EVALUATION AND DEVELOPMENT OF EARLY PREGNANCY
DIAGNOSIS IN DAIRY CATTLE

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of Requirements for the Degree of
Master of Science

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DIAGNOSIS IN DAIRY CATTLE

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<thead>
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<th>Full Form</th>
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<tbody>
<tr>
<td>ACY</td>
<td>Accuracy</td>
</tr>
<tr>
<td>CL</td>
<td>Corpus Luteum</td>
</tr>
<tr>
<td>COX2</td>
<td>Prostaglandin Synthase 2</td>
</tr>
<tr>
<td>cPKA</td>
<td>constitutive Protein Kinase A</td>
</tr>
<tr>
<td>DIM</td>
<td>Days in Milk</td>
</tr>
<tr>
<td>EP2</td>
<td>Prostaglandin E$_2$ Receptor 2</td>
</tr>
<tr>
<td>EP4</td>
<td>Prostaglandin E$_2$ Receptor 4</td>
</tr>
<tr>
<td>ESR</td>
<td>Estrogen Receptor</td>
</tr>
<tr>
<td>FP</td>
<td>Prostaglandin F$_{2\alpha}$ Receptor</td>
</tr>
<tr>
<td>FSH</td>
<td>Follicle Stimulating Hormone</td>
</tr>
<tr>
<td>FSHR</td>
<td>Follicle Stimulating Hormone Receptor</td>
</tr>
<tr>
<td>GAS</td>
<td>Gamma interferon activation site</td>
</tr>
<tr>
<td>GnRH</td>
<td>Gonadotropin Releasing Hormone</td>
</tr>
<tr>
<td>hCG</td>
<td>Human Chorionic Gonadotropin</td>
</tr>
<tr>
<td>HDL</td>
<td>High Density Lipoprotein</td>
</tr>
<tr>
<td>IFNG</td>
<td>Interferon Gamma</td>
</tr>
<tr>
<td>IFN</td>
<td>Type 1 Interferons</td>
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<tr>
<td>IFNT</td>
<td>Interferon Tau</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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</tr>
<tr>
<td>IL1β</td>
<td>Interleukin 1 beta</td>
</tr>
<tr>
<td>IL8</td>
<td>Interleukin 8</td>
</tr>
<tr>
<td>IMM</td>
<td>Inner Mitochondrial Membrane</td>
</tr>
<tr>
<td>IRF2</td>
<td>Interferon Regulatory Factor 2</td>
</tr>
<tr>
<td>IRF9</td>
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<tr>
<td>ISG15</td>
<td>Interferon-stimulated Gene 15</td>
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<td>ISGF3</td>
<td>Interferon-stimulated Gene Factor 3</td>
</tr>
<tr>
<td>LDL</td>
<td>Low Density Lipoprotein</td>
</tr>
<tr>
<td>LE</td>
<td>Luminal Epithelial Cells</td>
</tr>
<tr>
<td>LH</td>
<td>Luteinizing Hormone</td>
</tr>
<tr>
<td>LHR</td>
<td>Luteinizing Hormone Receptor</td>
</tr>
<tr>
<td>LLC</td>
<td>Large Luteal Cell</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>MnSOD</td>
<td>Manganese Superoxide Dismutase</td>
</tr>
<tr>
<td>MRP</td>
<td>Maternal Recognition of Pregnancy</td>
</tr>
<tr>
<td>Mx1</td>
<td>Myxovirus Resistance 1 Gene</td>
</tr>
<tr>
<td>Mx2</td>
<td>Myxovirus Resistance 2 Gene</td>
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<tr>
<td>NPV</td>
<td>Negative Predictive Value</td>
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<tr>
<td>OAS1</td>
<td>2′,5′-Oligoadenylate Synthetase 1 gene</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>OD</td>
<td>Optical Density</td>
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<tr>
<td>OXTR</td>
<td>Oxytocin Receptor</td>
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<tr>
<td>P4</td>
<td>P4</td>
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<tr>
<td>PAG</td>
<td>Pregnancy associated glycoproteins</td>
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<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
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<tr>
<td>PG</td>
<td>Prostaglandins</td>
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<tr>
<td>PGE₂</td>
<td>Prostaglandin E₂</td>
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<tr>
<td>PGF₂α</td>
<td>Prostaglandin F₂α</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
</tr>
<tr>
<td>PMN</td>
<td>Polymorphonuclear Leukocytes</td>
</tr>
<tr>
<td>PPV</td>
<td>Positive Predictive Value</td>
</tr>
<tr>
<td>PR</td>
<td>P4 Receptor</td>
</tr>
<tr>
<td>PTGS₂</td>
<td>Prostaglandin Synthase 2</td>
</tr>
<tr>
<td>RIA</td>
<td>Radioimmunoassay</td>
</tr>
<tr>
<td>ROC</td>
<td>Receiver Operator Characteristic</td>
</tr>
<tr>
<td>SLC</td>
<td>Small Luteal Cell</td>
</tr>
<tr>
<td>Sn</td>
<td>Sensitivity</td>
</tr>
<tr>
<td>Sp</td>
<td>Specificity</td>
</tr>
<tr>
<td>STAR</td>
<td>Steroidogenic Acute Regulatory Protein</td>
</tr>
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TNF-α  Tumor Necrosis Factor alpha
ABSTRACT OF THESIS

EVALUATION AND DEVELOPMENT OF EARLY PREGNANCY DIAGNOSIS
IN DAIRY CATTLE

Early chemical pregnancy tests for use in dairy cattle are necessary to aid in reducing the costs associated with days the animal is not pregnant. Identifying non-pregnant dairy cattle sooner would allow for efficient treatments and improve decision making for culling cows from the herd. A new rapid visual pregnancy associated glycoproteins test 25 days after insemination had equal sensitivity and accuracy to existing tests. With respect to on-farm application, the slightly lower specificity reinforces the need for a second pregnancy diagnosis (either chemical test or alternative method) after the period of embryonic loss has subsided. Measurement of interferon stimulated gene 15 in milk and blood cells was successful to detect non-pregnant cows but was not robust to serve as an imperfect early pregnancy biomarker. ISG15 expression in both blood and milk somatic cells was greater for pregnant compared with non-pregnant Holstein cows. The reason for increased ISG15 expression and variability among cows is still not understood. ISG15 expression in peripheral blood leukocytes was greater for pregnant compared with non-pregnant Holstein cows. ISG15 increased in the circulation during early pregnancy and responded to a minimal dose of 10 pg/ml when blood was tested in vitro. The in vivo response to pregnancy and in vitro responses to a known dose of IFNT, however, were not correlated. Individual cow differences for ISG15 were not explained by the sensitivity of PBL to IFNT. We failed to confirm a strong correlation between day 18 ISG15 expression and day 25 PAG concentration in pregnant dairy cows and heifers. Further investigation of novel biomarkers to develop a robust chemical pregnancy test in dairy cows 18 days after insemination is still needed.
CHAPTER 1
LITERATURE REVIEW

1.1. Introduction

Pregnancy is required for initiation of milk production which is the main income for dairy farms. Traditional methods of pregnancy diagnosis that include palpation, ultrasound (Fricke, 2002; Caraviello et al., 2006), or lab-based blood tests (Fosgate et al., 2017; Green et al., 2005) are used approximately 4 weeks after insemination and 2 weeks after the cow recognizes the embryo. The time that a cow remains not pregnant costs $3 to $5 per cow per day on average for U.S. dairy farms (De Vries, 2011; French and Nebel, 2003). Performing pregnancy diagnosis sooner after insemination could decrease days from calving to next pregnancy (days open) in dairy cows if non-pregnant cows are treated so that they come back into estrus and are re-inseminated (Green et al., 2011; Thompson et al., 2010). Thus, developing chemical pregnancy tests in blood or milk that can be performed on farm would aid in finding non-pregnant cows or embryonic loss (Pohler et al., 2016) sooner, beginning re-insemination quicker, and reducing costs associated with increased days open.

1.2. Bovine Estrous Cycle

Estrus is the period of sexual receptivity, when there is a peak in estrogen, and the luteinizing hormone (LH) surge before ovulation (Senger, 2005). The estrous cycle in cattle is composed of 2 to 3 follicular waves consisting of the recruitment, selection, dominance, and either atresia of non-dominant follicles or ovulation of the dominant
folllicle (Lucy, 2007). Each follicular wave is preceded by an increase in the gonadotrophin follicle stimulating hormone (FSH). This rise in FSH initiates recruitment of follicles, which contain oocytes (egg or female gamete). The dominant follicle is selected from the recruitment pool (Lucy, 2007). Changes in the luteinizing hormone receptor (LHR) and FSH receptor (FSHR) lead to the selection of the dominant follicle. As estradiol increases, gonadotropin releasing hormone (GnRH) and LH concentrations are decreased, and the dominant follicle develops (Lucy, 2007; Ginther et al., 1996). The dominant follicle develops while FSH declines.

Aromatase is necessary for estradiol production by the granulosa cells (Badinga et al., 1992). Estradiol is the stimulus for prostaglandin production during luteolysis. Estradiol is synthesized from androgens synthesized by theca cells (McNatty et al., 1984). Estradiol can then circulate in the blood to the brain (increasing estrous behavior) and reproductive tract (increasing blood flow, leukocyte secretion, smooth muscle motility for oocyte movement, myometrial tone and growth of uterine glands). Estradiol also causes a positive feedback loop with the surge center of the hypothalamus which leads to induction of the GnRH surge that triggers the preovulatory gonadotropin (LH) surge.

The LH surge causes resumption of meiosis, ovulation and eventually luteinization. Luteinization of the thecal and granulosa cells in the preovulatory follicle, ovulation and the formation of the corpus luteum (CL) lead to increased progesterone (P4) concentrations, synthesized by luteal cells, changing the pattern of LH secretion from low amplitude-high frequency to high amplitude-low frequency pulses of LH (Foster et al., 1975). As the dominant follicle develops follicular estradiol increases leading to induction of P4 receptors (PR) in the endometrium during the early luteal phase of the estrous cycle.
P4 then binds to its receptors inhibiting expression of estradiol receptors (ESR) in the uterine epithelial cells. Regression of the CL, luteolysis, due to increased levels of prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$) and decreased levels of P4, occurs when an antiluteolytic signal is not present to maintain P4.

Knowledge of follicular wave patterns can be advantageous to controlling reproductive events in cattle using timed artificial insemination (Pursley et al., 1995; Thatcher et al., 1989), resynchronization (Fricke et al., 2003), and superovulation (Hasler et al., 1992) for embryo transfer. Since follicular waves occur continuously they can be targeted with P4 (Bisinotto et al., 2010; Savio et al., 1993) or estradiol (Cerri et al., 2004) to induce follicular maturation of premature follicles or ovulation with an LH surge, respectively. Cystic follicles can be treated with P4 or GnRH or human chorionic gonadotropin (hCG). A series of GnRH (Thatcher et al., 1993) and PGF$_{2\alpha}$ (Pursley et al., 1995) injections can be given to cattle to synchronize follicular waves for ovulation among a group of cows. Synchronization allows producers to efficiently manage reproduction in their herds (Caraveillo et al., 2006).

Expression of estrus does not occur in all animals (Roelofs et al., 2005a; Lopez et al., 2004). Ovulation occurs approximately 31 ± 8 h after the onset of estrus. Dairy cows are polyestrous animals, meaning estrous cycles are uniform and regular throughout the year (Senger, 2005). Several factors can affect resumption of the estrous cycle or length of follicular or luteal phases within the estrous cycle (Lucy, 1999; Ouweltjes et al, 1996). Anestrus occurs in 33% of cows (Peter et al., 2009; Wiltbank et al., 2006; Hall, 1959). Thus, continuous monitoring of estrous cycles for individual cows is necessary for predicting optimal insemination time relative to ovulation time (Roelofs et al., 2005). Silent
ovulation is one of the most common reproductive dysfunctions in high yielding dairy cows and occurs more frequently in the first 60 days in milk (DIM) (Ranasinghe et al., 2010). Silent ovulations can easily affect dairy cow reproductive performance and decrease estrus detection rates (Roelofs et al., 2005).

1.3. Prostaglandin (Luteolytic) Action

Prostaglandins are lipid molecules that play a major role in reproduction. Prostaglandin F$_{2\alpha}$, from the uterus leads to the luteolysis (McCracken et al., 1973). This is important because the CL produces P4 and maintains pregnancy. Mechanisms leading to uterine PGF$_{2\alpha}$ pulsatility involve PR, ESR (Spencer and Bazer, 1995), and oxytocin receptors (OXTR; Wathes and Lamming, 1995). Increased binding of P4 in circulation to PR causes a downregulation of PR in the uterine epithelial cells. Downregulation of PR leads to increased expression of ESR (Geisert et al., 1992). Oxytocin receptor expression in the endometrium is then induced by follicular estradiol binding to endometrial (luminal epithelial cells) ESR.

Steroidogenesis of P4 is activated by a lipoprotein, high-density lipoprotein (HDL) or low-density lipoprotein (LDL), binding to its receptors on large luteal cells (LLC; Senger et al., 2005). Constitutive protein kinase A (cPKA) during this early period phosphorylates steroidogenic acute regulatory protein (STAR; rate limiting step in P4 synthesis) to transport cholesterol (product of lipoprotein breakdown) to the inner mitochondrial membrane (IMM). Once cholesterol is in the IMM, P450 side chain cleavage enzyme converts cholesterol to pregnenolone in the mitochondria that is then
transported to the smooth endoplasmic reticulum where 3β-Hydroxysteroid dehydrogenase converts pregnenolone in the LLC to P4.

The increase in circulating oxytocin binding to OXTR continues the demise of the CL (day 14-16; Niswender, 2002) as this induces a pulse of uterine PGF$_{2α}$. Uterine PGF$_{2α}$ then binds to its receptors (FP) on LLC which does 3 things: 1) stimulates prostaglandin synthase 2 (PTGS2 or COX2) to convert arachidonic acid in the LLC to luteal PGF$_{2α}$, 2) activation of protein kinase C (PKC) to inhibit P4 synthesis from lipoproteins, and 3) induce release of luteal oxytocin from the LLC that will bind to the OXTR on the small luteal cells (SLC). This activation of the OXTR will stimulate PKC to inhibit P4 synthesis resulting in functional luteolysis (inability to synthesize P4) in all luteal cells. At approximately day 17 of the bovine estrous cycle, luteal PGF$_{2α}$ binds to FP receptor on the LLC stimulating synthesis of more luteal PGF$_{2α}$, stimulation of oxytocin secretion from the LLC to bind to the OXTR on the SLC, and PKC to initiate an influx in Ca$^{2+}$ (Burns et al., 1998) leading to apoptosis of the LLC and SLC as part of structural luteolysis and regression of the CL.

These series of events increase the effect of PGF$_{2α}$ on luteolysis in changing the luteal vasculature in ruminants. Blood flow in the vessels leading to the CL, a highly vascularized structure, rapidly decreases during luteolysis because PGF$_{2α}$ is a vasoconstrictor and microvascular endothelial cells inside the CL have PGF$_{2α}$ receptors. A decrease in luteal blood supply because of vasoconstriction initiates apoptotic mechanisms (structural luteolysis; Niswender, 2002). Apoptosis is initiated by caspases, intracellular proteases, which are activated by binding of ligands to tumor necrosis factor (TNF) family receptors on the luteal cell membrane.
Immune cells (macrophages and T lymphocytes) also play a major role in luteolysis because they have major histocompatibility complex (MHC; Fairchild and Pate, 1989) molecules on their surface and are present in the CL prior to luteolysis (day 14) in cattle. Due to the MHC molecules, clonal expansion of immune cells occurs, stimulated by PGF$_{2\alpha}$, releasing cytokines such as tumor necrosis factor alpha (TNF-α; Benyo and Pate, 1992) and interferon gamma (IFNG). Tumor necrosis factor-α causes P4 to decrease because TNF-α decreases StAR, LHR, and manganese superoxide dismutase (MnSOD) mRNA. The apoptosis mechanism intracellularly is furthered by the decline in MnSOD that allows reactive oxygen species produced from activated lymphocytes. MnSOD aids in degrading luteal cells if exposed to interleukin 1 beta (IL1β; Townson and Pate, 1994), an important cytokine that mediates responses to inflammation – cell proliferation differentiation, and apoptosis.

