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Advances in reproductive management: pregnancy diagnosis in ruminants

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Abstract

During the last 10 years the U.S. dairy industry has experienced a reversal of the decades-long trend in declining fertility traits. In fact, there is evidence that, nationally, this is contributing to improvements in pregnancy rates. And while these measures are still close to their historical lows, there is reason for optimism that this reversal will continue into the future. The reasons for improved pregnancy rates are related to use of biotechnologies and improved management practices for high producing dairy cows as well as greater emphasis on genetic selection for fertility-related traits. Combined, these factors have resulted in a reduction in the average days to first service in our national dairy herd of approximately 10 days over the past decade and a reduction in calving interval of approximately 15 days. However, current challenges include accurate identification of cows that fail to conceive following insemination and their timely reinsemination. The primary metric for success of pregnancy diagnosis is the inter-service interval, or the number of days between insemination and the subsequent insemination in a cow that fails to conceive or that loses an established pregnancy. This trait is directly affected by the choice of pregnancy diagnosis method. Pregnancy diagnosis methods include estrous detection (visual or assisted), transrectal palpation of uterine contents, transrectal ultrasound visualization of uterine contents and assay for hormones in blood, milk or other body fluids. Each of these methods has advantages and disadvantages. Presently, ultrasound and blood hormone assay at 28 days after insemination offer the earliest specific diagnostics for determining pregnancy status. However, other methods are on the horizon that may provide opportunities to further reduce the interval between insemination and accurate diagnosis of pregnancy status of dairy cattle. One of these targets identification of failed inseminations 18 to 20 days after insemination. This approach, if successful, would allow identification of a portion of open cows prior to their expected return to estrus. The ultimate goal is to identify cows that fail to conceive to an insemination in time to reinseminate them at a normal cycle interval (21 to 23 days) while achieving high conception rates. Reproductive management programs that utilize early pregnancy diagnosis will reduce the interservice interval and improve pregnancy rate, which is a key metric in determining profitability on dairy farms.

Keywords: cattle, diagnostic, fertility, interferon, pregnancy, ultrasound.

Introduction

Poor reproductive performance remains one of the primary reasons for involuntary culling of dairy cows in the U.S. and globally. Roughly one third of dairy cows culled annually in the U.S. are culled due to reproductive problems (DeVries *et al*., 2010; Pinedo and DeVries, 2010). Lactating cows that fail to conceive are eventually culled for low production late in lactation at high cost to the dairy farmer (Britt, 1985). Low fertility results in reduced herd milk production, increased cost associated with multiple inseminations and increased number of replacement heifers needed to maintain herd size (Britt, 1985). Fortunately, during the last 10 years the U.S. dairy industry has experienced a reversal of the decades-long trend in declining fertility traits. In fact, trends in fertility traits are increasing in the U.S. (Fig. 1). And while fertility traits are still close to their historic lows, there is reason for optimism that this reversal will continue into the future. Pregnancy rate is the product of the estrous detection or submission rate (for dairies using timed artificial insemination) and the conception rate. It is a measure of how quickly cows that are eligible to become pregnant (i.e. after the voluntary waiting period) actually become pregnant. Improved fertility traits and use of technologies to improve submission rates have been largely responsible for the improved pregnancy rates during the last decade. For example, use of ovulation synchronization (e.g. Ovsynch) with timed artificial insemination (TAI) has resulted in more cows getting their first postpartum insemination closer to the end of the voluntary waiting period. One current challenge, however, is early and accurate diagnosis of pregnancy status to allow for cows that failed to conceive or maintain pregnancy to be reinseminated in a timely fashion.

