IMPROVING REPRODUCTIVE PERFORMANCE OF CATTLE THROUGH THE DEVELOPMENT OF NOVEL MANAGEMENT STRATEGIES AND TECHNOLOGIES

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

Magdalena Masello Souza

May 2020

IMPROVING REPRODUCTIVE PERFORMANCE OF CATTLE THROUGH THE DEVELOPMENT OF NOVEL MANAGEMENT STRATEGIES AND TECHNOLOGIES

Magdalena Masello Souza

Cornell University 2020

ABSTRACT

The objectives of the studies presented in Section I were to evaluate insemination dynamics, time to pregnancy, and profitability of replacement dairy heifers managed with first service programs that relied primarily on insemination at detected estrus (**AIE**), timed-artificial insemination (**TAI**), or a combination of both. The objectives of the experiments presented in Section II were; to develop and validate a tool integrating a disposable fluorescence-based lateral flow immunoassay (**LFIA**) coupled with a portable imaging device to determine the reproductive status of cows based on circulating progesterone (**P4**) concentration; to test the efficacy of intravaginal (**IVG**) administration of prostaglandin-F2α (**PGF**) to induce corpus luteum (**CL**) regression; and to develop and validate an electronically-controlled hormone delivery device for reproductive control of cattle.

The studies presented in Section I demonstrated that a reproductive management strategy designed to submit all heifers to TAI reduced time to first AI and time to pregnancy when compared with a strategy that relied primarily on AIE after induction of estrus with PGF treatments. The program that combined AIE and TAI resulted in intermediate performance. In spite of greater reproductive cost, programs that used TAI in combination with AIE or solely TAI reduced rearing cost and generated more revenue during first lactation, which translated in numerically greater cash flow under fixed or simulated market conditions.

The first experiment of Section II demonstrated that the developed LFIA system can accurately predict the presence of a functional CL (i.e., P4 ≥ 1 ng/mL) using bovine plasma samples. Additional improvements of this assay may lead to the development of a rapid, low-

cost, cow-side tool for determination of reproductive status of cattle. The other experiments presented in Section II demonstrated that two IVG treatments of PGF 12 hours apart resulted in similar luteal regression risk, estrus expression, ovulatory response to GnRH, and pregnancy per AI compared with that observed after a single intramuscular dose of PGF. Further, we demonstrated that the current IVG prototype device for hormone delivery can be programmed to automatically release PGF for successful induction of luteal regression in cattle. Once optimized, the developed IVG device may be an alternative too to the needle-injection methods presently used to synchronize ovulation of cows.

BIOGRAPHICAL SKETCH

Magdalena Masello Souza was born on May 17, 1990 in Montevideo, Uruguay. In August 2014 she graduated as a Doctor of Veterinary Medicine from the Universidad de la República (Uruguay). In January 2015 she joined Cornell University as a visiting scholar to conduct research in the Dairy Cattle Biology and Management Laboratory in the Department of Animal Science under the supervision of Dr. Julio Giordano. During that time, Magdalena participated in several research projects that investigated various aspects of dairy cow reproduction and health. She was then accepted as a Ph.D. student and began her doctoral studies in the spring of 2016 under the guidance of Dr. Julio Giordano. Her work focused on dairy cattle reproductive physiology and management, and in the development of novel technologies to optimize reproductive management of cattle.

ACKNOWLEDGMENTS

First and foremost, I would like to thank my advisor Dr. Julio Giordano for providing me with the invaluable opportunity of pursuing my PhD degree at Cornell University. I am also thankful for his great mentorship on how to conduct good research and for kindly sharing his expertise and knowledge with me. His guidance has been key to my training and has helped me become a better scientist. I would also like to express my appreciation to the other members of my PhD committee; Dr. Jerrie Gavalchin, Dr. David Erickson, and Dr. Daryl Nydam, for their contributions during my graduate studies.

I will be forever grateful to my colleagues and friends Robert Wijma and Matias Stangaferro, who have been incredibly helpful and supportive throughout my PhD program. I would also like to thank Susanne Pelton, for her valuable technical assistance and guidance in the laboratory. Her resourcefulness, patience, and knowledge were key for the successful completion of my research experiments. Special mention also goes to all of those that contributed to the realization of my research studies, including fellow graduate students, visiting scholars, undergraduate students, and farm owners and personnel.

Next, to all my Ithaca friends, for making me feel at home during these years in the US. I am especially grateful to Lu, for being the best officemate I could have ever asked for. Last and most importantly, I would like to thank Eduardo, who shares with me the love for science and has been an incredible support during this time, and to my family, for their encouragement to continuously enrich my education.

