SPECIFIC OBJECTIVES

To achieve the overall objective of this proposal, collaborative studies using technical and intellectual resources of members of this multi-state research group will be used to carry out the following specific objectives.

Objective 1: Determine the Impact of Altered Ovarian Function on Ruminant Reproductive Performance (IA, KY, MA, MS, NE, NH, NY, PA, VA, VT, WI , WV)

Objective 2: Identify Alterations in Embryo Development and Uterine and CL Function Associated with Declining Pregnancy Establishment in Ruminants – (NE, NY, PA, VA, WI)

Objective 3: Identify Changes in Genetics and Reproductive Management that Lead to Improved Pregnancy Rates in Ruminants (NY, PA, VA, VT, WI)

EXPERIMENTAL APPROACHES AND METHODS

Objective 1: Determine the Impact of Altered Ovarian Function on Ruminant Reproductive Performance (IA, KY, MA, MS, NE, NH, NY, PA, VA, VT, WI, WV)

Objective 1A. Role of specific signaling pathways in regulation of follicle development, granulosa and thecal cell function, and vascular development of follicles: Hedgehog, HIPPO, SMAD, CCN1, Fas, ERK, and MAPK pathways – (NH, NY, PA, VT, WI)

Objectives: To determine the role of specific, critical signaling pathways in regulation of follicle development, granulosa and thecal cell function, and follicle vascular development.

Specific Background and Rationale: Regulation of ovarian follicle growth is a dynamic process during which cohorts of follicles are recruited and grow in successive waves throughout the estrous cycle of the dairy cow [11-13]. Recruitment of each new wave is preceded and triggered by a transient rise in circulating FSH concentrations [14-19]. During each period of recruitment only a single antral follicle is selected to become dominant over the remaining subordinate follicles of the cohort. The dominant follicle then has one of two fates: attain preovulatory status and eventually ovulate, or be eliminated with the cohort of subordinate follicles through follicular atresia.

Apoptosis of granulosa cells (GCs) is a prominent mechanistic feature of follicular atresia that is widely-viewed as an early and necessary event [15, 118-123]. Follicular atresia occurs in part by an up-regulation of cytokine receptors on GCs [124-126], and is associated with the elimination of subordinate follicles in the bovine ovary [127]. The pattern of cytokine receptor expression on GCs is localized however to only certain cells [124, 125]. Notably, this pattern of expression is restricted to cells of the membrana granulosa, which are among the first to undergo apoptosis [125]. Thus, while overt changes in gonadotropin, steroid and cytokine secretion within the microenvironment of the follicle can certainly trigger the onset of apoptosis, these mechanisms cannot fully account for the specificity of cell loss observed during the onset of follicular atresia. Theca cells of atretic follicles are similarly eliminated by apoptosis, but the process occurs at a much slower, controlled rate [128, 129]. In dominant follicles selected for ovulation, some GCs remain vulnerable to apoptosis until the LH surge, but then abruptly become resistant as they differentiate into luteinized cells of the CL [130]. These observations indicate that cell-specificity of apoptosis within the granulosa layer is a critical part of follicular atresia. *However,*

our current understanding of the complex cellular mechanisms influencing continued growth or atresia of follicles is incomplete.

GC proliferation and function is critical for follicle growth and steroidogenesis. Many hormones regulate GC function including FSH, LH, insulin-like growth factor I (IGF-I), and estradiol which all act synergistically and at specific stages of follicle development to stimulate GC proliferation, provide resistance to apoptosis [15, 131, 132] and promote acquisition of dominance in the selected dominant follicle [133-135]. Yet systemic fluctuations or even intrafollicular alterations in these hormones do not address how the fate of individual GCs within the follicle is determined nor how the fate of the GC in individual follicles is translated to specific follicle growth patterns *in vivo*. As *in vivo* studies clearly show, there is cell-specificity to the process, even to the extent that in bovine follicles the terms "antral atresia" and "basal atresia" have been coined to describe two distinct patterns of GC loss observed during follicular atresia [136, 137].

In the current study, we propose that the combination of targeting (i.e., by immune mediators), and intracellular response are critical for guiding GC fate. The rationale for focusing on Fas-mediated apoptosis is based upon *in vivo* evidence that Fas expression is up-regulated in GCs of subordinate, but not dominant nor preovulatory bovine follicles [127]. The importance of investigating the MAPK/ERK signaling pathway stems from recent work showing ERK expression is up-regulated in GCs of future dominant bovine follicles prior to follicular selection [138]. Moreover, ERK signaling mediates FSH-stimulated estradiol synthesis by GCs [139], which also supports GC viability during follicular development. Acknowledging the PI3 kinase/Akt pathway, as well as other downstream signals of the MAPK/ERK pathway (e.g., P38, JNK), influence GC survival [138-141], there are several compelling arguments for selecting ERK signaling in this study. The ERKs have a known role in the functional differentiation of GCs at the time of follicle selection [142, 143]. The number and variety of ligands that affect ERK signaling is extensive, including cytokines, IGF-I, epidermal growth factors (EGF), transforming growth factor (TGF), gonadotropins, bone morphogenic proteins (BMP), and glucocorticoids. Lastly, differences in the duration, magnitude and subcellular localization of ERK activity generate variations in signaling that ultimately affect cell fate decisions [144].

In addition, we propose that two distinct pathways, Hippo and SMAD interact to determine the rate of GC proliferation and thus the rate of follicular growth, as well as GC differentiation and selection of an individual dominant follicle. The Hippo pathway regulates the balance between proliferative and apoptotic pathways in cells and tissues [145]. Although the upstream regulatory signals are not precisely known, they converge on the auto-phosphorylation of the Hippo kinases, STK4 (MST1) and STK3 (MST2) which then phosphorylate and activate the downstream kinases LATS1 and LATS2. Activated LATS, in turn, phosphorylate and inactivate the transcriptional co-activators YAP and TAZ [146, 147]. When phosphorylated by LATS1/2 on serine 127 (YAP) or S89 (TAZ), both proteins becomes sequestered in the cytoplasm by association with 14-3-3 proteins and eventually are degraded. Inactivation of Hippo signaling leads to de-phosphorylation of YAP and TAZ and translocation to the nucleus where they bind TEAD1-4 transcription factors and induce genes required for cellular proliferation and/or survival [146, 148, 149]. At the same time, YAP/TAZ proteins block cellular differentiation [150-152]. Deletion of the *Lats1* gene is associated with reduced number of ovarian follicles, no CL and development of ovarian stromal tumors and soft tissue sarcomas [153]. Moreover, *Lats1* mutant mice have fewer primordial follicles and increased germ cell apoptosis [154]. Recent

work in mice shows that fragmentation of mouse and human ovaries activates YAP in the somatic cells and this is required for increased cellular proliferation and follicular development [155]. Ovarian fragmentation increases actin polymerization which activates YAP in granulosa cells and increases follicular development [156, 157].