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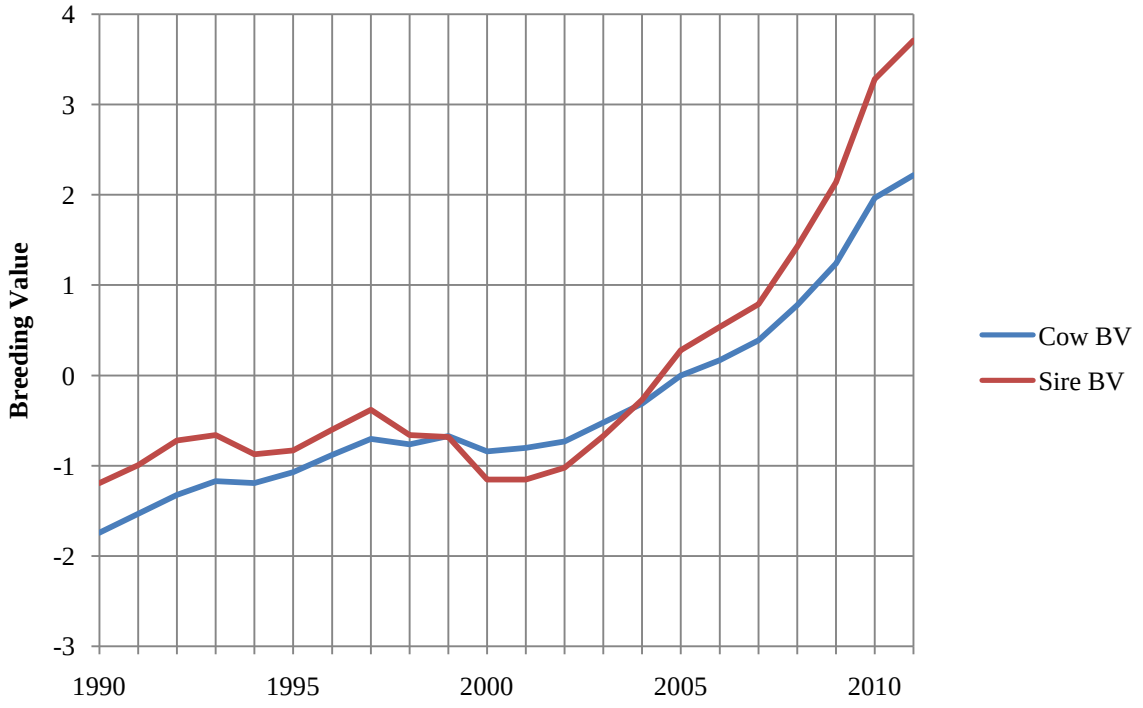

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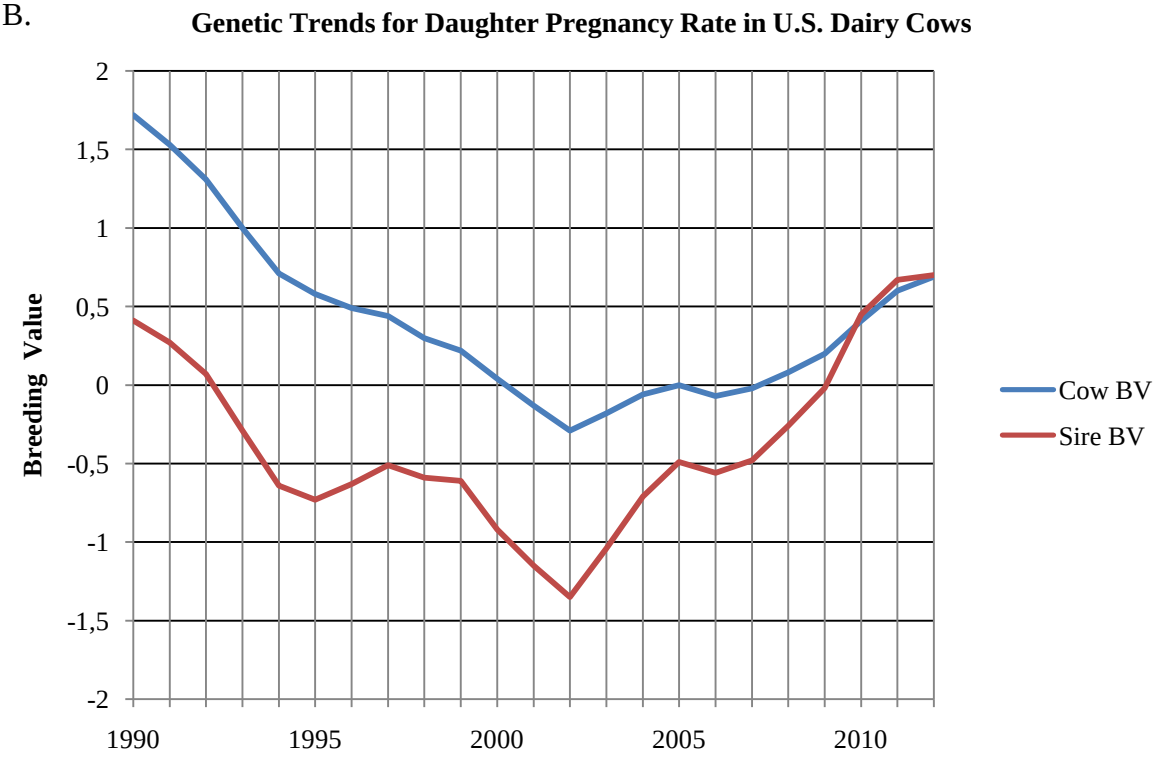