1.4. Maintenance of Pregnancy (Luteotrophic Action) in Domestic Species

Maternal recognition of pregnancy (MRP) is the release of molecules from the conceptus to signal extension of the CL lifespan for production of P4 (luteotrophic action), necessary for maintenance of pregnancy (Short, 1979; Rowson & Moor, 1967). The mechanisms of MRP differ by species and typically involve antiluteolytic hormones that prevent uterine prostaglandin synthesis. The MRP for rodents, humans, horses, pigs, and sheep have been studied extensively since the mid-20th century (Moor, 1968).

The MRP for rodents, mice and rats, is placental lactogen produced by the placenta after day 12 of pregnancy (Ben-Jonathan et al., 2008) and surges of prolactin produced by the anterior pituitary after mating stimulation by penile spines. The surges of prolactin
cause LH/choriogonadotropin receptor to increase on luteal cells for formation of a CL and suppression of aldo-keto reductase family 1, member 1 activity in the CL. Prolactin surges are abruptly stopped by placental lactogen which also affects the ESR and PR on the placenta necessary for conceptus development and maintenance. Both hormones maintain P4 secretion necessary for pregnancy.

The equine MRP is not known (Bazer, 1992). The antiluteolytic hormone is PGF$_{2\alpha}$ and the conceptus may inhibit endometrial PGF$_{2\alpha}$ around day 14 to 16 after insemination (Sharp et al., 1989). The production of uterine fluid and venous PGF$_{2\alpha}$ in mares is reduced in the presence of a conceptus due to absence of endometrial OXTR (Flemming et al., 2006; Goff et al., 1987). Estrogen and equine chorionic gonadotropin are produced during pregnancy in mares but are not the MRP signals. Instead the CL is protected on days 12 to 18 after insemination following frequent migration of the conceptus between uterine horns. Migration of the conceptus in mares is believed to be the antiluteolytic stimulus (Ginther et al., 1984).

The porcine MRP, estradiol, served as a model for ruminant MRP. However, besides estradiol presence, maternal recognition requires the positioning of at least two conceptuses in each uterine horn prior to day 12. Estradiol synthesis increases as the blastocyst begins rapid elongation. The ESR are not present in the uterus prior to day 12 of pregnancy in pigs. Estrogen is necessary for implantation of the conceptuses as ESR increase and PR decline in the uterine epithelium to block luteolysis of the CL by prostaglandins (PG). The endocrine exocrine theory (Bazer and Thatcher, 1977) exists in pigs due to the switch from endocrine PGF$_{2\alpha}$ secretions from the uterine myometrium during the estrous cycle to exocrine PGF$_{2\alpha}$ secretions from the uterine lumen during
pregnancy 10 to 12 d after insemination (Gross et al., 1988). Estrogens stimulate a release of uterine calcium which we know is associated with the reversed switch to exocrine secretions of PGF$_{2\alpha}$ days 11 to 13 and 15 to 30 after insemination. The relation of this switch to prolactin receptors for continued pregnancy maintenance is not clear.

Interferon tau (IFNT) from the conceptus is the MRP in sheep as well as cattle (Godkin et al., 1984; Martal et al., 1979). Ruminants have similar MRP mechanisms but MRP in sheep occurs on day 12 to 13, 2-3 days sooner than cattle (Spencer et al., 2007). On day 12 of pregnancy, the conceptus releases IFNT which binds to the interferon receptor on the luminal epithelium and the superficial glandular epithelium. This binding of IFNT activates interferon regulatory factor 2 (IRF2; Choi et al., 2001) that blocks ESR thus blocking OXTR that would normally bind to oxytocin for pulses of prostaglandin to regress the CL. These mechanisms extend the luteal lifespan and are hypothesized to stimulate other factors of uterine receptivity and antiviral properties (Ott et al., 1991).

1.5. Bovine Early Pregnancy

The CL forms shortly after ovulation in most mammals and synthesizes P4, a steroid hormone, necessary for pregnancy maintenance. In non-pregnant ruminants, PGF$_{2\alpha}$ is released from the uterus to cause regression of the CL so that the animal will return to estrus. In pregnant cattle the zygote develops into a blastocyst. The blastocyst is further remodeled by rapid cleavage divisions in ruminants (Betteridge and Fléchon, 1988; further reviewed by Peippo et al., 2011). The hatched ruminant blastocyst is made up of two distinct cell populations at day 8 of pregnancy (Winters et al., 1942), the inner cell mass and the trophectoderm. The inner cell mass will eventually become the embryo proper.
Ruminant extraembryonic membranes form following remodeling of the trophectoderm to the amnion and chorion eventually exposed to the endometrial epithelium. Elongation of the embryo and extraembryonic membranes occurs between day 14 and 18 after insemination (Betteridge et al., 1980). The bovine conceptus secretes IFNT (Bartol et al., 1985; Godkin et al., 1984) during this period. The IFNT is reduced 23 to 26 days of pregnancy (Ealy et al., 2001; Bartol et al., 1985). The ruminant placenta is synepitheliochorial (reviewed by Roberts et al., 2016) which involves exposure of the endometrial epithelium to the chorionic villi epithelium. This placenta type allows for antiluteolytic molecules, specifically, IFNT to signal the maternal side for further development of the embryo with P4 from the CL and interferon stimulated genes and proteins in an endocrine and paracrine matter (Hansen et al., 2017). Interferon stimulated genes are detectable with the purpose of improving maternal uterine receptivity (Forde et al., 2011).

During bovine embryo implantation there is a larger conceptus surface area (from 0.196 mm to 5.58 mm) in contact with endometrial epithelium. Implantation ends with the bovine embryo by day 27 of pregnancy (Leiser, 1975). The allantois is derived from the primary gut of the embryo during this same period. Fusion of the allantois and chorion creates the allantochorion, which will become the fetal side of the ruminant placenta. Ruminants form a syndesmochorial or cotyledonary placenta with a fetal (cotyledonary) and maternal (caruncular) fusion (known as placentomes) for nutrient, gaseous, and signal exchange. Continued growth of the embryo occurs as the first fetal cotyledons are observed at day 30 of pregnancy (Winters et al., 1942). As early as day 22 of pregnancy, pregnancy associated glycoproteins (PAGs) are detected in the bovine and similar timing in other
ruminants (Wallace et al., 2015). The following sections will discuss the endocrine and paracrine actions of IFNT, ISGs, and PAGs as necessary molecules for embryo survival.

1.5.1. Interferon Tau: The Ruminant MRP

Most of the work in trying to understand the mechanisms involved in ruminant luteolysis has been published in sheep since the early 1960’s and 1970’s (Rowson and Moor, 1967). The effect of IFNT directly on endometrial luminal epithelial cells (LE) and indirectly on endometrial stromal cells (ST), the uterine ovarian plexus (UOP) and LLC for prevention of luteolysis is still under study (Hansen et al., 2017 and Bazer and Thatcher, 2017). Interferon tau is a type 1 interferon (IFN) secreted from the ruminant (cattle and sheep) mononuclear trophectoderm cells of the elongating conceptus between 11 and 23 days of pregnancy. Key proteins, cells, and actions of type 1 interferons will be described.

Type 1 interferons are cytokines found in all nucleated cell types in most mammalian species that trigger antiviral immune responses to prevent replication of the virus in the host, including IFNα, IFNβ, IFNω, IFNκ, IFNδ, and limitin. The two major actions of interferons are 1) activation of specific and adaptive leukocytes, known as lymphocytes; and 2) increasing expression of MHC antigens. Type 1 IFN are secreted by lymphocytes present in circulation and tissues with or without a viral infection.

Lymphocytes include B lymphocytes that participate in the humoral immune response, T lymphocytes that are cell mediated further divided into T-helper (T_h) lymphocytes that bind to the MHC-II complex on the B lymphocytes and T-cytotoxic (T_c) lymphocytes that attack cells that have been infiltrated by virus and attracted by MHC-I. The MHC-1 is expressed in endometrial epithelial cells on days 10 and 12 of the estrous
cycle and pregnancy in sheep and after 20 days of pregnancy is expressed in endometrial stromal and glandular epithelial cells (Choi et al., 2003). MHC-I is an interferon stimulated gene (ISG) that requires regulation to avoid creating toxic conditions (killing of foreign or infected cells of the trophectoderm with Tc) for the embryo (Choi et al., 2001; Choi et al., 2003). Type 1 interferons enhance only MHC-I and not MHC-II as they are restricted to IFNγ and type 3 interferons. MHC-I gene expression is a prime example of why it is important to understand what affects the transcription of ISGs.

Type 1 interferons are proteins that act as ligands to activate cell surface receptor complexes. All type 1 interferons bind to the cell surface receptor interferon α/β receptor (IFNAR) which is expressed in the sheep uterine endometrial epithelial cells (Rosenfeld et al., 2002). The IFNAR has two polypeptide subunits IFNAR1 and IFNAR2. In mice and humans (Schreiber, 2017), IFNAR2 tends to have the highest binding affinity thus the working model of IFNT stimulation of ISG uses the IFNAR2 as the main receptor for the beginning of ISG regulation. Transcription regulation at the level of IFNAR includes the number and binding affinity of the receptors. Cell types differ in IFNAR expression in pig (Jang et al., 2017), sheep (Brooks and Spencer, 2015), and cattle (Wang et al., 2017). If binding affinity and receptor number is low, then the stimulation of the JAK-STAT pathway is reduced ultimately affecting the rate of transcription of ISG. Conformational changes of the receptor occur upon binding to IFN. It is not understood if IFNT binds to structurally similar IFNAR of IFNα and IFNβ. If they IFN bind in the same affinity during pregnancy, then the sole effect of IFNT specifically binding to IFNAR on endometrial epithelial cells is still not understood.
IFNT binds to interferon alpha receptors 1 / 2 (IFNAR1/2) on LE cells activating tyrosine kinase and the janus kinase signal transducer and activator of transcription (JAK-STAT) pathway proteins in ruminants (Binelli et al., 2001). Phosphorylation and dephosphorylation of STAT proteins are no longer the only difference in response to type 1 interferons since most ISG were not expressed in ovine endometrium models in vivo (Choi et al., 2003). Since stroma and glandular cells lack interferon regulatory factor 2 [(IRF2; a gamma-activation sequence (GAS) element)] the interferon regulatory factor 9 (ISGF3 or IRF9) complex is initiated and hyperactivates the ISG proteins. The presence of STAT1 and STAT2 in the stroma and glands allows GAS to be formed to translocate the nucleus and transactivate genes such as IRF1 to stimulate other ISG (Spencer et al., 2008). STAT proteins can be inhibited by suppressor of cytokine signaling (SOCS) proteins 1 & 3 through inhibition of STAT phosphorylation and STAT stimulation of GAS elements and other transcription factors on the promoter region of DNA.

The interferon regulatory factor 2 (IRF2) limits transcription of ISGs to the stroma and glandular epithelium and represses transcription of ISGs in the LE and superficial glandular epithelium. IRF2 blocks transcription of ESR1 thus preventing upregulation of OXTR along with PTGS2 to stimulate pulses of PGF2α (Danet-Desnoyers et al., 1994) that would regress the CL. Epidermal growth receptor 1 is also thought to bind to the LE to inhibit PG transporters suppressing the pulsatile PGF2α from the endometrium to the PGF2α secretions from LLC. The synthesis of PGE2 begins with arachidonic acid in the LE and ST that is then transported by PG transporter through the UOP to LLC where PGE2 binds to its receptors (EP2/EP4). Activation of EP2/EP4 leads to antiapoptotic pathways and protects the CL from structural luteolysis allowing steroidogenesis and survival of luteal
cells. The ultimate hypothesis is that a combination of IFNT and ISGs at varying magnitudes are needed for maternal-conceptus signaling. Specifically, increased transcription of ISGs in the endometrium is necessary for uterine receptivity of the elongating conceptus prior to attachment.

1.5.2. Interferon Stimulated Genes

The general mechanism of IFN stimulation of ISGs on specific cell types begins with the IFN1 binding to the specific cell surface receptor (IFNAR), receptor phosphorylation by Tyrosine kinase 2 and JAK1, STAT binding of phosphorylated receptor, STAT dimer formation and translocation to the nucleus of the specific cell for binding to DNA and changing transcription. IFNT initiates endocrine and paracrine effects on the uterine endometrium and CL. The ISGs, found in the uterus and circulating leukocytes (lymphocytes and neutrophils), prime the uterus to receive the conceptus and enable elongation, implantation, and establishment of pregnancy. The magnitude of the leukocyte response to IFNT, however, depends on a variety of cow-level factors that we do not fully understand.

IFNT is considered to have endocrine actions in sheep when detected in uterine vein serum with a radioimmunoassay (RIA) for IFNT (Romero et al., 2015). ISG15 mRNA is often measured as a marker of IFNT stimulation in the endometrium and peripheral blood mononuclear cells (PBMC). In sheep ISG15 increases by day 13 in the endometrium of pregnant ewes but IFNT was not detectable in uterine vein serum until day 16 post insemination. Further studies need to be completed to look at peripheral IFNT concentration to determine if the PBMCs with ISG stimulation are shed from the
endometrium and released into circulation or if there is a direct action of IFNT in
circulation beyond the uterine vein to the peripheral circulation. Stimulation of ISGs in the
CL has been reported (Romero et al., 2015; Bott et al., 2010). Regarding the induction of
ISGs in the CL, uterine veins have contact with ovarian arteries but the in vivo migration
of polymorphonuclear leukocytes (PMN) to the CL of pregnant animals is not well
understood. Shirasuna et al. (2015) found an increase in PMN migration in vitro when
stimulated with interleukin 8 (IL8) but not IFNT. There is still speculation if IFNT
stimulates IL8 or classical ISG directly in the bovine CL. Interestingly, the number of PMN
in the CL tripled in pregnant animals compared with non-pregnant animals. IFNT and IL8
had a positive association of increased P4 percent compared to the control ($P < 0.05$;
Shirasuna et al., 2015). PMNs aid in angiogenesis (formation of vasculature) in the CL
(Jiemtaweeboon et al., 2011) which is important for luteal development and luteolysis with
transfer of prostaglandins (PGE$_2$ and PGF$_{2\alpha}$, respectively).

A few of the ISGs ((Interferon Stimulated Gene 15, ISG15; Myxovirus resistance
1 and 2, MX1, MX2; and 2',5'-Oligoadenylate Synthetase 1, OAS1) that have been studied
in ovine and bovine models to determine their function during early pregnancy (Bazer et
al., 2006). The ISGs are divided into ISG that are only found in LE or superficial
epithelium, ISG in the stroma and glandular epithelium, and ISG that are found in both the
endometrium and distal tissues or cells. Interferon regulatory factor 2 (IRF2) is responsible
for repressing the expression of certain ISGs beyond the stroma. This leads to us pondering
the function of ISGs distal to the endometrium with respect to ruminant pregnancy. To
answer this question the following review will focus on four IFNT stimulated genes that
are greater than two-fold upregulated (ISG15, Mx1, Mx2, and OAS1) and have been
repeatedly reported in peripheral blood lymphocytes and tissues like the CL distal to the endometrium in ruminants.

A primary speculation of the role ISGs play in early pregnancy is to specify cell differentiation and proliferation in the circulation. Leukocytes may allow shunting of specific nutrients to the conceptus at specific time points during elongation and implantation. The inferred control of ISGs by IFNT has been reached from studies that replace IFNT with other type 1 interferons mainly interferon α (IFNA) (Shirozu et al., 2017; Spencer et al., 2008). Both studies found that ISG expression increased in ovine and bovine endometrial (BEND) cells when IFNA was added. The ISGs change in expression throughout the estrous cycle and pregnancy (Joyce et al., 2005). Fluctuations of IFNA and IFNT may also explain why ISGs including ISG15 and MX2 are not imperfect markers for early pregnancy testing in dairy cattle.

Interferon stimulated gene 15 also known as Ubiquitin cross reacting protein has two potential roles: 1) cross reaction with ubiquitin for rapid degradation in the proteasome or 2) cross reaction with ubiquitin for stabilizing the protein for long term modification. Joyce et al. (2005) reported ISG15 mRNA abundance from day 11 to 120 of pregnancy in sheep. Day 15 of pregnancy was the greatest with over 1600 x 10^3 RU ISG15 mRNA. The ISG15 mRNA declined every 2-3 days after but remained above 150 x 10^3 RU at 120 days of pregnancy. Ubiquitin is a small protein (76 amino acids) that is responsible for preparing proteins it conjugates for degradation (ubiquitination). Understanding ISG15’s cross reaction with ubiquitin may aid in determining its role in early and late pregnancy (Bebington et al., 2001). Binding with ubiquitin by stimulation of type 1 interferons produced by the conceptus indicates a need for increased energy (ATP) supply to the dam.
The possibility still exists for ISG15 to degrade cells that would support a viral response to the conceptus that would explain why cells that secrete IFN (lymphocytes, macrophages, fibroblasts, endothelial cells etc.) secrete more type 1 interferons during preimplantation.

