birth to postnatal day 13 increased uterine RXFP1 gene expression, and both ESR1 and VEGFA proteins at postnatal day 14 [12]. When uterine responses were examined in mature gilts at day 12 of pregnancy, endometrial RXFP1 mRNA remained elevated, while ESR1 protein was reduced in gilts that were postnatally treated with estradiol valerate. Early estradiol valerate treatment decreased neonatal uterine WNT7A but increased HOXA10 expression. The expression of WNT7A was reduced in estradiol valerate-treated adults. Transient EV exposure increased MMP9 transcripts at postnatal day 14, whereas both latent and active MMP9 activity was increased in adults at day 12 of pregnancy due to early estradiol valerate treatment. These findings support the hypothesis that transient estrogen-induced disruption of porcine uterine development from birth alters early programming events that lead to functional consequences in the adult.

The lactocrine regulation of uterine development in the bovine neonate is essentially unknown. As described above there are various growth factors in colostrum of cattle and in particular very high concentrations of IGF-1 [43]. Relaxin concentrations compared to the pig are low or perhaps non-existent in cattle; although a relaxin-like factor (RLF), a new member of the insulin-relaxin gene family, is expressed in bovine ovarian follicular thecal cells (33). Bartol *et al.* [3] demonstrated that exposure of neonatal heifer calves to progesterone and estradiol benzoate (PE) delivered from a commercial growth-promoting implant either at birth (day 0), 21 or 45 days of age altered adult (i.e., 15 mo of age in the luteal phase of an induced estrous cycle) uterine morphology and uterine luminal protein content compared to non-implanted control heifers. Treatment did not affect plasma progesterone concentrations (3.2ng/ml). Regardless of age at treatment, neonatal PE exposure reduced uterocervical wet weight by 35% (112.8 < 173.9 g), myometrial area by 23% (126.3 < 162.8 +8.5 mm^2), and endometrial area by 27% (33.3 < 45.4 +2.7 mm^2) compared with the

untreated controls. Endometrial gland density was reduced by 40% in treated heifers. This effect was related to age at implant placement. Uterine gland density was reduced by 66% in heifers treated at birth, while reductions of 22 and 33% were observed for heifers treated on neonatal days 21 or 45, respectively. Consistently, ULF protein content was lower in the treated heifers (2.67<4.98 mg/ total uterine luminal fluid). Thus, exposure of newborn calves to PE can have profound effects on adult morphology and environment of the uterus with the extent depending upon the developmental period when exposure occurs. Identification of the complete array and role of factors that define the uterine developmental program and the subsequent impact of reproductive tract development on fertility will provide critical management insight into factors regulating herd fertility. This is an area warranting further investigation in cattle.

III. OPTIMIZATION OF REPRODUCTIVE PROGRAMS FOR TIMED ARTIFICIAL INSEMINATION (TAI)

It is essential that dairy producers, farm staff, nutritionists, and veterinarians understand the physiological underlying reasons why certain components of the reproductive management program are able to improve reproductive performance or conversely why a misunderstanding of the program can lead to catastrophic pregnancy results. No one reproductive breeding program is practical and economically optimal for all dairy production units due to differences in available facilities, size of the unit, labor that places reproduction as a high priority, and a functionally dynamic record system. What is essential is the implementation of optimal programs that increase pregnancy rates and these programs become fertility programs that achieve more than just inseminating all cows.

Optimization of stage of the estrous cycle (i.e., days 5 to 9) at the onset of the Ovsynch protocol (i.e.GnRH_5-7days_$PGF_{2\alpha}$_2-3days-GnRH-16h-AI) is important to achieve a subsequent synchronized ovulation at the second GnRH preceding the TAI. Programming the stage of the estrous cycle at the time the Ovsynch protocol is implemented (e.g., Days 5-9 of estrous cycle) has multiple effects: increases the probability that the first injection of GnRH will induce ovulation of the first wave follicle and subsequent recruitment of a new follicle wave; that there is progesterone availability throughout the period between the first injection of GnRH and injection of $PGF_{2\alpha}$, that there is a CL to respond to the luteolytic injection of $PGF_{2\alpha}$, and producing a viable oocyte for fertilization and development of a robust CL upon induction of ovulation to the second GnRH. Indeed ovulation of the first follicle wave results in presence of both the original CL and an accessory CL, induced by the GnRH injection, which are responsive to the injection of $PGF_{2\alpha}$.

The Ovsynch protocol preceded by a $PGF_{2\alpha}$ program (Presync-Ovsynch) has become the nucleus program for reproductive management in the industry. Successful use of such a program is dependent highly upon obtaining good compliance in implementing all component parts of the protocol. The original Presynch-Ovsynch program entailed two injections of $PGF_{2?}$given 14 days apart with the Ovsynch protocol initiated 12 days after the second injection of $PGF_{2?}$for presynchronization [41]. This system increased pregnancy rates compared to Ovsynch alone, and it is essential to start the Ovsynch program between 10 to 12 days after presynchronization (i.e., the second injection of $PGF_{2\alpha}$) to obtain good pregnancy rates to the TAI. A 14-day interval may be convenient for producers but is not optimal to obtain maximal fertility. An 11–day interval after presynchronization (i.e., cows would be predominately on days 5-8 of the estrous cycle) was better than a 14-day interval to begin the timed AI protocol [23].

High-producing lactating dairy cows have a greater incidence of two waves of follicle growth during the estrous cycle compared with growing heifers that are more likely to have three follicular waves. The interval from follicle emergence to estrus is 3.5 days greater for cows with two follicular waves than for those with three follicular waves [7]; consequently the period of follicular dominance is greater and fertility to TAI in cows with greater periods of follicle dominance is reduced. One means of reducing the period of ovulatory follicle dominance is to shorten the interval from follicle recruitment to luteal regression (i.e., implement a 5-day interval between GnRH and $PGF_{2\alpha}$injection) to increase pregnancy per TAI in lactating dairy cows. Following two presynchronization injections of $PGF_{2\alpha}$at 36 and 50 days in milk, Santos *et al.* [50]randomly assigned 933 cows to a Cosynch 72 h protocol (CoS72: day 61 GnRH, day 68 $PGF_{2\alpha}$, day 71 GnRH) or to a 5

day-Cosynch 72 h with two injections of $PGF_{2\alpha}$(5 day-CoS2: day 61 GnRH, day 66 and day 67 $PGF_{2\alpha}$, day 69 GnRH). Regression of CL was lesser (91.5 vs. 96.3%) and pregnancy/TAI greater (39.3 vs. 33.9%) for 5 day-CoS2 than CoS72, respectively. It was essential to inject two doses of $PGF_{2\alpha}$ given 24 h apart (i.e., day 66 and day 67) to insure complete regression of the CL.