Figure 1. A) Genetic trends for cow and sire breeding value (BV) for productive life in U.S. dairy cows, and B) Genetic trends for daughter pregnancy rate in U.S. Dairy cows. From Council for Dairy Cattle Breeding, 2014.

Trends in genetic selection for fertility traits

Since its inception 20 years ago, the Lifetime Net Merit index (NM$) in the U.S. has increased genetic selection for fertility- and health-related traits. In 1994, when Net Merit Dollar index was introduced it was weighted roughly 75% for production traits (yield, fat and protein). Today's NM$ gives only ~35% weight to production traits with the remaining emphasis placed on health and fitness traits. These include productive life (PL; 22% current weight), which was introduced in 1994 and daughter pregnancy rate (DPR) which was introduced in 2003 with a weight of 6% and increased twice to its present weight in the index of 11%. While it will take some time to determine the full effects of these latest changes, it is clear they have resulted in improved genetic merit for fertility traits in the U.S. dairy herd. Currently, the difference in predicted transmitting ability (PTA) between the highest and lowest ranking bulls for DPR is approximately 8, which translates to daughters of the highest ranking DPR bulls conceiving, on average, 32 days sooner than those from the lowest ranking DPR bulls. Furthermore, with the routine collection of more fertility related data (e.g. cow conception rate, heifer conception rate) along with other health and fitness traits, it is likely that we will see continued evolution of NM$ towards more robust selection of fertility and health traits (Dechow, 2014).

Trends in reproductive management strategies

During this same 20 year span, approaches to reproductive management have also changed. In 1994, the majority of dairies bred cows based on estrous detection with few farms using estrous synchronization (Miller *et al*., 2007). With the advent of ovulation synchronization programs in the mid-1990's, farms that were struggling with estrous detection had another tool to manage reproduction (Pursley *et al*., 1995, 1997). This period was also accompanied by increased use of transrectal ultrasound and blood and milk hormone tests for pregnancy diagnosis. Ultrasound evaluation of ovarian structures also increased the ability to tailor reproductive management to ovarian status. Today, most dairies use a combination of insemination based on estrous detection and synchronization of ovulation coupled with TAI (Caraviello *et al*., 2006). Combining these approaches is the most economical way to improve pregnancy rates given typical rates of synchronization drug injection compliance and estrous detection efficiencies (Galvão *et al*., 2013). Fewer dairies are choosing to use on-farm bulls for mating cows, and for good reason. Bull breeding should be considered a choice of last resort for modern dairies. Aside from their lower genetic merit, on-farm bulls consume feed and occupy facilities, suffer from infertility and venereal diseases, require veterinary attention, and cause injuries and deaths on farms each year (Lima *et al*., 2010).

Efficiency and accuracy of estrous detection have also benefited from development of tools including simple tail head chalking/painting and glue-on mount detectors to higher tech pedometers and activity monitors containing accelerometers that continuously monitor a cow's activity (Van Eerdenburg, 2008; Fricke *et al*., 2014a). Use of activity monitors with accelerometers will likely continue to increase because of their automation and compatibility with mobile devices and cloud-based data storage and analysis. This technology is particularly attractive for dairies of intermediate size (150 to 500 cows) that struggle to maximize efficiency of labor use (Fricke *et al*., 2014b) and for dairies that prefer not to use hormonal synchronization (Neves *et al*., 2012). For example, in a large study comparing the use of automated activity monitors (AAM) with synchronization and timed artificial insemination, AAM reduced days to first service in two of the three large dairies examined (Neves *et al*., 2012). Recently, comparison was made between AAM and a presynchronization-ovulation synchronization program with TAI (Stevenson *et al*., 2014). Interestingly, pregnancies per AI were modestly lower with insemination based on AAM, but cows became pregnant quicker with AAM compared to TAI, probably due to the earlier VWP in the AAM group (Stevenson *et al*., 2014).