GDF9 and BMP15 are two members of the TGF beta superfamily of proteins that are secreted by the oocyte and regulate GC function. GDF9 signals through SMAD2/3 and BMP15 signals through SMAD1/5/8 to activate gene transcription in conjunction with Co-SMAD4. BMP15 and GDF9 signaling pathways have highly regulated mechanisms which ensure signal termination. There are two SMAD inhibitors currently known, SMAD6 and SMAD7 [158]. A number of single gene mutations have been identified that produce high ovulation rate in sheep [159-163]. Determination of the precise mutation has highlighted the critical role of members of the GDF9 and BMP15 and their signaling family in ovarian development and particularly in selection of one compared to multiple ovulatory follicles [159-174]. For example, Juengel et al. [175] reviewed 10 mutations that produce high ovulation rate in ewes and 6 are in the BMP15 gene, 3 are in the GDF9 gene, and one is in the BMP receptor, BMPR1B. As part of the previous research period, members of the NE-1227 project have been involved in evaluating a novel genotype, termed TRIO, that produces high ovulation rate in cattle (Garcia-Guerra, Wiltbank, Kirkpatrick, unpublished; [176]). The causative mutation lies within a 1.2 Mb region of chromosome 10 that contains a limited number of candidate genes including SMAD2 and SMAD 6. Preliminary RNA-Seq data revealed that SMAD6 mRNA was elevated 6-fold in GC of heterozygous carriers of the TRIO allele. We plan to continue to characterize the follicular and hormonal dynamics of this genotype in vivo, as well as evaluate the in vitro regulation of proliferation and differentiation in response to GDF9, BMP15, and SMAD regulators. In addition, the interaction of the Hippo pathways and SMAD pathways will be evaluated since both YAP and TAZ, downstream Hippo regulators, can interact with SMAD1/5/8 and SMAD2/3 proteins [177-181].

In addition to the role of GC in follicular function, regulation of thecal cell function and vascular development can have key roles in regulation and optimization of follicular development. One hypothesis to be tested is that a developmental signaling pathway, known as the hedgehog (HH) signaling pathway, regulates the development of the vasculature of the follicle and corpus luteum. There is extensive literature to support a role of HH signaling in regulating vascular development in multiple tissues [182-186] and limited information to suggest its ability to influence the follicular vasculature [187, 188]. Two secreted ligands within the pathway, Indian HH (IHH) and desert HH (DHH), are expressed by GCs beginning with the initiation of follicle growth. The membrane-associated receptor patched (PTCH) and downstream target genes are expressed in the mesenchymal tissue surrounding primary follicles, and continue to be expressed as the theca layer of the follicle is established [187, 189-191]. In mice conditional ovarian deletion of Ihh and Dhh prevented follicle development past the preantral stage and the theca layer was poorly developed [192]. In wild-type mice and rats, there is an acute decline in HH signaling to the theca in response to the preovulatory LH surge [187, 190, 191]. This decline is temporally associated with reported changes in the vasculature during the periovulatory period which may be essential to promote the increase in angiogenesis in the developing CL [193-196].

A second hypothesis to be tested is that the angiogenic inducer, Cysteine rich 61-Connective tissue growth factor- Nephroblastoma overexpressed (CCN1), may be an important regulator of angiogenesis in the periovulatory follicle. As an angiogenic inducer, CCN1 is a matricellular,

multi-functional protein that plays key roles in cellular proliferation, differentiation, angiogenesis, apoptosis and tumor growth [197, 198]. While expressed by fibroblasts and endothelial cells, CCN1 was shown for the first time to be expressed by steroidogenic luteal cells of the bovine CL [199]. Since then, it was shown that CCN1 was also expressed by bovine granulosa and theca cells [200] (abstract), and the human granulosa tumor cell line, KGN [201] (abstract). While much is known about the role of angiogenic regulators, such as vascular endothelial growth factor (VEGFa), basic fibroblast growth factor (FGF2), and matrix metalloproteinases (MMPs) in follicle function [202-208], the regulation of CCN1 in ovarian follicular cells (particularly granulosa cells) and its mechanisms of action on these cells, are unclear. Further, we also need to better understand how CCN1 interacts with other angiogenic regulators (e.g. VEGF and FGF2) in granulosa cells to switch on angiogenesis following ovulation, which results in the transformation of the collapsed follicular envelope to become a functional CL.

Methods: The experimental stations involved (NY, NH, PA, VT, WI) will conduct a series of *in vitro* experiments using fetal ovarian GCs and cortical tissue, human granulosa cell lines, KGN [209] and HGrC1 [210], and/or GCs collected at specific times either by ovariectomy, ultrasound-guided follicular aspiration, or from slaughterhouse ovaries.

Experimental Approach 1: Determine if Hippo signaling is activated during ovulation to block proliferation and promote differentiation of granulosa cells. The hypothesis to be explored is that Hippo signaling acts as a switch in GC's during ovulation. Primary GC cultures under control or luteinizing conditions will be employed and activation of the Hippo pathway determined by western blot to detect phosphorylation of the target proteins, YAP and TAZ. Cellular proliferation will be determined using an MTT-based assay and cellular differentiation will analyzed by upregulation of the P4 biosynthetic pathway using real-time PCR, western blot or measurement of P4 accumulation in the media of cultured cells.

Experimental Approach 2: Identify signaling pathways that regulate Hippo signaling during ovulation. The hypothesis that activation of the PKA and/or MAPK3/1 pathways induces Hippo pathway activation in GCs will be tested, since ovulatory signals detected in GCs due to LH receptor activation involves signaling directly or indirectly through PKA and MAPK pathways. To determine which pathway(s) regulate hippo signaling during ovulation, luteinizing GCs will be cultured with or without specific PKA or MAPK3/1 inhibitors and the status of Hippo pathway activation will be determined by qPCR of the Hippo target transcripts and western blot for pYAP and pTAZ

Experimental Approach 3: Determine if ERK signaling enhances GC resistance to FAS-mediated apoptosis as a cellular mechanism influencing follicle selection. The hypothesis that activation of the MAPK3/1 pathway induces ERK signaling and GC resistance to FAS-mediated apoptosis will be tested. Genetic manipulation of ERK signaling will be performed to gain insight about ERK signaling role(s) in immune-mediated apoptosis of GCs and growth selection of bovine follicles: primary cultures of bovine antral follicle GCs will be utilized and several qualitative (immunoblot analysis, confocal microscopy) and quantitative (qPCR, cell death assays) measures of ERK expression and apoptosis assessed. Also, a molecular approach (genetic overexpression/RNA interference) will be implemented to augment/inhibit ERK expression and determine the effects on FAS-mediated apoptosis.