TABLE OF CONTENTS

SECTION III

GENERAL CONCLUSION

LIST OF ABBREVIATIONS

Ab – antibody
AFC – age at first calving
AI – artificial insemination
AIC – Akaike information criterion
AIE – artificial insemination after detected estrus
AIP – artificial insemination period
AUC – area under the curve
BCS – body condition score
BSA – bovine serum albumin
BW – body weight
CF – cash flow
CIDR – controlled internal drug release insert
CL – corpus luteum
CV – coefficient of variation
DIM – days in milk
DM – dry matter
DMI – dry matter intake
DMW – daily milk weight
DO – Double Ovsynch protocol
ELISA – enzyme-linked immunosorbent assay
FCM – fat corrected milk
FLP – first lactation period
GnRH – Gonadotropin-releasing hormone
HI – heat index score
HR – hazard ratio
HRP – horseradish peroxidase
IBD – intelligent breeding device
IM – intramuscular
IOFC – income over feed cost
IVG – intravaginal
LFIA – lateral flow immunoassay

LH – Luteinizing hormone
LSD – Least Significant Difference
LSM – Least Squares Means
NPD – nonpregnancy diagnosis
NPV – negative predictive value
PCB – printed circuit board
P/AI – pregnancies per artificial insemination
PGF – Prostaglandin-F2α
PPV – positive predictive value
PVD – purulent vaginal discharge
P4 – Progesterone
RIA – radioimmunoassay
ROC – receiver operating characteristic
RP – rearing period
RPE – R-phycoerythrin fluorophore
Se – sensitivity
Sp – specificity
TAI – timed artificial insemination
T/C – test-to-control-line ratio
TP – transrectal palpation
TUS – transrectal ultrasonography
5dCP – 5-day Cosynch protocol

SECTION I

STRATEGIES TO MAXIMIZE REPRODUCTIVE AND ECONOMIC PERFORMANCE OF REPLACEMENT DAIRY HEIFERS

CHAPTER I

IMPACT OF HEIFER REPRODUCTIVE MANAGEMENT STRATEGIES ON THE PERFORMANCE OF REPLACEMENT DAIRY HEIFERS

1. General introduction

Nulliparous dairy heifers are an integral component of dairy operations since they represent the future revenue-generating units and provide the replacements needed to either maintain or increase herd size. Unfortunately, raising heifers from birth up to first calving (i.e., onset of productive life) represents a major financial investment for most farms. Total cost to raise heifers has increased substantially in the past decade (Heinrichs et al., 2013; Karszes, 2014; Akins et al., 2015), and can represent as much as $1,800 to $2,100 per individual heifer (Heinrichs et al., 2013; Karszes, 2014). This large economic burden is primarily explained by feed cost, which can account for as much as 70% of all rearing costs (Heinrichs et al., 2013). In addition, replacement heifers are financially non-productive until they join the milking herd, which occurs at first calving. Thus, management strategies aimed at minimizing days in rearing can have a direct effect on both, the total cost of rearing and the time taken for the individual heifer to achieve positive cash flow. Indeed, observational studies (Pirlo et al., 2000; Ettema and Santos, 2004) have shown that reducing age at first calving (**AFC**) from 26 to 24 months could result in as much as $42 to $99 of benefit per heifer when accounting for reduced rearing costs

and the difference in income generated during first lactation. However, only a limited number of dairy operations in the US have average AFC of ≤ 24 mo (NAHMS, 2007).

As for most mammalian species, gestation length in cattle is fixed (within a certain range). Therefore, variability in AFC within a herd is primarily driven by the age at initiation of the artificial insemination period (**AIP**) and reproductive efficiency. The optimal age to start breeding heifers it is still highly debatable in the dairy industry and can vary across farms. The criteria is often based on body weight (e.g., 55% of mature body weight), height (e.g., ~50 inches), age (e.g., 12 months of age) or a combination of these. As management strategies aimed at reducing age at puberty are limited, reducing time to pregnancy after the beginning of the AIP is one of the few management opportunities to optimize AFC. Therefore, implementation of proactive and effective reproductive management programs that hasten the generation of heifer pregnancies after the beginning of the AIP have the potential to reduce variability in AFC and thus minimize the number of animals in the replacement inventory with extended AFC.

Although achieving pregnancies in an efficient and timely manner is a very important part of a heifer-raising program, only a limited number of studies have evaluated the effect of different reproductive management strategies on reproductive and economic performance of replacement dairy heifers. In addition, the few economic analyses conducted only accounted for rearing cost without incorporating the opportunity cost of delayed lactation.

This chapter intends to review relevant information regarding current knowledge of reproductive management strategies available for dairy heifers and their effect on the economics of the heifer enterprise.