Together, estrous synchronization and ovulation synchronization with TAI are credited with reducing the average days to first service from 90 to 81 days over the last 10 years (Miller *et al*., 2007; Council on Dairy Cattle Breeding, 2014). This has resulted in a reduction of calving interval of about two weeks during this same period. In spite of these improvements in estrous detection technologies, low efficiency and accuracy of estrous detection remains the primary reason for dairies adopting ovulation synchronization and TAI programs (Goodling *et al*., 2005; Moore and Thatcher, 2006). Variations of the ovulation synchronization programs coupled with presynchronization (e.g. Presynch) provide a variety of options to fit producer needs. The Ovsynch 56 program (gonadotropin releasing hormone (GnRH) followed 7 days later by prostaglandin F2α (PGF), then a second GnRH injection 56 h later with TAI 16 h later), has proven to be the most effective ovulation synchronization program for maximizing pregnancy rates. This program is most effective when initiated at days 5 to 9 of the cows' estrous cycle (Vasconcelos *et al*., 1999; Moreira *et al*., 2000). To accomplish this, presynchronization programs have been developed using either PGF (Presynch: two injections of PGF 14 days apart followed by Ovsynch 11 to 14 days later) or a combination Ovsynch without TAI followed by Ovsynch started 7 days later (Double-Ovsynch; Moreira *et al*., 2000; Souza *et al*., 2008). The advantage of the

latter programs that include GnRH in the presynchronization is that cows that are not cycling will be induced to form a corpus luteum by use of GnRH (Herlihy *et al*., 2012; Ayres *et al*., 2013). In either case, use of presynchronization ensures that a greater number of cows will ovulate to the first GnRH of the ovulation synchronization program (Gumen *et al*., 2012; Ayres *et al*., 2013). Use of Presynch-Ovsynch programs on well-managed dairies yields pregnancy rates comparable to inseminating cows based on detected estrus (Rabiee *et al*., 2005; Stevenson *et al*., 2014), and a high percentage of large dairies in the U.S. use some form of hormonal synchronization with timed artificial insemination (Caraviello *et al*., 2006).

Improvements in estrous detection and estrous and ovulation synchronization coupled with TAI have resulted in more cows being inseminated in the first 21 days after the voluntary waiting period. Improvements in fertility traits, while modest, should result in improved conceptions rates to these earlier services. Thus, improvements in both submission rates and conception rates will likely continue to yield slow, but steady improvement in pregnancy rates. The current challenge is to reduce the interservice interval for those cows that fail to conceive or that lose an established pregnancy. This challenge requires accurate and early detection of pregnancy status.

Pregnancy diagnosis

Key to effective reproductive management programs is early and accurate pregnancy diagnosis following insemination. The goal is for cows to be reinseminated, on average, before 42 days after a failed insemination. Estrous detection following insemination remains a widely used approach to pregnancy diagnosis. However, dairies that struggle with accurate estrous detection often experience extended (>42 days) interservice intervals when relying on this method. In addition, inaccurate estrous detection increases the risk for insemination of pregnant cattle (Moore *et al*., 2005). Here again, use of second generation AAM may aid in detecting cows returning to estrus 21 to 24 days after insemination. Furthermore, for dairies that use ovulation synchronization and TAI for first services, some cows will not continue cycling after the first insemination and will not be detected in heat (Lucy *et al*., 2004).

Transrectal palpation and ultrasound visualization of uterine contents

Palpation of uterine contents per rectum is the most widely used method for pregnancy diagnosis in dairy cattle. The technique can be performed reliably after day 30 of pregnancy and is highly accurate when practiced by a skilled veterinarian or animal manager. Palpation does require training and experience to conduct with high accuracy and without damaging the embryo when conducted at very early stages gestation (<35 days; O'Connor, 1994). It is also possible to determine ovarian structures. However, this requires an even higher level of training and experience to conduct reliably. Transrectal palpation is relatively inexpensive compared to other methods for pregnancy diagnosis, but it does increase the risk of iatrogenic pregnancy loss, spreading diseases between cattle and is physically demanding for the technician.

With the development of ultrasound technology in the 1980's, dairies had a new, accurate and specific tool for determining pregnancy status (Pierson and Ginther, 1984). The cost of the technology initially limited its use for commercial dairies (O'Connor, 1994). However, as average dairy size has increased and the cost, ease of operation and reliability of ultrasound improved, the use of ultrasound for pregnancy diagnosis has increased (Quentela *et al*., 2012; Pereira *et al*., 2013). The advantages of ultrasound include ability to determine: 1) pregnancy status at earlier stages (>25 days in cattle); 2) presence of twins with increased accuracy; 3) fetal viability (e.g. heart beat); 4) fetal gender; and 5) ovarian structures (follicles and corpora lutea). Although many veterinarians now provide ultrasound service to dairies of all sizes, cost and frequency of veterinary visits is still limiting for smaller dairies.

