Assessment of a Test for Pregnancy-Associated Glycoproteins in Milk from Dairy Cows

by

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ABSTRACT

ASSESSMENT OF A TEST FOR PREGNANCY-ASSOCIATED GLYCOPROTEINS IN MILK FROM DAIRY COWS

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The first objective of this thesis was to evaluate the effect of storage temperature and duration on milk pregnancy-associated glycoprotein (PAG) ELISA results. PAG level was influenced by storage duration and condition. The change in PAG level was small and the misclassification of samples was minimal. The second objective was to describe the relationship between PAG at various stages of gestation and the likelihood of successful calving. Logistic regression models were constructed for the prediction of successful calving. In cows that tested pregnant > 25 and ≤ 45 days in gestation (DIG), there was a positive curvilinear relationship between PAG level and odds of calving. In cows > 45 and ≤ 75 DIG at the time of testing, PAG level was negatively associated with the odds of calving, and for cows tested > 75 and ≤ 290 DIG PAG level was positively associated with odds of calving.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1: LITERATURE REVIEW

Pregnancy-associated glycoproteins (PAG) are part of the aspartic proteinase family and are expressed in the placenta of species in the Cetartiodactyla order, which includes cattle, pigs, sheep, and goats. In cattle, the PAG gene family contains at least 22 intact genes as well as variants. The function of PAG is not fully understood. Through their abundant expression, presence at the feto-maternal interface, and accumulation in maternal circulation we can conclude they are essential to a successful pregnancy. As PAG are a useful marker of pregnancy, the published research on PAG largely focuses on the development of pregnancy tests for commercial use in ruminants. This literature review will discuss our current knowledge of PAG and the practical considerations surrounding the use of PAG tests for pregnancy diagnosis in dairy cattle.

Pregnancy-associated glycoproteins

Pregnancy-associated glycoproteins are members of the aspartic proteinase family and originate from mononucleate and binucleate cells of the embyronic trophoblast (Xie et al., 1997, Green et al., 2000). PAG have an N-linked glycosylation, this is an attachment of a sugar molecule, glycan, to an amide nitrogen of an asparagine residue of a protein (Klisch et al., 2008). It is a complex post-translational modification. Pregnancy-associated glycoproteins are a complex group of molecules and their role in the maintenance of pregnancy is not fully understood.

As noted in a recent review by Wallace et al. (2015), PAG were identified by three independent research groups (Butler et al., 1982; Sasser et al., 1986; Zoli et al., 1991; Mialon et al., 1993). Other names for PAG include pregnancy specific protein B and pregnancy serum protein 60. Phylogenetic analyses show that there are two separate groups of PAG, one ancient

group, having arisen ~87 million years ago and a second one that is believed to be a product of a more recent gene duplication, estimated to have arisen 52 million years ago (Hughes et al., 2000). The modern PAG are particularly abundant in ruminants.

To better understand PAG and their possible function, we must consider the placenta, where PAG is produced. To maximize surface area and increase substance exchange between the fetus and dam, areas of interdigitated attachment develop which are called placentomes. Placentomes are formed from the tuft of chorionic villi of the cotyledon enmeshed with corresponding crypts of the caruncles (Peter, 2013). This microvillar junction is where the PAG secreting trophoblast cells are located. Both mononucleate and binucleate trophoblast cells express PAG. The binucleate cells also produce placental lactogen, progesterone, estrogen, and prostaglandin. These cells develop granules to release these products into maternal circulation (Peter, 2013).

Pregnancy-associated gylcoproteins are part of the aspartic proteinase family but the function and extent of their proteolytic activity during pregnancy is unknown. This is in part due to the large number of PAG molecules. Some PAG have unusual amino acid substitutions around the catalytic centre, making proteolytic activity unlikely (Xie et al., 1991). Nonetheless, proteolytic activity has been confirmed for several PAG (Teluga and Green, 2008).

There is evidence for PAG having a role in adhesion and binding proteins (Wooding et al., 2005). PAG are perfectly situated between two epithelial surfaces. To facilitate **e**fficient transport of gases and nutrients between the trophoblast and uterine surfaces, the gap between the two surface must be as narrow as possible. In the interplacentomal areas, where there is less contact, PAG are not expressed or expressed only weakly (Wooding et al., 2005). PAG have a

possible binding cleft that could engage a protein and a carbohydrate (Wooding et al., 2005). At the maternal-fetal interface, PAG could provide a variety of peptide-bonding specificities.

Due to the relative abundance of PAG and their location, they likely play a role in local immune suppression during pregnancy. Some PAG are expressed from binucleate cells and released into maternal circulation but others accumulate extensively in the stromal layer within the maternal caruncles (Wooding et al., 2005). This localization is consistent with the formation of a barrier, either physical or immunological, that could protect the fetus (Wooding et al., 2005). There is some experimental evidence that PAG can influence the immune system. Certain PAG can decrease hematopoietic cell proliferation (Hoeben et al., 1999) and increase the release of chemotactic proteins from endometrial cells (Austin et al., 1999). Certain PAG molecules have a specific bisecting glycan that could reduce target-cell susceptibility for NK-induced cell lysis (Yoshimura et al., 1996). NK-cells are potentially hazardous for trophoblast cells in bovine placentomes. Zoli et al. (1992) found that recipient cows which are carrying a fetus of a different breed had PAG levels 3 times higher than females carrying a fetus of the same breed. Similarly, interspecies pregnancy of a Spanish ibex fetus in domestic goat recipients induced abnormally high plasma levels of PAG (Fernandez-Arias et al., 1999). The reason for higher PAG levels is unknown, although it has been hypothesized that it could be due to fetal distress (Fernandez-Arias et al., 1999). A different hypothesis is that PAG are suppressing the maternal immune response, and in these cases of genetic dissimilarity, PAG levels are increased.

Another hypothesis for the function of PAG is luteotrophic action. Experimental evidence shows PAG treatment of luteal cells increases measurable prostaglandin E_2 in vitro (Del Vecchio et al., 1996). A luteotophic mechanism may explain why PAG accumulates in maternal circulation, whereas if PAG only had an immune-suppressive role this abundance in

circulation would not be biologically advantageous (Wallace et al., 2015). In a recent review, Wallace et al. (2015) note the difficulty in reconciling these two very different functions, immunomodulation and luteotrophism. Due to the quantity of PAG produced, and the complexity of the molecules, it is likely they play multiple roles in placental development and function.

Few studies have examined cow-level factors associated with PAG level. Lopez-Gatius et al. (2007) reported associations between plasma PAG concentration, measured by radioimmunoassay, and day of gestation, milk production, number of fetuses, and sire. Plasma and milk PAG levels were found to be negatively correlated with milk production in both primiparous and multiparous cows (Ricci et al., 2015). Ricci et al. (2015) also noted temporal PAG levels throughout early gestation. Plasma and milk PAG levels, as tested by an ELISA, increased from 25 d post-AI to a peak at 32 d. A trough in PAG level was seen from 53 to 60 d in plasma and from 46 to 67 d in milk. The trough was followed by a gradual increase in PAG until 102 d post insemination, the last day PAG was measured. This circulating PAG profile containing two peaks, one early in gestation and another at parturition, has been demonstrated previously (Wallace et al., 2015). The reason for an initial peak in PAG levels at approximately 30 day of gestation followed by an apparent decline is not known. This could be due to PAG variants detected by the ELISA, as some PAG are expressed early in gestation and others only later in pregnancy (Green et al., 2000).

The use of tests for pregnancy-associated glycoproteins in reproductive management

The timely and accurate diagnosis of pregnancy in dairy cattle is economically important. Several methods exist to determine the pregnancy status of dairy cows. The most

commonly used method is rectal palpation, with or without ultrasound. The sensitivity and specificity of ultrasound diagnosis have been reported as, 97.7% and 87.7%, respectively, when conducted between 26 and 33 d post AI (Pieterse et al., 1990). However, determination of test accuracy is limited by subsequent pregnancy loss and is variable due to the experience of the palpator. There has always been interest in the development of a chemical pregnancy test for cattle. The possibility to diagnose cows as pregnant or open without the presence of a veterinarian could increase convenience, and potentially decrease costs associated with delayed diagnosis. Pregnancy diagnosis has been done by measuring concentrations of progesterone in blood and milk (Nebel et al., 1989). However, due to limitations in accuracy these tests were not widely adopted. Progesterone is an indirect measure of pregnancy and progesterone can be elevated for reasons other than pregnancy.

With the identification of PAG molecules as a useful and direct marker of pregnancy, many studies have focused on the development of a PAG test to reliably diagnose pregnancy (Sasser et al., 1986; Zoli et al., 1992; Green et al., 2005). PAG can be diagnostically useful starting at 28 d post-insemination (Green at el., 2005). The first commercially available PAG assay, (BioPRYN, Biotracking; Moscow, ID) was released in 2002. This assay is for serum and is limited by the requirement to collect blood. Currently, several plasma and serum PAG tests are commercially available and have been validated (Silva et al., 2007; Romano and Larson, 2010). There is concern with the relative long half-life of PAG and the possibility of false-positive results, for cows tested early in the post-partum period, due to residual PAG levels from a previous pregnancy. Due to the multiplicity of PAG, different PAG have different half-lives. Attempts have been made to develop assays that detect PAG with a shorter-half life (Green et al., 2005). Green et al. (2005) found PAG was undetectable in the serum of 38 out of 40 cows tested

by 8 weeks post-partum. Several different PAG tests are available, though it is not clear which PAG are most abundant in maternal circulation during the different stages of pregnancy. It is also uncertain which PAG are detected by the assays (Wallace et al., 2015).

Recently, an enzyme linked immunosorbent assay (ELISA) for the detection of PAG in milk has been developed and marketed to dairy producers (Idexx Milk Pregnancy Test, Idexx Laboratories Inc., Westbrook, ME). This test has been validated and is of interest due to the potential increased convenience of testing milk samples rather than blood. In a study that included 683 cows from 8 farms, the sensitivity and specificity of the test for confirmation of previously-diagnosed pregnancy were reported as 99.2% and 95.5%, respectively, with a prevalence of 97% pregnant cows in the sample (LeBlanc, 2013). Lawson et al. (2014) found similarly high sensitivity and specificity, 100% and 97.9%, in 112 cows tested between 33 and 52 days after insemination. In the Canadian provinces of Ontario, Manitoba, Saskatchewan, Alberta and British Columbia, the milk pregnancy test is available through CanWest Dairy Herd Improvement (DHI).

As farms incorporate chemical pregnancy tests into their reproductive management programs, more research is needed to better understand the impact of sample handling and storage as samples are collected on farms and shipped to laboratories for processing. In the case of the milk PAG test, during routine DHI milk testing, metered composite milk samples are collected during milking and shipped to a central milk testing laboratory where the milk PAG ELISA is conducted. The milk samples can be subjected to delays in transit time and exposed to quite variable and potentially extreme temperature conditions. Previous studies have investigated the effect of freezing on PAG ELISA results for blood and serum (Stahmann et al., 2013). The results of these studies showed that PAG levels in blood and serum decreased following freezing

and storage for 2 weeks. No published study has evaluated the impact of sample age and storage temperature on PAG milk ELISA results.

Pregnancy-associated glycoproteins and pregnancy loss

Though no clear function of PAG has been determined, there is much interest in using PAG as an indicator of fetal and placental viability. Giordano et al. (2012) examined PAG levels following different methods of experimentally induced pregnancy loss. Cows were administered an intramuscular injection of $PGF_{2\alpha}$ or administered an intrauterine saline infusion. Despite the different methods of inducing pregnancy loss, PAG levels decreased at a similar rate for both treatments. This suggests that the treatment with $PGF_{2\alpha}$ may have disrupted the maternal-placental interface.

Multiple studies have endeavored to describe the relationship between PAG concentrations in maternal circulation and the risk of pregnancy loss. Results of these studies have shown that pregnant cows that experience pregnancy loss have lower measured PAG levels prior to loss compared to cows that went on to calve successfully. Garcia-Ispierto et al. (2012) examined PAG levels in a group of cows infected with *Neospora caninum* that were therefore at a high risk of abortion. In pregnant cows, the odds of abortion were higher for cows with decreased PAG concentrations measured on day 120 of gestation. This study included 72 cows, of which 19 aborted. Pohler et al. (2013) found cows that suffered fetal loss (n=19) had reduced PAG concentrations on day 28 post-insemination compared to cows that maintained pregnancy (n=176). This finding was similar to Thompson et al. (2010), where cows that maintained pregnancy (n=36) had greater plasma PAG concentrations on day 30 of gestation compared to

cows that underwent embryo mortality between 32-60 days of gestation (n=7). No associations were found for PAG concentrations measured after 30 days in gestation.

Contrary to these results of only low PAG being associated with later pregnancy loss, a study examining PAG levels on day 35 of gestation found the odds of fetal loss were 10 and 6.8 times higher in cows with low and high PAG levels compared to medium PAG (Lopez-Gatius et al., 2007). In this study 98 pregnancies were investigated, of which 18 suffered fetal loss. It is possible both low and high PAG levels, particularly around 28-35 days in gestation, indicate fetal or placental abnormality.