An additional study tested timing of the GnRH associated with timed insemination at first service and development of a re-synchronization TAI program for second service of non-pregnant cows [6]. Following two injections of $PGF_{2?}$at 46 and 60 days in milk, 1227 cows were randomly assigned to a 5-day OVS56 h (day 72 GnRH, days 77 and 78 $PGF_{2\alpha}$, day 79 [56 h] GnRH TAI at 72 h or to a 5-dayCosynch72h (i.e., GnRH and TAI occurred concurrently). Pregnancy/TAI did not differ between groups when evaluated at either day 32 (45.9%) or day 60 (39.7%) after TAI. Thus, the 5-dayCosynch 72 h program with two injections of $PGF_{2\alpha}$is very efficient in getting cows pregnant. Cows diagnosed as nonpregnant by transrectal ultrasonography on day 32 after the first TAI postpartum (112 ± 3 days in milk) were blocked by parity and method of synchronization at first AI [6]. Within each block, cows were assigned randomly to one of two treatments. All cows received an injection of GnRH 2 days after the pregnancy diagnosis at day 34 after the previous AI. They were treated with an injection of $PGF_{2\alpha}$5 and 6 days after the first GnRH. A second injection of GnRH was administered 56 h after the first $PGF_{2\alpha}$, and insemination was performed 16 h later. Cows assigned to the control group (n = 334) did not receive any further treatment, whereas cows assigned to supplemental progesterone (n = 341) received an intravaginal insert containing 1.38 g of progesterone (Eazi-Breed CIDR Cattle Insert, Pfizer Animal Health, New York, NY) from the first GnRH to the first $PGF_{2\alpha}$of the resynchronization protocol. Treatment cows supplemented with progesterone had greater pregnancy/AI compared with unsupplemented cows (51.3 vs. 43.1%). Premature ovulation tended to be greater for control than progesterone treated cows (7.5 vs. 3.6%).

Presently in 2011, these systems have been developed to optimize the beginning of a TAI program, controlling the period of follicle dominance to improve fertility, recognition of the need to sustain progesterone exposure throughout the period of follicular synchronization, the essentiality of obtaining complete regression of the CL in lactating dairy cows, and finally the need to optimize timing of AI relative to induction of ovulation with GnRH. These advancements allow on farm pregnancy rates of 40 to 50% for first and second service. However, they require maximal compliance in protocol implementation, integration with on farm computer monitoring for lists of cows to be handled, treated, and monitoring the efficiency of the system. It is essential that studies designed to examine specific effects, in all areas of dairy management, on pregnancy per AI have sufficient number of animals per treatment group for example to have at least a 95% chance (P 0.05) of detecting a 7-percentage-unit increase in pregnancy per AI (e.g., 34 vs. 41%) with 90% protection against a type II error.

Implementation of such programs are successful as depicted by 21-day pregnancy rates for consecutive 21 day intervals from July, 2009 through the following March, 2010 in a Florida commercial dairy herd (Figure 2). The dairy was comprised of 4,000 cows managed in a free-stall facility with an average milk production of 10,000 Kg/cow. The bench mark 21-day pregnancy rate that was the goal to be achieved on the farm was 20%, whereas the average pregnancy rate obtained was 23% (Figure 2). Pregnancy rate during the hot periods of July through September was a mean of 18.5%, with a carry-over period of reduced pregnancy rates until early November. Pregnancy rates increased to 29% in January. The overall 23% pregnancy rate would be equivalent to achieving an overall 70% estrous detection rate and a 32.8% conception rate to inseminations in cows detected in estrus. Usual estrous detection rates are 50% with conception rates of 30%, which would approximate a 21 day conception rate of 15%. Heat detection rates and conception rates can drop to 15% and 12% in the summer periods of Florida, which is equivalent to a 2% pregnancy rate. Thus the chronic use of this fertility management program was quite effective.

Future advancements to further improve efficiency of the programs will entail early diagnosis of pregnancy and online monitoring of ovarian cycles and health status in the milking parlor with the use of nano-technology. Such technology combined with optimized housing to maximize animal comfort,

health and well-being will further allow high producing lactating dairy cows to successfully reproduce and yet sustain high levels of milk production. Assisted reproductive technology such as Timed Embryo Transfer with in vitroproduced embryos is further effective in improving fertility with implementation of improved culture media [8]. However, advancements in embryo freezing of in

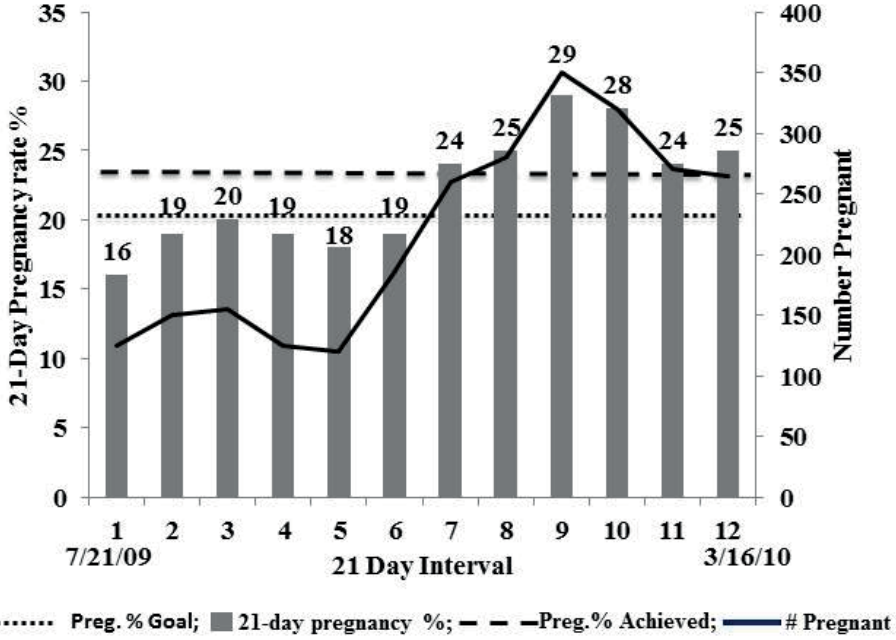

Figure 2. Twenty-one day pregnancy rate and number of pregnant cows per 21-day interval for twelve 21- intervals beginning 7/21/09 and ending 3/16-10 at Alliance Dairy, Florida. Mean 21-Day pregnancy rate of 23% compared to a pre-established management goal of 20%.

vitroproduced embryos to achieve normal pregnancy rates are still needed for more worldwide implementation of this technology.