Chemical pregnancy assays

Blood and milk progesterone assays have been available for over 20 years (O'Connor, 1994). However, because progesterone is not pregnancy-specific and the requirement for multiple assays to achieve acceptable specificity, these assays have not been widely adopted. However, development of sensitive automated inline milk progesterone assays should make this technology amenable to commercial application (Käppel *et al*., 2007; Fricke *et al*., 2014a). Automated inline testing should accelerate the adoption of progesterone assay for pregnancy diagnosis because of the high accuracy of sequential testing at insemination and then at 20 to 24 days after insemination for detecting failed inseminations (O'Connor, 1994). Testing at later intervals could then be used for confirming pregnancy status. Adoption of inline testing technology will likely take some time due to the high capital costs and will be dependent on the reliability of the automated inline tests.

The first reliable pregnancy-specific hormone assays were developed to measure placenta-derived proteins. The first of these measured circulating concentrations of pregnancy-specific protein B (PSPB; Butler *et al*., 1982). Pregnancy-specific protein B is produced by placental giant binucleate cells that form from mononuclear trophoblast cells starting around days 17 to 19 of pregnancy in cattle (Spencer *et al*., 2007). Pregnancy-specific protein B concentrations begin to be

reliably detectable in plasma starting at day 24, and by day 28 concentrations are sufficiently elevated to allow their use for a highly reliable test for pregnancy in ruminant animals (Sasser *et al*., 1986). Pregnancy-specific protein B is a member of the pregnancy associated glycoprotein (PAG) family of proteins which are encoded by a very large gene family (Xie *et al*., 1994). PAG are also secreted in milk and reliable milk PAG tests are now available in the U.S. for use after day 35 of early pregnancy (Green and Roberts, 2006). A number of commercial suppliers are now producing diagnostic tests for PAG family members in blood and milk and millions of samples are tested annually in the U.S. Currently, available PAG diagnostic tests require delivering samples to a centralized testing laboratory. Once these tests are adapted to inline milking systems and/or developed into "cow-side" diagnostics, they will be more widely adopted.

New opportunities for early diagnosis of pregnancy status

A large but ill-defined percentage of cows fail to conceive following insemination or lose embryos prior to rescuing CL function (Pereira *et al*., 2013). Theoretically, these cows could be detected in estrus and be reinseminated 21 to 24 days after their first insemination. However, as stated above, not all these cows will exhibit estrous behavior and those that do often go undetected resulting in less than 50% of open cows being detected in estrus 21 to 24 days after insemination. These cows have been called "phantom" cows (Lucy *et al*., 2004). Therefore, if a pregnancy-specific signal could be detected during early pregnancy it could be used to identify failed conceptions and allow for reinsemination of open cows at 21 to 24 day intervals.

Interferon tau (IFN-τ) is the conceptus signal responsible for rescuing CL function in ruminants (Bazer *et al*., 2009). With its discovery and characterization, a number of groups attempted to detect IFN-τ in systemic circulation as a method for determining conceptus signaling during early pregnancy (Stewart *et al*., 1992). With the exception of one study with a small number of pregnant sheep (Schalue-Francis *et al*., 1991), the outcomes of a number of studies supported the prevailing hypothesis that IFN-τ did not escape the uterus in appreciable quantities. It was generally accepted that IFN-τ acted locally on the uterine endometrium to alter the pattern of PGF release and maintain CL function (Spencer and Bazer, 2004). This is in contrast to humans where conceptus-produced chorionic gonadotropin directly supports CL function and can be measured in maternal blood and urine as soon as 6 to7 days following fertilization (Bazer *et al*., 1991). Early studies either used antiviral assay for detecting interferon activity (Pontzer *et al*., 1988) or RIA (Vallet *et al*., 1988) or ELISA (Zhu *et al*., 1996) to directly assay for IFN-τ. More recently, we addressed the question of systemic responses to conceptus signaling in ruminants using a different, indirect, approach of assaying for expression of interferon stimulated genes (ISG) in peripheral blood leukocytes (Yankey *et al*., 2001). Type I interferons such as IFN-τ induce a large number of ISG that are better known in the immune response to viral infection (Williams, 1991). Among these are the myxovirus resistance genes (MX1 and MX2; Ott *et al*., 1998) and interferon stimulated gene 15 (ISG15; Austin *et al*., 2004).