The studies reported here are subject to similar limitations. They have been conducted in single herds, with small numbers of cows suffering fetal loss. It is difficult to test and follow large numbers of cows, and only a small percentage of cows will lose pregnancies. It is also difficult to compare relative PAG concentrations across studies as different PAG testing procedures have been used. Due to the complexity of PAG molecules and the different tests used, it is likely they measured different PAG. Limited information is available on PAG levels throughout gestation and the incidence of pregnancy loss.

Economics of evaluating pregnancy

Dairy farm profitability relies on having a successful reproductive program. One component of reproductive management is the timely detection of non-pregnant cows so that they can be promptly re-inseminated. The value of a pregnancy was estimated by DeVries (2006) for a cow in a United States herd as $278. In sensitivity analysis, inputs that had the largest effect in reducing pregnancy value were increased probability of pregnancy, increased persistency of milk yield and smaller replacement heifer costs.

Pregnancy loss is common during early gestation. Between 28-45 days in gestation approximately 13% of pregnancies will be lost (Santos et al., 2004). The rate of pregnancy loss decreases as gestation progresses. From 60 days in gestation to term approximately 2.5% of pregnancies are lost (Santos et al., 2004). DeVries (2006) estimated the average cost of pregnancy loss to be $555. Due to this high percentage of loss and high cost, pregnancy diagnosis is typically done multiple times. The first pregnancy diagnosis is performed early in gestation, starting at approximately 28 days, to detect the majority of open cows. Pregnancy diagnosis is often repeated several weeks later to confirm that the cows are still pregnant. As mentioned previously, dairy producers have different options for pregnancy diagnosis and confirmation, including diagnosis by a veterinarian by rectal palpation, with or without ultrasound, and PAG assay of blood or milk.

As PAG testing is integrated into reproductive management programs in dairy herds, it is important to consider the possible economic impact. Cost-benefit evaluations of pregnancy diagnosis have been published (Oltenacu et al., 1990; Galligan et al., 2009; Ferguson and Galligan, 2011). These studies focused on early pregnancy diagnosis, from 19 to 35 days post-insemination. Regardless of the test used (chemical, ultrasound or palpation) these studies found value in detecting open cows and returning them to a re-insemination program. The calculated value of pregnancy diagnosis differed between the studies, but was consistently dependent on the accuracy of the test, the number of days open and conception rate. Giordano et al. (2012) used a dynamic model of a lactating herd to evaluate chemical tests as part of a resynchronization program. The results showed the value of a chemical test was affected by the accuracy of the test, risk of pregnancy loss, proportion of questionable diagnoses, proportion of cows removed from testing as a result of detected estrus, and the cost of the test. Pregnancy diagnosis method

should be considered as a potential area to improve herd reproductive performance and overall profitability.

Conclusions and thesis objectives

Pregnancy-associated glycoproteins are a complex and not well understood group of molecules. The use of PAG as a measure of placental viability is complicated by the changes in the type of PAG produced throughout gestation, and the lack of knowledge about which PAG are detected by the various assays. Further research is needed for the development of tests to better differentiate specific PAG. As we continue to learn about PAG we should be able to cease thinking of this varied and complex group as one type of molecule, and consider the specific functions of individual PAG.

While research continues in the biochemical classification and function of PAG, they can still be used in a practical application for diagnosis of pregnancy in dairy cattle. Therefore, the purpose of this thesis was to consider the practicalities surrounding the milk PAG pregnancy test. These include, impact of milk sample storage, if relative PAG level in milk can predict the viability of pregnancy, and the economic impact of milk PAG testing for pregnancy diagnosis.

Specifically, the research objectives were to:

1) Evaluate the effect of sample storage and storage condition on milk PAG ELISA results

2) Examine the relationship between relative milk PAG levels and pregnancy outcome in dairy cattle

3) Complete a cost-benefit analysis of the use of a milk PAG pregnancy test for confirmation of pregnancy

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CHAPTER 2. THE EFFECT OF STORAGE CONDITIONS AND STORAGE DURATION ON MILK ELISA RESULTS FOR PREGNANCY DIAGNOSIS

ABSTRACT

The objective of this study was to evaluate the effect of storage temperature and the time from sample collection to analysis on milk PAG ELISA results. No published studies have evaluated the impact of sample handling on milk PAG results. We evaluated sample storage at 5 temperature conditions: 37°C, 22°C, 4°C, -20°C, or -80°C. Sample aliquots were stored for 4, 7, 14, 28, 60, 90, or 365 days. The measured PAG level was influenced by storage duration and condition. Samples stored for 365 d increased slightly in PAG level whereas for all other storage durations the samples declined in PAG slightly compared to the initial result. The reason for an increase in PAG level following long-term storage is not known. This will not impact dairy producers using the test, but may be important in samples stored for research applications. The changes in PAG level were too small to cause a change in result classification, suggesting that although the changes were statistically significant, they were likely not biologically important.

INTRODUCTION

The timely and accurate diagnosis of pregnancy in dairy cattle is economically important. Several methods exist to determine pregnancy status of dairy cows. The most commonly used method is rectal examination with or without ultrasound. Chemical tests that detect pregnancy-associated glycoproteins (PAG) as a marker for pregnancy are now commercially available. Pregnancy-associated glycoproteins are members of the aspartic proteinase family and originate from mononucleate and binucleate cells of the embyronic trophoblast (Xie et al., 1997, Green et al., 2000). Pregnancy-associated glycoproteins can be detected in maternal circulation and are a useful marker of pregnancy (Sasser et al., 1986; Zoli et al., 1992). Pregnancy-associated

glycoproteins can be diagnostically useful starting at 28 d post-insemination (Green at el., 2005). Several plasma or serum PAG tests are commercially available and have been validated (Silva et al., 2007; Romano and Larson, 2010). Recently, an enzyme linked immunosorbent assay (ELISA) for the detection of PAG in milk has been developed and marketed to dairy producers (Idexx Milk Pregnancy Test, Idexx Laboratories Inc., Westbrook, ME). The test uses a microtiter plate coated with an anti-PAG antibody. Captured PAG is measured by colorimetric analysis following the addition of a chromogenic substrate. In the Canadian provinces of Ontario, Manitoba, Saskatchewan, Alberta and British Columbia, the milk pregnancy test is available through CanWest Dairy Herd Improvement (DHI). In a study that included 683 cows from 8 farms, the sensitivity and specificity of the test for confirmation of previously-diagnosed pregnancy were reported as 99.2% and 95.5%, respectively, with a prevalence of 97% pregnant cows in the sample (LeBlanc, 2013). Lawson et al. (2014) found similarly high sensitivity and specificity, 100% and 97.9%, in 112 cows tested between 33 and 52 days after insemination.

As farms incorporate chemical pregnancy tests into their reproductive management programs, more research is needed to better understand the impact of handling and storage condition of samples as they are collected on the farm and shipped to laboratories for processing. In the case of the milk PAG test, during routine DHI milk testing, metered composite milk samples are collected during milking and shipped to a central milk testing laboratory where the milk PAG ELISA is conducted. The milk samples can be subjected to delays in transit time and exposed to quite variable and potentially extreme temperature conditions. These delays can be due to the distance that samples must be transported or delays in shipping or processing at the lab. Temperature conditions vary due to season. In southern Ontario, average winter

temperatures are -7.0°C with average lows of -10°C and average summer temperatures are 20°C with average highs of 26°C (Environment Canada, Canadian Climate Normals, 2010).

Previous studies have investigated the effect of freezing on PAG ELISA results for blood and serum (Stahmann et al., 2013). The results of these studies showed that PAG levels in blood and serum decreased following freezing and storage for 2 weeks. No published study has evaluated the impact of sample age and storage temperature on PAG milk ELISA results.

The objective of this study was to determine if storage temperature or the interval from sample collection to analysis affect the results of milk PAG ELISA. The primary objective was to determine if storage condition could cause a significant change in test result classification; for example, a sample that originally tested pregnant that was stored, and due to a decrease in PAG level during storage or a freeze-thaw cycle had a final test classification of open.

MATERIALS AND METHODS

Milk samples were collected on dairy farms in Ontario by milk recording field staff using routine milk testing procedures followed by CanWest DHI. All samples were composite samples of all milked quarters for each cow, collected throughout milking, and preserved with bronopol. Samples were shipped to the CanWest DHI laboratory located in Guelph, Ontario, Canada. Milk samples underwent routine testing for fat, protein and somatic cell count (SCC) prior to PAG ELISA testing (Idexx Laboratories Inc., Westbrook, ME).

The ELISA reports a S-N value, which is the optical density (OD) of the sample (S) minus the OD of the negative control (N) at 450 nm. Based on the manufacturer's guidelines, samples with $S - N < 0.100$ are classified as not pregnant, $S - N \geq 0.100$ but < 0.250 as "recheck," and $S - N \geq 0.250$ as pregnant. Milk samples tested with the ELISA produce a

maximum S-N value of 4. Milk samples with a pregnant result within one standard deviation of the 'pregnant' cut-off of 0.25 were selected from those available. These samples had S - N values between 0.26 and 0.67. Samples within one SD were selected as they would be most at risk of misclassification if test result changed (decreased) over time or due to storage conditions. Samples that produced 'recheck' results were also included as a separate group. Using an effect size of 0.15 (S-N value), which was defined as the smallest difference of interest in the mean value of the outcome variable between pregnant and open samples, and a two-sided hypothesis test ($\alpha = 0.05$, power = 0.80), it was determined that 7 pregnant samples and 7 open samples would be the minimum number required to detect a change in status.

45 milk samples were selected. They were samples with test results classified as pregnant (n=20), open (n=10) and recheck (n=15). From each of the 45 samples, 15 aliquots were created, and a set of 45 aliquots were subjected to a different set of time and temperature exposures.

During a 4 week period in January and February 2014, all milk samples that were PAG tested at the CanWest DHI Laboratory were retained for 24 hours after testing. The following day, the PAG results were used to select samples for inclusion in the study. Only pregnant samples with an S - N $\leq$ 0.67 were eligible for selection. When multiple samples met the eligibility criteria, samples were selected purposively. The selected samples were aliquoted into 15 1.5mL tubes and stored for various time periods under 5 temperature conditions. The temperature conditions included incubation at 37°C to represent the extreme heat that samples might be subjected to during shipment in summer, controlled room temperature (22°C) representing normal spring or fall daytime temperatures, refrigerated (4°C) representing mild winter conditions or controlled storage, frozen (-20°C) representing cold winter conditions or planned storage and frozen (-80°C) representing research samples stored for future use. Aliquots

were stored for 4, 7, 14, 28, 60, 90 or 365 days (Figure 2.1). After storage the aliquots were tested using the same PAG ELISA protocol. Frozen samples were left to thaw at room temperature for 1 h prior to testing. Aliquots were discarded after testing. Technicians performing the ELISA were blinded to sample status.

The ELISA kit used for all milk samples in this study was the IDEXX Milk Pregnancy Test (IDEXX Laboratories, Inc., Westbrook, ME). The assay was performed according to the manufacturer's instructions. Briefly, the microtiter plates were prepared by coating an anti-PAG antibody onto the plate. After incubation of the test sample in the coated well, captured PAG was detected with a PAG-specific antibody horseradish peroxidase conjugate. Unbound conjugate was washed away, and the colour-developing substrate was added to the wells. Color development was proportional to the amount of PAG in the sample and was measured using a spectrophotometer. ELISA results were calculated from the optical density (OD) of the sample (S) minus the OD of the negative control (N) at 450 nm (with both values corrected by subtraction of the reference wavelength OD), which resulted in an S − N value. Each microplate included negative and positive control and reference samples. Based on the manufacturer's guidelines, samples are classified as not pregnant, "recheck," or pregnant according to S - N value.

Data were entered into Microsoft Excel and exported into SAS version 9.4 (SAS Institute Inc., Cary, NC). All variables were screened for abnormal or missing values. PROC MEANS was used to generate means, standard deviations and 95% CI for PAG results for pregnant, recheck and open samples at all time points. PAG level was modeled as a continuous variable using mixed linear regression (MIXED procedure in SAS). Categorical variables included storage temperature and time in storage. Sample (cow) was included as a random effect due to

multiple observations per sample. Based on univariable analysis, all explanatory variables that produced a P-value < 0.20 were retained for inclusion in the multivariable model. An interaction between temperature and storage time was tested. Manual backwards stepwise elimination was used to refine the model until only variables with P < 0.05 remained. Variables were considered confounders if there was > 20% change in the coefficient for PAG. If deemed a confounder the variable was retained in the model. The model was assessed graphically for outliers and normality of residuals. A 3 by 3 table was constructed with the initial sample classification (pregnant, open or recheck) against the sample classification after storage. Bland-Altman plots, graphing the mean PAG (S-N) of stored and fresh samples against the difference between PAG (S-N) for stored and fresh samples, were created (Bland and Altman, 1995).