IV. GENOMICS AND ENDOMETRIAL TRANSCRIPTOME ANALYSES OF THE ENDOMETRIUM

4.1 Evolutionary Genomics

Since completion of the human genome sequencing project in 2001, the genome for the bovine has been sequenced in 2004. It was further refined in 2009 to allow for evolutionary comparisons with other species and more detailed identification of expressed genes and their proteins. Sequencing of the bovine genome was a world-wide endeavor (for more information on the project and selected references see www.hgsc.bcm.tmc.edu/projects/bovine). It has been estimated that the cattle genome contains approximately 3 billion nucleotides with roughly 1% coding for functional genes. The high degree of conservation of genetic sequences across different species is providing valuable comparisons of genomic sequences to help in the discovery of genes and to map their location to bovine chromosomes. Latest estimates have identified at least 22,000 protein coding genes and 496 miRNA genes that are capable of differentially regulating gene expression.

What applications and inferences do technology associated with elucidation of the bovine genome offer to dairy producers First there are the evolutionary implications associated with the ruminant and importance of lactation [19]. Seventy-six percent (778 out of 1020) of sequential duplications corresponded to complete or partial gene duplications with high sequence identity (median 98.7%). This suggests that many of these gene duplications are specific to either the Bos lineage (i.e., wild and domestic cattle) and tend to encode proteins that often interface with the external environment, particularly immune proteins and sensory and/or olfactory receptors and include defensins, and pregnancy-associated glycoproteins. Duplications that are present exclusively in cattle may have functional implications for their unique physiology, environment that they subside in, and diet of cattle. An overrepresentation of genes involved in reproduction

in cattle is associated with several gene families expressed in the ruminant placenta. These gene families encode the intercellular signaling proteins pregnancy-associated glycoproteins, interferon tau (IFN-α) and prolactin-related proteins These genes regulate ruminant-specific aspects of early pregnancy recognition, fetal growth, maternal adaptations to pregnancy, and the coordination of parturition. Examples of genes varying in cattle relative to mouse include a cluster of b-defensin genes, which encode antimicrobial peptides. Compared to the human and mouse genome, the cattle genome has increased changes in the numbers of interferon genes and the number and organization of genes involved in adaptive immune responses. This extensive duplication and divergence of genes involved in innate immunity may be because of the substantial load of microorganisms present in the rumen of cattle, which increases the risk of opportunistic infections at mucosal surfaces and positive selection for the traits that enabled stronger and more diversified innate immune responses at these locations. Another possibility is that immunity may have been under selection due to the herd structure, which can promote rapid disease transmission. Also, immune function–related duplicated genes have gained nonimmune functions, e.g., IFN-α that in addition to regulating antiviral activity is involved in maintenance of the corpus luteum in early pregnancy by its actions on the uterus to ultimately suppress secretion of prostaglandin F2α; the C-class lysozyme genes, which are involved in microbial degradation in the abomasum.

A summary of these evolutionary comparisons among species indicate that the biological systems most affected by changes in the number and organization of genes in the cattle lineage include reproduction, immunity, lactation, and digestion. These changes in the cattle lineage probably reflect metabolic, physiologic, and immune adaptations due to microbial fermentation in the rumen, the herd environment and its influence on disease transmission, and the reproductive strategy of cattle. Mapping of the cattle genome and associated resources will facilitate the identification of novel functions and regulatory systems as well as the tools for genetic improvement within the dairy industry.

4.2 Functional genomics

Gene discovery says nothing of gene function. However, searching databases from other species and now the bovine genome is helping to predict gene function, particularly for single gene traits. Other methods are also helping us to identify functional roles of genes and include gene chips or microarrays, gene knockouts and gene knockdowns. For example Affymetrix Inc. now markets a bovine genotyping chip, allowing broader translation of the genome project into applications. Microarrays are nylon or glass slides or "chips", as they are commonly called, that are spotted with partial gene coding sequences. Chips are incubated with fluorescently tagged complementary DNA (cDNA) from tissues of interest to determine what genes are being expressed.

As a very specific integrated example for the use of this technology, we have taken a conceptus/endometrial analytical transcriptome approach to elucidate potential biological effects of cycle versus pregnancy of nonlactating (NL) and lactating (L) dairy cows at day 17 after a programmed LH surge [10,61]. We first characterized an experimental platform to evaluate lactation and pregnancy effects for subsequent global transcriptome analyses. Pregnant heifers (n=33) were assigned randomly after calving to L (n=17) and NL (n=16) groups. The L group was fed a total mixed ration (1.65 Mcal NEL/kg, 16.5% CP) and the NL group fed a maintenance ration (1.45 Mcal NEL/kg, 12.2% CP). Blood was collected thrice weekly for 8 weeks and analyzed for insulin, IGF-1, NEFA, BHBA, glucose, and BUN. Rectal temperatures, ovarian ultrasonography, body weight (BW) and body condition score (BCS) were measured during the study. All cows were pre-synchronized and enrolled in a timed artificial insemination (TAI) protocol; 10 cows in the L and 12 in the NL were TAI. On d 17 after GnRH/TAI, all cows were slaughtered and endometrial and conceptus tissues collected. The Bovine was used to assess conceptus and endometrial gene expression.

Temporal changes in BCS and BW did not differ between L and NL cows. L cows had higher body temperature than NL cows (38.4 vs 38.2ºC), and NL cows cycled earlier than L (26.3 vs 34.7 days postpartum). Concentrations of NEFA did not differ between NL and L cows; however, cows in L group had greater concentrations of BHBA (4.90 vs 2.97 mg/dL) and BUN (11.6 vs 6.5 mg/dL) and lower

concentrations of glucose (74.0 vs 79.9 mg/dL) than NL cows. Mean plasma concentrations of insulin postpartum did not differ between NL and L (1.28 vs 1.24 ng/mL). Concentration of IGF-1 was lower ($P < 0.01$) for L compared with NL (140.5 vs 198.2 ng/mL), and also different ($P = 0.01$) between cyclic and pregnant (147.6 vs 191.0 ng/mL). Insulin was not correlated ($P > 0.10$) with any of the metabolites measured in both simple and partial correlations. Concentrations of IGF-1 had a +0.25 correlation ($P < 0.01$) with glucose, but this correlation was not significant when adjusted for lactation. Negative correlations ($P < 0.01$) between IGF-1 and NEFA (r = -0.33), and BUN (r = -0.25) were detected. Among metabolites, the highest correlation was between BHBA and BUN ($P < 0.01$; r = +0.59). Concentration of progesterone from GnRH or TAI (d 0) until d 17 was lower for L cows than NL cows.

Metabolomic responses such as concentrations of NEFA, BHBA, BUN, glucose, insulin and IGF-1 in plasma are indicative that L cows underwent metabolic changes associated with homeorhetic processes in response to lactogenesis and galactopoiesis. The alterations in metabolites reflect mobilization of lipids and proteins during a period of negative energy balance postpartum. In the present study, metabolic changes related to lactational status were observed even though there were no differences in BW and BCS between LC and NL cows.