Experimental Approach 4. Investigate whether the hedgehog (HH) signaling pathway plays a role in directing follicle development. The hypothesis to be tested is that HH signaling regulates the development of the vasculature in the growing ovarian follicle and corpus luteum. In previous work, the NY lab used a transgenic mouse model that determined the identity and fate of follicular cells responding to HH signaling. This information will now be applied to determining the role of HH signaling in the bovine follicle and corpus luteum with a focus on vascular development. Cortical tissue from fetal ovaries containing primordial follicles will be cultured and the development of follicles to primary and secondary stages and the associated development of the vasculature will be followed. In some experiments, cultures will be treated with reagents that stimulate or inhibit HH signaling and effects on follicular and vascular morphology and on gene and protein expression patterns associated with vascularization determined. Techniques will include quantitative analysis of changes in mRNA levels and immunohistochemistry and imaging by confocal microscopy. In additional experiments, cultures of dispersed theca tissue and dispersed cells of early corpora lutea, containing the mixtures of steroidogenic and vascular cells present in these tissues, will be used to examine the development of vascular tubes and complexes and the interactions among vascular cell types in response to manipulation of HH signaling.

Experimental Approach 5. Determine cellular mechanisms that regulate the expression of **C**ysteine rich 61-**C**onnective tissue growth factor- **N**ephroblastoma overexpressed (CCN1) in GCs during the folliculo-luteal transition that lead to the development and maintenance of the functional bovine CL? Steroid hormones, gonadotropins and prostaglandins orchestrate a series of coordinated cellular and structural changes that lead to ovulation, and subsequent transition of the follicle to become the corpus luteum [211]. We have used two human granulosa cell lines, KGN [209] and HGrC1 [210], to characterize the regulation of CCN1 mRNA expression by steroid hormones, gonadotropins and prostaglandins. This approach will be utilized to investigate CCN1 expression regulation by estradiol, gonadotropins and prostaglandins in bovine GCs. Midcycle cows will be ovariectomized at 0 hr (control) or 24 and 48 hrs after a single injection of Lutalyse (25 mg). GCs will be isolated from follicles that are >10 mm in diameter, and treated for two hrs with various concentrations of estradiol, LH, FSH, PGF2α and PGE2. RNA will be extracted, cDNA generated and real-time PCR for CCN1 performed. Conditioned medium will be collected and analyzed for steroid hormones (estradiol and P4) by radioimmunoassay and for matrix metalloproteinases (MMPs; specifically gelatinases) by gelatin zymography. Because PGF2α stimulated CCN1 expression in granulosa cells, we will determine PGF2α's downstream signaling pathways, using KGN and HGrC1 cells, which are more readily available. Following a two hr treatment with PGF2α, cell extracts will be prepared to determine the expression of signaling molecules, including extracellular signal-regulated kinase (ERK), rapidly accelerated fibrosarcoma (RAF) and the Rho GTPase, RhoA, using protein arrays, immunoblotting or in-cell westerns. Since P4 production by granulosa-lutein cells increases during the folliculo-luteal transition, we will determine the effect of P4 on CCN1 expression, using KGN and HGrC1 cells. After granulosa cells reach confluency, KGN and HGrC1 cells will be treated with various concentrations of P4 followed real-time PCR to quantify CCN1 abundance.

Experimental Approach 6. Investigate roles of SMAD and Hippo pathways in regulating proliferation and differentiation of GC from carriers and non-carriers of the TRIO genotype. A series of collaborative experiments will be performed using GCs collected from follicles of carriers and non-carriers of the TRIO genotype using ultrasound-guided follicular aspiration.

Pathways will be activated by treatment with activators of SMAD (GDF9, BMP15) and Hippo and activation of SMADs, YAP, and TAZ will be evaluated in the two genotypes by western blotting.

Outcomes of interest: We anticipate that we will be able to provide more depth of knowledge related to the specific cellular signaling pathways that regulate GC proliferation and differentiation, thecal cell function, and vascular development. In particular, the interaction of these pathways during *in vitro* experiments and using *in vivo* approaches will allow a fuller understanding of how follicular development is regulated in cattle and how this process can be potentially optimized during ART procedures.

Objective 1B. Role of periconceptional stress (LPS, heat stress, and fescue toxins) and how it alters the ovarian reserve, follicular environment (steroidogenesis), CL development (vascularization) and ultimately how this impacts oocyte quality (early programming of methylation) and early embryo development--(IA, KY, MA, NE, VA)

Specific Background and Rationale: Multiple extrinsic and intrinsic stresses including heat stress (HS) and metabolic dysfunction causes anovulation, decrease conception rates and compromise the ability to maintain pregnancy in agriculturally important animals. Reproductive dysfunction due to thermal stress is an enormous economic burden and represents a food security issue world-wide. Globally, HS is the primary factor limiting efficient animal protein production for human consumption, and the deleterious effects of HS on reproduction are difficult to mitigate because information on the causative mechanism(s) is scant. Additionally, as climate change continues, seasonal infertility will likely become even more of an economic burden and animal welfare issue in the future. Likewise, metabolic disturbances due to negative energy balance associated with increased milk yield have contributed to decreased fertility particularly in the dairy cow [30]. It is clear from the existing epidemiological data and multiple mouse models, that metabolic dysfunction reduces oocyte quality [31-33]. Indeed, obesity-related metabolic dysfunction has been correlated to mitochondrial dysfunction [31, 34], endoplasmic reticulum stress [35, 36], and increased abundance of oocyte mRNAs [33, 37]. Endoplasmic reticulum reticulum stress is also associated with reduced Ca^{2+} store content [212, 213] and low developmental competence [212, 214]. Interestingly, recent studies by our group have demonstrated altered circulating NEFA and sex-hormone binding globulin (a liver-expressed protein) concentrations and expression of metabolic-related genes in theca cells of beef cows that are sporadically or chronically anovulatory suggesting that metabolic dysfunction may alter ovarian follicular growth and oocyte quality in beef and dairy cows. What is not clear are the mechanistic links between these external stressors and abnormalities in follicular growth and oocyte quality. Understanding these mechanisms is important in order to circumvent this growing problem