Results of Yankey *et al*. (2001) were the first to show that early pregnancy resulted in increased abundance of mRNA and protein for MX1 in peripheral blood leukocytes (PBL) of pregnant compared to non-pregnant ewes on day 15 after insemination (Fig. 2). The effect of early pregnancy signaling on ISG expression in PBL was subsequently confirmed in cattle (Han *et al*., 2006; Gifford *et al*., 2007; Stevenson *et al*., 2007; Oliveira *et al*., 2008; Green *et al*., 2010; Ribeiro *et al*., 2014). Figure 3 shows abundance of mRNA for MX2 and ISG15 in PBL of dairy cattle following insemination (Gifford *et al*., 2007). Messenger RNA for ISG15 was increased in PBL of pregnant compared to bred, nonpregnant, dairy cows on day 18 and 20 after insemination and MX2 mRNA abundance was greater at day 16, 18 and 20 after insemination. Importantly, differences in expression of ISG occurred prior to the expected time of return to estrus (day 21 to 24). These results caused a careful reevaluation of hypothesis that IFN-τ acted solely in a paracrine fashion to alter uterine PGF production and maintain CL function (Oliveira *et al*., 2008).

Consistent with previous results, Oliveira *et al*. (2008) demonstrated that expression of interferon stimulated genes increased in the peripheral blood of pregnant sheep at day 15. Furthermore, they showed that antiviral activity (a measure of IFN) was elevated in uterine vein plasma, but not plasma obtained from the uterine artery during early pregnancy. These results strongly suggested that there was endocrine release of IFN-τ or a related IFN from the uterus in response to conceptus IFN-τ. This hypothesis was confirmed in subsequent studies using implanted mini-pumps delivering low doses of IFN-τ into the uterine vein that protected the CL from a subluteolytic dose of PGF (Bott *et al*., 2010; Antoniazzi *et al*., 2013). These experiments strongly supported the hypothesis that IFN-τ exits the uterus, induces ISG in blood cells and peripheral tissues including CL and liver and that this mechanism may contribute to protecting the CL from the luteolytic effects of PGF (Antoniazzi *et al*., 2013).

From a practical standpoint, the fact that the presence of a conceptus induces ISG in peripheral blood prior to expected return to estrus provides an opportunity to detect failed inseminations during a period when the ovary should contain a second or third wave dominant follicle that could be induced to ovulate. This would allow reinsemination of open cows at 21 to

24 day intervals (Lucy *et al*., 2004). In this model, cows detected open at day 18 to 20 after insemination would receive injection of a luteolytic dose of PGF and be bred on detected estrus or receive TAI accompanied by a GnRH injection 48 to72 h after the PGF injection (Lucy *et al*., 2004). The effectiveness of such a program for reinsemination of open cows has not yet been determined. It would be highly dependent on the percentage of open cows that are detectable at 18 to 20 days after insemination, which has not been determined in replicated large-scale studies. However, estimates are that greater than 50% of failed inseminations have occurred by 20 days after insemination (Pereira *et al*., 2013). Any diagnostic test developed for this purpose would need to exhibit a high degree of specificity because it would trigger a management decision to lyse the CL with PGF and return the cow to estrus. Of course, cows with elevated expression of ISG at this time would be presumed pregnant, but pregnancy would need to be confirmed later in gestation to account for later embryo losses. A diagnostic used as described here could not be considered a pregnancy test, due to the relatively high degree of embryo loss that occurs between day 20 and 45 after insemination (Pereira *et al*., 2013). The usefulness of such a diagnostic would be for detecting open cows.