RNA from conceptus and intercaruncular endometrial tissues was extracted using TRIzol®reagent (Invitrogen Corporation, Carlsbad, CA, USA) according to instructions provided by the manufacturer. Samples were purified (PureLink®Micro-to-Midi kit; Invitrogen Corporation, Carlsbad, CA) and RNA concentrations and purity were determined (Agilent 2100 Bioanalyzer, Agilent Technologies, Inc., Santa Clara, CA, USA). All samples were further processed for amplification and labeling and had a RNA integrity number > 7.5, which is related to the ratio of 18S and 28S ribosomal subunits. Samples were placed in aliquots and stored at -80°. Amplification and biotin labeling were performed with an initial 200 ng of RNA by using the MessageAmp™ III (Applied Biosystems, Inc., Foster City, CA, USA) according to manufacturer's guidelines. Samples were then tested in the bioanalyzer for quality determination and subsequently submitted for fragmentation and hybridization in the bovine microarray (Affymetrix® Bovine Genome Array, Affymetrix®, Inc., Santa Clara, CA, USA).

Only pregnant (L, n=8; NL, n=6) and non-inseminated cyclic (L, n=7; NL, n=4) cows were analyzed. Differentially expressed genes were selected with *P*-value < 0.01 and absolute expression > 40. In addition, a fold effect > 1.5 was used as a criterion for genes affected by pregnancy. Analyses of the endometrium detected 210 genes differentially regulated by lactation (136 down-regulated and 74 up-regulated), 702 genes differentially regulated by pregnancy (407 down-regulated and 295 up-regulated) and 61 genes were responsive in an interactive manner between pregnancy and lactation. Genes up- and down-regulated in pregnant cows were associated with several gene ontology (GO) terms, such as defense response (GO:0006952), interferon regulatory factor (IPR001346), cell adhesion (GO:0007155) and extracellular matrix (GO:0031012). Gene ontology (GO) analyses of up- and down-regulated genes of lactating cows revealed terms related to immunoglobulin-like fold ((IPR013783), immune response (GO:0006985), COMM domain (IPR017920) and non-membrane bounded organelle (GO:0043228).

For purposes of this presentation, we have chosen to focus on expression of Early Pregnancy Associated Glycoproteins (PAGs), also known as Pregnancy Specific Protein B (PSPB; [9]) and Pregnancy Serum Protein of Mr 60 kDa (PSP60; [39]) in early pregnancy at day 17 in both conceptus and endometrial tissues. Adequate synchronization of embryonic development and remodeling of the endometrium are crucial to support conceptus-placental development throughout gestation and avoid pregnancy failure. The conceptus-derived placental cells (trophoblasts) fuse to the endometrium and deliver secretory products into the maternal system. Numerous molecules, including proteins, cytokines, hormones, and growth factors coordinate the conceptus-maternal interface and systemically moderate maternal anatomy, endocrinology, immunology and physiology to create an appropriate environment for conceptus development and survival [5,22]. In cattle, 18 distinct PAG genes and 14 pseudogenes have been identified [56]. The measurement of PAG in maternal blood is an alternative reproductive management tool that can

be used for early pregnancy diagnosis in cattle (i.e., day 27) and may be an indicator of conceptus/fetal wellbeing and pregnancy loss [26,51,62]. However, the functional role (s) of these molecules is still unclear. The function (s) of PAG family genes may be combined with their spatial and temporal expression throughout pregnancy and may be involved in adhesion, implantation and remodeling of the fetal-maternal unit [67], immune regulation[1,16,31] and prostaglandin synthesis and regulation [14, 65].

Around 40% of total embryonic losses are estimated to occur between d 8 and 17 of pregnancy [58]. An important event on d 16 to 17 after estrus is the maintenance of the CL. This process is established by the ability of the conceptus to secrete sufficient amounts of IFN-?, which regulates secretion of $PGF_{2?}$in the uterine endometrium [57]. Changes involved in the process from a cyclic to a pregnant state not only depend on adequate production of antiluteolytic signals from the conceptus, but also the response of the endometrium to those signals. A precise understanding of the dialogue between conceptus-maternal-placental units is needed to reduce these early losses and improve reproductive efficiency of cattle. Thus, elucidating the mechanisms that control embryo and endometrial development in early gestation is important for identification of

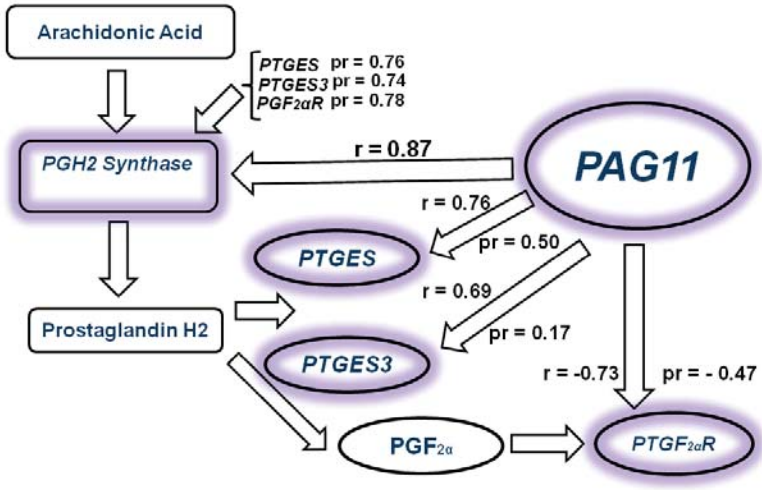

Figure 3. Conceptus gene expression; simple (r) and standard partial (pr) correlations of PAG11 with prostaglandin regulatory genes: PGH2 synthase (prostaglandin H2 synthase); PTGES (prostaglandin E synthase; PTGES3 (prostaglandin E synthase 3 [cytosol]); and PGF2αR (prostaglandin F2α receptor). Grey shadow represents genes that were correlated with PAG 11 in both simple and partial correlation analyses.

Table 1. Conceptus and endometrial expression of PAG genes at d 17 of pregnancy.

PAG Genes	Gene Expression Level		
	Conceptus (13)[a]	Endometrium Cyclic Cows	Endometrium Pregnant Cows
2	28,818.95 ± 1,435	4.22 (11)	52.78 ± 36 (4/14)[a]
8	10,352.84 ± 1,892	4.22 (11)	8.87 (1/14)
11	53,831.91 ± 2,620	4.23 (11)	84.15 ± 39 (7/14)
12	9,187.08 ± 1,170	4.22 (11)	11.73 ± 5.9 (3/14)

[a]Number of animals in parenthesis.

genes regulating implantation, placentogenesis, and maintenance of pregnancy in lactating dairy cows.