Despite hallmarks traditionally associated with hypoinsulinemia, such as 1) marked reductions in feed intake, and 2) rapid body weight loss; we have demonstrated that basal insulin concentration increase in a variety of heat-stressed agriculturally important animals [215]. Heat-stressed animals redistribute blood to the periphery in an attempt to maximize radiant heat dissipation. To maintain blood pressure during HS, the gastrointestinal tract vasculature constricts and blood flow to the splanchnic tissues is markedly reduced. Enterocytes are extremely sensitive to oxygen and nutrient restriction [216] and HS causes striking conformational changes and reduces intestinal integrity. Metabolic endotoxemia (ME) results from compromised intestinal barrier function, where bacteria and their associated toxins, such as LPS, breach the intestinal barrier

and enter systemic circulation [217-219]. This gut dysbiosis, which is also a characteristic of metabolic dysfunction, disrupts the symbiotic relationship between gut microbial populations and intestinal lumen cells [220, 221]. Changes in the relative abundance of microbial populations in the gut (e.g. decreased ratio of the *Bacteroidetes* to *Firmicutes* phyla of bacteria) have been correlated with increased intestinal and systemic inflammation [222-225]. Impacts of gut microbial communities on the metabolic and digestive health of an individual are the subject of numerous reviews [226-230]. In addition, there is evidence that the gut microbiome may be impacting female fertility. Specifically, diet dependent bacterial metabolites can cause sterility in certain strains of *C.elegans* [231, 232] and shifts in the relative abundance of microbial communities have been correlated with increased ovarian inflammation in a mouse model of obesity [233]. *It is intriguing to consider modification of gut microbial communities as a therapy for female infertility; however, understanding the mechanistic relationship between relative gut microbial populations and the function of the reproductive tract in mammalian systems is needed*.

Physiologically LPS compromises folliculogenesis and steroidogenesis in the bovine ovary. Bovine ovarian cortical explants exposed to LPS had reduced number of primordial follicles after 6 d, concomitant with increased atresia of the ovarian reserve [234]. Similarly, mice exposed to LPS *in vivo* had reduced primordial follicle number which was described as a TLR4-mediated effect, since *Tlr4*$^{-/-}$ mice were refractory to LPS-mediated primordial follicle depletion [234]. LPS also alters the level of anterior pituitary hormones, through direct or indirect mechanisms. LPS infusion decreased LH but stimulated systemic prolactin (PRL) and cortisol levels in anestrous ewes. Additionally, mRNA abundance of genes encoding LH (LHβ) and the LH receptor (LHR) were reduced by approximately 60% in both cases [235]. Interestingly, the FSH and FSH receptor as well as PRL and PRL receptor genes were increased by LPS infusion [235].

LPS exposure did not impact cell number or androstenedione production from cultured theca cells from either small, medium or large ovarian follicles, but did reduce the amount of E_2 produced from cultured GCs isolated from all three follicular sizes [236]. In an *in vitro* system where ovarian cortical explants were cultured with LPS and provided with FSH or androstenedione, E_2 and P_4 conversion was reduced potentially due to the observed decreased expression of *Cyp19a* mRNA and protein [237]. Cultured GCs had increased expression of TLR4 to mediate LPS signaling, and negative impacts of LPS on E_2 production were demonstrated [238]. While no overall impact of LPS on E_2 was observed *in vivo*, a temporal decrease in bovine P_4 concentrations and lower ovulation rates resulted from LPS treatment [236]. In agreement with reduced E_2 level (needed to induce the LH surge), when LPS was infused into the uterine lumen, the pre-ovulatory LH surge was attenuated [239, 240]. Furthermore, LPS-treated females had delays in the time to the LH surge [241].

Follicular fluid that surrounds and nourishes the maturing oocyte also contains LPS levels reflective of the systemic circulation. Thus, LPS is reaching the ovary via the systemic circulation and directly interacts with the oocyte proportionately as extra-ovarian tissues [238]. LPS binding to toll-lie receptor 4 (TLR4) in non-ovarian tissues leads to downstream activation of the p65 subunit of the nuclear factor-κB (NF-κB [242]) via phosphorylation (p65NF-κB), and bovine granulosa cells exposed to LPS in culture display p65NF-κB [237]. Activation of this pathway also increases the expression of pro-inflammatory cytokines TNF alpha and interleukin-6 (IL6) [243]. These cytokines also activate NFκB as well as JAK-STAT signaling [244, 245].

The end result of these pathway activations is phosphorylation and nuclear translocation of transcription factors STAT and NFκB p65, respectively resulting in transcriptional regulation of cytokine, anti-apoptotic, and cell survival genes [246]. While the bulk of these signaling studies have been performed using metabolic or immune cells, it is important to note that the ovary is infiltrated by macrophages which play important roles in its normal physiological function [247]. Furthermore, there is growing evidence that chronic inflammation associated with obesity leads to ovarian dysfunction that may be mediated, in part, by these macrophages [248, 249]. However, *despite demonstration that NFκB p65 and STAT3 are expressed in the ovary [248, 250]; little is known about their role in transcriptional and post-transcriptional regulation of mRNAs in oocytes and/or somatic cells of the ovary*.

HS-related reductions in feed intake, milk production and conception rates have been observed and recorded for decades. The mechanisms responsible for these responses are complex, and many are not well understood. Furthermore, there are less obvious consequences of HS that have only recently been described. HS experienced during very early gestation (periconceptional) or during late gestation is detrimental to both the dam and the resulting offspring [251-254]. In fact, the heifer calves that were conceived during periods of HS eventually go on to produce less milk than their thermoneutral-conceived counterparts [253, 254]. The impact of periconceptional HS is particularly interesting because it suggests that the impact of HS on the oocyte or early embryo is long-lasting and persists into adulthood. The mechanisms responsible for these differences are currently under investigation.

Methods: The experimental stations involved (IA, KY, NE, VA) will conduct a series of experiments using dairy cows synchronized to begin the experimental treatment at the same phase of the estrous cycle as described below:

Experimental Approach 1: Determine role of chronic low-level LPS on ovarian function and hepatic clearance of steroid hormones. The hypothesis to be tested is that chronic exposure to LPS, due to compromised intestinal integrity or infection, alters steroid hormone production in the ovary and metabolism of steroid hormones in the liver thereby affecting fertility in dairy cows. Jugular catheters will be surgically placed in twelve multiparous dairy cows to facilitate blood collection and LPS infusion. An infusion pump will be used to deliver chronic LPS (0.02-0.15 μg/kg/h) continuously over 24h for a 7d period. Dominant follicle diameter will be recorded daily 4 days prior to ovulation. At the time of predicted ovulation, follicular fluid will be aspirated for steroid hormone analysis (estradiol and P_4). On the day of predicted ovulation, hepatic biopsies will be obtained and flash frozen for analyses of steroid hormone clearance enzymes.