Myxovirus resistance 1 and 2 are antiviral proteins forming an early defense against viruses and type 1 interferons (Toyokawa et al., 2007) found in the CL (Romero et al., 2013) uterus, and peripheral blood leukocytes (PBL) of sheep (Romero et al., 2015) and cattle (Green et al., 2010). The function of Mx proteins in the uterus seems to be trafficking of proteins and regulating uterine secretions of other ISGs due to its similarities with dynamin. However, the function is still unknown outside of the uterus other than the potential for antiviral activity. The potential function of Mx proteins in circulation may be to attract proteins to the blood vessels of the CL to increase lipid concentration for P4 synthesis from cholesterol. The increased abundance of Mx2 mRNA as early as day 14 of pregnancy in primiparous dairy cows PBL may function to prevent viral infection.

2′,5′ ‑Oligoadenylate Synthetase 1 regulates ribonuclease L antiviral responses and unwanted apoptosis of vital cells during implantation (Johnson et al., 2001). Johnson et al. (2001) reported a P4 dependent response of increased expression of OAS1 following an intrauterine infusion of roIFNT in ovine. In relation to other studies in bovine (Schmitt et al., 1993; Short et al., 1991), OAS1 is upregulated after direct IFNT endometrial stimulation. Interestingly, two of the three isoforms of OAS1 are not stimulated by IFNT in LE. This brings up a potential limitation with measuring ISG for detection of pregnancy as researchers have not completely understood which isoforms of the ISGs make it to circulation and how they get there. The work done in ovine CL (Kiyma et al., 2016) brings up the possibility that the CL is a hub for shunting ISGs through the highly vascular CL to
the peripheral bloodstream after an interaction with P4. Distal to the endometrium, OAS1 may serve the same functions as it is inferred to have in the uterus to prevent apoptosis of leukocytes in the circulation.

**1.5.3. Pregnancy Associated Glycoproteins**

Pregnancy associated glycoproteins, also known as pregnancy specific protein B (PSPB) and PSP60, is a gene family with highly conserved sequence in ruminants and other domesticated species with an epitheliochorial placenta including pigs (Hughes et al., 2003). The PAG gene family evolved following duplications and expansion of the gene pepsinogen F (Wallace et al., 2015; Hughes et al., 2003). PAGs were originally localized in secretory granules of binucleated giant trophoblasts derived from mononucleated trophoblasts in cattle (Wooding et al., 2005), other ruminants, and pigs (Mialon et al., 1993; Zoli et al., 1992; Sasser et al., 1989). Cattle, unlike sheep do not form a syncytium from binucleate cells, in which more than 21 and 9 PAGs are primarily detected in cattle and sheep respectively (Green et al., 2000). The bovine PAGs (more than 21) were identified numerically in bovine based on immunolocalization in placental tissue. There are more PAGs than identified loci (Green et al., 2000). Originally, the most commonly studied PAG for pregnancy detection in cattle was bovine PAG1. Like many of the PAGs, bovine PAG1 is not always consistently elevated throughout pregnancy (Green et al., 2009; Green et al., 2000). The three main research groups that worked on immunolocalization of PAGs in binucleate cells of the placenta reached similar conclusions that it remains unclear which PAGs are more prominent at stages from early pregnancy through parturition (Wallace et al., 2015).
The function of PAGs is unclear. Researchers have reported potential immunomodulatory and luteotrophic functions for certain groups of PAGs. However, the abundance of PAGs near parturition is greater than early pregnancy. This lead researchers to think that there is an immunomodulatory role in suppressing immune function at parturition to allow for easier detachment of the placenta. Luteotrophic function was found when PSPB was added to bovine endometrial explants then PGE$_2$ and P4 concentrations measured in spent media were increased (Del Vecchio et al., 1990; Humbolt et al., 1988). When these studies were repeated by other research groups, however, P4 concentrations were similar to control samples for some of the PAGs. PSPB increased P4 concentration early in estrous cycle (day 14 in cattle) but not later (220 days) in pregnancy (Humbolt et al., 1988).

The pathway for PAG stimulation of target cells is still unknown. Immunolocalization demonstrated exocytosis of secretory granules traveling through gap/tight junctions to maternal endometrial stromal cells (Green et al., 2005; Zoli et al., 1992). PMN migration and activation in maternal circulation is possible due to secretory granules, carrying PAGs, being released from binucleated trophoblasts near maternal ST (Zoli et al., 1992). Some of the PAGs are predictive of embryonic loss at 25 to 35 days of pregnancy (Pohler et al., 2016; Pohler et al., 2013). As will be discussed later, on PAGs in certain combinations can predict pregnancy or pregnancy loss as soon as 25 days after insemination (DAI). One of the most important findings since Sasser et al. (1989) is that even with the same sample isolates, the antibody combinations for PAGs can result in different PAG concentrations by different labs. Studies to elucidate functions of specific PAGs at specific points are still ongoing (Wallace et al., 2015).
1.6. Dairy Cattle Early Pregnancy Diagnosis

Early pregnancy diagnosis in dairy cattle is necessary for shortening interbreeding intervals, reducing days open (French and Nebel, 2003) and implementing rebreeding protocols (Lucy et al., 2004). Traditional methods are typically performed by a veterinarian no earlier than 28 days after insemination. Traditional methods of pregnancy diagnosis in dairy cattle include measuring PAGs in milk or blood 25 to 37 DAI (Mayo et al., 2016; LeBlanc, 2013; Green et al., 2005), consecutive measurements of milk P4 manually (Nebel, 1998) or in-line (Friggens and Chagunda, 2005), and manual palpation of the uterus and ultrasonography 26 to 35 DAI (Fricke, 2002). All of these methods are used 2 to 4 weeks after MRP. Pregnancy is signaled to the mother during conceptus elongation through IFNT and ISGs. Chemical pregnancy diagnosis using proteins such as ISGs on day 18 to 22 has not been commercialized in blood (Green et al., 2010; Gifford et al., 2007) or milk (Mo et al., 2014) but has been patented (Ott, 2006). The average early pregnancy loss 20 to 70 days after insemination is 13% (Pohler et al., 2013) increasing the importance of finding non-pregnant cows sooner than current commercial pregnancy tests.

1.6.1. PAG Testing in Blood and Milk

The PAG can be detected in either plasma, serum, or milk (Silva et al., 2007; Lawson et al., 2014) using an ELISA originally developed and patented by Green et al. (2005). The accuracies of commercial PAG tests for plasma, serum, or milk range from 89% to 96% (Ricci et al., 2015; LeBlanc, 2013; Silva et al., 2007). The accuracy of PAG tests makes them suitable alternatives to traditional methods of pregnancy diagnosis (palpation or ultrasound).
The following discussion will use terminology associated with assessing efficacy of tests for diagnosis compared to a reference standard. The sensitivity (Sn) is the \[
\frac{\text{number of cows correctly diagnosed positive with a chemical pregnancy test (CPT)}}{\text{the number of cows with a positive ultrasonographic pregnancy test or other reference standard}} \times 100\%.
\]
The specificity (Sp) is \[
\frac{\text{number of cows correctly diagnosed negative with a CPT}}{\text{number of cows with negative ultrasonographic pregnancy test}} \times 100\%.
\]
The positive predictive value (PPV) is \[
\frac{\text{number of cows correctly diagnosed pregnant with a CPT}}{\text{number of cows correctly diagnosed pregnant with a CPT} + \text{the number of cows incorrectly diagnosed pregnant with a CPT}} \times 100\%.
\]
The negative predictive value (NPV) is \[
\frac{\text{number of cows correctly diagnosed not pregnant with a CPT}}{\text{number of cows correctly diagnosed not pregnant with a CPT} + \text{the number of cows incorrectly diagnosed not pregnant with a CPT}} \times 100\%.
\]
The accuracy (ACY) is \[
\frac{\text{number of correct CPT}}{\text{total number of CPT}} \times 100\%.
\]

The original monoclonal-based PAG ELISA measuring PAG-1 (Green et al., 2005) was developed to reduce the number of false positives (FP; number of animals incorrectly diagnosed pregnant postpartum) due to PAGs long half-life in serum and plasma. The assay was developed using placental tissue from cattle 24, 27, 29, and 34 DAI and cotyledons on 80 and 150 DAI from pregnant cows. Purification methods of PAGs involved pepstatin-A agarose columns. PAGs were separated by 12.5% SDS–PAGE and western blotting was done with antibodies created with rabbits. An indirect sandwich ELISA was validated with five serum samples, assayed five times in quadruplicate to determine inter- and intra-assay variation. Similar to studies in sheep and bovine (Xie et al., 1994) there was high variability in the molecular weights of PAGs. The concentration of PAGs was greater in day 150 pregnant cotyledons compared with days 24 to 34 and day 80 (Green et al., 2005). This
observation of increasing PAG concentration from 15 to 35 days, a decrease after 45 days and a 10-fold increase after 150 days of pregnancy has been repeated in plasma and serum from thousands of pregnant cows (Ricci et al., 2015; Silva et al., 2007; Lopez-Gatius et al., 2007; Zoli et al., 1992).

A false positive result is a limitation of early PAG pregnancy diagnosis. Approximately 10 to 20% of pregnant dairy cows will undergo embryonic loss after wk 4 (Giordano et al., 2013). A cow with embryonic loss during this period will initially test pregnant and later be found not pregnant when the gold standard test is applied (Giordano et al., 2012; Pohler et al., 2016). PAG testing is done approximately 30 d after a negative pregnancy diagnosis in resynchronized cows. There is a high possibility, therefore, that residual PAG from a cow previously experiencing embryonic loss can be carried into the next sampling period and cause a false positive result (Szenci et al., 2003; Giordano et al., 2012). As with all early pregnancy tests based on PAG or ultrasound there is a need to recheck cows for pregnancy after the initial period of embryonic loss (Giordano et al., 2013). The concentration of PAGs can differ in maternal circulation depending on type of embryo transferred (SCNT or control) or compared to artificial insemination (AI; Ricci et al., 2015; Mercandante et al., 2016; 2013) due to the structure of the binucleated trophoblast interface with the uterine epithelium and P4 concentrations.

Prior to recent studies, there were limitations for on farm use of bovine PAG pregnancy tests. Plasma or serum-based tests require the collection of a blood sample and subsequent centrifugation. The centrifugation step requires equipment that is typically not available on-farm. Milk testing does not require centrifugation but the initial assay step requires a thermally-controlled platform shaker for the ELISA plate (LeBlanc, 2013). Shaking is not
required for assays performed with plasma or serum but an incubator is needed. Regardless of whether plasma, serum, or milk is used, there is also a requirement for a microtiter plate reader to measure the OD for individual wells in the ELISA plate (Green et al., 2005). Given the need for specialized equipment, PAG testing is most-likely done in a veterinary clinic or other centrally located laboratory. A final impediment to the on-farm test is the time from sample collection to pregnancy test result. Traditional tests have a series of incubations with antibody and reagent solutions that can require from 2 to 3 h or as long as overnight to complete the test (Stahman et al., 2015; Ricci et al., 2015). In many herds, treatments are administered to non-pregnant cows after pregnancy diagnosis; usually before they are released from animal restraint (headlocks, squeeze chute or palpation rail). The length of time from sampling to test completion is too long if cows are to remain restrained or kept in separate pens. A lesser time from sampling to test result would theoretically improve on-farm utility of a PAG test.

1.6.2. P4 Measurements

Milk P4 (MP4) concentrations in postpartum dairy cows are indicative of ovarian cyclicity (Darwash et al., 1999; Darwash and Lamming, 1998). Most would think that P4 would serve as a pregnancy diagnosis hormone since it is the “hormone of pregnancy maintenance from the CL”. However, Bulman and Lamming (1978) reported mean P4 levels in dairy cows (n = 535; non-pregnant and pregnant) 21 days before until 13 days after first insemination were not different (P > 0.05). Non-pregnant cows at 15 DAI declined from 15 ng/ml to less than 1 ng/ml at 25 DAI. Pregnant cows increased to approximately 22 ng/ml at 25 DAI. Darwash and Lamming performed some of the first
milk P4 RIA studies reporting variability in temporal P4 patterns of pregnant cows 9 days before insemination and between calving to first insemination. Observations of irregular temporal P4 patterns also belong to repeat service cattle (Tenghe et al., 2015; Bulman and Lamming, 1978). Commercial milk P4 tests are available with an 89 to 98.9% accuracy (Nebel et al., 1989) but serve more for determining cyclicity and non-pregnancy instead of pregnancy diagnosis 21 days after insemination.

Within the last decade, automatic or in-line sampling and measurements of MP4 have become more accessible to farmers to predict successful timing of insemination by a decrease in MP4, indicative of estrus. Different models have been tested to determine the efficacy of using consecutive MP4 measurements for detection of estrus (Friggens and Chagunda, 2005; Blavy et al., 2016). Friggens et al. (2008) analyzed robotic MP4 measurements (1 per day) during first 120 d post-partum; 55,036 MP4 measurements from 578 lactations of 380 cows and 121 confirmed pregnancies. There was 93.3% sensitivity and 93.7% specificity for detection of estrus. The greatest proportion of detected estrus was with daily MP4 measurements and decreased to 75% with 2.5 d interval from 1 d between MP4 measurements. These studies also confirmed the variation in threshold of MP4 (below above 1 to 6 ng/ml). Consecutive measurements therefore, are needed to make more accurate predictions of estrus or pregnancy. Measurement of a more robust biomarker is still needed for accurate pregnancy diagnosis before 28 DAI.

The adoption of automated in-line MP4 monitoring on farm has created the need for benchmarks to better understand MP4 data across cows, parities, farms, etc. In a study to establish useful fertility benchmarks for in-line MP4 records. MP4 records (n=135,588; Mayo et al., 2016) from an automated milk P4 sampling system (Herd Navigator; DeLaval
International, Tumba, Sweden) were studied. The mean interval to commencement of luteal activity was 28.7 ± 14.6 d for all farms. More aged cows (28/114, 25%) failed to achieve commencement of luteal activity by 50 d postpartum compared with primiparous cows (13/148, 8.8%; \( P < 0.05 \)). Highest producing cows (Q4) failed to achieve commencement of luteal activity by 40 d postpartum compared with cows producing less than 33kg/day \( (P < 0.001) \). Length of estrous cycle differed \( (P < 0.001) \) for primiparous, mature, and aged cows, 21.3 ± 0.2 d, 22.7 ± 0.2 d, and 23.0 ± 0.5 d, respectively. Results of this study enhanced our curiosity for a better understanding how interactions of cow (age and parity specifically) and embryo factors affect ISG expression during early pregnancy.

1.6.3. ISG Chemical Pregnancy Tests in Blood and Milk

The use of ISGs expression in maternal peripheral blood for pregnancy detection in research settings in sheep (Yankey et al., 2001) and dairy cattle (Monteiro et al., 2014; Green et al., 2010; Gifford et al., 2007) has increased within the past decade. The optimum day for measuring ISGs for non-pregnancy diagnosis would be 18 DAI to align with commercial resynchronization protocols (Lucy et al., 2004). Candidate biomarkers were selected from microarray results from 15 and 18 DAI pregnant cow PBL RNA (Green et al., 2010). The increased ISG expression on day 18 in pregnant cows compared with 14 to 17 DAI found by Green et al. (2010) agrees with the timing of increased abundance of IFNT measured by 2D SDS PAGE gel (Bartol et al., 1995). Multiple research groups have measured selected ISGs in PBL of cows from different commercial herds and environments (Carvalho et al., 2016; Monteiro et al., 2014; Green et al., 2011). ISGs were also
differentially expressed in RNA from pregnant and non-pregnant bovine endometrial biopsies submitted for RNA sequencing (Forde et al., 2011). As discussed previously, the mechanism for IFNT stimulation of ISGs expressed in endometrial tissue compared with PBL is not fully understood. Non-pregnant cow MX2 and ISG15 expression from 14 to 20 DAI was less (1.5 ± 0.75-fold change for days 14 to 20) than pregnant cows (4 to 18-fold change on day 18 and 20; Green et al., 2010; Gifford et al., 2007). There is variation in pregnant cow expression of ISG so the measurement of ISGs in PBL is an imperfect pregnancy test but measuring ISG may be reliable for detecting non-pregnancy in cows and heifers (Carvalho et al., 2016; Green et al., 2011 and 2010; Gifford et al., 2007). Green et al. (2010) reported 82% and 94% true positive rate in detecting pregnant animals with MX2 and OAS1 respectively in heifers and primiparous cows 18 DAI. An attempt to measure ISGs in milk from dairy cows has only been reported (Mo et al., 2014) but was unsuccessful due to the quality of cell and RNA isolation methods.