In the present study all conceptuses (n = 13) expressed PAG 2, 8, 11 and 12, which are all members of the ancient PAG group indicating that these genes are expressed by both trophectoder mmononucleated and binucleated cells (Table 1). The PAG11 was the PAG family gene member most abundantly expressed by d 17 conceptuses (i.e., expression level = 53,831 ± 2,620). Additional PAG family genes were expressed by some of the conceptuses: PAG 1 (n=1), PAG 7 (n=3), PAG 9 (n=1), PAG 10 (n=1), PAG 17 (n=2), PAG 18 (n=1), PAG 20 (n=1) and PAG 21 (n=5). These reflected other ancient and modern (i.e., expressed in binucleate cells) PAG family genes expressed by some of the conceptuses.

Simple correlation (r) analyses were analyzed with PAG 11 that was present and most abundant in all conceptuses. The following correlations of PAG 11 with genes expressed in conceptuses were detected: PGH2 synthase (prostaglandin H 2 synthase [Cox-2]; r = 0.87; $P < 0.01$), PTGES (prostaglandin E synthase; r = 0.76; $P < 0.01$), PTGES3 (prostaglandin E synthase 3 [cytosol]; r = 0.69; $P < 0.02$) and $PGF_{2\alpha}$(prostaglandin $F_{2\alpha}$receptor; r = -0.73; $P < 0.01$). Standard partial correlation (pr) analyses (Figure 3) holding PGH2 synthase as a constant showed a decrease in the correlations of PAG 11 with PTGES (pr = 0.50; $P < 0.09$), PTGES3 (pr = 0.17; $P < 0.59$) and $PGF_{2\alpha}$(pr = -0.47; $P < 0.11$). Moreover, PAG11 and PGH2 synthase were still highly correlated when standard partial correlation analyses were run holding PTGES (pr = 0.76; $P < 0.01$), PTGES3 (pr = 0.74; $P < 0.01$) or $PGF_{2\alpha}$(pr = 0.78; $P < 0.01$) constant (Figure 3). Prostaglandin related expression of genes in the conceptus was not affected by lactation. These series of analyses suggest that PAG 11 is highly associated with gene expression of PGH2 Synthase in the conceptus and that downstream influences of PAG 11 on expression of enzymes in the PGE cascade as well as the $PGF_{2\alpha}$were possibly manifested by the upstream regulation of PGH2 Synthase expression.

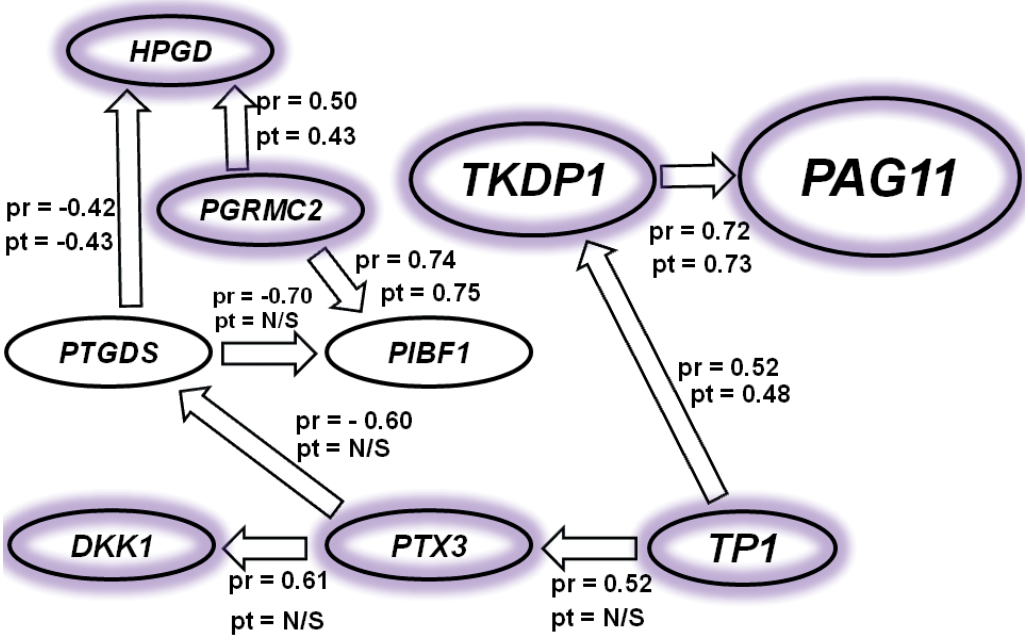

Figure 4. Endometrium gene expression; standard partial correlations (pr) associations of PAG 11 with other functional genes. TKDP1 (trophoblast Kunitz domain protein 1); TP1 (trophoblast protein 1); PTX3 (Pentraxin-Related Gene); DKK1 (Dickkopf Homolog 1); HPGD (Hydroxyprostaglandin dehydrogenanse 15); PGRMC2 (progesterone receptor membrane component 2); PTGDS (prostaglandin D2 synthase) and PIBF1 (progesterone induced blocking factor 1). Grey shadow represents the genes that were correlated with PAG11 in simple correlation analyses. The standard partial correlation, pt is the correlation between the expression of two genes with expression of a third gene held constant and also adjusted for treatments (N/S = not significant).

The PAG genes, which are expressed exclusively by trophoblast cells (i.e., ancient PAG) were also observed in the endometrium of pregnant cows at d 17 of pregnancy. Among the PAG genes expressed in the endometrial tissue were PAG 2 (n=4), PAG 8 (n=1), PAG 11 (n=7) and PAG 12 (n=3) (Table 1). PAG 11 was expressed in a greater number of cows and at a higher level than the other PAG genes observed in the endometrial tissue at day 17 of pregnancy (i.e., expression level = 84.15 ± 39), which is well before detection in the blood at day 27 [62].

Analyses of simple correlations in endometriual tissue detected associations of PAG 11 with expressed genes of trophoblast cells such as TKDP1 (trophoblastKunitz domain protein 1; r = 0.86; $P < 0.01$) and TP1 (trophoblast protein 1; r = 0.67; $P < 0.01$). PAG 11 also was correlated with genes expressed in the endometrium associated with conceptus invasion and implantation, PTX3 (pentraxin-related gene; r = 0.50; $P < 0.01$) and DKK1 (dickkopf homolog 1; r = 0.43; $P < 0.02$); prostaglandin regulatory genes such as HPGD (hydroxyprostaglandin dehydrogenanse 15; r = 0.56; $P < 0.01$), and a gene related to progesterone regulation, PGRMC2 (progesterone receptor membrane component 2; r = 0.35; $P < 0.07$).