Experimental Approach 2: Investigate effects of HS on oocyte quality and store content. We hypothesize that altered insulin and glucose concentrations that dairy cattle experience during HS are detrimental to the oocyte and that the resultant changes are permanent and long-lasting. These outcomes could be mediated through changes in the Ca^{2+} content of the endoplasmic reticulum affecting the transcriptome, proteome or epigenome. Potential extranuclear effects could be equally devastating to the oocyte and resulting embryo, prompting us to investigate the effects of HS on the ultrastructure of the oocyte and function of the mitochondria and endoplasmic reticulum. Lactating dairy cows will be subjected to four sequential treatments: 1) thermoneutral environment, 2) thermoneutral environment + hyperinsulinemic hypoglycemic clamp, 3) HS environment and 4) HS environment + euglycemic clamp. Ovarian follicular contents, including the oocyte, follicle fluid and GCs will be collected from each cow twice

during each treatment. Estradiol, P4, insulin and glucose will be measured in the follicular fluid from each collection. Glucose uptake and glucose oxidation will be measured in the GCs. The oocyte and additional GCs will be submitted for gene expression and transmission electron microscopy analyses. Ca^{2+} store content will be assessed using microfluorometry and the fluorescence dye Fura-2.

Experimental Approach 3: Determine impact of fescue on ovarian function. We hypothesize that consumption of endophyte-infected fescue alters ovarian function and confers direct effects on the ovary and ovarian follicle. Cows will be fed either endophyte-infected fescue seed (KY31) or fescue seed largely devoid of endophyte (Low Endophyte Seed) for six weeks, and then follicular fluid will be aspirated every week for six weeks. Blood samples also will be collected throughout the study to evaluate circulating prolactin concentrations. The collected follicular fluid will be added to *in vitro* oocyte maturation media, and maturation/development of those oocytes will be monitored. Changes in cleavage, morula or blastocyst development following *in vitro* fertilization will be indicative of the quality of the follicular fluid, and thus, one factor contributing to the follicular environment.

Outcomes of interest: We anticipate that both ovarian steroid hormone production and hepatic clearance thereof will be reduced in LPS exposed cows investigated in experimental approach 1. In experimental approach 2, we expect changes in the oocyte, follicular fluid and GCs to be directly related to the altered insulin and glucose concentrations that dairy cattle experience during HS. We expect the Ca^{2+} content of the store to be lower or the baseline Ca2+ levels increased. In experimental approach 3, we anticipate that follicular fluid from cows consuming the endophyte-infected fescue seed to yield fewer cleaved oocytes and fewer blastocysts. Taken together, these findings will enhance our understanding of the impacts of environmental stressors on ovarian function and fertility in dairy cattle and may identify targets for amelioration strategies to improve fertility in dairy cows.

Objective 1C. Evaluate the intracellular pathways utilized by Prostaglandin F2a (PGF) in regulating function of the corpus luteum -- (PA, VT, WV, WI)

Specific Background and Rationale: In the absence of the establishment and maintenance of a pregnancy, the dam reinitiates the estrous cycle to provide another opportunity for pregnancy at the earliest possible time. In a regularly cycling animal, this is accomplished by uterine-derived PGF which causes CL regression. In ruminants, specifically the cow and ewe, CL of the estrous cycle become sensitive to PGF on day 5-6 after estrus [255, 256]. The difference in sensitivity of the early and later CL has been enigmatic, because quantity of PTGFR did not differ with stage of the estrous cycle [257]. Recently, Kim et al. (2015) [258] found evidence in primate luteal cells (Cynomolgus monkey) that estrogen promoted luteolysis by redistributing receptors for $PGF_{2}\alpha$ (PTGFR) from throughout the cytoplasm to the perinuclear area. Specifically, PTGFR were located throughout the cytoplasm in monkey GCs, but perinuclear in luteal cells by mid-late luteal phase. An analog of $PGF_{2}\alpha$ (fluprostenol) decreased secretion of P_4 by cells from mid-late to late luteal phase, but not in cells from younger CL or GC. Similarly, in human granulosa cells, PTGFR were located throughout the cytoplasm on the day of aspiration, but were perinuclear 2 days after luteinization in culture, at which time fluprostenol decreased P_4 secretion. Treatment with estrogen caused PTGFR to move from being distributed throughout the cytoplasm to a perinuclear location in human luteinizing GC; both the effect of estrogen on

location of PTGFR and the decrease in P_4 secretion in response to $PGF_{2\alpha}$ were blocked by treatment with an estrogen receptor antagonist or an inhibitor of estrogen synthesis. Inhibitors of synthesis of P_4 (trilostane) or $PGF_{2\alpha}$ (indomethacin) did not block the translocation of PTGFR. Follicular estradiol is necessary for normal luteolysis, as demonstrated by studies in which follicles were destroyed by X-irradiation [259]. The effect observed in primate luteal cells [258] could be the mechanism by which estrogen produces sensitivity of the CL by day 5 in ruminants, as follicular estrogen secretion is rising during the first 4 days of the cycle, when P_4 is low.

Methods: Experiments will be conducted at the stations involved in Objective 1C (PA, VT, WV, WI) to understand the role of estradiol and PGF in the luteolysis process.

Experimental Approach 1: The hypothesis to be tested is that PGF acts on luteal cells via an intracellularly located receptor, PTGFR, and that the function of this receptor is developmentally regulated by and dependent on estrogen. Three objectives will be pursued to interrogate this hypothesis.

Objective 1: Determine which intracellular compartments contain PTGFR. Estrous cycles of beef cattle located at West Virginia University will be synchronized. Corpora lutea will be collected on Day 4 and Day 11 of the estrous cycle, representing CL that lack or have acquired luteolytic capacity, respectively. Penn State University will process these CL for immunoelectron microscopy to visualize the intracellular location of the PTGFR, using a commercially available PTGFR antibody. Preliminary data have been collected that validate the method and the antibody specificity, and have indicated that PTGFR is located inside the luteal cells. In addition, tissues from these CL will be processed for immunohistochemistry to determine cellular location of PTGFR and quantify differences in expression and localization in Day 4 vs Day 11 CL.