The reason for lesser ISG expression in pregnant multiparous cows 18 to 20 DAI compared with pregnant primiparous cows and heifers is still unknown (Green et al., 2010). Two popular speculations are that there is a difference of embryo size or metabolism that affect IFNT affecting expression in PBL. The basal level of IFNT or sensitivity to IFNT in pregnant cows may be less in multiparous cows. The most obvious difference between heifers and multiparous dairy cows is that multiparous cows have gone through changes in energy balance (NEFA, DMI, glucose, insulin, etc.) associated with gestation, parturition, and lactation (Lucy et al., 1992, Rhoads et al., 2008, Berry et al., 2016).

The concept for improved fertility by increasing P4 prior to TAI or during resynchronization is based on the improved uterine receptivity and conceptus development
reported in high P4 compared to low P4 environments (Forde et al., 2009 and 2012; Bisinotto et al., 2013; Mann and Lamming, 1999). However, P4 supplementation in dairy cows (n = 1,498) did not increase mRNA abundance for ISG15 on day 16 and tended to decrease ISG15 mRNA abundance on 19 DAI (Monteiro et al., 2014). The measurement of ISG15 in PBL (78% ACY) is still more effective in detecting non-pregnant cows than P4 17 to 25 DAI (58% ACY; Han et al., 2006). Han et al. (2006) measured P4 and ISG15 15, 16, 17, 18, 19, 20, 21, 25, and 32 DAI. They found that cows differed in rise and decline of both ISG15 and P4 from 15 to 32 DAI. Studies are needed to determine if the slow rise in ISG15 is due to slow P4 rise or slower growth of the conceptus.

1.7. Resynchronization and Re-Insemination of Dairy Cows

The use of timed artificial insemination programs allows for management of ovarian dynamics in dairy cattle including anovulatory cows or cows with follicular cysts that affect cyclicity and conception (Thatcher et al., 2006; Pursley et al., 1995). The key components of TAI includes exogenous GnRH, PGF$_{2\alpha}$ and/or an intravaginal device releasing P4 (CIDR). TAI is often more effective than visual observation for estrus alone because less than 60% of dairy cows express estrus due to lower levels of estradiol associated with high milk production, lameness, and postpartum metabolic disease (Roelofs et al., 2006). The addition of a pre-synchronization to a TAI program improves P/AI because it groups cows to between day 5 to 10 of the cycle which is the best time to start Ovsynch (Moreira et al., 2001; Thatcher et al., 2006; 1989). GnRH is used to stimulate LH leading to ovulation and luteinizing of the largest follicle present on the ovaries. PGF$_{2\alpha}$ is used to regress the CL and begin a new cycle. Supplemental P4 is used in cattle with
multiple failed breeding services to improve ovulation percentage (Bisinotto et al., 2010; 2013).

The majority, > 60%, of lactating dairy cows will not conceive from the first insemination regardless of the TAI program (Moreira et al., 2001; Pursley et al., 1997). Thus, Wisconsin (Fricke et al., 2002) and Florida (Moreira et al., 2000) research groups have implemented hormonal protocols that begin before or after early pregnancy diagnosis. These are known as resynchronization (resynch) protocols for ovulation. In a study with 711 lactating Holstein cows on a commercial farm, a resynch protocol (100 μg GnRH, d 0, 25 mg PGF$_{2α}$, d 7, 100 μg GnRH + TAI, d 9) was started either 19, 26, or 33 days after first TAI (Fricke et al., 2003). GnRH administration to pregnant cows did not induce embryonic loss. This made resynch a viable option to initiate a TAI earlier than 28 to 35 DAI. There was not a difference in fertility in cows that started the resynch 26 d compared to 33 d. Cows that had a CL at PGF$_{2α}$ (day 18 cows) or GnRH of the resynch (26 or 33 d) had more P/AI than cows without a CL (Fricke et al., 2003). Resynchronization occurs in 77% of large U.S. commercial dairy farms (Caraveillo et al., 2006). The success of resynchronization protocols is measured by pregnancies per artificial insemination (P/AI). The P/AI for protocols using a combination of progestins (Bisinotto et al., 2010; Chebel et al., 2006), estradiol cypionate (Bartolome et al., 2005; Stevenson et al., 2003), or PGF$_{2α}$ (Silva et al., 2007) ultimately depends on the presence of a CL either at the first or subsequent PGF$_{2α}$ treatment.

The success of resynch protocols can also be affected by body condition score and milk production at time of protocol start. High milk production cows tend to have lower expression of estrus due to increased metabolism of estradiol 17-β and increased incidence
of anovulation (Wiltbank et al., 2006; Thatcher et al., 2006; Lucy et al., 2001). High production dairy cows also tend to have a lower P4 at the beginning of synchronization protocols. Resynchronization is also effective in increasing P4 levels at first GnRH of resynch and P/AI in pasture based or feedlot cattle (Ribeiro et al., 2011; Lucy et al., 2004). Lopes et al. (2013) reported GnRH presynchronization only slightly increased low P4 levels and did not differ in P/AI from non-presynchronized cows (38.9 vs. 33.8% respectively) and was lower than 69.1% P/AI in cows with a high P4 environment at first GnRH of the resynch. The reason for this is that lower levels of circulating P4 leads to changes in the uterine endometrium of increased activation of ESR1 that stimulate oxytocin and PGF$_{2\alpha}$ that regresses the CL. Lower levels of P4 at the beginning of synchronization has been reported by Florida and Wisconsin research groups to affect maintenance of pregnancy and conceptus development.

1.8. Conclusions

Early pregnancy in cattle involves a series of developmental changes in the conceptus. Conceptus development includes the expression and release of signaling molecules (e.g., IFNT) or other molecules (PAG) that can be detected in the maternal circulation. The presence of PAG in the circulation is used to identify pregnant cattle as early as 25 DAI. The PAG test has been commercialized but commercial tests were confined to the laboratory. A more useful test could be done “at home “or cow side. Chapter 2 describes a PAG test that could be done at home. ISGs are detected earlier than PAG and would be more useful if a reliable test were developed. Furthermore, a milk test for ISG would be very valuable to the farmer because of collection convenience compared to blood tests.
Chapter 3 describes an attempt to validate a milk ISG test. Finally, although blood ISG tests can be used, they are less sensitive in older cows. Chapter 4 describes our attempt to understand why older cows provide a less intense signal than heifers or first lactation cows. Ultimately, early pregnancy tests have a tremendous utility to the farmer because they enable the early indication of non-pregnant cows. Our hope for this work is that it will eventually lead to effective tests for earlier pregnancy detection.
CHAPTER 2.

Validation of a chemical pregnancy test in dairy cows that uses whole blood, shortened incubation times, and visual readout

2.1. ABSTRACT

Chemical pregnancy testing is an alternative to traditional methods of pregnancy diagnosis (either manual palpation or ultrasound) in postpartum dairy cows and heifers. The objective was to validate a new chemical pregnancy test that confers the advantages of using whole blood, rapid incubation times, and visual readout. Blood and milk samples were collected from Holstein dairy cows \([n = 320; 162 \pm 62 \text{ (mean } \pm \text{ SD) DIM}]\) on a confinement farm in Northeast Missouri at 28 d after AI. The samples were assayed for pregnancy-associated glycoproteins (PAG) by using a new rapid visual test as well as traditional plasma- and milk-based tests. Transrectal ultrasonography diagnosis for pregnancy at 35 d to 38 d after AI was the reference (gold) standard for all PAG tests. There were 159 cows diagnosed pregnant by the reference standard (pregnancies per AI = 49.7\%). The tests were ELISA and either optical density (OD; measured with a microtiter plate reader; plasma, milk, and rapid tests) or visual readout (rapid test) were used to diagnose pregnancy. When the OD was used, the percentage of pregnant cows classified correctly (sensitivity) for the plasma, milk, and rapid tests were 97 ± 1, 96 ± 2, and 95 ± 1\% (± SE), respectively. The sensitivity of the rapid test when assessed visually was 98 ± 1\%. The specificity (proportion of non-pregnant cows classified correctly) for the plasma, milk, and rapid was 94 ± 2\%, 94 ± 2\% and 93 ± 2\% when an OD was used. When read visually, the specificity of the rapid test was lesser (85 ± 3\%) because some cows with faint visual signals yielded false positive diagnosis. The overall accuracy (proportion of
pregnant and non-pregnant cows diagnosed correctly) was similar for all tests (plasma, milk, rapid OD, and rapid visual; 96 ± 1, 95 ± 1, 94 ± 1, and 92 ± 2%, respectively). In a second experiment, lactating Holstein cows (n=291) from 4 commercial confinement dairy farms in western Kentucky were tested 25 to 95 d after AI using the rapid visual test. The OD of the rapid visual test followed the known profile for PAG in circulation during the first trimester of pregnancy. The conclusion is that the new rapid visual test has equal sensitivity and accuracy to existing PAG tests. A slightly lower specificity was found when the rapid test was read visually. The lesser specificity was explained by a small number of non-pregnant cows with a weak signal (false positive result). The same wells yielded a negative result when the OD was measured mechanically with a microtiter plate reader.

2.2. INTRODUCTION

Pregnancy diagnosis within 4 to 6 wk after AI in postpartum dairy cows is critical for identifying non-pregnant cows eligible for rebreeding so that time from calving to conception (days open) is less (Silva et al., 2009; Giordano et al., 2013). Traditional methods of pregnancy diagnosis such as manual palpation and ultrasonography are typically performed 32 d after AI (Romano et al., 2006). Earlier tests are based on pregnancy associated glycoproteins in the circulation that are detected as early as 25 d after AI in cattle (Green et al., 2005; Green et al., 2009; Wallace et al., 2015). The PAG can be detected in either plasma, serum, or milk (Silva et al., 2007; LeBlanc, 2013; Lawson et al., 2014). The accuracies of commercial PAG tests for plasma, serum, or milk range from 89% to 96% (Silva et al., 2007; Ricci et al., 2015). The accuracy of PAG tests makes them suitable alternatives to traditional methods of pregnancy diagnosis (palpation or
ultrasound). There are limitations, however, to their use. Plasma or serum-based tests require the collection of a blood sample and subsequent centrifugation. The centrifugation step requires equipment that is typically not available on-farm. Milk testing does not require centrifugation but the initial assay step requires a thermally-controlled platform shaker for the ELISA plate. Shaking is not required for assays performed with plasma or serum but an incubator is needed. Regardless of whether plasma, serum, or milk is used, there is also a requirement for a microtiter plate reader to measure the optical density (OD) for individual wells in the ELISA plate. Given the need for specialized equipment, PAG testing is most-likely done in a veterinary clinic or other centrally located laboratory. A final impediment to the on-farm test is the time from sample collection to pregnancy test result. Traditional tests have a series of incubations with antibody and reagent solutions that can require from 2 to 3 h or as long as overnight to complete the test. In many herds, treatments are administered to non-pregnant cows after pregnancy diagnosis; usually before they are released from animal restraint (headlocks, squeeze chute or palpation rail). The length of time from sampling to test completion is too long if cows are to remain restrained or kept in separate pens. A lesser time from sampling to test result would theoretically improve on-farm utility of a PAG test. We tested a new PAG test (Rapid Visual Pregnancy Test; IDEXX, Westbrook, ME; 2016 release date) that addresses some of the limitations listed above. Specifically, whole blood (EDTA) can be used in addition to plasma (EDTA) or serum, total test time is reduced to approximately 30 min, and the plate can be read visually. The objective of this experiment was to validate the new test performed 28 d after AI by using transrectal ultrasound on d 35 to 38 as the gold standard. We also compared the results obtained from the new test with those obtained using
traditional PAG tests based on plasma or milk. Finally, the new PAG test was used to assay a series of samples collected during the first trimester of pregnancy (d 25 to 95).

2.3. MATERIALS & METHODS

The validation experiment was performed at a confinement dairy farm in northeast Missouri by using two cohorts of lactating Holstein cows (n = 320) in January 2016. The cows were 162 ± 62 (mean ± SD) DIM on the day of blood and milk sample collection. Both primiparous (n = 95) and multiparous (n = 225) cows were used (mean ± SD = 2.3 ± 1.2 calvings). Cows were housed in a standard free-stall (mattresses or sand bedding), milked 3 times daily, and fed a TMR. Cows were treated with a “Presynch Ovsynch _56” protocol (PGF$_{2\alpha}$, 14 d, PGF$_{2\alpha}$, 14 d, GnRH, 7 d, PGF$_{2\alpha}$, 56 h, GnRH, 16 h, timed AI) so that first the timed AI was 70 to 76 d postpartum. The PGF$_{2\alpha}$ was Lutalyse (5 mL; 25 mg; Zoetis Animal Health, Florham Park, NJ) and the GnRH was Fertagyl (2 mL; 100 μg; Intervet, Milan, Italy). Cows submitted to a timed AI (n = 114) or resynchronization timed AI (n = 206) were enrolled in the study. The resynchronization protocol began with a GnRH treatment 32 d after timed AI and concluded with a resynchronized timed AI on d 42 (GnRH, 6 d, ultrasound pregnancy diagnosis, 1 d, and then for non-pregnant cows, PGF$_{2\alpha}$, 56 h, GnRH, 16 h, timed AI). The number of inseminations at the time of pregnancy diagnosis averaged 2.3 ± 1.4 (mean ± SD).

Blood and milk samples were collected 28 d after timed AI. Cows eligible for pregnancy diagnosis from the herd were identified by RFID upon exit from the parlor and marked with tail chalk before being directed into a standard palpation rail that was used to restrain cows during sampling. Blood and milk samples were collected while cows stood
in the palpation rail. Blood was collected by coccygeal venipuncture from the median caudal vein or artery into a Monoject™ tube containing 100 μL of a 15% solution of EDTA (K$_3$) (Covidien, Minneapolis, MN). Milk was collected into a 20 mL disposable polyethylene scintillation vial (Fisherbrand, Pittsburgh, PA). Blood and milk samples were placed on ice immediately after collection, transported back to the laboratory when collection was complete, and stored at 4°C overnight. The following morning, blood tubes were inverted to mix and approximately 2 mL of whole blood was transferred to a 12 x 75 mm borosilicate glass tube for subsequent use in the rapid visual test. The remaining blood was centrifuged at 1,500 x g for 15 min. Plasma for use in the conventional test was aspirated into a 12 x 75 mm polypropylene tube. Milk was mixed thoroughly by inversion before use in the milk test.

All assays were performed according to the manufacturer’s instructions found on the insert within the package. The purpose of this study was to validate the performance of the new rapid visual test (IDEXX Rapid Visual Pregnancy Test) relative to transrectal ultrasonography as the reference (gold) standard for pregnancy diagnosis. A conventional test that can use either plasma or serum (IDEXX Bovine Pregnancy Test) and a milk-based test (IDEXX Milk Pregnancy Test) were also compared to the reference standard as well as to the rapid visual test.

The rapid visual test is a sandwich-style ELISA. It has a similar format to conventional (plasma or serum) and milk tests but some steps are combined and incubation times are shorter. Although performance of the test with whole blood (EDTA) is presented, plasma (EDTA) or serum can also be tested. The manufacturer provides 96 well assay plates comprised of twelve 8-well assay strips. For the rapid test, 100 μL of whole blood and 3
drops (approximately 100 µl) of reagent 1 detector solution were added to individual wells of the PAG antibody-coated ELISA plate. The plate was covered, tapped gently to mix, and incubated for 7 min at room temperature (23° to 26°C). After 7 min, the solution in the wells was removed by inversion and then the wells were washed thrice with distilled water. For washing, individual wells were gently filled until overflowing by using a 500 mL wash bottle (no. 414004-227; VWR, Radnor, PA) and a flow rate of approximately 0.68 mL/sec. Three drops of conjugate solution containing horseradish peroxidase were then added to the wells and a second 7 min incubation followed by washing was completed. Three drops of tetramethylbenzidine (TMB) substrate solution was then added and the reaction was stopped after 10 min by adding 3 drops of a stop solution. Each individual assay consisted of 28 unknown samples and negative and positive control wells at the beginning and end of each assay (32 wells total; 4 ELISA plate assay strips). Test results were interpreted according to the manufacturer’s recommendations. Absence of color (clear solution) was interpreted as non-pregnant. Cows were classified pregnant if the sample was any shade darker visually than the negative controls. Cows were classified not pregnant if the sample was not darker visually than the negative controls. After the wells were interpreted visually, the OD (absorbance) was read at 650 nm using a microtiter plate reader (BioTek Synergy HT, Winooski, VT).