2. Reproductive management of replacement heifers

2.1. Insemination after spontaneous estrus or prostaglandin-F2α

For dairy operations that use artificial insemination (**AI**) as the sole method to get heifers pregnant, AI after detected estrus (**AIE**) and synchronization of ovulation for timed AI (**TAI**) are the two main methods available for submission to AI. Historically, reproductive management programs for dairy heifers have been based primarily on AIE rather than submission to TAI. According to a survey conducted by the National Animal Health Monitoring System (NAHMS, 2018), approximately 60% of dairy farms in the United states use AIE as the sole method to manage reproduction of the heifer herd. High estrus expression and pregnancies per AI (**P/AI**) for AIE services (50 to 75%; Tanabe and Hann, 1984; Kuhn et al., 2006; Stevenson et al., 2008) for dairy heifers combined with the relatively low cost of implementation (Stevenson et al., 2008; Lopes et al., 2013) likely explains the preference for this type of program. The success of AIE-based programs is highly dependent on the efficiency and accuracy of estrus detection which depends on two main factors; i.e., the ability of heifers to express estrus and of farm personnel to detect estrus on a regular basis (i.e., at least once or twice per day).

A strategy used to facilitate detection of estrus is to administer prostaglandin-F2α (**PGF**), a hormone commonly used for reproductive management in cattle that induces corpus luteum (**CL**) regression (Louis et al., 1973; Lauderdale et al., 1974). Administration of PGF promotes estrus expression by reducing circulating progesterone (**P4**) concentration which enables a series of well-orchestrated hormone-release patterns that culminate with estrus expression and ovulation. Thus, dairy producers can hasten estrus expression rather than waiting for heifers to express estrus spontaneously. Indeed, a single PGF treatment at random stages of the estrous cycle may result in 50 to 70% of heifers displaying estrus 2 to 11 d after treatment (Hafs et al., 1975; Fogwell et al., 1986; Stevenson et al., 2008), with the vast majority displaying estrus

within 3 to 4 d (Hafs et al., 1975; Stevenson et al., 2008). Studies which evaluated estrous behavior in dairy heifers also suggested that inducing the formation of sexually active groups (i.e., more heifers coming into estrus concomitantly) with PGF treatments can increase the intensity (Helmer and Britt, 1985) and duration (Kaim et al., 1990) of estrus, which can potentially increase the success of estrus detection programs. Therefore, administration of PGF to heifers eligible for pregnancy can help reduce days to AI and improve estrus detection efficiency. Moreover, as the fertility of AIE services after PGF-induced estrus is similar to that of heifers bred to a natural estrus (Kaim et al., 1990), ultimately the pregnancy rate is expected to increase for heifers managed with PGF-based programs.

One limitation of using PGF for induction of estrus is that complete luteal regression is not induced unless the CL is fully responsive to PGF (Louis et al., 1973; King and Robertson, 1974). This is relevant when heifers are to be treated with PGF at random stages of the estrous cycle. To circumvent this issue, PGF-based estrus synchronization schemes usually include two PGF treatments administered at either 11- (Stevenson et al., 1984; Fogwell et al., 1986; Chebel et al., 2007) or 14-d (Pursley et al., 1997; Stevenson et al., 2008; Lopes et al., 2013) intervals. The reasoning behind this scheme is that the first of the two PGF treatments induces luteal regression in about 70% of heifers, whereas the second treatment is expected to induce luteal regression in the initially unresponsive group (King and Robertson, 1974). Moreover, heifers that respond to the initial PGF are expected to have a responsive CL at the time of the second treatment (King and Robertson, 1974). Dairy producers can conduct AIE after each PGF treatment (Chebel et al., 2007; Stevenson et al., 2008; Lopes et al., 2013) or concentrate estrus detection efforts after the second PGF treatment (Kaim et al., 1990; Colazo and Mapletoft, 2017). When using the latter approach, it has been reported that approximately 85 to 88% of heifers displayed estrus and were

inseminated within 6 d of the second PGF treatment (Hafs et al., 1975; Kaim et al., 1990; Colazo and Mapletoft, 2017). Thus, under commercial farm conditions it is expected that a vast majority of heifers would be inseminated in a relatively short period after PGF treatments, potentially increasing the insemination rate compared with insemination after spontaneous estrus. In this regard, Stevenson et al. (2008) demonstrated that insemination of heifers using PGF treatments every 14 d resulted in faster insemination relative to AI after spontaneous estrus expression. As there were no deleterious effects on fertility for heifers inseminated after PGF-induced estrus, the synchronized group became pregnant at a faster rate. These results indicated that submission of heifers to AI using PGF-based synchronization of estrus protocols might be reasonable alternative for dairy farms.