RESULTS

Due to a lab error, results were not available for 6 samples that were stored for 4 days under the incubated, ambient, refrigerated and frozen (-20°C) conditions (24 aliquots). Six replacement samples were selected, stored and tested. Two aliquots were removed from the analysis; one stored at -20°C condition for 90 days soured and was not tested, and one stored at -80°C condition for 365 days did not have a valid result due to lab error. Descriptive means of the ELISA results across time in storage are presented in Table 2.1. The results of the univariable analysis are presented in Table 2.2. The final PAG multivariable model is presented in Table 2.3. Storage condition (P < 0.01) and days (P < 0.001) were significant in the model. There was no significant interaction between storage temperature and time (P = 0.67). Comparison of sample interpretation is presented in Table 2.4 and shows that no samples changed classification from open to pregnant. One sample changed from pregnant to open. All other samples that changed

classification changed within the recheck range. From the Bland-Altman plot of all stored samples (Figure 2.2), the mean difference between fresh and stored samples was 0.01, with a 95% confidence interval of 0.15 to -0.13. Figure 2.3 and 2.4 show Bland-Altman plots for the subset of samples stored at ambient temperatures for 4 days and samples stored at frozen (-80°C) temperature for 365 days, respectively, to illustrate the disparity in mean and confidence intervals for samples stored short-term compared to long-term.

DISCUSSION

There is evidence that sample handling can impact ELISA results (Holten-Anderson et al., 2003) though no studies have been published that address PAG results in milk. Milk samples taken on farm and shipped to a laboratory for testing may experience delays during transport and changes in temperature. Long term storage of samples is common practice for some research applications. However, little research exists on the validity of results from stored samples.

The results of the multivariable analysis show that both storage duration and temperature had small effects on milk PAG results. PAG level increased slightly following storage at 4, -20, or -80°C, between 0.01 and 0.02 PAG (S - N) units, compared to samples stored at 22°C for 4 d, 7 d, and fresh samples. For samples stored at 37°C, PAG level decreased slightly, by 0.02 PAG (S - N) units, compared to samples stored at 22°C for 4 d, 7 d, and fresh samples. For storage duration, in samples stored for 4, 7, 14, 28, 60, or 90 days, PAG levels decreased slightly, between 0.01 and 0.05 PAG (S-N) units, compared to the initial result. The change for samples stored for 365 days followed a different pattern: these increased in 0.09 PAG (S-N) units compared to the initial PAG result. The reason for an increase in PAG level following long-term storage is not known. It is possible during storage protein degradation released bound PAG or

denatured proteins cross-reacted with the test enzymes. The 365 day samples changed the most and in a different direction than the samples stored for shorter durations. The modelled change in PAG level due to storage condition and duration were too small to cause a major change in sample result classification for most of the samples tested, suggesting that though the changes were statistically significant, they were not likely to be biologically important.

The Idexx Milk Pregnancy Test has good repeatability, though small changes are expected due to interplate and interlot variability. The intraplate coefficient of variation for the test is approximately 3.75%-5.28% with S - N SD of 0.023-0.034 (IDEXX, 2014). The differences we observed in PAG level following storage are within this expected variation. The small differences could be due to normal variation and not due to storage duration or temperature.

Table 2.4 shows that 1 sample changed classification from pregnant to open. The initial test value for this sample had a low pregnant result. Nothing unusual was found about the sample. No samples changed from open to pregnant. Seventy-three samples changed classification within the recheck range (pregnant or open to recheck; recheck to pregnant or open). These samples were examined for commonalities. The initial result for the majority of these samples was close to the cut point and only had to change a small amount to switch classification. There was no pattern related to misclassification and storage duration or temperature. The recheck result exists due to biological variation among cows and limitations in repeatability of the test. When a sample receives a recheck result it is recommended that the cow be re-tested or examined by a veterinarian. For this reason, samples changing classification around the limits of the recheck range are expected.

The Bland-Altman plot for all the milk samples showed slight fanning, which indicates that samples with higher PAG values showed higher variability when retested. The two Bland-Altman plots for subsets of the data illustrate the difference in pattern for samples stored at -80°C for 365 days and the samples stored at 22°C for 4 days. The latter represents more likely circumstances under field conditions and there was little bias or change in results.

Due to the noted changes in PAG level for the 365 day samples and the Bland-Altman plot results, it appears long-term storage of milk samples at -80°C for PAG testing is not appropriate and may introduce meaningful error, especially for samples in the 'pregnant' range. Bland-Altman plots are used to measure agreement between two tests and we acknowledge that using the Bland-Altman plots to compare results before and after storage is not the exact application of this statistical measure.

Stahmann et al. (2013) examined the effect of freezing serum and blood samples for 2 weeks on PAG level as measured by an ELISA. They noted a decline in PAG levels that was concentration dependent, with higher variability and higher declines at larger initial PAG levels. In contrast to the small PAG changes measured in milk, this study found biologically significant declines in PAG. Milk appears to be more stable than blood and serum in regard to PAG level following storage.

Limitations of this study include the assumption that the initial PAG test result captured the true reproductive status (pregnant or open) of the cow. We found no consistent changes in classification among all aliquots for a single sample. This is additionally mitigated by the high sensitivity and specificity of the test. Another limitation was the sample selection process. Samples were selected after arrival and testing at the lab. Samples were approximately 3 days old

when they were selected for inclusion in the study and aliquoted. We do not know the conditions that the original samples were exposed to prior to arrival at the lab.

Overall, PAG level following storage was influenced by storage duration and condition. The measured change in PAG level was small and the misclassification of pregnant and open samples was minimal. PAG level in milk appears robust to many storage temperatures and durations, though frozen storage past 90 days is not recommended.

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Table 2.1. Descriptive means for milk samples initially classified as pregnant, S -N > 0.25 and < 0.67 (n=337), recheck (n=180) and open (n=208) over various storage times.

	Mean S-N		
Time (days)	**Pregnant (n=337)**	**Recheck (n=180)**	**Open (n=208)**
	Mean (SD) (95% CI)	Mean (SD) (95% CI)	Mean (SD) (95% CI)
1 (initial test)	0.47 (0.11) (0.43-0.52)	0.17 (0.04) (0.14-0.19)	0.02 (0.03) (0.01-0.04)
4	0.39 (0.09) (0.37-0.41)	0.15 (0.04) (0.14-0.16)	0.03 (0.04) (0.02-0.04)
7	0.48 (0.13) (0.45-0.52)	0.16 (0.05) (0.14-0.17)	0.03 (0.03) (0.02-0.04)
14	0.47 (0.12) (0.41-0.52)	0.17(0.04) (0.14-0.20)	0.03 (0.04) (0.01-0.06)
28	0.46 (0.12) (0.42-0.50)	0.15 (0.04) (0.14-0.17)	0.03 (0.04) (0.02-0.04)
60	0.43 (0.09) (0.40-0.45)	0.16 (0.03) (0.15-0.17)	0.03 (0.04) (0.01-0.05)
90	0.44 (0.09) (0.41-0.47)	0.17 (0.04) (0.15-0.19)	0.03 (0.04) (0.02-0.05)
365	0.72 (0.63) (0.46-0.97)	0.18 (0.04) (0.15-0.22)	0.02 (0.02) (0.01-0.04)

Table 2.2. Results of the univariable linear regression models to determine the impact of storage time or temperature on milk PAG ELISA result (cow included as random effect).

			95% CI		
Effect	**Estimate**	**SE**	**Lower**	**Upper**	**P-value**
Intercept	0.26	0.03	0.20	0.32	<0.0001
Storage temperature (°C)					
22	Referent				
37	-0.04	0.01	-0.06	-0.02	<0.0001
4	0.00	0.01	-0.01	0.01	0.87
-20	-0.01	0.01	-0.02	0.01	0.33
-80	0.03	0.01	0.02	0.04	<0.0001
Intercept	0.28	0.03	0.22	0.34	<0.0001
Days					
1	Referent				
4	-0.04	0.01	-0.05	-0.02	<0.0001
7	-0.01	0.01	-0.02	0.01	0.32
14	-0.01	0.01	-0.03	0.01	0.42
28	-0.01	0.01	-0.03	0.00	0.06
60	-0.03	0.01	-0.05	-0.02	<0.0001
90	-0.02	0.01	-0.04	-0.01	0.001
365	0.11	0.01	0.09	0.13	<0.0001

Table 2.3. Results of the multivariable linear regression model to determine the impact of storage time or temperature on milk PAG ELISA result (cow included as random effect).

			95% CI		
Variable	**Estimate**	**SE**	**Lower**	**Upper**	**P-value**
Intercept	0.28	0.03	0.22	0.34	<0.0001
Storage temperature (°C)					
22	Referent				
37	-0.02	0.01	-0.03	0.002	0.08
4	0.01	0.01	-0.01	0.02	0.3083
-20	0.01	0.01	-0.0002	0.03	0.05
-80	0.02	0.01	0.005	0.04	0.01
Storage time (d)					
1	Referent				
4	-0.04	0.01	-0.06	-0.02	<0.0001
7	-0.01	0.01	-0.03	0.002	0.09
14	-0.01	0.01	-0.03	0.01	0.21
28	-0.03	0.01	-0.05	-0.01	0.002
60	-0.05	0.01	-0.07	-0.03	<0.0001
90	-0.04	0.01	-0.06	-0.02	0.0002
365	0.09	0.01	0.06	0.11	<0.0001

Table 2.4. Contingency table comparing the initial classification of samples to the classification following storage (n=673).

	Initial			
After Storage[1]	**Pregnant**	**Recheck**	**Open**	**Total**
Open	1	46	149	313
Recheck	6	158	1	165
Pregnant	292	20	0	196
Total	299	225	150	673

[1]After storage includes samples stored at all temperatures and times evaluated

Figure 2.1. Outline of the storage temperatures and times evaluated for effect on the results of a ELISA test for PAG in milk samples

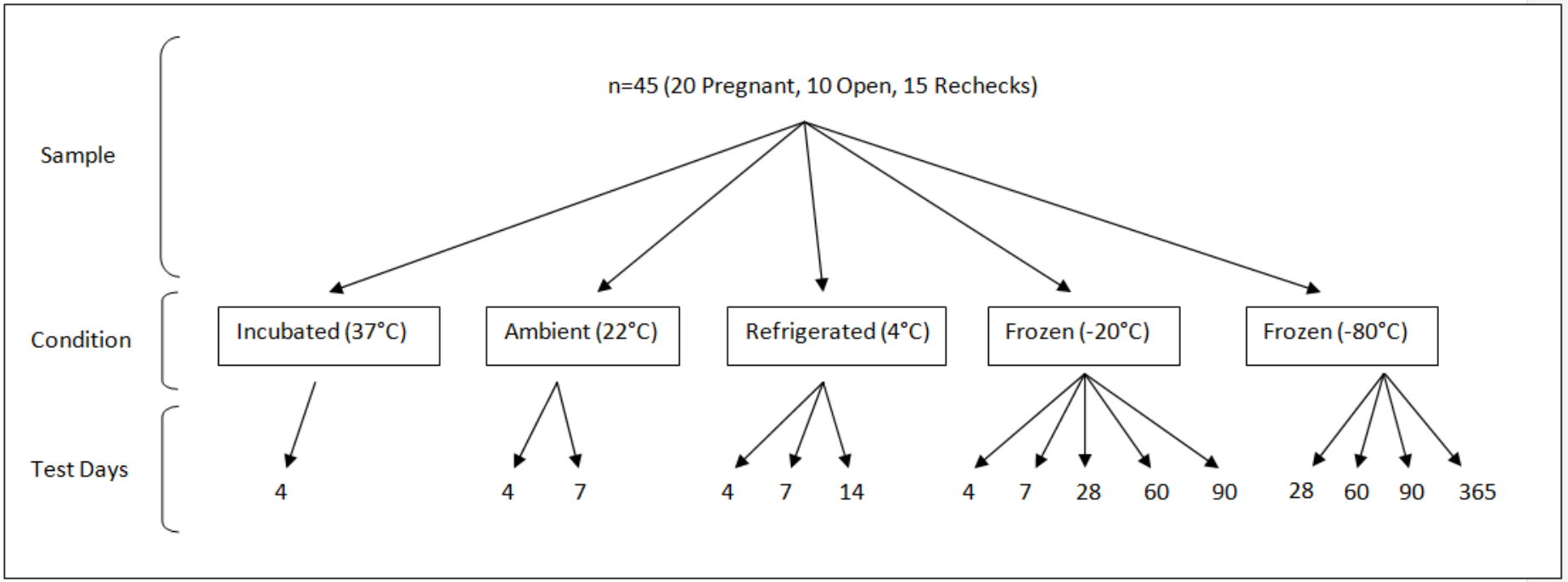


Figure 2.2. Bland-Altman plot of 673 PAG ELISA milk samples after storage for 4 to 365 d at 37, 22, 4, -20 or -80°C.