Finally, studies on conceptus-uterus-immune cell cross-talk during early pregnancy have raised new questions related to the function of these ISG both locally in the uterus and in the peripheral tissues during early pregnancy (Ott and Gifford, 2010). Interferon stimulated genes generally function as part of the innate immune response to viral infection. For example, MX1 blocks the replication of negative-stranded RNA viruses by interfering with generation of viral transcripts or by inhibiting assembly of mature viral particles depending on the species of animal (Haller and Kochs, 2011). Whether activation of ISG in early pregnancy is to elevate innate immunity (during a period when some aspects of immune function are down regulated to protect the allogeneic conceptus) is a question currently under investigation (Ott and Gifford, 2010). Furthermore, assaying for expression of interferon stimulated genes in blood during early pregnancy provides a non-invasive window on conceptus-uterine cross-talk during early pregnancy (Gifford *et al*., 2008). For example, Ribeiro *et al*. (2014) recently showed that treatment of lactating cows with sequential low doses of recombinant bovine somatotropin 14 days apart starting at insemination improved conceptus growth and pregnancy rates (Ribeiro *et al*., 2014). Enhanced conceptus growth was also reflected in increased abundance of ISG15 mRNA in peripheral blood leukocytes at day 19 after insemination in cows that maintained their pregnancies (Fig. 4). Interestingly, not all ISG responded in a similar fashion suggesting that much more remains to be learned about conceptus-uterus-immune cell cross-talk during early pregnancy in ruminants (Ribeiro *et al*., 2014).

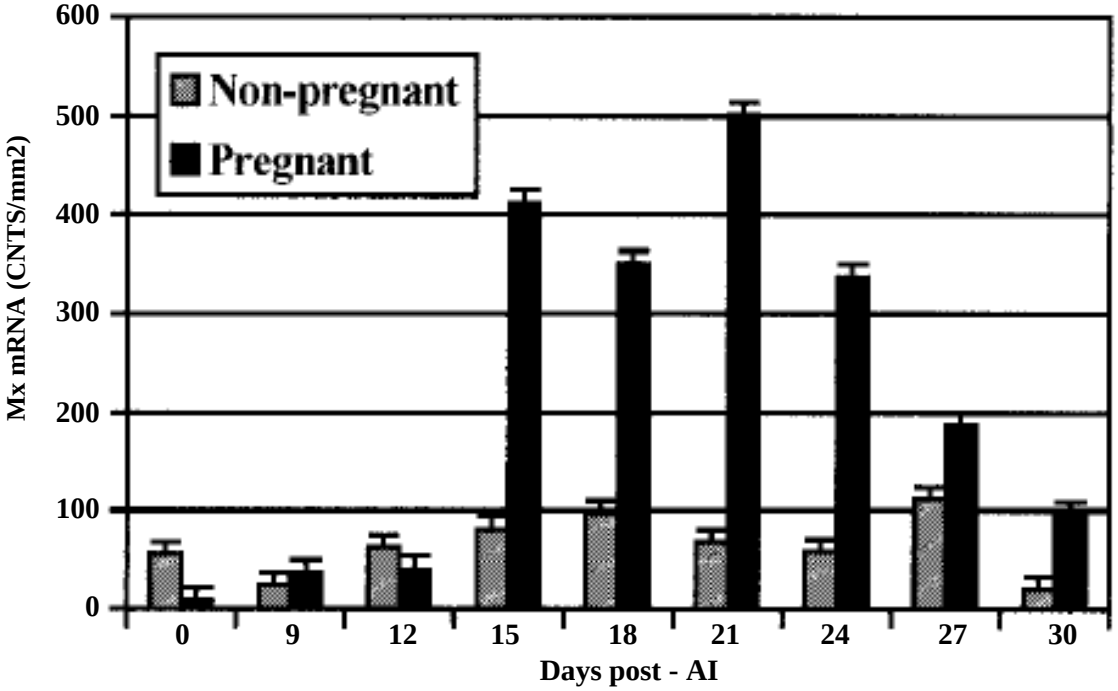

Figure 2. Steady state abundance of myxovirus resistance 1 (Mx) mRNA in peripheral blood lymphocytes of pregnant (black bars) and bred, non-pregnant (grey bars) ewes from insemination (day 0) to 30 days after insemination. Mx mRNA was increased in pregnant ewes from day 15 to 30 after insemination. From Yankey *et al*., 2001.