Objective 2: Determine if PGF actions occur primarily at the plasma membrane or inside the cell. This experiment will allow us to distinguish if the different effects of PGF on luteal cells are mediated through receptors in different cellular compartments (plasma membrane or intracellular). Midcycle CL will be collected, dissociated, and placed into culture plates for short term incubations or long term cultures. A PGF-biotin conjugate will be used to determine if PGF actions require its transport into luteal cells. The PGF-biotin is biologically active, and can bind to the PTGFR at the cell membrane, but we also have preliminary data indicating that it can diffuse into cells (more rapidly than what would be expected by receptor-mediated internalization). However, binding of PGF-biotin to streptavidin will form a stable complex, and this large protein will prevent diffusion into the cells. Using this approach, the effects of PGF on luteal cells that will be tested including a) activation of protein kinase C, b) mobilization of calcium, and c) inhibition of LH-stimulated P4. The first two effects are rapid effects of PGF, whereas inhibition of P4 occurs only after chronic exposure of luteal cells to PGF *in vitro*.

Objective 3: Evaluate the effect of estrogen exposure on luteal cell responses to PTGFR. Cultured luteal cells will be obtained as described above. Day 4 and Day 11 CL will be collected at West Virginia University. These tissues will be transported to Penn State University for processing and culture. Cells will be treated with estrogen to determine if estrogen promotes translocation of PTGFR to the nucleus of the luteal cells, as has been proposed by Kim et al. (2015). Localization of PTGFR will be by immunohistochemistry at 1000X magnification using the antibody that we have optimized for this work.

Experimental Approach 2: Determine if CL rescue can be achieved by PGE production. The hypothesis to be tested is that interferon-tau rescues CL through stimulation of PGE production by the uterine endometrium. Cows will be treated with interferon-tau or control protein into the uterine horn ipsilateral to the CL. After 12 h of treatment, cows will be challenged with low doses of PGF administered directly into the uterus. In a second experiment, the uterine horn will be treated with or without interferon-tau and with or without an inhibitor of PGE synthesis. Biopsies of the CL will be taken at 30 min after each PGF pulse for evaluation of mRNA expression (RNA-Seq) and physiological endpoints, such as immune cell infiltration.

Outcomes of interest: We anticipate that these studies will allow understanding of the molecular mechanisms involved in the absence of PGF action in the early CL and the mechanisms that mediate PGF action in the mature CL. In addition, we anticipate that these studies will allow us to further understand the mechanisms involved in rescue of the CL during early pregnancy.

Objective 2: Identify Alterations in Embryo Development and Uterine and CL Function Associated with Declining Pregnancy Establishment in Ruminants – (NE, NY, PA, VA, WI)

Specific Background and Rationale: A major emphasis for several multistate participants (NE, NY, PA, VA, WI) is investigating the core physiologic, cellular and molecular processes that control early embryonic development and pregnancy maintenance or loss in cattle. Early embryonic wastage is a major problem in lactating cattle [53], and opportunities exist to greatly improve reproductive efficiency of cattle by limiting early embryonic loss. A central concept of this research is that there are two fundamental goals during early pregnancy in ruminants. First, initiate, establish, and maintain a healthy pregnancy. Second, in the absence of success in the first process, reinitiate the process to provide another opportunity for pregnancy at the earliest possible time. The embryo, therefore, encounters physiological gateways during pregnancy and the uterus/dam makes a "decision" about the "health" of the pregnancy and eventually either continues the current pregnancy or resets the whole process by eliminating the "substandard" conceptus and the source of P4, the CL. These gateways during pregnancy establishment and maintenance involve an intricate and remarkable communication betwee n three distinct structures, the developing conceptus, the uterus, and the CL.

As part of our previous regional research project, we have identified a number of "pivotal periods" when pregnancy loss occurs. In addition, we made the novel observation that when an accessory CL is produced by ovulation of the dominant follicle of the first follicular wave that there is a local effect of the pregnancy on maintenance of this CL. If the accessory CL is on the same side as the pregnancy (ipsilateral) then the CL is maintained throughout pregnancy but if the accessory CL is on the opposite ovary (contralateral) then this CL will generally regress during pregnancy. The accessory CL regresses during 2 distinct times: early, from day 19 to 25, or late, from day 35 to 60. After careful consideration, we have realized that this observation may provide substantial insight into the mechanisms that underlie pregnancy loss since the major periods of pregnancy loss also correspond to these periods [3]. This proposal focuses on understanding pregnancy loss by utilizing the contralateral, accessory CL model employing both an intensive collaborative study (Experiment 1) to discover the whole-animal physiological mechanisms associated with this process and also a large field study on commercial dairies (Experiment 2) to test the practical utility of this model.

Our studies focus on two critical "gateways" in which the pregnancy either continues or is lost. The first period is from Days 18 to 24 when the CL is maintained by the embryonic signal, interferon-tau. A few contralateral, accessory CL will regress during this first period and this primarily occurs in multiparous cows that have a much larger uterus [260]. We postulate that contralateral CL regression during this early period is due to insufficient IFN-T reaching the contralateral horn and therefore there is no alteration in the PGF and PGE patterns in the contralateral horn during this early CL regression period. The CL that do not regress during the first critical period, must undergo a second critical period to determine whether the pregnancy is sufficiently healthy to allow continuation and maintenance of the CL. Interestingly, most of the contralateral, accessory CL will regress during this second critical period indicating the local nature of the signal(s) that maintain the CL during this critical period (second month of pregnancy).

Methods: These studies will be collaborative studies that will be carried out in each of the participating experiment stations (NY, PA, VA, WI). In order to perform these critical, but animal intensive, experiments, it is imperative that multiple experiment stations are involved in collecting the data.

Experimental Approach 1: A total of 16 non-pregnant (non-bred; 4/experiment station) and 48 potentially pregnant (Bred by AI; 12/experiment station to produce at least 4 primiparous and 4 multiparous pregnant cows on each experiment station) Holstein dairy cows will be used in this study. Cows will be synchronized using the Double-Ovsynch protocol: 100ug GnRH – 7 d later – 25 mg PGF – 3 d later GnRH – 7 d later – GnRH – 7 d later - PGF – 1 d later PGF – 32 h later – GnRH – 16 h later – Timed AI (or no AI in non-pregnant cows). All cows will receive GnRH (200 ug) on Day 5 after AI and only cows with a contralateral accessory CL will be used in the study. Cows will be evaluated by daily ultrasound and blood sampling from Day 13 to 60 of pregnancy or until CL regression occurs. Blood samples will be taken on Day 19, 20, and 21 to determine ISGs in white blood cells and circulating PGFM and PGEM. Samples will also be taken on Day 22, 24, 26, and 28 to determine PAGs.