The procedures for the conventional (plasma) and milk tests were similar to those described for the rapid visual test but there were methodological differences with respect to incubation conditions and times. Although functionally-related reagents had similar names they were not necessarily identical in terms of chemical composition or antibody concentration. Solutions used, therefore, were only used with the respective kit. The
conventional and milk assay plates were not interpreted visually. Per manufacturer instructions, OD was measured at 450 nm and a reference wavelength of 650 nm was used. Total incubation times (not including pipetting, washing, or microtiter plate reading) were 21, 135, and 200 min for the rapid visual, conventional (plasma), and milk tests.

Receiver operator characteristic (ROC) curves were generated from OD using the LOGISTIC procedure in SAS 9.3 (SAS Institute, Inc., Cary, NC). The event was the result of the reference (gold) standard. The gold standard was defined as the result of pregnancy diagnosis at 35 to 38 d after AI (approximately 7 to 10 d after the chemical testing) as performed by a single veterinarian using transrectal ultrasonography (equipment used was an Easi-Scan; BCF Technology, Rochester, MN, USA). Insemination number and parity were included as fixed effects in the model. Cutpoints for pregnancy diagnosis were selected that had the minimal distance from the ROC curve to the “perfect” point at the upper left corner where sensitivity = 1 and 1-specificity= 0). The GLM procedure in SAS 9.3 was used to test for the effects of parity, insemination number, and days open at the time of sampling on the OD of individual samples.

The sensitivity [(number of cows correctly diagnosed positive with a blood pregnancy test / the number of cows with a positive ultrasonographic pregnancy test) x 100], specificity [(number of cows correctly diagnosed negative with a blood pregnancy test / number of cows with negative ultrasonographic pregnancy test) x 100], positive predictive value [PPV; (number of cows correctly diagnosed pregnant with a test / number of cows correctly diagnosed pregnant with a test + the number of cows incorrectly diagnosed pregnant with a test) x 100], negative predictive value [NPV(number of cows correctly diagnosed not pregnant with a test / number of cows correctly diagnosed not pregnant with
a test + the number of cows incorrectly diagnosed not pregnant with a test) x 100] and accuracy [ACY; (number of correct blood pregnancy tests/total number of blood pregnancy tests) x 100] were calculated for each assay. Differences between assays for sensitivity, specificity, PPV, NPV, and ACY were tested for significance using Fisher’s Exact Test. The data are reported as the proportion ± SE where SE = square root \( \frac{pq}{n} \) (Snedecor and Cochran, 1989). The FREQUENCY procedure of SAS 9.3 was used to calculate the Cohen’s kappa statistic which measures the agreement between all methods of pregnancy diagnosis (chemical tests and transrectal ultrasonography).

2.4. RESULTS & DISCUSSION

There were 159/320 (49.7%) cows that were pregnant at the time of diagnosis by ultrasound. The area under the ROC curve for the conventional (plasma) test was 0.9754 with a 95% confidence interval of 0.9592 to 0.9917 (Figure 1A; \( P < 0.001 \)). A cutpoint of 1.72 for OD at 450 nm had a sensitivity of 97 ± 1% and a specificity of 94 ± 2%. The area under the curve for the milk-based test for pregnancy was 0.9658 with a 95% confidence interval of 0.9430 to 0.9887 (\( P < 0.001 \); Figure 1B). A cutpoint of 0.86 for OD at 450 nm had a sensitivity of 96 ± 2% and a specificity of 94 ± 2%. The area under the curve for the rapid visual test was 0.9710 with a 95% confidence interval of 0.9527 to 0.9892 (Figure 1C; \( P < 0.001 \)). A cutpoint of 0.304 for OD at 650nm had a sensitivity of 95 ± 2% with a specificity of 93 ± 2%. The ROC curves that were generated based on OD for plasma, milk and rapid were similar with respect to area under the curve (ranging from 0.9658 to 0.9754) and also sensitivity (ranging from 95 to 97%) and specificity (ranging from 93 to 94%). The area under the curve for the conventional plasma and milk test using the sample OD
minus negative control OD was identical to that using the sample OD alone. We chose not to subtract the negative control OD, therefore, as previous studies have done (LeBlanc, 2013; Ricci et al., 2015) because a reference wavelength was not used for the rapid test interpretation. The conclusion was that a useful cutpoint could be found for all tests when the test result was an OD as measured by a microtiter plate reader. Assuming 8.9% pregnancy loss, Giordano et al. (2013) concluded that the sensitivity and specificity of the chemical tests at d 31 needed to be 96.4% and 95.1% (respectively) for an equal return on investment with respect to rectal palpation at 39 d after AI. The sensitivity and specificity of plasma, milk, and rapid tests when the results were measured with a microtiter plate reader (OD) were nearly identical to the break-even numbers proposed by Giordano et al. (2013).

When interpreted visually, the sensitivity of the rapid visual test was 98 ± 1% (Table 1). The sensitivity for the test was not different ($P > 0.10$) for the rapid visual test when a cutpoint based on OD was used for diagnosis (95 ± 2%) and was not different ($P > 0.10$) from plasma (97 ± 1%) or the milk (96 ± 2%) tests. The specificity of the rapid visual test interpreted visually was 85 ± 3% and was less ($P < 0.05$) than the specificity of the rapid visual test interpreted using OD or the specificity of the other tests. The PPV for the rapid, plasma, and milk tests when OD was used for interpretation did not differ ($P > 0.10$; Table 1). The PPV for the rapid visual test interpreted visually (87 ± 3%) was less ($P < 0.05$) than other tests. The NPV and ACY for the rapid visual test interpreted visually was 98 ± 1 and 92 ± 2% (respectively) and did not differ from the NPV and ACY of the rapid, milk, or plasma tests when OD was used for interpretation.
There were 15 cows with a false negative test result for any test and 26 cows with a false positive test result for any test (Figure 2). Two of the 15 (13%) cows had a false negative test result on all 4 tests (Figure 2A). The remaining false negatives were primarily found within a single test indicating that there was no relationship between the tests with respect to false negative results. There were 8 cows that were false positive on all 4 tests possibly explained by embryonic loss between d 28 (PAG sample) and d 35 to 38 (transrectal ultrasonography; Figure 2B). A false positive result is a recognized limitation of early pregnancy diagnosis performed within 4 wk after AI because approximately 10 to 20% of pregnant dairy cows undergo embryonic loss after wk 4 (Giordano et al., 2013). A cow with embryonic loss during this period will initially test pregnant and later be found not pregnant when the gold standard test is applied (Giordano et al., 2012; Pohler et al., 2016). The PAG testing was done approximately 30 d after the negative pregnancy diagnosis in resynchronized cows. There is a slight possibility, therefore, that residual PAG from a cow previously experiencing embryonic loss could have carried into the next sampling period and caused a false positive result as well (Szenci et al., 2003; Giordano et al., 2012). As with all early pregnancy tests based on PAG or ultrasound there is a need to recheck cows for pregnancy after the initial period of embryonic loss (Giordano et al., 2013).

The reason for the lesser specificity and PPV of the rapid visual test (Table 1) is that 12 cows were diagnosed pregnant based on visual interpretation but were not pregnant based on the OD interpretation of the exact same well (Figure 2B). The average OD for these 12 cows was 0.22 ± 0.03 compared with the pregnancy OD cutpoint of 0.304 and average OD of the remaining pregnant cows of 0.51 ± 0.01 ($P < 0.001$). The cows correctly
classified not pregnant had an average rapid OD of 0.11 ± 0.01 \((P < 0.001)\). When these 12 samples were reanalyzed and interpreted visually, the result was negative and the specificity improved to 93 ± 2\% for the rapid visual test. The cumulative interpretation is that the microtiter plate reader had a greater capacity to interpret faint color when compared with the visual interpretation. This finding is perhaps not unexpected. Repeating the assay for cows with a faint signal may resolve false positives. With respect to on-farm application, the slightly lower specificity means that cows that are not pregnant are diagnosed as pregnant and this phenomenon reinforces the need for rechecking cows with a positive test result after the period of embryonic loss has subsided. The sensitivity and NPV of the visual test was nearly 100\% and not different from other tests. This indicates that very few truly pregnant cows are misdiagnosed as not pregnant.

Cohen’s kappa statistics were calculated to assess the agreement between the different tests with respect to the diagnosis of pregnancy (Table 2). Agreement was extremely high for tests based solely on OD. Agreement for the rapid test assessed visually and other tests based on OD was slightly less but would be considered almost perfect (> 0.81; Viera et al., 2005). Insemination number and days open at the time of sampling did not affect the OD for any test. Pregnant primiparous cows had greater OD (0.54 ± 0.02) than pregnant multiparous cows (0.49 ± 0.01; \(P < 0.05\)) when the rapid test was used. There was no effect of parity on OD for the plasma or milk tests.

The circulating concentrations of PAG increase approximately 25 d after AI in pregnant cows (Green et al., 2005). The concentrations continue to increase until approximately d 35 and then decrease for a period of 3 to 4 wk before increasing again until the end of pregnancy (Ricci et al., 2015). The underlying cause for the changes in circulating PAG
are unclear but the interpretation of the test result can be affected in primiparous and multiparous cows (Ricci et al., 2015). We examined whether the PAG signal changed during early gestation when the rapid visual test was used. In a separate study using a prototype kit with identical chemical reagents, lactating Holstein cows (n = 291) from 4 commercial confinement dairy farms in central and western Kentucky were enrolled in May and June 2015. Cows were between 25 to 95 d after AI on the sampling day. For statistical analysis, cows were grouped by week of pregnancy beginning on d 25. Blood was collected and tests were performed using procedures similar to those described for the Missouri study. The result of the rapid visual test was determined visually and the OD was also measured. The average OD for cows classified not pregnant was 0.21 ± 0.03 compared with pregnant OD of 0.37 ± 0.01 (P < 0.001). The OD was greatest for pregnant cows sampled from d 25 to 32 and then declined to its lowest level on d 46 to 60 after AI (Figure 3; effect of sampling day; P < 0.01). There was a subsequent increase in PAG OD (Figure 3). The interpretation was that the OD for the rapid visual test demonstrated the same pattern for PAG during the first trimester of pregnancy as reported for alternative PAG tests (Ricci et al., 2015).

2.5. CONCLUSIONS

In conclusion, a new rapid visual test had equal sensitivity and accuracy to existing tests. A slightly lower specificity was found when the rapid test was read visually. The lower specificity was explained by a small number of cows with a weak signal and false positive result. The same wells yielded a negative result (below an established cutpoint) when the OD was measured mechanically with a microtiter plate reader. With respect to
on-farm application, the slightly lower specificity reinforces the need for a second pregnancy diagnosis (either chemical test or alternative method) after the period of embryonic loss has subsided. The sensitivity and NPV of the visual test was nearly 100% and not different from other tests. This indicates that very few truly pregnant cows are misdiagnosed as not pregnant when the rapid visual test is used.

2.6. ACKNOWLEDGEMENTS

The authors thank Heartland Dairy Farm for access to their cows and for the assistance of their employees. The authors also thank Roger Molina-Coto and Holly Ann Thibodeaux for their assistance in sample collections in Northeast Missouri. The authors thank Billy Riney, Josh Duvall, Mike Hunt, and Bradley Elkins for access to their cows in Kentucky. The authors greatly appreciate Susan Hayes, Hugo Hamilton, Denise Ray, Georgia Skelton, Karmella Dolecheck, Nicky Tsai, and Tatiana Muñoz for their assistance in sample collection in central and western Kentucky. ELISA test kits used in this work were provided by IDEXX (Westbrook, ME).
Table 2.1. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy (% ± SE) for pregnancy-associated glycoproteins (PAG) tests using plasma and milk and a rapid test using whole blood with test results interpreted by using either optical density (OD) or visually when transrectal ultrasonography was used as the reference (gold) standard for pregnancy diagnosis in lactating Holstein cows 28 d after timed AI.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Sensitivity¹</th>
<th>Specificity²</th>
<th>PPV³</th>
<th>NPV⁴</th>
<th>Accuracy⁵</th>
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<tbody>
<tr>
<td></td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
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<tr>
<td></td>
<td>(no./no.)</td>
<td>(no./no.)</td>
<td>(no./no.)</td>
<td>(no./no.)</td>
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<tr>
<td>Plasma OD⁶</td>
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<td>95 ± 2</td>
<td>97 ± 1</td>
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<td>(155/164)</td>
<td>(152/156)</td>
<td>(307/320)</td>
</tr>
<tr>
<td>Milk OD⁶</td>
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<td>(152/161)</td>
<td>(152/161)</td>
<td>(152/159)</td>
<td>(304/320)</td>
</tr>
<tr>
<td>Rapid OD⁶</td>
<td>95 ± 2</td>
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<td>93 ± 2</td>
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<td>(137/161)</td>
<td>(156/180)</td>
<td>(137/140)</td>
<td>(293/320)</td>
</tr>
</tbody>
</table>

¹Number of cows with a correct positive test / the number of cows with a positive ultrasonographic test.
²Number of cows with a correct negative test / the number of cows with a negative ultrasonographic test.
³Number of cows with a correct positive test / number of cows with a correct positive test + the number of cows with an incorrect positive test.
⁴Number of cows with a correct negative test / number of cows with a correct positive test + the number of cows with an incorrect negative test.
⁵Number of correct blood pregnancy tests / total number of blood pregnancy tests.
⁶Optical density measured by a microtiter plate reader for all samples at a wavelength of 650 nm for whole blood and 450 nm for plasma and milk. In this study, the ROC-determined cutoffs for pregnancy were 0.304, 0.860, and 1.716 for the rapid, milk, and plasma assays respectively.
⁷Cows were classified pregnant if the sample was any shade darker visually than both negative controls. Cows were classified not pregnant if the sample was not darker visually than both negative controls.
Table 2.2. Cohen’s kappa statistic for bovine chemical pregnancy tests (n=320) and transrectal ultrasonography measuring inter-test agreement at 28 d after insemination for PAG and 35 to 38 d after AI for transrectal ultrasonography.