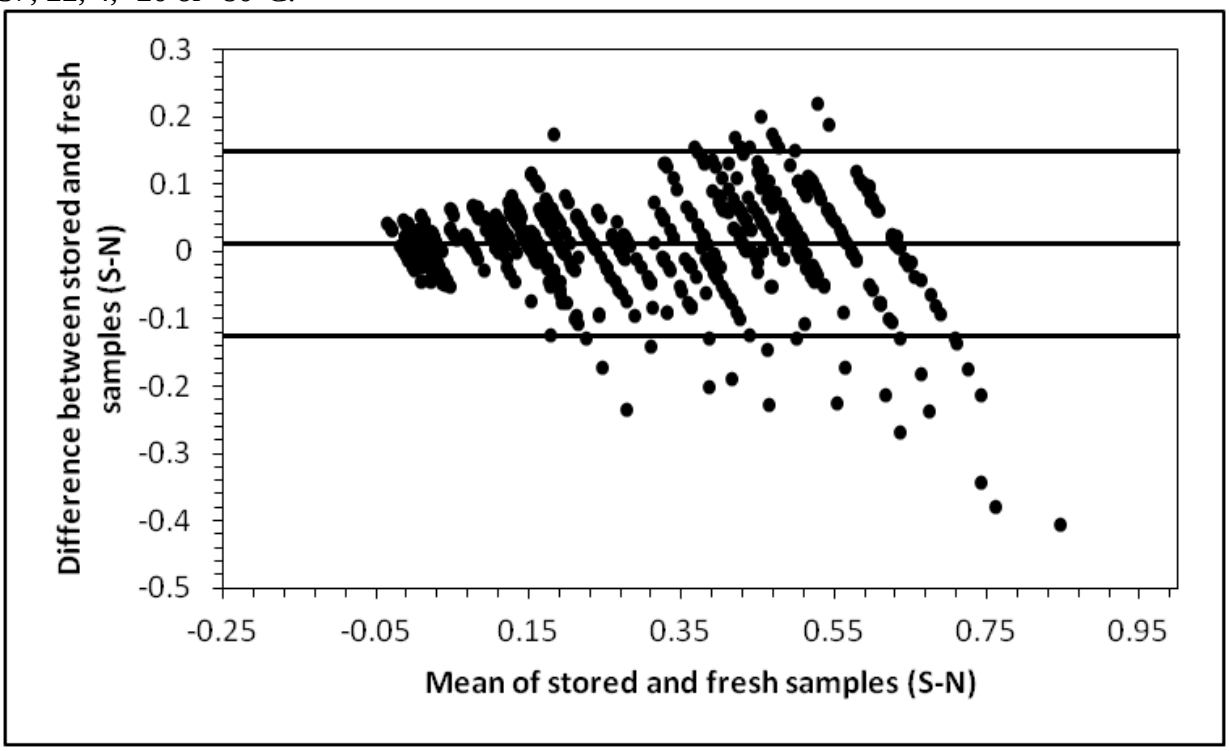


Figure 2.3. Bland-Altman plot of 45 PAG ELISA milk samples after storage at 22°C for 4 days.

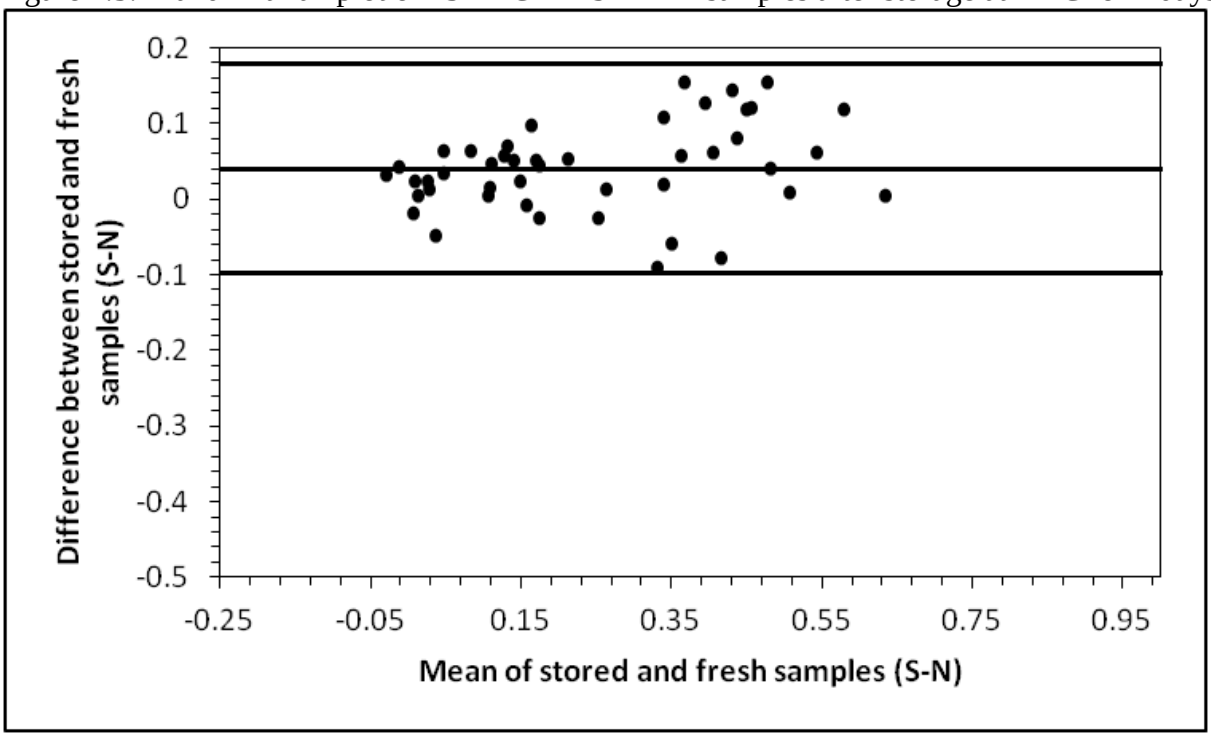
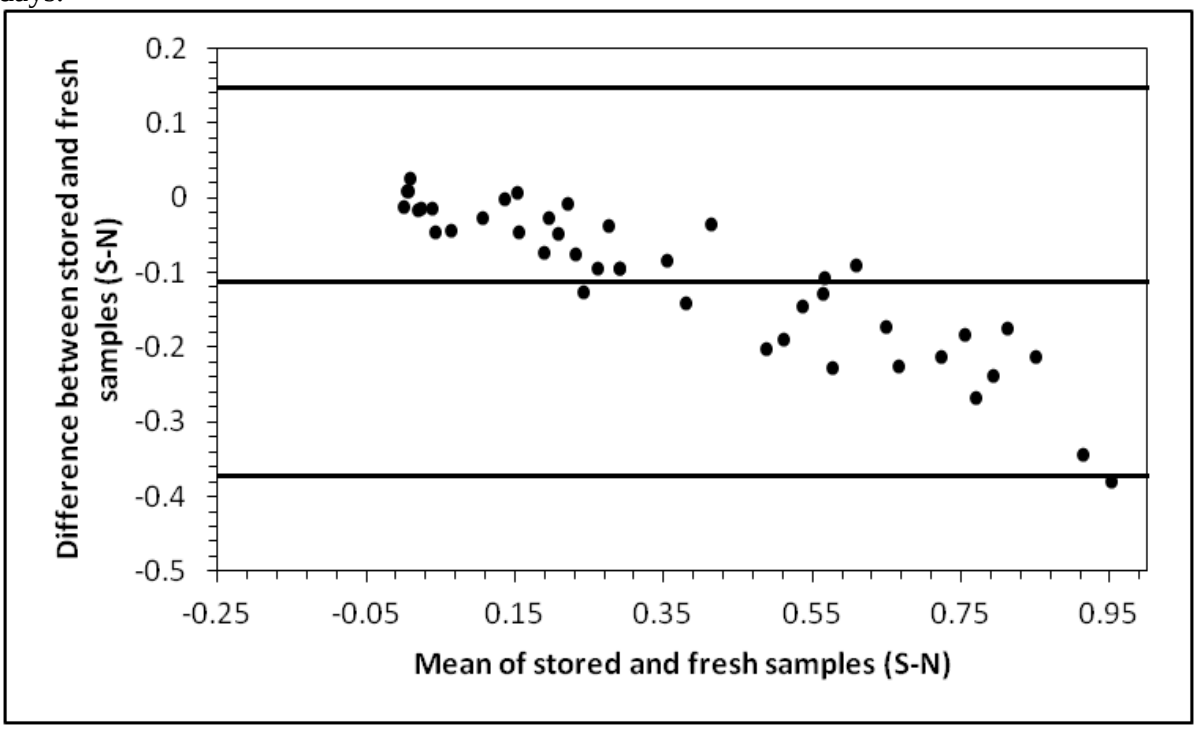


Figure 2.4. Bland-Altman plot of 44 PAG ELISA milk samples after storage at -80°C for 365 days.



CHAPTER 3: PREGNANCY OUTCOMES BASED ON MILK PREGNANCY-ASSOCIATED GLYCOPROTEIN LEVELS IN DAIRY COWS

ABSTRACT

The objective of this study was to evaluate the cow-level factors associated with milk PAG level and describe the relationship between PAG at various stages of gestation and the likelihood of successful calving. Data were collected from CanWest DHI for all milk PAG ELISA tests run from January 1 to May 31, 2013. Cows that tested pregnant were included in the analyses (n=6119). Milk PAG levels were higher after insemination, lower between 45 and 75 days in gestation (DIG), then higher again for the remainder of gestation. Pregnancy-associated glycoprotein levels were negatively correlated with milk production in both primiparous and multiparous cows. In the logistic regression model for the prediction of successful calving there was an interaction between PAG level and DIG, therefore data were stratified by DIG. In cows that tested pregnant > 25 and ≤ 45 DIG, there was a positive curvilinear relationship between PAG level and odds of calving. In cows > 45 and ≤ 75 DIG at the time of testing, PAG level was negatively associated with the odds of calving. More intuitively, in the model for cows tested > 75 and ≤ 290 DIG PAG level was positively associated with odds of calving. It is difficult to interpret these results as the function of PAG and the reason for a decrease in PAG from 45 to 75 d of gestation is unknown. It appears as measured by this test, at this time, that a decline in PAG level is desirable for successful pregnancy. However, our dataset included only one test per cow, so these data do not directly address the dynamics of PAG over time.

INTRODUCTION

The diagnosis of pregnancy and detection of pregnancy loss is important to dairy herd profitability. Early diagnosis of pregnancies allows non-pregnant cows to be re-inseminated in a timely manner. Pregnancy loss is common during early gestation. Between 28 and 45 days in gestation approximately 13% of pregnancies will be lost (Santos et al., 2004). The rate of pregnancy loss decreases as gestation progresses. From 60 days in gestation to term approximately 2.5% of pregnancies are lost (Santos et al., 2004). Due to this high percentage of loss, when diagnosing pregnancy early in gestation it is important to confirm the diagnosis later in gestation. Dairy producers have different options for pregnancy diagnosis and confirmation, including rectal palpation, ultrasound and blood or milk-based chemical tests.

Pregnancy-associated glycoproteins (PAG) are part of the aspartic proteinase family and originate from mononucleate and binucleate cells of the embyronic trophoblast (Xie et al., 1997, Green et al., 2000). In cattle, there are at least 24 transcribed PAG genes (Wallace et al., 2015). Though no clear function of PAG has been identified, they can be detected in maternal circulation and are a useful marker of pregnancy (Sasser et al., 1986; Zoli et al., 1992). PAG can be detected in both serum and milk. PAG levels begin to increase at approximately 24 days of gestation and can be diagnostically useful starting at 28 days (Green at el., 2005).
Several plasma and serum PAG tests are commercially available and validated (Silva et al., 2007; Romano and Larson, 2010). Recently, an ELISA for the detection of PAG in milk has been developed and marketed to dairy producers (Idexx Milk Pregnancy Test, Idexx Laboratories Inc., Westbrook, ME). The ELISA reports a S-N value, which is the optical density (OD) of the sample (S) minus the OD of the negative control (N) read by a spectrometer at 450 nm wavelength. Based on the manufacturer's guidelines, samples with $S - N < 0.100$ are classified as not pregnant, $S - N \geq 0.100$ but < 0.250 as "recheck," and $S - N \geq 0.250$ as

pregnant. The sensitivity and specificity of the test for confirmation of pregnancy have been reported as 99.2% and 95.5%, respectively (LeBlanc, 2013). Lawson et al. (2014) found similarly high sensitivity and specificity, 100% and 97.9%.

In addition to being a marker for pregnancy, PAG has been used as an indicator of fetal and placental viability (Giordano et al., 2012; Pohler et al., 2013). Pohler et al. (2013) found that cows that suffered fetal loss had lower PAG concentrations on day 28 post-insemination compared to cows that maintained pregnancy. Similarly, a study examining PAG levels on day 35 of gestation found the odds of fetal loss were 10 and 6.8 times more likely in cows with low and high PAG levels, respectively, compared to medium PAG (Lopez-Gatius et al., 2007). Limited information is available on the association of PAG levels throughout gestation with the risk of pregnancy loss.

Few studies have examined cow-level factors associated with PAG in milk and the potential impact on pregnancy test results. Lopez-Gatius et al. (2007) reported associations between plasma PAG concentration, measured by radioimmunoassay, and day of gestation, milk production, number of fetuses, and sire. Plasma and milk PAG levels were found to be negatively correlated with milk production in both primiparous and multiparous cows (Ricci et al., 2015).

The two specific objectives for this study were to identify cow-level factors associated with milk PAG level at the time of testing, and to describe the relationship between PAG at various stages of gestation and the likelihood of successful calving in cows with a positive pregnancy test result.