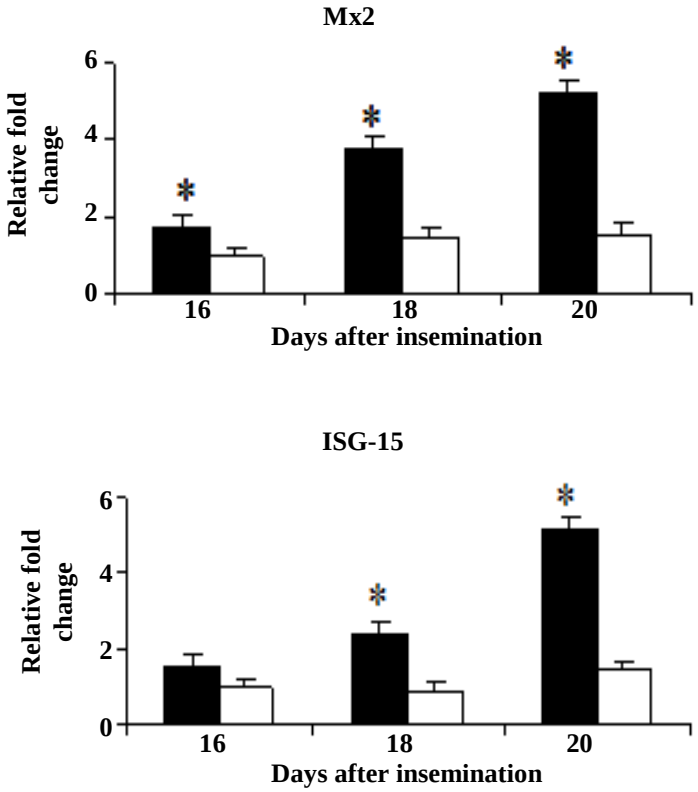

Figure 3. Relative fold change in steady state abundance of mRNA for interferon stimulated gene 15 (ISG-15) and myxovirus resistance 2 (Mx2) in peripheral blood leukocytes at 16, 18 and 20 days after insemination in pregnant (filled bars) and bred, non-pregnant dairy cows (open bars). *indicates statistical difference between pregnancy statuses ($P < 0.05$). From Gifford *et al*., 2007.

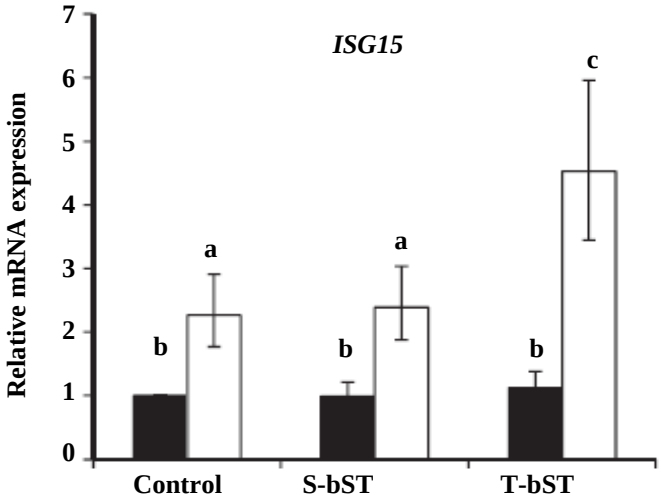

Figure 4. Effects of a single low dose (325 mg) of bovine somatotropin (S-bST) given at insemination or two sequential S-bST at insemination and 14 days later on relative mRNA abundance of ISG15 at day 19 after insemination in cows diagnosed pregnant (open bars) and open (filled bars) 31 days after insemination. From Ribeiro *et al*., 2014.

Conclusions

Obtaining optimal pregnancy rates is a key to success in the dairy business. Technologies developed over the last 25 years have improved the ability of producers to accurately detect estrus and monitor pregnancy status while maximizing labor efficiency. Coupled with an increase focus on reproductive and fitness traits in selection indexes, dairy producers are beginning to reverse the decades-long trend in declining reproductive performance in the U.S. Automated continuous activity monitors containing accelerometers will allow real-time evaluation of health status and improve estrous detection rates. Integration of these systems with cloud computing and mobile device communication will provide producers with continuous information about the status of cows in the herd. The challenge will be with handling large data sets and distilling down information in a form that is useful to support on-farm decision making. There will likely be increased adoption of pregnancy-specific hormone assays, especially if these assays can be adapted to inline or "cow-side" diagnostic platforms as has been done for the milk progesterone assay. Ultrasound will remain the gold-standard for evaluating reproductive status and affordability and ease of use are likely to continue to improve. Optimized hormonal synchronization protocols currently allow producers to precisely target first inseminations. However, detection of failed inseminations and timely reinsemination of cows continues to be a challenge that increases days open and reduces profitability. New diagnostic approaches are targeting detection of open cows 18 to 20 days after insemination. If successful, they should allow a large proportion of open cows to be identified and reinseminated at 21 to 24 day intervals.

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