<table>
<thead>
<tr>
<th></th>
<th>Ultrasound</th>
<th>Rapid visual</th>
<th>Rapid OD</th>
<th>Milk OD</th>
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</thead>
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<td>0.89</td>
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<tr>
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<tr>
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<td>Ultrasound</td>
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<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
Figure 2.1. Receiver Operator Characteristic (ROC) curves for three bovine chemical pregnancy tests; A, conventional plasma PAG test (area under the curve = 0.9754); B, milk PAG test (area under the curve = 0.9658); and C, rapid visual test (area under the curve = 0.9710).
Figure 2.2. Venn diagrams for bovine chemical pregnancy test in lactating Holstein cows 28 d after timed AI with transrectal ultrasonography at 35 to 38 d after timed AI as the reference (gold) standard. The number of individual cows within and between each individual test is shown for cows that were either false negatives (A; n = 15 cows total) or false positives (B; n = 26 cows total).
Figure 2.3. Optical density at 650 nm for a rapid visual pregnancy test from pregnant lactating Holstein cows in central and western Kentucky 25 to 95 d after insemination. Bars with different superscripts differ at \( P < 0.05 \). Cows were grouped by week with 25 d including cows that were 25 to 31 d pregnant; 32 d including cows that were 32 to 36 d pregnant, etc. The average OD for cows classified not pregnant in the same study was 0.21 ± 0.03.
CHAPTER 3:
Development and use of method to measure of ISG15 in milk cells for pregnancy diagnosis in dairy cows

3.1. ABSTRACT
Performing pregnancy diagnosis sooner after timed artificial insemination (TAI) could decrease days open in dairy cows if non-pregnant cows are enrolled in resynchronization programs. Methods for accurate pregnancy diagnosis within 3 weeks after TAI are based on interferon tau-stimulated gene (ISG) expression in blood but milk samples are often more convenient to obtain than blood samples. The overall objective was to assess the utility of measuring ISG15 expression in milk somatic cells as a method to diagnose pregnancy in cows after TAI. We hypothesized that cows would differ in ISG expression in milk cells compared to blood cells and among cows. To test these hypotheses, three studies were conducted to measure milk cell ISG expression in vivo (study 1 and 2) and in vitro (study 3). Blood (10 mL) and composite milk (200 mL) samples were collected from 48 primiparous and 13 multiparous Holstein cows (study 1; n = 61; 102 ± 12 DIM; 36 ± 20 kg/d milk) at 18, 20, and 22 d after TAI. Samples were placed on ice after collection and RNA was extracted on the same day. RNA samples were used for cDNA synthesis and cDNA was used in RT-PCR analysis of gene expression for ISG15 and cyclophilin (reference gene). Ratios of ISG15 to cyclophilin (ICR) were calculated. Transrectal ultrasonography diagnosis for pregnancy at 33 d or 35 d after TAI was the reference standard. The ICR (study 1) of blood and milk cells were tested for the effects of pregnancy status, day, parity, and interactions using the MIXED procedure of SAS 9.4 (Cary, NC).
The REG procedure of SAS was used to determine the correlation between milk and blood ICRs. The ICR was greater in blood of pregnant cows (0.58 ± 0.07; n = 28 compared with non-pregnant cows (0.12 ± 0.06; n = 33) on d 18, 20, and 22 (P < 0.0001). In same cows and on the same days, milk somatic cell ISG15 expression was also greater in pregnant (0.64 ± 0.17) compared with non-pregnant (0.20 ± 0.16) cows (P < 0.059). In study 2, the uterine horn ipsilateral to the CL of each cow received an intrauterine infusion of embryo flush media (10 ml) containing 0, 500, or 1000 µg of recombinant ovine IFNT (roIFNT). Blood (10 ml) and milk (150 ml) samples were collected at -1, 5, 9, and 13 h relative to infusion (0 h). In milk samples, ISG15 mRNA abundances increased across sampling times independent of roIFNT infusion dose (P < 0.001) and differed between cows (P < 0.001). In contrast to blood, an increase in ISG15 mRNA was not detected (P > 0.10) in milk at 4 h but was at 12 h (P < 0.05) after roIFNT infusion. In study 3 milk samples were taken from mid-lactation Holstein cows (n = 7) using a DHI approved sampler during the morning milking and pooled. Whole milk samples (50 mL) were treated with increasing doses of roIFNT (0, 1, 10, 100, and 1000 pg/ml) for 2 h with continuous rotation in a 37°C incubator. The ISG15 to cyclophilin and GAPDH ratios in pooled milk cells increased 25 to 50-fold, 2 and 4 h respectively after addition of 100 or 1,000 pg roIFNT in vitro. Overall, ISG15 expression in both blood and milk somatic cells was greater for pregnant compared with non-pregnant Holstein cows. Milk cells were very sensitive to roIFNT indicating more research is needed with larger sample sizes to determine efficacy of measuring ISGs in milk cells 20 d after insemination to identify non-pregnant cows. Testing milk for ISG15 expression may be an alternative to ISG15 testing in blood.
3.2. INTRODUCTION

Pregnancy is required for initiation of milk production which is the main income for dairy farms. Traditional methods of pregnancy diagnosis that include palpation, ultrasound (Fricke, 2002; Caraviello et al., 2006), or lab-based blood tests (Fosgate et al., 2017; Green et al., 2005) are used approximately 4 weeks after insemination and 2 weeks after the cow recognizes the embryo. The time that a cow remains not pregnant costs $3 to $5 per cow per day on average for U.S. dairy farms (De Vries, 2011; French and Nebel, 2003). Performing pregnancy diagnosis sooner after insemination could decrease days from calving to next pregnancy (days open) in dairy cows if non-pregnant cows are treated so that they come back into estrus and are re-inseminated (Green et al., 2011; Thompson et al., 2010). Developing chemical pregnancy tests in blood or milk that can be performed on farm sooner after AI would aid in finding non-pregnant cows or embryonic loss cows (Pohler et al., 2016) sooner, beginning re-insemination quicker, and reducing costs associated with increased days open. The overall objective was to assess the utility of measuring ISG15 expression in milk somatic cells as a method to diagnose pregnancy in cows after TAI. We hypothesized that cows would differ in ISG expression in milk cells compared to blood cells and among cows. To test these hypotheses, three studies were conducted to measure milk cell ISG expression in vivo (study 1 and 2) and in vitro (study 3).

3.3. MATERIALS & METHODS

The following studies followed the research animal care and welfare regulations established by the Institutional Animal Care and Use Committee (IACUC) of the University of Missouri.
Milk leukocyte isolation and RNA isolation

Milk leukocyte isolation and RNA isolation were used for all studies as described here. While samples were on ice, 0.5 M EDTA (~300 μL) was added to the milk and inverted. Milk samples were centrifuged using conical cushions at 1,800 x g for 15 min at 4°C. Fat and whey were removed using a vacuum flask with a new 25 mL tip for each sample until only 10 mL of whey and the cell pellet remained. Cell pellets were resuspended in 20 mL of molecular grade phosphate-buffered saline (PBS; Fisher BioReagents, Fair Lawn, NJ) and 20 μL of 0.5 M EDTA then transferred to a new 50 mL centrifuge tube (Corning Falcon, Corning, NY). Samples were centrifuged at 2,100 x g for 15 min at 25°C. Supernatant was decanted and tubes were left upside down for 2 min to remove remaining whey and fat. Cells were resuspended in 1 mL Trizol (Invitrogen, Carlsbad, CA) until viscous and then homogenized (Tissue-Tearor, Biospec, Bartlesville, OK). Homogenized samples were stored in -80°C freezer until RNA extraction.

RNA extraction was completed according to Trizol manufacturer’s instructions with the following adjustments. Only 500 μL of the aqueous phase was removed to avoid disrupting the interphase. The RNA was precipitated from the aqueous phase by the addition of 1.5 μL of GlycoBlue Coprecipitant (15 mg/mL; Applied Biosystems, Foster City, CA), 50 μL of room temperature 3 M NaAc (pH 5.5), and 400 μL of room temperature 100% Isopropanol (Fisher Scientific, Hampton, NH). Supernatant was aspirated with a new glass Pasteur pipet. Cold 75% ethanol was used for washing the RNA pellet twice. The pellet was resuspended in 20 μL of room temperature nuclease-free water (Invitrogen, Carlsbad, CA) then incubated in a 55°C water bath for 10 min. Samples were then placed on ice immediately and evaluated for quantity and quality on a NanoDrop.
Spectrophotometer (Thermo Scientific, Waltham, MA). Samples were stored in -80°C freezer until gel electrophoresis for RNA quality verification.

3.3.1. Study 1 Design. Measurement of ISG15 in milk somatic cells for pregnancy diagnosis 18, 20, and 22 days after timed artificial insemination (TAI) in dairy cows. Blood (10 mL) and composite milk (200 mL) samples were collected from 48 primiparous and 13 multiparous Holstein cows (Figure 3.2.0; n = 61; 102 ± 12 DIM; 36 ± 20 kg/d milk yield) at 18, 20, and 22 d after TAI. Samples were placed on ice after collection and RNA was extracted on the same day. RNA samples were used for cDNA synthesis and cDNA was used in RT-PCR analysis of gene expression for ISG15 and cyclophilin (reference gene). Ratios of ISG15 to cyclophilin (ICR) were calculated. Transrectal ultrasonography diagnosis for pregnancy at 32 d or 34 d after TAI was the reference standard. The ICR of blood and milk cells were tested for the effects of pregnancy status, day, parity, and interactions using the MIXED procedure of SAS 9.4 (Cary, NC). The REG procedure of SAS was used to determine the correlation between milk and blood ICRs. The ISG15 ratio data were analyzed and receiver operator characteristic (ROC) curves were produced. Data were analyzed for each independent day (d 18, 20 and 22 after timed AI). The Logistic procedure of SAS was used with positive pregnancy diagnosis based on PAG outcome as the event. The sensitivity was defined as the proportion of cows identified pregnant by ISG15 ratio from the cows identified pregnant by PAG. The specificity was defined as the proportion of cows identified not pregnant by ISG15 ratio from the cows identified not pregnant by PAG. PROC FREQ was used to evaluate the agreement between the pregnancy
diagnosis made by each individual day by ISG15 versus the pregnancy outcome from PAG. The effects of parity and PAG were also tested for the ISG15 ratio.

3.3.2. Study 2 Design. Interferon stimulated gene 15 (ISG15) expression in milk and blood following intrauterine infusion of recombinant ovine interferon tau (roIFNT) in dairy cows. Lactating Holstein cows (n = 4) were randomly assigned in a Latin Square Design (Figure 3.1.0) to receive intrauterine infusions on days 11, 13, and 15 of a synchronized estrous cycle. The uterine horn ipsilateral to the CL of each cow received an intrauterine infusion of embryo flush media (10 ml) containing 0, 500, or 1000 µg of roIFNT (gift from Gilles Charpigny of the French National Institute for Agricultural Research). Rectal temperature was taken every 4 h (08:30, 12:30, 16:30, and 20:30) for a 12 h period for 7 days to detect any increases in temperature. In previous studies ± 0.4°C differences in rectal temperature 4 h were found after cows were given intrauterine infusions 5 mg of roIFNT (Meyer et al., 1995b). Even though the amount given did not exceed 1 mg, as a precaution, we took rectal temperatures in order to detect any potential fever in the cows. Cows with rectal temperatures above 39.3°C were assessed by a veterinarian, and immediately given Flunixin Meglumine (Banamine, Merck, Kenilworth, NJ).

Preparation of materials for intrauterine infusions of roIFNT occurred in the lab using sterile procedures. Recombinant ovine IFNT (70 mg) was produced in yeast according to methods in Martal et al. (1990), put through ultrafiltration, concentration, and ion exchange HPLC separations. The roIFNT used was successful in stimulating expression of ISGs in the CL with minimal adverse effects in sheep and cattle (Forde et al., 2012; Martal et al., 1990; Charpigny et al, 1988). The roIFNT was stored at -20°C until
addition to buffer solution. Immunological cross-reactivity was tested with bovine endometrial cells to ensure effectiveness and dose response to roIFNT before being used in vivo.

Prior to roIFNT intrauterine infusions, manure was removed from the rectum and vulva with several paper towels soaked in warm water and chlorhexidine to prevent manure from entering the vagina. Infusions of roIFNT were given using sterile procedures with a standard artificial insemination (AI) pipette covered in a clean protective plastic sheath for each cow. The AI pipette was inserted through the vulva, vagina, cervix. One half of the solution was deposited in each uterine horn. A new AI pipette was used for each cow.

Blood (10 mL) and milk (150 mL) samples were collected at -1, 5, 9, and 13 h relative to infusion (0 h). Total RNA was isolated from blood (QIAamp RNA Blood Mini Kit, QIAGEN, Hilden, Germany) according to manufacturer’s instructions and from milk samples (TRIzol Reagent, Life Technologies). Isolated RNA was used for cDNA synthesis (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems™, Foster City, CA). The abundance of ISG15 mRNA were quantified with cDNA using quantitative RTPCR with peptidylprolyl isomerase A (PPIA) as a reference gene (SYBR® Green PCR Master Mix, Applied Biosystems; Table 3.1.). Data were analyzed using the MIXED procedure of SAS 9.4 for effects of dose, time, cow and their interactions.

3.3.3. Study Design. Interferon stimulated gene 15 (ISG15) expression in milk cells following in vitro addition of recombinant ovine interferon tau (roIFNT) to whole milk from dairy cows. Milk samples were taken from mid-lactation Holstein cows (n = 7) using a DHI approved sampler during the morning milking. Whole milk samples (50 mL) were
treated with increasing doses of roIFNT (0, 1, 10, 100, and 1000 pg/ml) for 2 h with continuous rotation in a 37°C incubator (Figure 3.3.0). Total RNA was isolated from cell pellets derived from whole milk samples dosed with roIFNT for each animal and time period (0, 2, and 4 h). Data were analyzed using the MIXED procedure of SAS 9.4 for effects of treatment, time, and their interactions.

3.4. RESULTS

3.4.1. Study 1 Results. Measurement of ISG15 in milk somatic cells for pregnancy diagnosis 18, 20, and 22 days after timed artificial insemination (TAI) in dairy cows. The average concentration of RNA isolated from cells derived from 200 ml of whole milk was 79.7 ± 95.7 ng/µl. This study used two cohorts of cows (n = 61). There was no effect of cohort among ISG15 expression from not pregnant and pregnant cows (P > 0.05). The ISG15 expression results were heterogeneous thus data were log transformed before testing for effects of diagnosis and DAI. Pregnant cows (0.64 ± 0.17) tended to have greater ISG15 mRNA abundance in milk cells than non-pregnant cows (0.20 ± 0.16; P = 0.059). The ISG15 expression was greater in blood of pregnant cows (0.58 ± 0.07; n = 28 compared with non-pregnant cows (0.12 ± 0.06; n = 33) on d18, 20, and 22 (P < 0.0001). The ROC curve area under the curve (Figure 3.2.4) was lesser in milk (0.6295, 0.5714, and 0.6180) compared with blood (0.8929, 0.9219, and 0.8731) for use of ISG15 expressions a detection method for pregnancy in dairy cows on d 18, 20, and 22 respectively. The sensitivity (correct identification of pregnant cows) was greater for blood (89.3%, 92.9%, and 80.8%) than milk (54.2%, 50%, and 64.3%), on 18, 20, and 22 DAI respectively. The specificity (correct identification of non-pregnant cows) was greater for blood (87.1%,
87.5%, and 83.3%) than milk (71.4%, 71.9%, and 60.6%) on 18, 20, and 22 DAI respectively. Day of sampling did not affect ISG15 expression for either milk or blood. Day 20 ISG15 expression tended to peak compared to d 18 and 22 ($P = 0.08$).

3.4.2. Study 2 Results: Interferon stimulated gene 15 (ISG15) expression in blood following intrauterine infusion of recombinant ovine interferon tau (roIFNT) in dairy cows. In blood samples, an effect of roIFNT dose by time was detected (Figure 3.1.1.; $P < 0.0001$) as ISG15 mRNA abundances did not increase after intrauterine infusion of embryo flush media alone as a control but did increase substantially after infusion of either 500 µg or 1000 µg of roIFNT with a peak response at 4 h. However, the peak ISG15 response varied between cows (Figure 3.1.2.; $P < 0.0001$) with respect to blood ISG15 mRNA abundances in response to roIFNT infusion (0.7, 54.5, 1.2, and 61.6 for cows 1 to 4; SEM = 14.9; 1000 µg dose). In milk samples, ISG15 mRNA abundances increased across sampling times independent of roIFNT infusion dose (Figure 3.1.3; $P < 0.001$) and differed between cows (Figure 3.1.4; $P < 0.001$). In contrast to blood, an increase in ISG15 mRNA was not detected ($P > 0.10$) in milk at 4 h but was detected at 12 h ($P < 0.05$) after roIFNT infusion.

3.4.3. Study 3 Results. Interferon stimulated gene 15 (ISG15) expression in milk cells following in vitro addition of recombinant ovine interferon tau (roIFNT) to whole milk from dairy cows. The milk cell pellets contained cells after 2 and 4 h incubation at 37°C. The ISG15 to cyclophilin and GAPDH ratios in pooled milk cells increased 25 to 50-fold, 2 and 4 h respectively after addition of 100 or 1,000 pg roIFNT ($P < 0.05$; Figure 3.3.2.). The expression of cyclophilin and GAPDH were not different ($P < 0.05$). Lesser doses of
roIFNT were added to individual cow milk samples (n = 4) due to the ISG15 expression in the pooled milk being much greater than physiological responses to IFNT in milk cells d 18, 20, and 22 after insemination. Cows 3332 and 3320 were excluded from the analyses due to low quantity of RNA obtained after incubation. The milk cells obtained from cows 3265 and 3318 were very sensitive to the 0.10, 1.0, 10, and 100 pg/ml roIFNT with 0.6 to 1.44-fold change in ISG15 expression after a 2 h incubation period (not shown).