MATERIALS AND METHODS

Data collection

CanWest Dairy Herd Improvement (DHI) began offering a milk PAG ELISA (IDEXX Laboratories, Inc.) for the detection of pregnancy in January 2013. It was available to customers in Ontario, Manitoba, Saskatchewan, Alberta and British Columbia. Producers could choose to enroll all cows at a certain day post-AI or individually select cows for testing. All milk ELISA results from January 1 to May 31, 2013 were made available for this analysis (Figure 3.1). Milk samples were collected on dairy farms by milk recording field staff as per routine milk recording procedures (CanWest DHI). All samples were composites of all milked quarters for each cow, collected throughout milking using a standard sampling device, and preserved with bronopol. Samples were shipped by courier to the CanWest DHI laboratory located in Guelph, Ontario, Canada. Milk samples underwent routine testing for fat, protein and somatic cell count (SCC) prior to PAG ELISA testing.

The ELISA kit used for all milk samples in this study was the IDEXX Milk Pregnancy Test (IDEXX Laboratories, Inc.). The assay was performed according to the manufacturer's instructions. The test uses a microtiter plate coated with an anti-PAG antibody. Captured PAG is measured by colorimetric analysis following the addition of a chromogenic substrate. The PAG milk assay results were reported as relative PAG levels (S-N value) on a continuous scale from 0 to 4, and categorized into three potential outcomes: pregnant, recheck, or open.

Data obtained from CanWest DHI included PAG ELISA result, individual cow test day milk, fat and protein production, and SCC for the PAG test date, as well as projected 305 day lactation milk, fat and protein production (m305, f305, p305), breed, all insemination dates, and the next calving date, and date of removal from the herd if applicable. Information about abortion date, or the date(s) pregnancy was diagnosed by a veterinarian was not available.

Data management

Data were stored and manipulated in SAS version 9.4 (SAS Institute Inc., Cary, NC). For all cows a 'relevant insemination' was chosen. For cows that were PAG tested and did not subsequently calve, the relevant insemination selected was the insemination that occurred immediately prior to the PAG test. For cows that were PAG tested and did subsequently calve, their calving date was used to select the relevant insemination. The interval in days between each insemination prior to the PAG test and the calving date was calculated. The insemination that was between 270 and 290 days before calving was chosen as the relevant insemination. If two inseminations fell within 270 to 290 days, the one closest in time to the PAG test was chosen. This method was used to determine the relevant insemination, as it is possible for a cow to be pregnant to an insemination that preceded the insemination immediately prior to the PAG test. Once the relevant insemination was chosen for all cows, several new variables were generated. A variable for the days in gestation at the time of PAG test (DIG) was generated by calculating the number of days between the relevant insemination date and the PAG test date. A dichotomous outcome of calving between 270 and 290 d after the relevant insemination was determined for each cow (CALV). A cow classified lost to follow-up if she left the herd < 290 days after her relevant insemination.

Statistical analysis

Statistical analyses were performed using SAS version 9.4 (SAS Institute Inc., Cary, NC). All variables were screened for abnormal or missing values. PROC FREQ was used to examine the frequency of observations for all categorical variables, including the outcome. PROC MEANS was used to generate means and standard deviations for all continuous variables. Cows with a PAG result of 'pregnant' (S-N > 0.25) were included in further analyses.

PAG level was modeled as a continuous variable using mixed linear regression (MIXED procedure). The primary predictor of interest was test day milk yield. Categorical variables included breed (Holstein or other) and parity (primiparous or multiparous). Continuous variables were DIM at the time of PAG test, DIG, test day linear score (log transformed SCC), test day milk production (kg), and m305 (predicted 305 d lactation yield at time of PAG test). Herd was included as a random effect.

Logistic regression models were fitted for the outcome CALV (dichotomous outcome of calving successfully) using the GLIMMIX procedure. The PAG ELISA test was considered the experimental unit. Categorical variables included breed (Holstein or other), and parity (primiparous or multiparious). Continuous variables were DIM at the time of PAG test, DIG, PAG level, test day linear score (log transformed SCC), test day milk production (kg), and m305 (predicted lactation yield at time of PAG test). Herd was included as a random effect. The model building process was similar for both outcomes. Continuous variables were tested for collinearity (PROC CORR) prior to model building. All variables offered to the univariable model that resulted in a P-value <0.20 were entered into the multivariable model. Biologically plausible interactions were offered into multivariable models. Manual backwards stepwise elimination was used to refine the model until only variables with $P < 0.05$ remained. Variables were considered confounders if there was >20% change in the PAG coefficient if the variable was removed from the model. If deemed a confounder, the variable was retained in the model. Linearity of continuous variables was statistically assessed with the inclusion of a quadratic term. Each model was assessed graphically for outliers and linear regression models for normality of residuals.

RESULTS

CanWest DHI performed 9401 milk PAG tests between January 1 and May 31, 2013, representing 8747 cows from 592 herds. Of these, 587 PAG tests had a recheck result, of which 58 were removed due to being repeat tests, or having missing service dates or incorrect DIG. The proportions of recheck and pregnant results that went on to calve successfully are presented in Table 3.1. Further analyses used only the 6795 pregnant test results. Repeat tests and tests with missing information were removed, leaving initial pregnant results from 6119 cows in 512 herds for analysis. Descriptive statistics for continuous explanatory and outcome variables are presented in Table 3.2. The distribution of PAG values over stage of gestation at the time of the PAG test is presented in Figure 3.2. Milk PAG levels increased after insemination, decreased between 45 and 75 DIG, then increased through the remainder of gestation.

Factors associated with PAG Level

Considering PAG as the outcome in the model, there was a significant interaction ($P = 0.01$) between parity and test day milk production. Separate models were built for primiparous and multiparous cows (Table 3.3). PAG levels in milk were negatively correlated with milk production ($P < 0.0001$) for primiparous and multiparous cows when days in gestation was controlled for ($P < 0.0001$).Figures 3.3 and 3.4 show the negative relationship between predicted PAG level and test day milk yield for primiparous and multiparous cows, respectively.

The model for the prediction of successful calving included parity, test day LS, test day milk, and a significant interaction between DIG at the time of the PAG test and PAG level. Due to this interaction, the data were stratified by DIG. Strata were chosen based on graphical evidence of an initial peak in PAG followed by a decrease, and based on previous research showing a nadir in milk PAG levels between 46 and 67 days (Ricci et al., 2015). Separate logistic models were built for each of the three strata.

Calving success for cows tested pregnant >25 and ≤45 DIG

The final multivariable model for the association of PAG level and calving for cows tested at >25 and ≤ 45 DIG included parity, PAG and a PAG quadratic term (Table 3.4). The inclusion of the quadratic term was deemed sufficient to model the curvilinear relationship. For PAG values between 0.25 and 1.25, increasing PAG levels were associated with increased odds of calving. There was an inflection point and PAG values higher than 1.26 were associated with decreased odds of calving. The majority of the observations (86%) fell within the 0.25-1.25 PAG range.

Calving success for cows tested pregnant >45 and ≤75 DIG

The final multivariable logistic regression model for cows > 45 DIG and ≤ 75 DIG on the test day included parity, test day milk yield, LS, and PAG (Table 3.4). Primiparous cows had 1.4 times higher odds of successful calving compared to multiparous cows. Test day milk yield was positively associated with the odds of calving, and LS was negatively associated with the odds of calving. PAG was negatively associated with the odds of calving. For every 1 unit increase in PAG value, the odds of calving were over 50% lower (odds ratio of 0.45).

Calving success for cows tested pregnant > 75 and ≤ 290 DIG

The final multivariable model for cows > 75 and ≤ 290 DIG included parity, test day milk, LS, and PAG (Table 3.4). The associations with parity, LS and test day milk were similar to the results for > 45 and ≤ 75 day model, but the association with PAG changed direction. Increasing PAG level was associated with increased odds of calving.

Models for the prediction of successful calving were run without the inclusion of cows lost to follow-up (n=570; Table 3.5) and the pattern and magnitudes of effects were similar to the models above including all cows.

DISCUSSION

PAG level during gestation had an initial peak, then a trough from 45 d to 75 d, and finally increased for the remainder of gestation (Figure 3.2). Similar temporal PAG patterns have been described in both plasma and milk (Lawson et al., 2014; Ricci et al.,2015). A study that used the same milk ELISA test noted PAG levels increased from 25 d after insemination, had an early peak at 32 d after insemination, then reached a nadir between 46 and 76 d. This transient decrease in PAG is not well understood. It is possible that the apparent decline in PAG is due to a shift to a PAG variant that is not recognized by the ELISA.

Milk PAG levels in cows that tested pregnant were negatively correlated with test day milk production for both primiparous and multiparous cows. This finding is consistent with other studies (Lopez-Gatius et al., 2007; Ricci et al., 2015). This effect has also been seen in plasma and is therefore unlikely to be a result of dilution of PAG when milk production increases (Ricci et al., 2015). It was suggested by Ricci et al. (2015) that this reduction in PAG was due to lower progesterone in higher-producing cows leading to slower conceptus growth and lower levels of secreted PAG.

Of the 529 cows that tested 'recheck', 58% went on to calve successfully. The proportion of successful outcomes was higher, 70%, for cows that tested 'recheck' between 45 and 75 DIG. For cows that tested 'pregnant' 83% were determined to have calved successfully. This number is lower than expected. It increases when the loss to follow-up cows are removed, to 89%, but still shows a higher pregnancy loss rate than is expected.

The relationship between the odds of calving and milk PAG level depended on the stage of gestation when the test is run. The model for cows >25 and ≤ 45 DIG included parity and a

positive curvilinear relationship between PAG level and odds of calving. The majority of the observations (86%) were prior to the inflection point in the curve. Similarly, a study examining PAG level and the odds of fetal loss found that pregnancy was 10 and 6.8 times more likely in cows with low and high PAG compared to medium PAG on day 35 of gestation (Lopez-Gatius et al., 2007). PAG levels above 1.26 S-N during early gestation may indicate placental malfunction or failure of the cow to respond to embryonic signals.

The model examining this relationship for cows >45 DIG and ≤ 75 DIG showed that higher PAG levels during this period were negatively associated with odds of calving. It is difficult to interpret this result as the function of PAG and the reason for a decrease in PAG during this period of gestation is unknown. It appears, as measured by this test, at this time, that a decline in PAG level is desirable for successful pregnancy. However, our dataset included only one test per cow, so these data do not address the dynamics of PAG over time. More intuitively, the model for cows >75 DIG and ≤ 290 DIG showed a positive relationship between PAG level and odds of calving.

All models were run with cows lost to follow-up included or removed. Cows that left the herd for any reason before 290 days after the relevant insemination were removed. It is most likely these cows were not pregnant. Pregnant cows are unlikely to be culled from a herd (Grohn et al., 1998). For this reason we analyzed the data with the lost-to-follow-up cows included as unsuccessful outcomes. The associations with PAG did not change with the lost-to-follow-up cows retained in the analysis.

There are inherent limitations to using an existing dataset for secondary analysis. One limitation was that we did not have information on when or why pregnancy loss occurred. Therefore the outcome we could determine was limited. We were able to classify cows as having

calved or not. Abortion information was not recorded for the majority of herds, and not standardized between herds. Some DHI herds do not use DHI to record reproductive data. If herds do a poor or inconsistent job of recording data it can result in misclassification. In particular, there is no way to determine if some insemination dates were not recorded or cows were pregnant to an insemination prior to the insemination immediately before the PAG test. Relevant insemination dates were used to calculate the DIG for cows at the time of PAG test. If the relevant insemination were incorrect, there could be misclassification of DIG. For cows that had a subsequent calving date, the DIG was corrected, choosing the insemination that matched the next calving date. This correction could only be applied to cows that calved successfully. This misclassification is unlikely to bias the results because only a small number of tests would be affected.

The results of this study provide insight into the relationship between PAG level at various stages of gestation and the odds of calving. Due to the milk PAG ELISA recently becoming commercially available we were able to access a relatively large population of cows in many herds. This is the first study of this magnitude to examine milk PAG levels.

CONCLUSION

Among cows with a "pregnant" milk PAG test result, higher PAG levels are positively associated with a positive calving outcome in general, but a lower PAG level around 45 to 75 d in gestation was associated with a successful pregnancy outcome. The reason for lower PAG levels between 45 and 75 d is not known but it appears to be consistent with successful pregnancy. Due to the temporal pattern of PAG through gestation, when testing cows between 45 and 75 DIG a higher percentage of recheck results are to be expected.

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Table 3.1. The association of the result of a single measurement of PAG in milk with recorded calving between 270 and 290 d after insemination.

	Percent Successful Outcome (n)			
	Total	**Cows >25 and ≤45 DIG[1]**	**Cows >45 and ≤75 DIG**	**Cows >75 and ≤290 DIG**
PAG ELISA with Recheck results (n=529)	58.4 (529)	20.3 (64)	70.3 (357)	41.7 (108)
PAG ELISA with Pregnant result (n=6119)	82.7 (6119)	73.5 (751)	80.4 (1672)	85.6 (3696)

[1]Days in gestation at the time of collection of the milk sample

Table 3.2. Description of test day data for cows classified pregnant by a milk ELISA test for PAG

	Mean (SD)			
	Total (n=6339)	**Cows >25 and ≤45 DIG (n=758)**	**Cows >45 and ≤75 DIG (n=1698)**	**Cows >75 and ≤290 DIG (n=3883)**
PAG (S-N)	1.03 (0.77)	0.83 (0.40)	0.60 (0.34)	1.26 (0.86)
DIG	104 (54)	39 (4)	61 (9)	136 (46)
DIM	252 (102)	196 (96)	217 (92)	278 (98)
LS	2.90 (1.66)	2.63 (1.63)	2.85 (1.77)	2.97 (1.61)
Milk (kg)	27.1 (9.4)	29.8 (9.6)	28.4 (9.1)	25.9 (9.3)
m305	9455 (2406)	9301 (2312)	9184 (2318)	9603 (2449)

Table 3.3. Results of multivariable linear regression models for PAG level in milk from cows tested once and classified pregnant.