A series of standard partial (pr) correlation analyses (Figure 4) revealed a direct correlation of PAG11 with TKDP1 (pr =0.72; $P < 0.01$) when holding TP1 constant (Figure 4). Moreover, when pr were run holding PAG 11 as a constant, TKDP1 was correlated with TP1 (pr= 0.52; $P < 0.01$). However, no correlation was observed between PAG11 and TP1 when TKDP1 was hold as a constant (Figure 4). In addition, associations between TP1 and PTX3 were observed when TKDP1 was held constant (pr = 0.52; $P < 0.01$). Moreover, having TP1 as a constant, showed that PTX3 was highly correlated with DKK1 (pr = 0.61; $P < 0.01$). A negative association between PTX3 and PTGDS (prostaglandin D2 synthase; pr = -0.60; $P < 0.01$) was observed when PIBF1 (progesterone-induced blocking factor 1) was held constant. Furthermore, PIBF1 was negatively correlated with PTGDS holding PGRMC2 constant (pr = -0.70; $P < 0.01$). PIBF1 also had a positive correlation with PGRMC2 (pr = 0.74; $P < 0.01$) when PTGDS was held constant. In addition, results of standard partial correlations showed that HPGD was correlated negatively with PTGDS (pr = -0.42; $P < 0.04$) when holding PGRMC2 constant and positively correlated with PGRMC2 (pr = 0.50; $P < 0.01$) when holding PTGDS constant (Figure 2). In general the absence of a pr+trt (i.e., partial correlation adjusted for both a third expression gene and treatment; not significant [N/S]) reflected that the pr correlation was mainly due to the treatment effects (e.g., pregnancy).

Endometrial expression of HPGD and PGRMC2 was not affected by pregnancy or lactation. Pregnant cows had lower expression of PTGDS compared with cyclic cows. Moreover, pregnancy enhanced the expression of PIBF1, DKK1 and PTX3. In addition, DKK1 expression was inhibited by lactation. Presence of genes expressed exclusively in trophoblast cells of the endometrium, such as TP1, TKDP1 and PAG11, were observed only in pregnant cows and their expression did not differ between LC and NL cows.

All conceptuses expressed PAG 2, 8, 11 and 12, which are all members of the ancient PAG group indicating that these genes are expressed by both trophectoderm mononucleated and binucleated cells. Correlation analyses were conducted with conceptus and endometrial expression levels of genes with the intent of identifying associations that might be important functionally for pregnancy and to provide a basis for future studies to identify the possible function(s) of PAG genes. Different possible functions of PAG genes may be related to the diverse localization of the ancient and modern PAG groups. Ancient PAG genes, mainly expressed at the microvillar junctions of the fetal-maternal interface, may be involved in binding together the fetal maternal surfaces or establishing an immunological barrier. In contrast, the expression of modern PAG genes (i.e., exclusively expressed in trophoblast binucleated cells) occurs mainly in the developing maternal villi of the placentomes which ideally positions them to manipulate the maternal immune system [67].

In the present study, no correlations between conceptus expressed PAG11 and genes involved in immune control were observed at d 17 of pregnancy. However, simple correlation analyses showed that endometrial PAG11 was correlated with PTX3, a gene that is produced by various tissues in response to proinflammatory signals. In humans, both trophoblast conditioned medium and trophoblast explants increased PTX3 mRNA expression in endometrial stromal cells [46]. In addition, Tranguch *et al*. [63]

documented that PTX3 null mice had compromised implantation and decidualization processes. Endometrial PAG11 of the present study was correlated with DKK1, a gene that antagonizes Wnt/ -catenin signaling. DKK1 is involved in early development of head structures anterior to the midbrain and promotes trophoblast cell invasion [42,44]. PTX3 was correlated positively with DKK1, suggesting that PTX3 might be acting in combination with DKK1 in the establishment of implantation. In addition, we observed a negative correlation between PTX3 and PTGDS, which might suggest an alternative way to attenuate $PGF_{2\alpha}$like effects at this stage of pregnancy. It has been documented that prostaglandin D2 is converted to a biologically active $PGF_{2\alpha}$stereo-isomer (9, 11 $PGF_{2\alpha}$; [60]), which has vasoconstrictive and smooth muscle contractile properties, therefore, possibly deleterious for the establishment of pregnancy. Moreover, results of standard partial correlation analyses showed a negative association between PTGDS and HPGD. HPGD is known to be involved in the degradation of prostaglandins such that a sequential reduction in PTGDS would enhance HPGD to increase $PGF_{2\alpha}$metabolism in early pregnancy. Endometrial expression of HPGD is possibly associated with the tight regulation of prostaglandin activity (i.e., production and degradation) in the endometrial tissue.

HPGD also was correlated positively with the expression of the PGRMC2 gene, which encodes a protein that binds progesterone. Thus, this correlation indicates that endometrial progesterone binding might be associated with prostaglandins degradation in early pregnancy. Interestingly, simple correlation analyses showed that endometrial PAG 11 was positively correlated with HPGD and PGRMC2. These associations may be important in the elucidation of functions for PAG genes. PAG genes might be involved in the prostaglandin and progesterone regulation of early pregnancy.

Early expression of PAG genes within the conceptus and endometrium of pregnant cows and the association with other genes infer a possible role of PAG in pregnancy maintenance and implantation by regulation of embryo development, trophoblast cell invasion, immune regulation, and prostaglandin metabolism. The associations detected are suggestive of potential pathways for investigation in early pregnancy at day 17 involving potential direct and indirect effects of PAG 11 produced by the conceptus, but in no means do they prove cause and effect. Future investigations involving use of proteomics, metabolomics, lipidomics, lazer dissection, and in vitrocell culture experiments will help to elucidate these suggested control systems inferred from a transcriptone analytical approach [20]. Nevertheless, use of a classical statistical approach of standard partial correlation analyses is insightful in sorting out potential interrelationships of conceptus and endometrial tissues.

4.3 Genomic selection

Mapping of the bovine genome has facilitated the ability to complement direct genetics with traditional quantitative genetics that will benefit the dairy industry. The genetic contribution of many multi-gene traits in cattle (e.g., milk production) is well documented, and this knowledge has provided the basis for the identification and mapping of a growing number of quantitative trait loci (QTL). The only limitation to performing direct genetic experiments and identifying genes underlying these traits is the lack of a complete genome sequence, which is now available for the bovine. Sequencing the bovine genome and identifying "Single Nucleotide Polymorphisms" (SNPs) will provide additional polymorphic markers and positional candidate genes derived from the human and bovine genomic maps. Indeed due to the higher homology between the bovine with the human genome compared to the genome of the mouse, the functional genomics of the bovine is probably more applicable than using the mouse as an experimental model. The populations with designed mating generated by natural reproduction, artificial insemination or assisted reproductive technologies provides unique opportunities for selection and propagation of efficient dairy cattle in the future that can perhaps both produce milk and reproduce efficiently. Clones can also be generated from fibroblasts or stem cells and cryopreserved.