3.5. DISCUSSION

The measurement of ISGs in milk somatic cells has only been tested by one other group to our knowledge (Mo et al., 2014). In testing the same methods described by this research group, we found similar difficulty in obtaining high concentration and quality RNA after using isolated cells or whole milk snap frozen with liquid nitrogen. The overall objective of our studies was to assess the utility of measuring ISG15 expression in milk cells as a method to diagnose pregnancy in cows after TAI. We hypothesized that cows would differ in ISG expression in milk cells similar to blood cells. We also hypothesized that ISG expression differed among cows due to different concentrations of IFNT released by the conceptus. To test these hypotheses, three studies were conducted to measure milk cell ISG expression in vivo (study 1 and 2) and in vitro (study 3).

Cánovas et al., (2014) compared different RNA sources (milk somatic cells, laser microdissected mammary epithelial cells, milk fat globules, and antibody-captured milk mammary epithelial cells) to improve RNA quality for RNA sequencing of the mammary gland transcriptome. The simplest and most effective RNA source was from milk somatic cells or milk fat globules (Canovas et al., 2014). However, when we attempted isolation of
milk fat globules with the RNA quantity and quality was much less than isolation of milk cells. Histology of milk samples used in this study showed that our methods targeting MEC and milk somatic cells was effective (Figure 3.3.1).

Three studies were conducted. Study 1 we isolated RNA from both blood and milk from the same cows. ISG15 ratio was determined by RTPCR. As expected we found that the ISG15 ratio was significantly increased in the blood of pregnant cows. There was an increase in ISG15 ratio in milk during pregnancy, but it was considerably less than blood.

The current theory for ISG15 detection in blood is that IFNT leaves the uterus and stimulated PBL to increase ISG15. This could also happen as cells move through the uterus to the maternal circulation. The cells in the milk are comprised of PBL and MEC. One possibility is that the PBL could enter the milk from circulation. The other possibility is that IFNT may be in the circulation and it enters the milk to stimulate both PBL and MEC. Most hormones that circulate in blood are found in milk so it is possible that the IFNT from the conceptus is found in milk. Regardless, the amount of ISG15 in milk is much less than blood and this makes it difficult to use as a pregnancy test.

One confounding aspect of our studies is that we are unsure exactly how much IFNT is being made by the embryo in the uterus. Cows could differ for embryo size and IFNT production. We infused known doses of roIFNT into the uterus to circumvent this problem (study 2). Again, the expression in blood was greater than milk. Another observation was that some cows had a robust response to IFNT infusion but other cows did not. So, the cow appears to be the major contribution to the overall ISG15 response. Perhaps the immune cells differ or perhaps the capacity of IFNT to leave the uterus differs. We attempted to examine the former response (immune cells) in chapter 4. Again, the milk
response was much less when known doses of IFNT were infused. This indicated that either
the stimulated cells enter the milk or that the IFNT must enter the milk and the efficiency
for transfer from the milk to blood is very low. We tested a third possibility in study 3,
specifically do milk cells fail to respond to IFNT. In study 3, known doses of IFNT were
used to treat milk cells in vitro. We observed a robust ISG15 response in milk cells during
this study. So, if the IFNT does reach the milk then it will cause ISG15 expression.

Collectively, our work demonstrates that there is an ISG15 pregnancy response in
milk but it is considerably less than blood. Milk cells do respond to roIFNT (study 3) so
the possibilities are that too few PBL are present in the milk of pregnant cows and that too
little IFNT enters the milk. These observations will make it challenging to develop an
ISG15 based pregnancy test in milk. Pregnancy can be detected with ISG15 expression in
milk but the overall sensitivity (Figure 3.2.4) was less than blood making it not viable for
a pregnancy test 18 to 22 DAI. Giordano et al. (2013) reported a chemical pregnancy test
Sn greater than 85% is needed to be economically efficient and the use of milk cell ISG15
expression was 66%. Similar to PBL ISG15 expression, milk cell ISG expression was less
variable in non-pregnant cows compared with pregnant cows 18, 20, and 22 DAI. There
was also less variation within and between cows at hour zero before IFNT intrauterine
infusions. There was no difference ISG15 expression by parity in milk cells or PBL (Figure
3.2.3). Previous studies resulted in no correlations between in vitro and in vivo ISG15
expressions in blood (Chapter 4). Due to time, the same comparisons were not completed
in milk cells in vivo compared to in vitro. Future research is needed to determine whether
“enough” IFNT leaves the uterus in order to stimulate an ISG expression in milk somatic
cells to become a viable early pregnancy test in dairy cattle.
3.6. CONCLUSIONS

Overall, ISG15 expression in both blood and milk somatic cells was greater for pregnant compared with non-pregnant Holstein cows. The reason for increased ISG15 expression and variability among cows is still not understood. Further investigation for novel biomarkers in milk and blood to develop a robust chemical pregnancy test in dairy cows 18 days after insemination is still needed.
Table 3.1. Gene, GenBank number, primer sequence (forward and reverse primers; 5’ to 3’) and the location of the primer within the GenBank sequence for genes amplified during the RTPCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank</th>
<th>Primer</th>
<th>Primer Sequence</th>
<th>Primer Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>ISG15</td>
<td>NM_174366</td>
<td>Forward</td>
<td>5’-CAGCCAACCAGTCTGCAGAGA-3’</td>
<td>14–36</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>5’-CCAGGATGGAGATGCAGTTCTGC-3’</td>
<td>284–306</td>
</tr>
<tr>
<td>Cyclophilin A</td>
<td>NM_178320</td>
<td>Forward</td>
<td>5’-CACCCTGTTCTTGACATCG-3’</td>
<td>23–42</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>5’-ACAGCTCAAAAGAGACCGG-3’</td>
<td>65–84</td>
</tr>
</tbody>
</table>
Table 3.2. Receiver Operator Characteristic (ROC) curve cut point, area under the curve, sensitivity, and specificity for use of interferon stimulated gene 15 expression to detect pregnancy in milk somatic cells and peripheral blood leukocytes 18, 20, and 22 days after timed artificial insemination of dairy cows

<table>
<thead>
<tr>
<th></th>
<th>Milk</th>
<th>Day 18</th>
<th>Day 20</th>
<th>Day 22</th>
</tr>
</thead>
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<tr>
<td>No. Cows</td>
<td></td>
<td>53</td>
<td>60</td>
<td>61</td>
</tr>
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<td>ISG15 to</td>
<td>Cut point</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclo Ratio</td>
<td>0.43096</td>
<td>0.43147</td>
<td>0.43963</td>
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</tr>
<tr>
<td>AUC</td>
<td>0.6295</td>
<td>0.5714</td>
<td>0.6180</td>
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</tr>
<tr>
<td>Sensitivity</td>
<td>54.2%</td>
<td>50%</td>
<td>64.3%</td>
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<tr>
<td>Specificity</td>
<td>71.4%</td>
<td>71.9%</td>
<td>60.6%</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Blood</th>
<th>Day 18</th>
<th>Day 20</th>
<th>Day 22</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. Cows</td>
<td>59</td>
<td>60</td>
<td>56</td>
</tr>
<tr>
<td>ISG15 to</td>
<td>Cut point</td>
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<td>0.35271</td>
</tr>
<tr>
<td>Cyclo Ratio</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>AUC</td>
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<td>Sensitivity</td>
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<tr>
<td>Specificity</td>
<td>87.1%</td>
<td>87.5%</td>
<td>83.3%</td>
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**Figure 3.1.0.** Study 1 design to measure ISG15 in milk somatic cells for pregnancy diagnosis 18, 20, and 22 days after timed artificial insemination (TAI) in dairy cows (n = 61).
Figure 3.1.1. Quality of RNA isolated from milk cells determined by ethidium bromide stained RNA gels. 28S to 18S bands are intact. 28S is approximately twice the intensity of 18S band.
Figure 3.1.2. Interferon stimulated gene 15 (ISG15) expression in milk somatic cells (A) and peripheral blood leukocytes (B) day 18, 20, and 22 after insemination in not pregnant (n = 33) and pregnant (n = 28) lactating cows.
**Figure 3.2.0.** Study 1 design to measure interferon stimulated gene 15 (ISG15) expression in milk somatic cells and peripheral blood leukocytes following intrauterine infusion of recombinant ovine interferon tau (roIFNT) in dairy cows.
Figure 3.2.1. RTPCR results of ISG15 expression to cyclophilin (PPIA) expression in peripheral blood leukocytes after intrauterine infusions of roIFNT. Different letters indicate difference (n = 4; P < 0.05) in means ± SE from hour zero.
Figure 3.2.2. Individual cow RTPCR results of ISG15 expression to cyclophilin (PPIA) expression [log10 (ISG15: PPIA)] in peripheral blood leukocytes after intrauterine infusions of roIFNT. Asterisk indicates difference (n = 4; P < 0.05) in means ± SE compared with hour zero.
Figure 3.2.3. RTPCR results of ISG15 expression to cyclophilin (PPIA) expression in milk somatic cells after intrauterine infusions of roIFNT. Different letters indicate difference (n = 4; $P < 0.05$) in means ± SE compared with hour zero.
Figure 3.2.4. RTPCR results of individual cow ISG15 expression to cyclophilin (PPIA) expression in milk somatic cells after intrauterine infusions of 1000 µg roIFNT. Asterisk indicates difference (n = 4; P < 0.05) in means ± SE compared with hour zero.
**Figure 3.3.0.** Setup of whole milk (50 ml) aliquots treated with recombinant ovine interferon tau then placed on a rotor in a 37°C incubator for 2 and 4 h.
Figure 3.3.1. Cell population of pellet containing neutrophils, milk fat globules, and mammary epithelial cells (MEC) obtained from pooled whole milk (50 ml) from Holstein cows 18 d after insemination.
**Figure 3.3.2.** RTPCR results of ISG15 expression in milk somatic cells from pooled whole milk from early lactation Holstein cows (n = 7) then aliquoted and treated with recombinant ovine interferon tau (roIFNT) then placed on a rotor in a 37°C incubator for 2 and 4 h. Different letters indicates difference (P < 0.05) in means compared with hour zero.
CHAPTER 4

Measurement of interferon stimulated gene 15 in peripheral blood leukocytes for pregnancy diagnosis in dairy cows

4.1. ABSTRACT

Pregnancy can be detected by measuring ISG15 mRNA (an IFNT stimulated molecule) in circulating leukocytes 18 to 22 d after AI or blood PAG 25 d after AI. Individual animals differ with respect to the increase in ISG during pregnancy. Whether individual differences in ISG are explained by the sensitivity of peripheral blood leukocytes (PBL) to IFNT or the amount of IFNT entering the circulation during pregnancy is unknown. The hypothesis was that the amount of ISG15 mRNA in PBL during early pregnancy in vivo would be correlated with the response of PBL to known doses of IFNT in vitro. If true, then individual cow differences would be explained by the amount of IFNT in circulation during early pregnancy. The objectives were to test whether some known varying doses of roIFNT would elicit a similar response in PBL in vivo and in vitro and 2) compare ISG15 expression 18, 20, and 22 d after insemination with PAG concentration 22, 25, and 28 d after insemination. These objectives were tested with measurement of ISG15 in blood samples collected 14, 18, 20, and 22 days after timed artificial insemination from Holstein dairy cows (n = 19 primiparous and n = 23 multiparous) and heifers (n=19). Total RNA was isolated from blood and the abundance of ISG15 mRNA was quantified using RTPCR. The same samples were analyzed for plasma PAG concentration. The d 14 samples were treated with increasing doses of recombinant ovine IFNT (roIFNT; 0, 1, 10, 100, and 1000 pg/ml) for 2 h at 37°C. Total RNA was isolated from blood from d 14 samples dosed with
roIFNT and from blood collected from the same cows on d 14, 18, 20, and 22. As expected, ISG15 mRNA increased \((P < 0.005)\) in pregnant cows from d 14 (baseline; 0.022 ± 0.011) to d 18 (0.050 ± 0.008), 20 (0.066 ± 0.007), and 22 (0.083 ± 0.029) but there was no effect of parity. There was an effect of dose \((P < 0.001)\) because log transformed ISG15 increased from 0 to 1000 pg/ml doses [-1.96, -1.83, -1.54, -1.05, and -0.19 (SEM=.09) for 0, 1, 10, 100, and 1000 pg/ml doses, respectively]. In pregnant cows, PAG concentrations on d 22 were correlated with PAG concentrations on d 25 \((r^2 = 0.58; P < 0.001)\).

4.2. INTRODUCTION

Interferon tau is produced by the elongating ruminant conceptus and has paracrine effects on the endometrium to signal pregnancy recognition and prevent embryonic loss. IFNT has endocrine effects as well and there is greater expression of ISGs in bovine PBL and other tissues during early pregnancy (Green et al., 2012; Hansen et al., 2017). The use of ISG expression in maternal peripheral blood for pregnancy detection in research settings in sheep (Yankey et al., 2001) and dairy cattle (Monteiro et al., 2014; Green et al., 2010; Gifford et al., 2007) has increased within the past decade. The optimum day for measuring ISGs for non-pregnancy diagnosis would be 18 days after insemination (DAI) to align with commercial farm timing of resynchronization protocols (Lucy et al., 2004). The increased ISG expression on day 18 in pregnant cows compared to 14 to 17 DAI found by Green et al., (2010) agrees with the timing of increased abundance of IFNT measured by a 2D SDS PAGE gel (Bartol et al., 1995). Multiple research groups have measured these selected ISGs in PBL of cows from different commercial herds and environments (Carvalho et al., 2016; Monteiro et al., 2014; Green et al., 2011). ISGs were also differentially expressed in
RNA from pregnant and non-pregnant bovine endometrial biopsies submitted for RNA sequencing (Forde et al., 2011).

Individual animals differ with respect to the increase in ISG during pregnancy (Mayo et al., 2016 & 2017). Whether individual differences in ISG are explained by the sensitivity of PBL to IFNT or the amount of IFNT entering the circulation during pregnancy is unknown. Therefore, the objective of the first study was to determine if the amount of ISG15 mRNA in PBL during early pregnancy in vivo was correlated with the response of PBL to known doses of IFNT in vitro. If true, then individual cow differences could be explained by the sensitivity of PBL to IFNT in circulation during early pregnancy. We then examined if the lymphocyte ISG15 mRNA was correlated with the circulating PAG later in pregnancy. If true, then a larger embryo achieving greater ISG15 during early pregnancy could be larger later in pregnancy and synthesize and secrete more PAG (Green et al., 2010).

**4.3. MATERIALS AND METHODS**

**4.3.1. Study 1: In vivo interferon stimulated gene 15 (ISG15) expression.** Blood (10 mL) samples were collected from 2 cohorts (2017 and 2018) of primiparous and multiparous Holstein cows (Figure 4.1.0; n = 61; 102 ± 12 DIM; 36 ± 20 kg/d milk yield) at 18, 20, and 22 d after TAI. Samples were placed on ice after collection and RNA was extracted on the same day. RNA samples were used for cDNA synthesis and cDNA was used in RT-PCR analysis of gene expression for ISG15 and cyclophilin (reference gene). Ratios of ISG15 to cyclophilin (ICR) were calculated. Transrectal ultrasonography diagnosis for pregnancy at 33 d or 35 d after TAI was the reference standard. The ICR of PBL were tested for the
effects of pregnancy status, day, parity, and interactions using the MIXED procedure of SAS 9.4 (Cary, NC).