Variable	**Estimate**	**SE**	**P-value**
Primiparous (n=2617)			
Intercept	0.51	0.06	<0.0001
Test day milk (per 1 kg increase)	-0.01	0.0002	<0.0001
DIG (per 1 day increase)	0.01	0.002	<0.0001
Multiparous (n=3755)			
Intercept	0.54	0.04	<0.0001
Test day milk (per 1 kg increase)	-0.01	0.0002	<0.0001
DIG (per 1 day increase)	0.01	0.001	<0.0001

Table 3.4. Results of multivariable logistic regression models for predication of calving 270 to 290 d after insemination.

Variable	**Estimate**	**SE**	**P-value**
>25 and ≤ 45 DIG (n=751)			
Intercept	-0.85	0.36	0.02
Lactation			
1	0.60	0.19	<0.01
2	Referent	-	-
PAG (S - N)	3.56	0.78	<0.001
PAG*PAG	-1.45	0.36	<0.001
>45 and ≤ 75 DIG (n=1628)			
Intercept	1.52	0.36	<0.0001
Lactation			
1	0.31	0.15	0.03
2	Referent	-	-
Test day milk (kg)	0.02	0.01	0.02
PAG (S - N)	-0.80	0.19	<0.0001
LS	-0.09	0.04	0.02
> 75 and ≤ 290 DIG (n=3559)			
Intercept	1.21	0.30	<0.0001
Lactation			
1	0.26	0.12	0.02
2	Referent	-	-
Test day milk (kg)	0.02	0.01	<0.01
PAG (S - N)	0.23	0.08	<0.01
LS	-0.08	0.04	0.03

Table 3.5. Results of multivariable logistic regression models for predication of calving 270 to 290 d after insemination with cows lost-to-follow-up removed.

Variable	**Estimate**	**SE**	**P-value**
>25 and ≤ 45 DIG (n=641)			
Intercept	0.27	0.46	0.56
PAG (S - N)	3.06	0.97	0.002
PAG*PAG	-1.21	0.44	0.01
>45 and ≤ 75 DIG (n=1479)			
Intercept	3.02	0.18	<0.0001
PAG (S -N)	-1.22	0.22	<0.0001
> 75 and ≤ 290 DIG (n=3506)			
Intercept	1.55	0.27	<0.0001
Test day milk (kg)	0.02	0.01	<0.01
PAG (S - N)	0.18	0.09	<0.05

Figure 3.1. Flow-chart with all milk PAG ELISA results between January 1 and May 31, 2013.

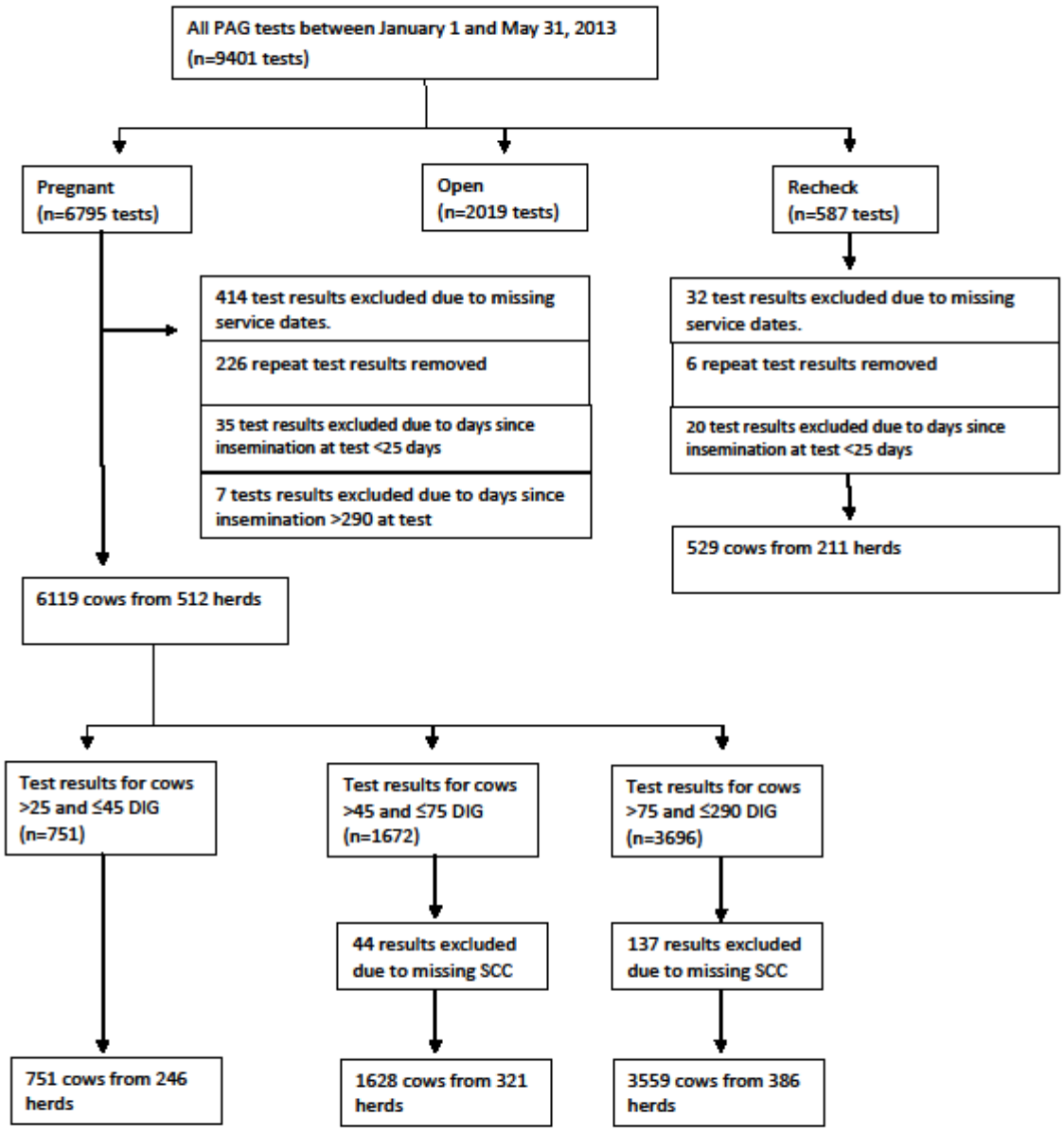


Figure 3.2. Milk PAG levels (S-N) and stage of gestation at the time of the ELISA. n=6119 tests (one test per cow) that were classified pregnant.

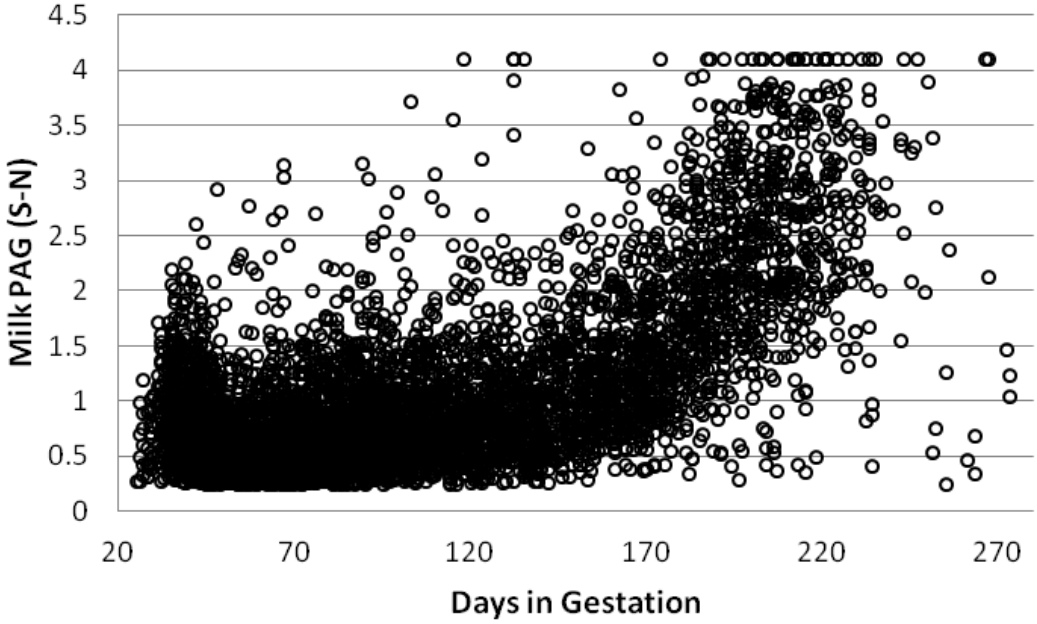


Figure 3.3: Predicted milk PAG levels by test day milk yield for primiparous cows (n=2615).

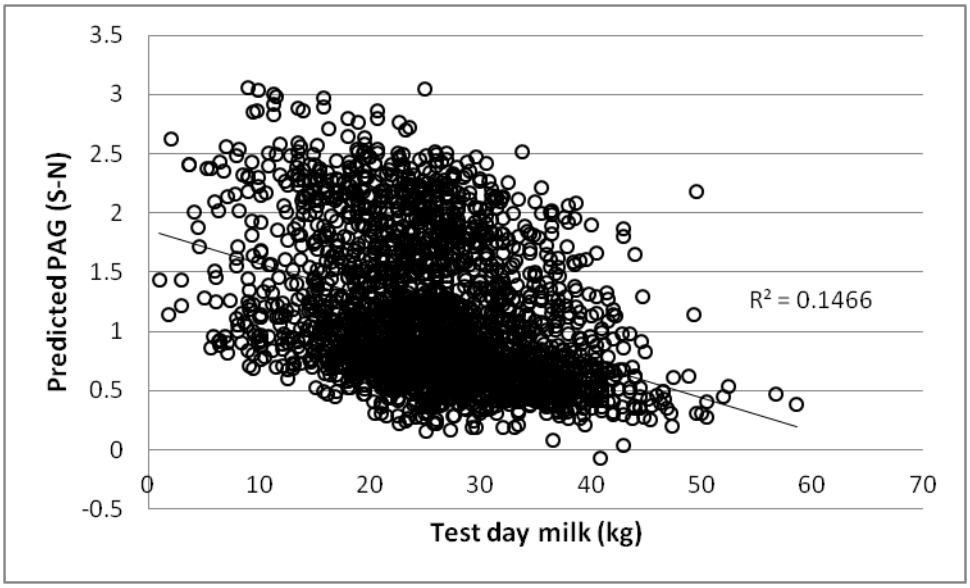
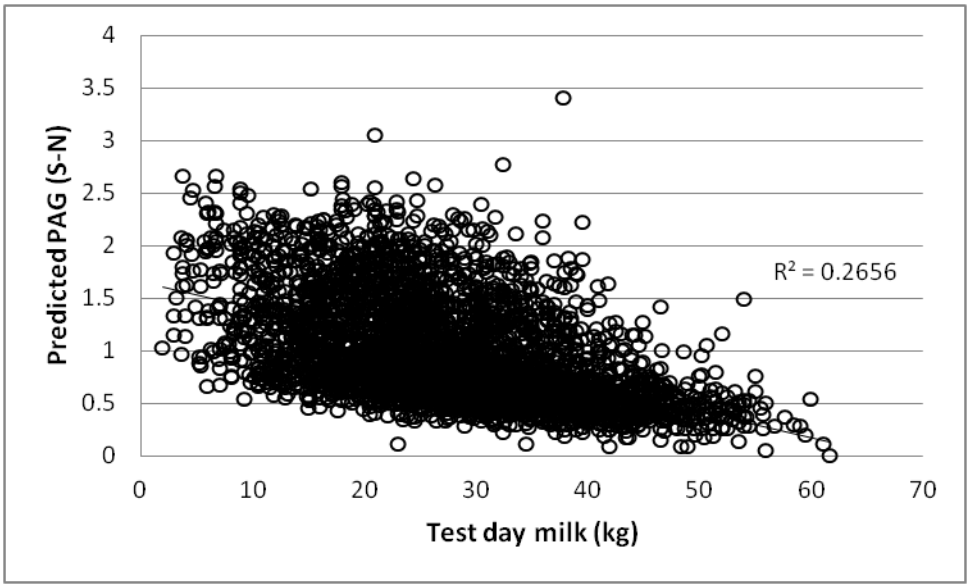


Figure 3.4: Predicted milk PAG levels by test day milk production for multiparous cows (n=3753).