A deep draft sequence assembly of shotgun reads from a single Hereford female and comparative sequences sampled from six additional breeds were used to develop probes to interrogate 37,470 single-nucleotide polymorphisms (SNPs) in 497 cattle from 19 geographically and biologically diverse breeds

[25]. These data show that cattle have undergone a rapid recent decrease in effective population size from a very large ancestral population, possibly due to domestication, selection, and breed formation. Domestication and artificial selection appear to have left detectable signatures of selection within the cattle genome, yet the current levels of diversity within breeds are at least as great as exists within humans.

The availability of high-throughput assays for genotyping single nucleotide polymorphisms (SNP) has led to the genotyping of thousands of dairy cattle using the BovineSNP50 BeadChip (Illumina, Inc., San Diego, CA, USA) or similar platforms. The SNP markers represent single base changes (A, T, C, or G) within the DNA sequence of a bull or cow. This technology provides the ability to carry out 54,000 DNA SNP marker tests simultaneously; SNPs are throughout the bovine genome of approximately 3 billion base pairs. Consequently, the SNPs become genetic markers for individual animals such as progeny tested bulls in artificial insemination (AI) programs or young bulls that are candidates for such programs. A study at the USDA-ARS Beltsville Agricultural Research Center established the SNP genotypes for 5,369 Holstein bulls and cows [64,66]. The genotype data of the bulls were used to estimate the effects of 38,416 SNP markers on production, type, longevity, udder health and calving ability. Based on the estimated SNP associations on these phenotypic traits from this parent population, a genomic predicted transmitting ability (PTA) was determined for each of 2,035 young Holstein bulls born from 2000 to 2003 that had no progeny. In 2009 the PTA of each young bull was determined from its progeny and compared with the traditional PA (Parental Average) and the genomic PTA computed from the 2004 data. The same process was performed in the Jersey breed (1361 older animals and 388 young bulls) and the Brown Swiss breed (512 older animals and 150 young bulls). Results in Table 2 show the increase in reliability (REL) due to genomic information, as compared with the REL from parent average information only. Gains in REL from genomic information were positive for almost all responses. Gains in REL for Jerseys and Brown Swiss were not as large as for Holsteins and this is largely due to a fewer number of progeny tested bulls that were genotyped. For each trait, a young animal's PA can be combined with information from the BovinSNP50 bead Chip to obtain a genomic PTA of much greater accuracy. For a bull calf, REL of the genomic PTA is equivalent to what could be obtained by measuring performance on 25 or 30 test daughters.

Weigel *et al*. [66] compared how well genomic evaluations were performing for the young bulls of 2000-2003 that in 2009 had both genomic data and at least 50 milking daughters. Parent averages (PA), Genomic Predicted Transmitting Abilities

Table 2. Changes in reliability due to the inclusion of genomic data in national genetic evaluations in the United States.

Trait	Holstein	Jersey	Brown Swiss
Net Merit	+24%	+8%	+9%
Milk Yield	+26%	+6%	+17%
Fat Yield	+32%	+11%	+10%
Protein Yield	+24%	+2%	+14%
Fat Percentage	+50%	+36%	+8%
Protein Percentage	+38%	+29%	+10%
Productive Life	+32%	+7%	+12%
Somatic Cell Score	+23%	+3%	+17%
Daughter Pregnancy Rate	+28%	+7%	+18%

Table from VanRaden *et al*. [64].

Table 3. Comparison of January 2009 parent averages (PA) and genome-enhanced predicted transmitting abilities (GPTA) for milk, fat, protein, somatic cell score (SCS), and daughter pregnancy rate (DPR) with August 2009 daughter yield deviations (DYD) for US Holstein bulls whose first-crop daughters calved between January and August.

	Milk	Fat	Protein	SCS	DPR
No. Bulls	238	238	238	237	60
Reliability (Jan '09 PA)	42%	42%	42%	39%	36%
Reliability (Jan '09 GPTA)	72%	72%	72%	67%	62%
No. Daughters (Aug '09 DYD)	71	71	71	71	62
Reliability (Aug '09 DYD)	84%	84%	84%	67%	52%
Correlation (Jan '09 PA, Aug '09 DYD)	0.444	0.540	0.476	0.376	0.213
Correlation (Jan '09 GPTA, Aug '09 DYD)	0.624	0.695	0.632	0.531	0.341

Table from Weigel K.A. *et.al*. 2010Available at: http://www.aipl.arsusda.gov/publish/other/2010/submit_9wcgalp_vanraden_kw.pdf [66].

(GPTA); and Daughter Yield Deviations (DYD; contains no genomic information) of the bulls. A total of 238 Holstein bulls had official genomic PTAs for milk, fat, protein, somatic cell score (SCS) in January 2009 that were based solely on genomic information and the bulls had at least 50 milking daughters in August 2009. Only 60 bulls had at least 50 daughters in their genetic evaluations for daughter pregnancy rate (DPR). Comparisons of reliability (REL) for PA (Jan. 2009), GPTA (Jan. 2009) and DYD (Aug. 2009) are very insightful and are presented in Table 3. The average January 2009 RELs for PA was 42% for yield traits, 39% for SCC, and 26% for DPR; whereas RELs of the genomic PTA, which include both pedigree and genomic information, averaged a higher 72%, 67% and 62%, respectively. When examining actual production responses of the daughters (DVD in August, 2009), the average REL(s) were 84% for yield traits, 67% for SCS and 62% for DPR. The correlations between August 2009 DYD from progeny testing and January 2009 PA and GPTA for each trait were much higher with the inclusion of genomic information (Table 3). The lower REL for DPR and SCS illustrates the greater difficulty in improving lower heritable fertility and health traits through genetic selection although progress can be made. However, with good reproductive management, as described earlier, the opportunities to improve reproductive performance through selection will be enhanced. The ability to estimate genomic PTA of young bulls via genotyping of SNPs without progeny test estimates allows for the use of young bulls with some degree of confidence. This would allow dairy producers to use a larger number of young bulls that would lower the risk associated with the use of lower REL bulls. Producers who supplement their traditional sire selections with a group of superior genome-tested bulls (i.e., each used in moderation) will achieve the greatest genetic progress.

Traditional reproductive fertility traits have been considered to be lowly heritable (e.g., < 0.05). Undoubtedly this has been due to fertility measurements (e.g., days open) that do not completely reflect the physiological characteristics of the cow and are confounded with environmental effects such as farm management expertise and decisions made by management (e.g., voluntary waiting period, culling, nutritional programs etc.). Royal *et al.* [47] pointed out that although traditional measures of fertility have low heritabilities they have relatively high coefficients of genetic variation indicative of a potential for genetic improvement.