Continuous values (hormone concentrations, follicular/CL sizes) will be analyzed by the MIXED procedure (SAS, version 8.2, 2001) using a model that includes treatment and parity and interaction of treatment with parity. Categorical data (pregnancy per AI at each pregnancy diagnosis) will be evaluated by logistic regression using the LOGISTIC procedure (SAS, 2001) with a model that includes treatment and parity and interaction of treatment with parity.

We anticipate that non-pregnant cows will have normal timing of CL regression (Day 19-21), no elevation in ISGs or PGEM, and an elevation in PGFM on days 19-21, as the CL regresses due to uterine PGF pulses. In pregnant cows, we anticipate elevated ISGs, elevated PGEM, and reduced PGFM from Days 19-21 and a subsequent elevation in PAGs. We anticipate that contralateral CL regression will occur in 2 distinct periods, as observed in our previous results from our project.

Experimental Approach 2: Determine potential for altered pregnancy outcome dependent on CL location. The hypothesis to be tested is that production of accessory CL will increase fertility if they are ipsilateral to the pregnancy but that they may reduce fertility if the accessory CL is contralateral to the pregnancy and regresses during the period of maternal recognition of pregnancy (Day 16-25). A total of 2,400 Holstein dairy cows on 4 commercial dairy farms (4 experiment stations) will be used in this study. Cows will be synchronized for first AI using the

Double-Ovsynch protocol as described above. Cows will be blocked by parity and randomized to receive GnRH (200 ug) or saline on Day 5 after AI. Cows will be randomized in a 2:1 amount since half of cows ovulating to GnRH would have the new CL ipsilateral to the pregnancy and half of the CL would be contralateral to the pregnancy. Cows will have ultrasound on d of AI (Day 0) and 5 d later to assure ovulation to the Double Ovsynch protocol. In addition, ultrasound will be done again on Day 12 after AI to determine ovulation to the GnRH treatment and location of accessory CL (ipsilateral vs. contralateral). In a subset of cows (n = 400), blood samples will be taken on Day 12 (P4), 19 (P4 and interferon-stimulated genes [ISGs] in white blood cells for determination of very early pregnancy), and 26 (P4 and Pregnancy-associated glycoproteins [PAGs] for early pregnancy determination). Ultrasound will also be performed on Days 19, 26, 32, 46, and 60 to determine timing of accessory CL regression and pregnancy.

Continuous values (hormone concentrations, follicular/CL sizes) will be analyzed by the MIXED procedure (SAS, version 8.2, 2001) using a model that includes treatment, parity, farm, and interaction of treatment with parity. Categorical data (pregnancy per AI at each pregnancy diagnosis) will be evaluated by logistic regression using the LOGISTIC procedure (SAS, 2001) with a model that includes treatment, parity, farm, and interaction of treatment with parity.

We anticipate that ipsilateral and contralateral accessory CL will increase fertility in primiparous cows because these cows will not regress the contralateral or ipsilateral accessory CL during early pregnancy (Day 16-25). In multiparous cows we anticipate a more complex response. We anticipate that ipsilateral accessory CL will not regress during early pregnancy and will increase fertility. However, we hypothesize that contralateral CL in multiparous cows will reduce fertility due to early regression of the contralateral CL (Day 19-26) and this will cause regression of the primary CL and early loss of pregnancy. Thus these cows will have elevated ISGs at Day 19 but early pregnancy loss will be higher in multiparous cows with accessory CL that are contralateral to the pregnancy compared to ipsilateral to the pregnancy.

Experimental Approach 3: Determine contribution of uterine factors to early embryonic development. Production level, disease and environmental conditions can create suboptimal uterine conditions that compromises early embryo development. Much of the early embryonic pregnancy failures may be attributed to a poor uterine environment. Research will be performed to identify specific uterine-derived growth factors and cytokines that mediate embryo development. These factors may serve as markers for fertility and may also be used as supplements for *in vitro*-produced embryos. *In vitro* produced embryos are less able to sustain a pregnancy to term than *in vivo*-generated embryos, and we hypothesize this to be due to lack of proper cues from uterine growth factors. Supplementing these factors to *in vitro*-produced embryos may, therefore, improve embryo competency.

A complete evaluation of the uterine fluid (UF) will be performed in synchronized cows that will be sampled on Day 0 (at the time of AI, estrogen influence), Day 10 (blastocyst in uterus, P4 influence), Day 14 (interferon tau secretion, presumptive period of maternal recognition of pregnancy), and Day 18 (peak interferon tau secretion, preimplantation period). Cows will be synchronized together and randomly assigned to two groups (bred or non-bred at Day 0). Embryo survival and mortality will be assessed using values of pregnancy specific protein B (PSPB) and flushing embryos during UF collection. Comparisons will be made both in series and between the bred and non-bred groups. The sample analysis will use both gas chromatography/time-of flight mass spectrometry (GC-TOF MS) and charged surface hybrid-quadrupole/time-of-flight mass spectrometry (CSH-QTOF MS) for analysis of metabolites.

Experimental Approach 4: Investigate the immune system contribution to pregnancy failure. It is clear that the early embryo alters the proportions and function of uterine resident immune cells. In other species these changes have been shown to be essential for establishment of pregnancy and to support development of an optimal placenta. Relatively little is known about the changes in uterine immune cells and immune mediating molecules during early pregnancy [76, 77]. We will test the hypothesis that a portion of the nearly 50% reduction in conception rates between heifers and mature lactating dairy cattle is due to immune dysregulation at the fetal-maternal interface. We will define the changes in immune cell proportions and expression of immune mediators during early pregnancy and compare these changes between fertile heifers and sub-fertile lactating dairy cows.

Experimental Approach 5: Delineate how embryonic cell lineages segregate during early development. The formation of definitive epiblast (i.e. embryo/fetal), trophoblast (i.e. placental) and endoderm (i.e. yolk sac) lineages by day 8 after fertilization is crucial for embryo survival. We aim to establish the molecular systems that control the emergence of these lineages and to identify the paracrine/autocrine factors that mediate the relative proportion of each cell type in embryos.