CHAPTER 4: ECONOMIC EVALUATION OF PREGNANCY CONFIRMATION IN DAIRY COWS

ABSTRACT

The objective of this study was to complete a cost-benefit analysis of a milk pregnancy test for confirmation of pregnancy. The test can be used as an alternative to the typical diagnosis by palpation or ultrasound by a veterinarian. CanWest DHI currently recommends using the milk pregnancy test for confirmation of pregnancy at ≥ 60 days in gestation, so for this reason the economic analysis assumed cows had been previously diagnosed pregnant. The model included 4 simulated pregnancy confirmation strategies: no confirmatory testing, confirmation by milk PAG test, confirmatory examination by a veterinarian, and confirmation using a combination of the milk test and veterinary exam. With reasonable estimates of inputs, there was a $6.10 difference per cow tested between the milk test and the veterinarian, with the milk test being the more expensive option. The milk test had a higher total cost due to the frequency of testing and the increased cost of the test. The most costly option was no confirmatory testing. The models were most sensitive to the proportion of cows found open and culling for reproductive failure. Pregnancy loss is a costly event but the cost can be limited by pregnancy confirmation testing.

INTRODUCTION

Dairy farm profitability relies on having a successful reproductive program. One component of reproductive management is the timely detection of non-pregnant (open) cows so they can be re-inseminated. Pregnancy loss is common during early gestation. Between 28 and 45 days in gestation approximately 13% of pregnancies will be lost (Santos et al., 2004). The rate

of pregnancy loss decreases as gestation progresses. From 60 days in gestation to term, approximately 2.5% of pregnancies are lost (Santos et al., 2004). Due to this high risk of loss, pregnancy diagnosis is commonly done more than once. Typically, an early diagnosis starting at approximately 28 days, is done to detect the majority of open cows, and a confirmatory diagnosis at approximately 60 to 80 days of gestation is done to confirm that the cows are still pregnant. Dairy producers have different options for pregnancy diagnosis and confirmation, including rectal palpation and ultrasound by a veterinarian, and chemical tests applied to blood or milk.

The value of testing and knowing a cow is open or pregnant is dependent on the subsequent management decisions that can be made. By finding an open cow sooner, the cow can be rebred to limit costly days open and reduce culling due to reproductive failure. Cost-benefit evaluations of pregnancy diagnosis have been completed (Oltenacu et al., 1990; Galligan et al., 2009; Ferguson and Galligan, 2011). These studies focused on early pregnancy diagnosis, from 19 to 35 days post-insemination. Regardless of the test used (chemical, ultrasound, or palpation) the studies found value in detecting open cows and returning them to a re-insemination program. The calculated value of pregnancy diagnosis differed among the studies. Galligan et al. (2009) estimated $1.70 return per test dollar for early pregnancy testing. Reported relative values for ultrasound, rectal palpation, and a blood test were $37.00, $35.10 and $37.80 to $34.96, respectively (Ferguson and Galligan, 2011). The values are similar and suggest no strong economic difference among the methods. In all studies, the profitability of testing was consistently dependent on the accuracy of the test, the number of days open and conception risk. Giordano et al. (2012) used a dynamic model of a lactating herd to evaluate chemical tests as part of a resynchronization program. The results showed the value of a chemical test was affected by

the accuracy of the test, risk of pregnancy loss, proportion of questionable diagnoses, proportion of cows removed from testing due to detection of estrus, and the cost of the test.

CanWest DHI, a milk recording service, offers a milk test for the diagnosis of pregnancy. The test detects pregnancy-associated glycoproteins (PAG) in milk as a marker for pregnancy. The test can be used as an alternative to the typical diagnosis by a veterinarian by palpation or ultrasound. CanWest DHI recommends using the milk pregnancy test for confirmation of pregnancy at ≥60 days in gestation. The objective of this study was to estimate the economic costs and benefits of using the milk pregnancy test for confirmation of pregnancy.

MAETRIALS AND METHODS

A partial budget model was developed using Excel (Microsoft Office 2010, Microsoft, Redmond, WA) with the add-on @Risk version 6.3 (Palisade Corporation, Ithaca, NY). The model included 4 simulated pregnancy confirmation strategies. These included no confirmatory testing, confirmation by milk test, confirmatory rectal palpation by a veterinarian, and confirmation using a combination of the milk test and palpation, depending on whichever occurred closest to 60 days in gestation. Model assumptions were that all cows were diagnosed pregnant by a veterinarian at 30 days in gestation, that cows became eligible for pregnancy confirmation at 60 days in gestation, and that the herd had biweekly veterinary visits and was enrolled in CanWest DHI milk recording with a milk test every 5 weeks.

The final model considered total net economic cost to be the cost of the test + losses due to days open + losses due to culling for reproductive failure. For each scenario, 1000 @Risk simulations were run generating a minimum, maximum and mean value.

Inputs for cost of confirmatory test

As it was a model assumption that the herd was enrolled in milk recording and had biweekly veterinary visits, the baseline costs for these services were not included. The additional charge for the milk pregnancy test was $6 per test. The milk test gives 3 possible results; open, pregnant or recheck. The recheck result is an uncertain diagnosis and it is recommended these cows be examined by a veterinarian. The proportion of recheck results is highest between 45 and 75 days of gestation, when PAG levels are lower than at 25 to 45 or > 75 days. The proportion of recheck results and the additional cost of veterinary examination are included in the model. The cost of pregnancy confirmation by a veterinarian was $2. For the combination scenario of milk test and veterinary confirmation, the test used was chosen based on whichever happened closest to 60 days, as this was the earliest cows were considered eligible for confirmatory testing. There was no test cost for the no-confirmation testing scenario.

Inputs for premature culling

Inputs for the model that were used to calculate the loss attributable to culling for reproductive failure (i.e. cows found not to be pregnant that would be too late in lactation for reinsemination to be economically feasible) remained the same for all scenarios and were not varied. They included the percentage of the herd that is in first lactation (30%), replacement heifer value ($2000), cull cow value ($1500), milk yield (9300 kg), milk reduction for a heifer (1860 kg), and Canadian milk price ($0.78 per L). Average milk yield was estimated from CanWest DHI milk recording data. The milk reduction for a heifer represents a 20% decrease in milk production of a primiparous cow compared to a multiparous. It was assumed a culled cow would be replaced by a heifer.

Inputs for increased days open

In the model following an open diagnosis, cows can be culled due to reproductive failure or rebred. The increased number of days open applies only to cows that will be rebred. It differs depending on the test used due to the differences in frequency of testing. A discrete uniform distribution was used to represent the possible longer number of days open, with each value having an equal probability. Given the assumptions, the possible values for the milk test were 1 to 35 days. The possible values for the veterinary confirmation were 1 to 14 days. For the combination scenario of milk test and veterinary confirmation, the test that resulted in the confirmation being performed closest to 60 days was selected. For no confirmation testing, a discrete uniform distribution was also used to represent the possible number of days open. The number of days open ranged from 1 to 220. In this scenario, as with the others, cows became eligible to be confirmed pregnant at 60 days in gestation. The wide range of possible days encompasses the possibility of finding an open cow quickly by estrus detection or much later via failure to calve. The cost of a day open was $2 (LeBlanc, 2007). As this is a confirmatory pregnancy diagnosis, cows would be over 100 DIM at the time of open diagnosis.

Inputs for percentage of cows found open and eligible to rebreed

The percentage of cows found open was set at 12% (Santos et al., 2004) for all scenarios. The cows found open were then eligible to be rebred. The percentage of cows eligible to be rebreed was 80% for the milk test, veterinarian, combination and no confirmatory testing scenarios. As the testing scenarios would be applied in a dynamic herd with cows at all stages of lactation, the 80% value was chosen to represent a system in which the majority of cows would go on to be rebred following an open diagnosis, with a smaller proportion (20%) being culled.

Sensitivity analysis

Sensitivity analysis was completed to explore the model sensitivity to changes in inputs. The change in outcome was observed when one input at a time was changed from the baseline input assumed in the model and all other inputs remained constant (Table 4.1). As the model was stochastic, the mean value was used to represent the result over many iterations. For the additional days open input, the minimum and maximum values used for sensitivity analysis were the minimum and maximum from the defined range in the selected distribution. For the herd reproductive inputs, percent cows found open and percent cows eligible to rebreed, reasonable minimum and maximum values were chosen to represent the breadth of herd reproductive performance.

RESULTS

In all of the modeled scenarios, pregnancy loss is costly to the herd. In the modeled scenario for no confirmatory testing, the partial cost was $57.60 per pregnancy (Table 4.2). The total cost for the milk test was $45.80 per pregnancy, a difference of $11.80 compared to no confirmatory testing. The total net loss for the veterinarian was $39.70 per pregnancy, very similar to the total net loss for the combination scenario, $39.90 per pregnancy. In all of the models, the benefit of confirmatory testing compared to no confirmatory testing was between $11.80 and $17.90. The sensitivity analysis for the no confirmatory testing model showed it to be sensitive to all inputs (Figure 4.1). The sensitivity analysis for the veterinarian and milk test showed the model to be most sensitive to the proportion of cows eligible to be rebred and the proportion of cows found open (Figures 4.2 and 4.3). These 2 models were much less sensitive to the cost of a day open, the number of days open, cost of the test, and the proportion of recheck results.

DISCUSSION

Previous economic models have examined the value of pregnancy diagnosis in dairy cows (Oltenacu et al., 1990; Galligan et al., 2009; Ferguson and Galligan, 2011). This previous research focused on initial diagnosis of pregnancy, and the use of chemical tests on blood samples collected by farm staff as an alternative to examination by a veterinarian. The current study examines a milk test within the constraints of the test being offered through a milk recording company during routine testing. As it is only available during scheduled milk recording visits, the frequency of testing is constrained to the typical 10 tests per year. No studies exist examining the economics of the milk pregnancy test for confirmation of pregnancy.

The results of the cost-benefit analysis show that it is beneficial to do confirmatory pregnancy testing. Pregnancy loss is costly and its cost can be limited by confirmatory testing which leads to a reduced number of days open and reduced number of cows culled due to reproductive failure due to undetected pregnancy loss. When comparing the milk test to pregnancy confirmation by a veterinarian, the value of confirmation by a veterinarian was $6.10 higher per pregnancy. This is due to the increased cost of the milk test and the testing frequency. The combined approach, selecting either the veterinarian or the milk test based on the test closest to 60 days in gestation, had equal economic benefit to pregnancy confirmation only by the veterinarian.

In the 4 modeled scenarios, the baseline proportion of cows eligible to rebreed remained the same. It is not unreasonable to assume in a scenario that results in longer time until detection of non-pregnancy the proportion of cows eligible to rebreed would decrease, because cows would be later in lactation when found open and a higher proportion would be culled. This possibility is represented by a change in values to the percent eligible to rebreed input (Table 4.2). A decrease of 1% for the no confirmation scenario and an increase of 1% for the milk test

results in a difference of cost between these two scenarios of $15.00, compared to a difference of $11.80 when the percent eligible to rebreed remains constant. The model likely under-estimates the cost of the no confirmation scenario.

Sensitivity analysis showed the no confirmatory testing model was sensitive to all inputs. This is due to the large range of days open, and with these much longer days open the cost per day is more influential. The sensitivity analysis for the veterinarian and milk test showed the model to be most sensitive to the percentage of cows eligible to be rebred and the percentage of cows found open, and much less sensitive to the cost of a day open, the number of days open, the cost of the test, and the proportion of recheck results. In the model, as more open cows are found, more cows are culled due to reproductive failure, and pregnancy loss and culling due to reproductive failure are costly. Other research has shown models to be sensitive to the accuracy of the test (Giordano et al., 2012). These were not included as inputs for this model. The milk pregnancy test has a high sensitivity and specificity for confirmation of pregnancy (sensitivity and specificity of 99.2% and 95.5%, respectively; LeBlanc, 2013). The test sensitivity and specificity would have a lesser impact in this model due to the population considered, where we expect the majority of cows to test pregnant. In this population where the expected prevalence of pregnancy is high the positive predictive value of the test will also be high.

This model was set assuming biweekly veterinary visits. If a herd had once-monthly veterinary visits instead of biweekly, the value of the milk test would increase. This is due to an increase in losses for days open for the veterinary scenario as open cows would not be detected as quickly. Other factors, unaccounted for in the model, may increase the value of using the milk test. As the milk samples are collected as part of herd monitoring there is no additional time or labour for the producer. If a herd had poor cattle handling facilities and it was therefore more

difficult to conduct herd health or took longer, the milk test could be cost-effective.

Improvements to the model could be made by developing better estimates for inputs. The relationship between finding an open cow and her outcome (rebreed or culling) depends on many factors unaccounted for in the model. These include cow (e.g., DIM, production, age, and health) and herd (pregnancy risk) factors. There is a relationship between the number of days open and increased culling due to reproductive failure. In the current model these inputs can be manipulated independently, but the relationship between them should be explored and included.

In conclusion, with reasonable estimates of inputs there was a $6.10 difference per cow tested between the milk test and veterinary examination, with the milk test being the more expensive option. The most costly option was no confirmatory testing. This was driven by the proportion of cows found open and the proportion of these that would be culled.

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Table 4.1. Inputs for calculating the cost of pregnancy confirmation testing and the range of values for sensitivity analysis.