A more specific physiological response study reported heritabilities for anovulation and pregnancy loss of 0.171 and 0.489, respectively when estimated in commercial herds of California and a research herd in Wisconsin [2]. A series of studies from the University of Nottingham documented that more specific measurements of reproductive responses

measured as progesterone profiles in milk are moderately to highly heritable [47,48]. Estimates of heritability for interval to commencement of luteal activity postpartum, length of the first luteal phase postpartum, and occurrence of a certain type of persistent CL were 0.16, 0.17, and 0.13, respectively [48]. Furthermore, significant and strong genetic correlations existed between endocrine fertility traits and production traits. Heritability for the percentage of milk samples with luteal activity during the first 60 d postpartum was 0.30 (3x/wk) and decreased with more infrequent sampling to 0.25, 0.20, and 0.14 for weekly, twice monthly, and monthly sampling, respectively [45]. Incorporation of endocrine parameters of fertility, such as commencement of luteal activity measured in repeated samples at test days, into a fertility selection index may offer the potential to improve the accuracy of estimating predicted transmitting ability for fertility.

Ever evolving and exciting technological strategies are now in place for development of gene markers offered by continual advances in DNA arraying technologies, the bovine genome mapping program, and deep RNA sequencing to more extensively explore the transcriptomes of conceptus and reproductive tissues. Use of genomic technology has identified a potential gene or associated locus that is related to bull fertility [21]. A Phase I comprehensive genome wide analysis of SNPs for bull fertility identified a total of 97 SNPs that were significantly associated with fertility ($P < 0.01$). In Phase II, the four most significant SNPs of Phase I were tested in 101 low fertility and 100 high-fertility bulls. One of the SNPs, rs41257187 (C-T) is in the coding region of the integrin beta 5 gene on chromosome 1. The SNP rs41257187 induces a synonymous (Proline - Proline) suggesting disequilibrium with the true causative locus. However, incubation of bull spermatozoa with integrin beta 5 antibodies significantly decreased the ability to fertilize oocytes. These insightful findings indicate that the bovine sperm integrin beta 5 protein plays a role during fertilization and could serve as a positional or functional marker of bull fertility. This genomic approach enters into the tool box for strategies to improve dairy cattle fertility.

Seven sequence variants (SVs) have been identified in exon 1 and in the promoter region upstream of the bovine gonadotrophin releasing hormone (GnRH) receptor gene [15]. The g.-108T > C allelic variants were associated with an approximately 0.4 day reduction in predicted transmitting ability for days to first service. This relationship infers that selection for animals carrying the g.-108T>C group of alterations may improve fertility in the dairy cow. Furthermore, cattle with the homozygous (CC) genotype for the calpastatin gene had an additional 0.82 and 0.57 PTA greater units of Daughter Pregnancy Rate (i.e., equivalent to 3.28 and 2.28 days open) compared with the TT homozygous and CT heterozygous animals [24].

The laboratory of H. Khatib at the Univerisity of Wisconsin constructed an *in vitro*fertilization (IVF) system that has the advantages of a unified environment and well-isolated components of the embryonic development process. Utilizing this system, SNP in several genes and interactions between them have been found to be associated with fertilization and early embryonic survival rates until day 7 [34,35,37]. In order to determine SNP genotpyes, the ovaries of origin for the oocytes and sperm from bulls were genotyped for genes of interest. The candidate genes with their SNP alleles were associated differentially with *in vitro*fertility of the embryos. This in vitroassessment of potential fertility genes were then examined *in vivo*in which bulls were genotyped for SNP alleles and fertility was assessed [36]. Estimated relative conception rate (ERCR) data from 222 young and mature Holstein bulls were obtained from April through June 2001. Estimated relative conception rate was the difference in conception rate (nonreturn rate at 70 d) of a sire compared with other AI sires used in the same herd for first insemination of lactating cows. The ERCR values for the 222 bulls ranged from 4.99 to +5.23. Semen for each of the bulls was SNP genotyped for the following genes: FGF2 = fibroblast growth factor 2; POU1F1 = pituitary-specific positive transcription factor 1; GH = growth hormone; PRL = prolactin; GHR= growth hormone receptor; PRLR = prolactin receptor; STAT5A = signal transducer and activator of transcription 5A; OPN = osteopontin; UTMP = uterine milk protein, and the data were analyzed for association with ERCR. The ERCR was associated with FGF2 and STAT5A polymorphisms. This *in vivo* validation of previous *in vitro* assessments of fertility

suggests that these genes can be used in gene-assisted selection programs for reproductive performance in dairy cattle.

Collectively the various studies cited identify a number of candidate genes for inclusion in a fertility array. Such strategies involving various technological approaches and cell-animal models will likely lead to development of fertility arrays that will allow for the identification of animals at a young age with potentially high fertility (i.e., male and female) and high production. The challenge will be to manage the lactating cows to achieve their reproduction and production potentials.

Markers for several recessive diseases have been developed through the use of Marker Assisted Selection. Examples of diseases that severely impact reproductive performance, but that have been reduced to minor concerns because of the use of genetic markers, are BLAD (Bovine Leukocyte Adhesion Deficiency), DUMPS (Deficiency of Uridine - 5-Monophosphate Synthase) and CVM (Complex Vertebral Malformation).

Sequencing the bovine genome and further advances in functional genomics promises great benefits to the dairy industry. As genes for production traits are identified, genetic selection strategies can be improved. One can envision making improvements in milk yields and milk fat and protein composition, as well as herd health and reproductive performance. As genes for production traits are identified, gene selection will be reduced to simply running a genetic test for the complement of gene alleles associated with the characteristics of interest.

V. CONCLUSION

Epidemiological data analyses are a powerful tool to identify reproductive inefficiencies and potential causative associations, but do not prove cause and effect. Healthy postpartum lactating dairy cows are indeed fertile.

Induction of ovarian quiescence in response to chronic exposure of a GnRH agonist induced postpartum uterine atrophy and warrants additional investigation relative to potential impacts on improved uterine health.

Dietary supplementation with polyunsaturated omega-6 and omega-3 fatty acids improves postpartum innate immune function and subsequent reproductive performance.

Colostrum feeding contains lactocrine secretions that influence uterine developmental programming in the immediate postpartum period and neonatal exposure to estrogens/progesterone alters early programming of the uterus leading to dysfunctional reproductive tract consequences in the adult.

Reproductive management programs that optimize ovarian and uterine function permit a single timed insemination to an induced ovulation that increases pregnancy per insemination to both first insemination and resynchronized inseminations of cows diagnosed non-pregnant.

The sequencing of the bovine genome has led to thorough characterizations of the endometrium and conceptus transcriptomes in response to key physiological periods such as pregnancy and lactation. Early expression of PAG genes within the conceptus and endometrium of pregnant cows and their association with other genes determined by standard partial correlation analyses infer a possible role of PAG in pregnancy maintenance and implantation by regulation of embryo development, trophoblast cell invasion, immune regulation, and prostaglandin metabolism.

Candidate genes have been identified that are related to fertility based upon *in vitro* and *in vivo* approaches. The array of SNPs across the bovine genome and specific SNPs within candidate genes related to reproductive processes and fertility will

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