Outcomes of interest: We anticipate that these studies will provide substantial insight into mechanisms associated with maintenance or loss of pregnancy and regression or rescue of the CL during the first (Days 16-25) and second (Days 26-60) pivotal periods of pregnancy. In addition, we will understand the key changes in UF and uterine immune cells that are associated with early pregnancy and improved fertility in cattle.

Objective 3: Identify Changes in Genetics and Reproductive Management that Lead to Improved Pregnancy Rates in Ruminants

Objective 3A. Evaluate genetic and genomic methods for improving reproduction in dairy cattle –(NY, PA, VA, WI)

Specific Background and Rationale: With the development of powerful molecular techniques to identify key genes involved in reproduction and genomic approaches that allow precise selection of high fertility genotypes, it will be possible during the next 5 years of this project to understand and select for higher fertility in dairy cattle.

Experimental Approach 1: Investigate associated between fertility outcomes and SNP in candidate genes. We will test the hypothesis that high *vs* low fertility in dairy cows is associated with individual or combined SNP's in genes linked to reproductive activity. At the 2014 annual meeting of the NE-1227 Multistate project committee, a collaborative project was discussed and initiated at multiple stations. Blood samples were obtained for DNA genotyping and pregnancy/fertility phenotype information collected from ~1000 lactating dairy cows. Thus far, 490 samples and fertility information from 4 stations have been collected. DNA will be extracted from blood to genotype each cow for these candidate genes: GHR, TNFα, and IGF-1. Data analyses will be conducted to determine the effects of genotypes (GHR, etc.) on pregnancy rates to 1st AI and through 210 days of lactation.

Experimental Approach 2: Determine association between genomic evaluation and reproductive phenotypes. We hypothesize that there are specific genomic associations that contribute to reproductive success in cattle. A genomic approach will be utilized to evaluate specific physiological traits of dairy cattle that we evaluate in the previous objective. Thus, the

2,400 cows that will be evaluated in that objective will also complete genomic evaluations performed. Since all cows will be bred using the same reproductive management protocol we expect to determine SNPs that are associated with specific traits, such as circulating P4 concentrations.

Objective 3B. Development of reproductive management protocols (ART) – **(**NY, PA, VA, VT, WI)

Specific Background and Rationale: Our overall objective is to develop novel reproductive management strategies that maximize dairy cattle reproductive performance and farm profitability through improved P/AI and a reduction of the interbreeding interval. Fertility will be improved by developing novel synchronization of ovulation strategies that optimize synchrony of ovulation and the hormonal milieu before and after AI. The interbreeding interval will be reduced by integrating novel methods to identify non-pregnant cows after a previous insemination with synchronization of ovulation protocols.

Our *working hypotheses* are that 1) optimizing the response to synchronization of ovulation protocols through improved ovulatory response to GnRH and luteal regression to PGF will increase pregnancy per AI after TAI services, 2) reproductive management programs designed to optimize the response to synchronization of ovulation protocols while reducing the interbreeding interval for second and greater AI services will improve herd reproductive performance, and 3) development and implementation of early pregnancy diagnosis technologies will further reduce the interbreeding interval and improve reproductive performance.

Experimental Approaches: The experimental stations involved (NY, PA, VA, VT, WI) will conduct a series of experiments in University herds or commercial dairy farms to develop and test reproductive management protocols including synchronization of estrus and/or ovulation and methods for early identification of non-pregnant cows. We will use the latest advancements in the understanding of estrous cycle physiology and the biology of pregnancy to develop protocols for submission of cows for first service postpartum and protocols for second and greater AI services.

First service postpartum. Optimizing the response to GnRH through presynchronization of the estrous cycle [96, 97, 261], improving luteal regression through sequential PGF treatments [100, 105, 262], and improved hormonal milieu during and after synchronization through P4 supplementation [263-265] can dramatically improve fertility of TAI services. In a series of experiments conducted in commercial dairy farms, cows will receive first service TAI after synchronization of ovulation with protocols that vary in the type of hormonal treatments (i.e., GnRH, PGF2α, intravaginal P4 releasing devices, FSH, LH, and hCG), interval between treatments, and hormone dosage. We will use transrectal ultrasonography to evaluate ovulation and follicular wave dynamics after GnRH treatments. Hormone profiles (e.g., P4, estradiol, LH, FSH) will be used to confirm ovulation, determine luteal regression, and to characterize the hormonal milieu before, during, and after synchronization of ovulation protocols.

Second and greater AI services: We will develop reproductive management strategies that integrate detection of estrus and synchronization of ovulation protocols customized to the ovarian physiological status of cows. These strategies will improve response to the protocol and fertility after TAI while reducing the interval between inseminations. Specifically, we will use transrectal ultrasonography or hormone concentrations in blood or milk to assign cows to synchronization protocols based on the type and status of ovarian structures present on the

ovaries (e.g., functional or non-functional CL and presence or absence of follicles with ovulatory capacity). The synchronization treatment will be developed following the same premises as for first service.

Pregnancy testing: Early detection of failed inseminations is key to improving reproductive performance. Elevated concentrations of interferon stimulated genes (e.g., ISG15 and MX2) and PAG are predictive of pregnancy in cattle. These technologies will be coupled with synchronization of ovulation protocols for second and greater AI services to identify open cows earlier and reduced the interservice interval. Also, circulating ISG expression levels and PAG concentrations will be assessed to inform the project on the timing of embryonic and fetal pregnancy losses in cattle.

Outcomes of interest: In our studies, we will monitor reproductive performance through determination of fertility after AI services (P/AI), time to pregnancy during lactation (survival analysis), and pregnancy rates (21d-preganncy rate and total pregnancy up to the end of lactation). Accuracy (sensitivity, specificity, positive predictive value, and negative predictive value) of pregnancy testing methods developed based on interferon stimulated genes and PAG will be determined using validated pregnancy testing methods as the gold standard. Herd profitability will be determined by evaluating the economic value of pregnancy at different stages of lactation (profitability per cow per lactation and per year) and the effect of reproductive performance on herd replacements dynamics (cost of replacing non-pregnant cows) and reproductive program costs (synchronization, estrus detection, insemination, and pregnancy testing costs).

Anticipated results: We anticipate an overall improvement in herd reproductive performance and profitability by improving P/AI to all AI services, reducing the interbreeding interval, and reducing reproductive program costs. Collectively, these changes will reduce time to pregnancy during lactation as well as the variation in time to pregnancy allowing dairy farms to select the time of lactation at which most of their cows will become pregnant. Given the central role of reproductive performance on the economic viability of dairy farms, optimizing timing of pregnancy in dairy cows will ensure farm profitability and sustainability.