	Base	Minimum	Maximum
Percentage of herd that is first lactation	30%		
Replacement heifer value	$2000		
Cull cow value	$1500		
Milk yield	9300 kg		
Milk reduction for a heifer[1]	1860 kg		
Milk price	$0.78		
Cost of milk test	$6	$2	$7
Cost per pregnancy diagnosed by veterinarian	$2	$1	$6
Additional days open (milk test)		1	35
Additional days open (veterinarian)		1	14
Additional days open (no confirmation)		1	220
Cost of a day open	$2	$1	$5
Proportion of cows found open[2]	12%	5%	20%
Proportion of cows eligible to rebreed[3]	80%	60%	90%
Percent 'recheck' milk result	8%	1%	15%

[1]Represents a 20% decrease in milk production of a primiparous cow compared to a multiparous as it was assumed a culled cow would be replaced by a heifer

[2]Proportion of cows previously diagnosed pregnant that are found not to be pregnant

[3]Proportion of cows found not pregnant at confirmatory testing that are eligible to be re-inseminated

Table 4.2. The total net loss for pregnancy confirmation testing for different scenarios. Column 1 shows the total net loss at baseline values. Column 2 shows the total net loss after changes to the percent cows eligible to rebreed input. Values in the parentheses are the percent cows eligible to rebreed.

	Total net loss (baseline)	**Total net loss (% eligible to rebreed)**
No confirmation	$57.60	$59.10 (79%)
Milk test	$45.80	$44.10 (81%)
Veterinarian	$39.70	$37.90 (81%)
Combination	$39.90	$38.10 (81%)

Figure 4.1. A tornado plot based on the estimated cost per pregnant cow of using no confirmatory testing of pregnancy. The plot depicts the change in the cost for 1 pregnancy due to the change of 1 input at a time from the minimum to the maximum value. Values in parentheses are baseline values used in model building. The line in the middle of the plot represents the losses for 1 pregnancy at the baseline values. Values on the sides of the bars are the minimum and maximum values used for sensitivity analysis.

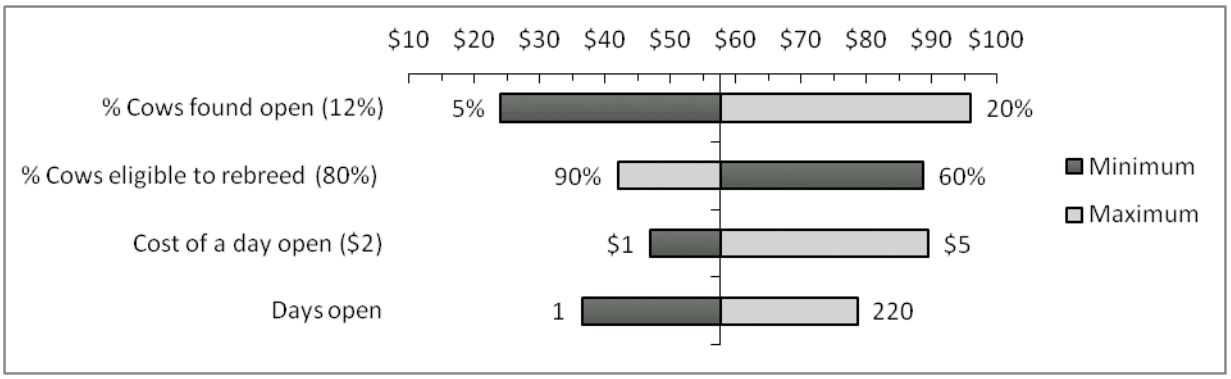


Figure 4.2. A tornado plot based on the estimated cost per pregnant cow using veterinary examination. The plot depicts the change in the cost of 1 pregnancy due to the change of 1 input at a time from the minimum to the maximum value. Values in parentheses are baseline values used in model building. The line in the middle of the plot represents the losses for 1 pregnancy at the baseline values. Values on the sides of the bars are the minimum and maximum values used for sensitivity analysis.

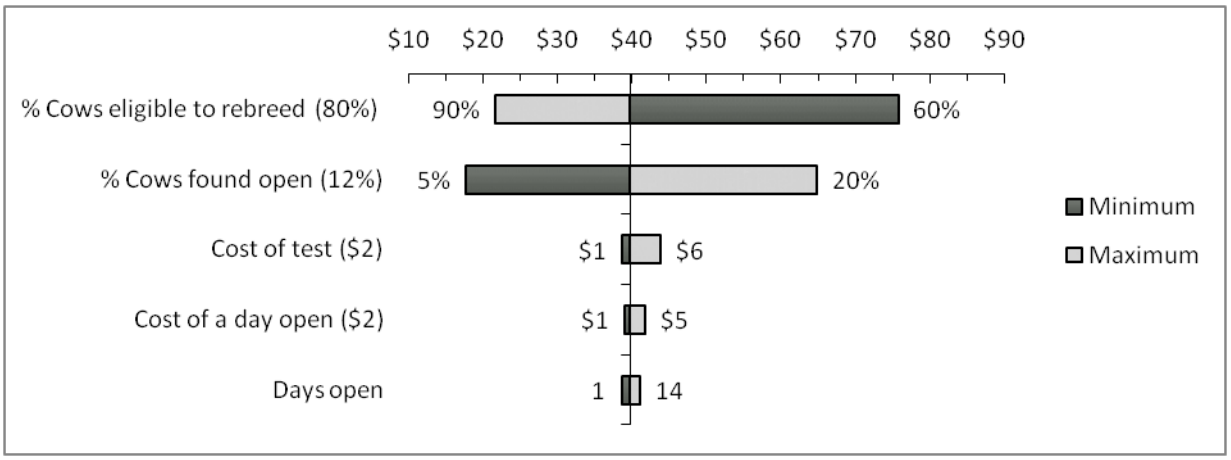
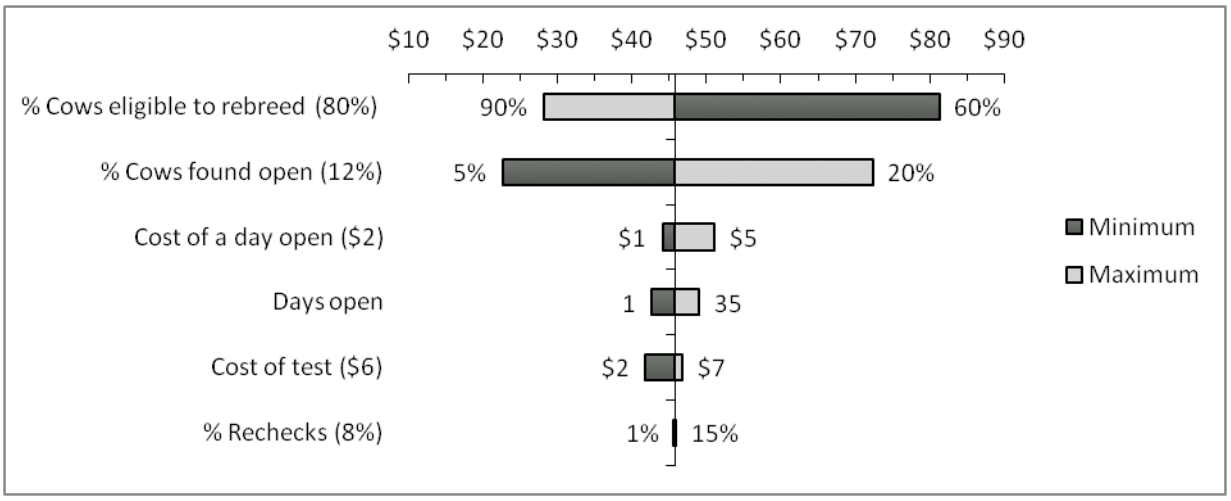


Figure 4.3. A tornado plot based on the estimated cost per pregnant cow using the milk test. The plot depicts the change in the cost of 1 pregnancy due to the change of 1 input at a time from the minimum to the maximum value. Values in parentheses are baseline values used in model building. The line in the middle of the plot represents the losses for 1 pregnancy at the baseline values. Values on the sides of the bars are the minimum and maximum values used for sensitivity analysis.



CHAPTER 5: GENERAL CONCLUSIONS

Pregnancy-associated glycoproteins (PAG) are a complex group of molecules and their full function is not understood. They are produced by the mononucleate and binucleate cells of the embryonic trophoblast. They can be detected in maternal circulation in both serum and milk. They have proven to be a useful marker of pregnancy. Much of the research surrounding PAG is focussed on the development and evaluations of tests for pregnancy diagnosis in dairy cattle. A milk PAG pregnancy test is commercially available. In the Canadian provinces of Ontario, Manitoba, Saskatchewan, Alberta and British Columbia, the test is available through CanWest DHI, a milk recording service. The overall objective of this thesis was to assess practical considerations surrounding the use of the milk pregnancy-associated glycoprotein test in dairy cows.

The first objective of this thesis was to evaluate the effect of storage temperature and the time from sample collection to analysis on milk PAG ELISA results. No published studies have evaluated the impact of sample handling on milk PAG results. We evaluated sample storage at 5 temperature conditions: 37°C, 22°C, 4°C, -20°C, or -80°C. Sample aliquots were stored for 4, 7, 14, 28, 60, 90, or 365 days. The measured PAG level was influenced by storage duration and condition. Samples stored for 365 d increased slightly in PAG level whereas for all other storage durations the samples declined in PAG slightly compared to the initial result. The reason for an increase in PAG level following long-term storage is not known. This will not impact dairy producers using the test, but may be important in samples stored for research applications. The modelled changes in PAG level due to storage condition and duration were too small to cause a change in result classification, suggesting that although the changes were statistically significant, they were likely not biologically important. Limitations for this study include the sample

selection process. Milk samples were approximately 3 days old when they were selected for inclusion in the study and aliquoted. The conditions the original samples were exposed to prior to inclusion in the study are unknown but represent the reality of field conditions. Overall, the measured change in PAG level was small and the misclassification of samples was minimal. PAG level appears robust to many storage temperatures and durations, though frozen storage past 90 days is not recommended.

The second objective was to evaluate the cow-level factors associated with milk PAG level and describe the relationship between PAG at various stages of gestation and the likelihood of successful calving. We wanted to examine the change in PAG levels throughout gestation and determine if PAG levels convey meaningful information about the maintenance of pregnancy. Data were collected from CanWest DHI for all milk PAG ELISA tests run from January 1 to May 31, 2013. Cows that tested pregnant were included in the analyses (n=6119). Milk PAG levels were higher after insemination, lower between 45 and 75 days in gestation (DIG), then higher again for the remainder of gestation. Pregnancy-associated glycoprotein levels were negatively correlated with milk production in both primiparous and multiparous cows. In the logistic regression model for the prediction of successful calving there was an interaction between PAG level and DIG, therefore data were stratified by DIG. In cows that tested pregnant > 25 and ≤ 45 DIG, there was a positive curvilinear relationship between PAG level and odds of calving. In cows > 45 and ≤ 75 DIG at the time of testing, PAG level was negatively associated with the odds of calving. More intuitively, in the model for cows tested > 75 and ≤ 290 DIG PAG level was positively associated with odds of calving. It is difficult to interpret these results as the function of PAG and the reason for a decrease in PAG from 45 to 75 d of gestation is

unknown. This temporal PAG pattern has implications for producers using the test, when testing cows between 45 and 75 DIG a higher proportion of recheck results are to be expected.

It appears as measured by this test, at this time, that a decline in PAG level is desirable for successful pregnancy. However, our dataset included only one test per cow, so these data do not directly address the dynamics of PAG over time. This study used an existing dataset for secondary analysis and as a result there are limitations. One limitation was that we did not have information on when or why pregnancy loss occurred. Therefore the outcome we could determine was limited. Additionally, if insemination dates were missing, incorrect, or a cow was pregnant to an insemination prior to the one immediately before the PAG test, there could be misclassification of days in gestation. Due to the milk PAG ELISA recently becoming available we were able to access a relatively large population of cows in many herds. This is the first study of this magnitude to examine milk PAG levels.

The third objective was to complete a cost-benefit analysis of the milk pregnancy test for confirmation of pregnancy. The test can be used as an alternative to the typical diagnosis by palpation or ultrasound by a veterinarian. CanWest DHI currently recommends using the milk pregnancy test for confirmation of pregnancy at ≥ 60 days in gestation, so for this reason the economic analysis assumed cows had been previously diagnosed pregnant. The model included 4 simulated pregnancy confirmation strategies: no confirmatory testing, confirmation by milk PAG test, confirmatory examination by a veterinarian, and confirmation using a combination of the milk test and veterinary exam. With reasonable estimates of inputs, there was a $6.10 difference per cow tested between the milk test and the veterinarian, with the milk test being the more expensive option. The milk test had a higher total cost due to the frequency of testing and the increased cost of the test. The most costly option was no confirmatory testing. The models were

most sensitive to the proportion of cows found open and culling for reproductive failure. Pregnancy loss is a costly event but the cost can be limited by pregnancy confirmation testing. Future economic research could evaluate the milk PAG test under different conditions, for example, as used for initial pregnancy diagnosis.

In this thesis we assessed a test for pregnancy-associated glycoproteins in milk from dairy cows. Chapters 2 and 4 provide valuable information on two practical considerations, the impact of sample handling on test results and an economic evaluation of the test. The results from Chapter 3 are more difficult to interpret, as the varied PAG levels at different stages of gestation are not well understood. Future research on pregnancy-associated glycoproteins should focus on the function of PAG and the role of PAG in the maintenance of pregnancy.