4.3.2. Study 2: In vitro interferon stimulated gene 15 (ISG15) expression. Holstein dairy cows (n = 19 primiparous and n = 23 multiparous) and heifers (n = 19) were enrolled in a synchronization protocol and inseminated artificially. Blood samples were collected 14, 18, 20, 22, and 25 days after insemination (Figure 4.2). The d 14 samples were treated with increasing doses of recombinant ovine IFNT (roIFNT; 0, 1, 10, 100, and 1000 pg/ml) for 2 h at 37°C in a shaker incubator at 130 rpm. Total RNA was isolated from blood from d 14 samples dosed with roIFNT and from blood collected from the same cows on d 14, 18, 20, and 22 (Green et al., 2010). Aliquots of all original blood samples on d 18, 20, 22, and 25 were centrifuged for plasma and assayed according to manufacturer’s instructions (IDEXX, Westbrook, ME; Silva et al., 2007). Pregnancy was diagnosed by using ultrasonography on d 32 after insemination (48% conception rate; Fricke, 2002). The RNA from 29 pregnant cows (7 nulliparous, 9 primiparous, and 13 multiparous) was assayed for ISG15 by using RTPCR. The ISG15 mRNA in systemic circulation (in vivo) on d 14, 18, 20, and 22 were tested for the effects of day, parity and parity by day. The ISG15 in vitro (dose response study) was log transformed to eliminate heterogeneous variance and tested for the effects of dose, parity, and dose by parity. The REG procedure of SAS was used to determine the correlation between in vitro doses of IFNT with 18, 20, and 22 DAI ISG15 expression.
Study 3: Expression of interferon-stimulated gene 15 (ISG15) mRNA is partially predictive of pregnancy-associated glycoprotein (PAG) concentrations during early pregnancy in dairy cows and heifers. Holstein dairy cows (primiparous and multiparous) and heifers were enrolled in a synchronization protocol and inseminated artificially. Blood samples were collected 14, 18, 20, 22, and 25 days after insemination (Figure 4.2.0.). Total RNA was isolated from blood. Aliquots of all original blood samples on d 18, 20, 22, and 25 were centrifuged for plasma and assayed for PAGs according to manufacturer’s instructions (IDEXX, Westbrook, ME; Silva et al., 2007). Pregnancy was diagnosed by using ultrasonography on d 32 after insemination (48% conception rate; Fricke, 2002). The RNA from 29 pregnant cows (7 nulliparous, 9 primiparous, and 13 multiparous) was assayed for ISG15 by using RTPCR. The ISG15 mRNA in systemic circulation (in vivo) on d 14, 18, 20, and 22 were tested for the effects of day, parity and parity by day. The ISG15 in vitro (dose response study) was log transformed to eliminate heterogeneous variance and tested for the effects of dose, parity, and dose by parity.

4.4. RESULTS

4.4.1. Results: In vivo interferon stimulated gene 15 (ISG15) expression. Only the 2017 cohort is included here because all samples from open cows are not assayed (Figure 4.1.1). As expected, ISG15 mRNA increased ($P < 0.005$) in pregnant cows from d 14 (baseline; $0.022 \pm 0.011$) to d 18 ($0.050 \pm 0.008$), 20 ($0.066 \pm 0.007$), and 22 ($0.083 \pm 0.029$) but there was no effect of parity. There was more variation among pregnant cows than non-pregnant cows (Figure 4.1.2.; B and A respectively). There is also a peak in ISG15 expression on 20
DAI in pregnant cows compared to 18 and 22 DAI but was not statistically different (Figure 4.2.1.2; \( P > 0.05 \)).

4.4.2. Results: *In vitro interferon stimulated gene 15 (ISG15) expression.* There was an effect of dose (\( P < 0.001 \)) because log transformed ISG15 increased from 0 to 1000 pg/ml doses [-1.96, -1.83, -1.54, -1.05, and -0.19 (SEM = 0.09) for 0, 1, 10, 100, and 1000 pg/ml doses, respectively]. The in vitro sensitivity (minimal effective dose) of ISG15 to roIFNT was 10 pg/mL (Figure 4.2.1.4.; \( P < 0.001 \)). There was no effect of parity on the ISG15 dose response to roIFNT and the ISG15 response to the 100 pg/ml dose in vitro was not correlated (\( P > 0.10 \)) with maximum ISG15 in vivo on d 18, 20, or 22 of pregnancy.

4.4.3. Results: Expression of interferon-stimulated gene 15 (ISG15) mRNA is partially predictive of pregnancy-associated glycoprotein (PAG) concentrations during early pregnancy in dairy cows and heifers. The conception rate was 45%. As expected, ISG15 mRNA increased (\( P < 0.001 \)) in pregnant cows from d 14 (baseline; 0.012 ± 0.007) to d 18 (0.046 ± 0.007), 20 (0.068 ± 0.007), and 22 (0.052 ± 0.007) and were greater (\( P = 0.014 \)) in primiparous cows compared with multiparous cows and heifers (Figure 4.1.3). There was an increase (\( P < 0.001 \)) in PAG on d 25 after AI in pregnant cows but no effect of parity. In pregnant cows, PAG concentrations on d 22 were correlated with PAG concentrations on d 25 (\( r^2=0.58; P < 0.001 \)). PAG concentrations on d 25 were not correlated (\( P > 0.10 \)) with maximum or mean ISG15 mRNA on d 18 to 22 or ISG15 mRNA on individual days. PAG concentrations on d 22 were modestly correlated with ISG15 on
d 20 (r² = 0.20; \( P < 0.053 \)) and maximum ISG15 mRNA on d 18 to 22 (r² = 0.18; \( P < 0.066 \)).

### 4.5. DISCUSSION

ISG15 expression increased in the circulation during early pregnancy and responded to a minimal dose of 10 pg/ml of PBL in PBS in vitro. In vivo response to pregnancy and in vitro responses to a known dose of IFNT, however, were not correlated. Individual cow differences for ISG15 were not explained by the sensitivity of PBL to IFNT.

As discussed previously, the mechanism for IFNT stimulation of ISGs expressed in endometrial tissue compared with PBL is not fully understood. Due to the variation in pregnant cow expression (Figure 4.2.1.2), the measurement of ISGs in PBL is not a imperfect pregnancy test in multiparous cows but is a reliable test for non-pregnant cows and heifers (Carvalho et al., 2016; Green et al., 2011 and 2010; Gifford et al., 2007). Green et al. (2010) reported 82% and 94% true positive rate in detecting pregnant animals with MX2 and OAS1 respectively only in heifers and primiparous cows 18 DAI.

The reason for decreased ISG expression in multiparous cows 18 to 20 DAI compared to pregnant primiparous cows and heifers is still unknown (Molina-Coto, 2016; Green et al., 2010). Two popular speculations are that the difference of embryo size or metabolism of IFNT affects the ISG expression in PBL. The basal level of IFNT or ISGs or embryonic loss carryover may lead to the decreased sensitivity of ISGs in pregnant cows, specifically multiparous cows due to difference in response by immune cells to IFNT. We were not able to test this specifically. The most obvious difference between heifers and dairy cows is that cows have gone through changes in energy balance (NEFA, DMI,
glucose, insulin, etc.) associated with gestation, parturition, and lactation (Lucy et al., 1992, Rhoads et al., 2008, Berry et al., 2016). These reasons could indirectly affect the upregulation or downregulation of transcription modulating genes (STAT1 & 2, OXTR, ISGs, etc.) and stimulation of ISGs (IRF9, STAT1 & 2) in uterine endometrial epithelial cells (Hansen et al., 1997, Johnson et al., 1998, Binelli et al., 2001) but we do not know for sure. The cell type used to measure mRNA abundance of ISG15 and OAS1 is also important. There is more variation in ISG expression in pregnant cow PBMC than PMN 18 and 21 DAI (Shirasuna et al., 2012). The rise in PMN ISG15 and OAS1 expression began as soon as 5 DAI in pregnant cows.

Matsuyama et al., (2012) reported strong positive correlations with ISG15 (r = 0.88) and Mx2 (r = 0.83) mRNA abundance in PBMC of multiparous crossbred beef cows with intrauterine IFNT (µg/kg body weight) infusions. The P4 concentrations and estrous cycle length did not differ between AI and embryo transfer (ET) pregnant cows. However, the ISG15 and Mx2 expression fold-change was greater in pregnant AI cows compared to pregnant ET cows ($P < 0.05$; Matsuyama et al. (2012). To our knowledge, similar tests have not been completed in dairy heifers and cows. It still remains to be determined if metabolization of IFNT is associated with cow size or uterine condition. Future studies with reproductive tract scoring may find relationships with uterine capacity and quality with endometrial cell populations, IFNT concentrations escaping the uterine epithelial cells via exocytosis of secretory granules. The cell populations that ISGs are predominantly found (endometrial epithelial and stromal cells) are also different in presence than binucleated trophoblasts that secrete PAG during early pregnancy compared to later pregnancy. Differences in the uterine-placenta interface may be why we did not find a
stronger correlation in ISG15 expression and PAG concentration. Future research is needed to compare endometrial cell and PBL populations at 18 DAI compared to 25 DAI to determine if there is an association with ISG15 expression and PAG concentration. Until the reason for differences within and among parities is resolved, ISG15 is not a suitable biomarker of pregnancy in PBL.

We and others have found that there is considerable variation in the ISG responses across cows. We see this when cows are tested in vivo and also when known doses are infused into the uterus (chapter 3). In these studies, we examined whether a cow’s own immune cells explained the differences in ISG response. To do this we tested known amounts of IFNT into blood in vitro. Our work revealed three things. First, blood cells are very responsive to IFNT. We detected a minimum threshold of 10 pg/mL for stimulation of ISG15. This is well within the expected physiological range. Second, we find that cows differed with respect to their ISG responses to known doses. This agrees with what we see in vivo (cows are different). Finally, the in vivo response was not correlated with the in vitro response. So, the cows sensitivity to IFNT in vitro did not explain the difference in vivo ISG response. This leads us to the important conclusion that the immune system response is not dictating the overall ISG response in vitro. Thus, in all likelihood it is the amount of IFNT escaping the uterus that dictates the ISG response.

The amount of IFNT escaping the uterus could be explained by the uterus itself or the amount of IFNT being made by the embryo. There is not a lot known about the aging cow uterus but perhaps older cows have more fibrosis (scarring) and this leads to less IFNT escaping the uterus. We tested the second possibility that a larger embryo was found in some cows in study 2. When we correlated ISG15 with PAG, we only found weak
correlations. Study 2 is, of course, not a direct test of the size of the embryo because PAG is only weakly correlated with early embryonic development.

4.6. CONCLUSIONS

ISG15 expression in peripheral blood leukocytes was greater for pregnant compared with non-pregnant Holstein cows. ISG15 increased in the circulation during early pregnancy and responded to a minimal dose of 10 pg/ml when blood was tested in vitro. The in vivo response to pregnancy and in vitro responses to a known dose of IFNT, however, were not correlated. Individual cow differences for ISG15 were not explained by the sensitivity of PBL to IFNT. We failed to confirm a strong correlation between ISG15 and PAG during early pregnancy in dairy cows and heifers. Further investigation of novel biomarkers to develop a robust chemical pregnancy test in dairy cows 18 days after insemination is still needed.
**Figure 4.1.0.** Study design for measurement of interferon stimulated gene (ISG) 15 in dairy cattle peripheral blood leukocytes (Study 1) following Presynchronization-Ovsynch (PGF$_{2\alpha}$, 14 d, PGF$_{2\alpha}$, 14 d, GnRH, 7 d, PGF$_{2\alpha}$, 56 h, GnRH, 16 h, timed artificial insemination (TAI)). **PGF$_{2\alpha}$**: prostaglandin F$_{2\alpha}$; **PAG**: pregnancy associated glycoprotein assay; **US**: transrectal ultrasonography pregnancy diagnosis.
**Figure 4.1.1.** Interferon stimulated gene 15 (ISG15) expression in peripheral blood leukocytes from **pregnant and non-pregnant** lactating Holstein cows in cohort 1 (2017) 18, 20, and 22 d after insemination following a Presynch Ovsynch timed artificial insemination (TAI). Pregnancy was diagnosed by pregnancy associated glycoprotein assay on d 25 and transrectal ultrasonography on d 32.
Figure 4.1.2. Individual expression of interferon stimulated gene 15 (ISG15) in peripheral blood leukocytes from (A) non-pregnant and (B) pregnant lactating dairy cows 18, 20, and 22 d after insemination following a Presynch Ovsynch timed artificial insemination (TAI). Pregnancy was diagnosed by pregnancy associated glycoprotein assay on d 25 and transrectal ultrasonography on d 32.
Figure 4.1.3. Interferon stimulated gene 15 (ISG15) expression (LSMEAN ± SE) in peripheral blood leukocytes from pregnant heifers and lactating (primiparous and multiparous) Holstein cows 14, 18, 20, and 22 d after insemination following a Presynch Ovsynch timed artificial insemination (TAI). Pregnancy was diagnosed by pregnancy associated glycoprotein assay on d 25 and transrectal ultrasonography on d 32.
Figure 4.1.4. ISG15 expression from PBL after treatment of roIFNT of 0, 1, 10, 100, and 1000 pg/mL to whole blood aliquots and 2h incubation at 37°C.
Figure 4.2.0. Study design (Study 2 and 3) for comparing measurement of interferon stimulated gene (ISG) 15 in dairy cattle peripheral blood leukocytes in vitro (recombinant ovine interferon tau (roIFNT) treatment of whole blood samples on d 14) and in vivo (d 18, 20, and 22 after insemination) in comparison to concentration of pregnancy associated glycoproteins (PAG) following Presynchronization-Ovsynch (PGF$_{2\alpha}$, 14 d, PGF$_{2\alpha}$, 14 d, GnRH, 7 d, PGF$_{2\alpha}$, 56 h, GnRH, 16 h, timed artificial insemination (TAI)). PGF$_{2\alpha}$: prostaglandin F$_{2\alpha}$; US: transrectal ultrasonography pregnancy diagnosis.
Chapter 5

CONCLUSIONS

A series of studies were conducted. In chapter 2 we validated a commercial PAG ELISA. As expected, the commercial PAG ELISA was as accurate and sensitive method for pregnancy detection in dairy cattle. A goal of chemical pregnancy testing is to test cows earlier after AI and using milk instead of blood. In chapter 3 we asked whether ISG15 could be measured in milk. We used blood as a positive control. We found that we could detect ISG15 in milk from pregnant cows but the test was not very sensitive. We clearly showed that milk cells respond to IFNT (chapter 3) so the cells in milk can react to a pregnancy signal or hormone. The interpretation is that either too few blood cells enter the milk or too little IFNT enters the milk for a reliable test. It may be possible to improve the milk test by further refinement of the methods for cell isolation from milk. Better quality cells used for RNA isolation may give cleaner results. In the last chapter, we tried to understand more about the variability between cows. It did not appear that immune cell response explained why cows differ. It may be that the amount of IFNT leaves the uterus is too inconsistent. This may also change as the cow ages.

In respect to where this leaves us with respect to ISGs and pregnancy detection, there is ongoing work in Troy Ott’s lab. Dr. Ott’s lab is using flow cytometry to isolate specific immune cells from blood and stain for ISG. If the most responsive cells can be isolated then a more sensitive test could be developed. There was also a recent paper in the Journal of Dairy Science by Kunii et al. (in press) that showed a very large ISG response on day 17 and 18 in cervical and vaginal tissue. Sampling closer to the uterus may overcome some of the limitations. There is both basic biology to be done (how much IFNT
leaves the uterus and why) and applied work to be done (how much IFNT enters the milk and why) before a viable test is developed in milk. Current efforts by the Ott lab and new exciting data in JDS suggest that we will eventually have a reliable test for detecting the early embryo. Once this test is developed, it will in all likelihood see rapid commercialization as we have witnessed with PAG tests. This will provide farmers with an additional tool in reproductive management to help them achieve highly efficient reproduction.
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**VITA**

*Lauren Mayo* is a Riverview, FL native with a strong passion for the dairy industry. Growing up, Lauren was heavily involved in 4H and FFA projects in poultry, dairy and swine. She graduated from the University of Florida in 2013 with a B.S. in Animal Sciences specializing in Dairy Industry and a minor in Agricultural Communications. She gained her interest for a potential career in extension from competing in Dairy Challenge on the regional and national level. In the summer of 2013, she completed an internship at the W.H. Miner Agricultural Research Institute in dairy farm management and the Southern Great Plains Dairy Consortium. She completed her Master’s degree in Animal Science at the University of Kentucky in July 2015, under the guidance of Dr. Jeffrey Bewley and Dr. William Silvia. Her research focused on the efficacy of parameters measured and alerts generated for estrus by precision dairy technologies. She was a graduate student at the University of Missouri under the guidance of Dr. Matthew Lucy with a focus in reproductive biology and early pregnancy detection using dairy cattle as a model. Lauren is a 2015-2017 NIH Initiative for Maximizing Student Development Fellow under the guidance of Dr. Mark Hannink. Lauren coached the MU Intercollegiate Dairy Challenge Team and mentors the MU Dairy Club along with 10 undergraduate research assistants. Lauren is a member of the American Dairy Science Association, Dairy Cattle Reproduction Council, and American Registry of Professional Animal Scientists. She hopes to pursue a career with research and extension or dairy consulting. Additionally, she hopes to someday own a dairy farm.
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