2.2. Potential advantages of incorporating timed AI for managing heifer reproduction

A potential limitation of relying solely on AIE for managing reproduction of heifers is that it may lead to suboptimal reproductive performance for farms with poor estrus detection efficiency. This is particularly relevant for replacement heifers as they usually have lower priority for management relative to the milking herd (Harsh et al., 2001), or for farms that do not have enough personnel or time to dedicate to daily observation of estrus. In addition, dedicating time and effort to estrus detection might not be economically feasible for many farms. Since the advent of the Ovsynch protocol for timed AI in the early 1990s (Pursley et al., 1995; Pursley et al., 1997), dairy producers have an alternative to AIE-based strategies. Such strategy consists of synchronizing ovulation of cows or heifers to conduct AI by appointment regardless of estrus expression (i.e., TAI). Ovsynch uses gonadotropin-releasing hormone (**GnRH**) and PGF to sequentially-control ovarian follicular dynamics, luteal regression, and ovulation. Briefly, the

protocol consists of 4 steps: 1) a dose of GnRH administered to induce ovulation and reset follicular growth, 2) seven days later, a PGF treatment to induce luteal regression, 3) 56 h later, a second GnRH treatment to induce ovulation, and finally 4) 16 to 18 h later cows receive TAI. When Ovsynch is modified so that TAI is performed concurrently with the last GnRH treatment, it is referred to as "Cosynch" (Geary et al., 1998). With such protocols, dairy operations can now set aside specific days of the week for reproductive management rather than dedicating labor and effort to daily estrus detection. Moreover, producers may increase the insemination rate of the herd by defining when animals are inseminated or improve P/AI using synchronization of ovulation protocols designed to optimize fertility of TAI services.

Because of the benefits of incorporating TAI, reproductive management strategies including synchronization of ovulation protocols have been widely adopted by the dairy industry to mitigate problems associated with low estrus expression in lactating dairy cows (Ferguson and Skidmore, 2013; Wiltbank and Pursley, 2014). Indeed, TAI-based programs are now used to manage cow reproduction in 56% of dairy operations in the US, and the vast majority (69%) have used it for at least 9 years or more (NAHMS, 2008). Conversely, less than 35% of dairy operations in the US use TAI-based programs for managing reproduction of the heifer herd (NAHMS, 2008). The limited use of TAI protocols in heifers can be explained, at least in part, by the reduced P/AI obtained with the standard Ovsynch protocol relative to AI after spontaneous or induced estrus (Pursley et al., 1997). In part, this is because Ovsynch-type protocols have been developed based on assumptions about the reproductive physiology of lactating cattle rather than considering potential differences with nonlactating animals, as it is the case for replacement heifers. In the last decade, however, a better understanding of the physiological differences between nulliparous heifers and lactating cows led to the development

of synchronization of ovulation protocols for TAI specifically tailored to heifers. Implementation of such protocols could potentially increase adoption of TAI by dairy operations, which in turn, may significantly improve reproductive performance of heifers.

2.3. Synchronization of ovulation protocols for timed AI and factors that affect fertility

2.3.1. The role of progesterone supplementation and duration of the proestrus period

In part, the historically low adoption of TAI in replacement heifers can be explained by the poor fertility response to Ovsynch (Schmitt et al., 1996; Pursley et al., 1997). It is important to note, however, that in the few studies conducted to evaluate Ovsynch in dairy heifers (Schmitt et al., 1996; Pursley et al., 1997), the time between PGF and final GnRH (~30 to 48 h) was shortened relative to the standard Ovsynch protocol (i.e., 56 h), which may have negatively affected fertility after TAI. Although the mechanisms that underlie the reduction in fertility in heifers synchronized with the Ovsynch protocol have not yet been fully elucidated, a possible explanation may be that heifers are more likely to have 3 rather than 2 follicular waves during the estrous cycle (Savio et al., 1988; Sartori et al., 2004). This might limit the likelihood of presenting a dominant follicle responsive to a GnRH-induced luteinizing hormone (**LH**) surge at any given time during the estrous cycle. Furthermore, nulliparous heifers have greater circulating P4 concentrations than lactating cows (Sangsritavong et al., 2002; Sartori et al., 2004), which may compromise LH secretion in response to GnRH and thus hinder ovulatory response (Colazo et al., 2008; Giordano et al., 2012; Lima et al., 2013). Because of these differences, it is likely that standard Ovsynch protocols are inadequate to properly synchronize follicle growth and ovulation in heifers. Indeed, 66 to 75% of heifers fail to ovulate in response to a GnRH treatment given at random stages of the estrous cycle (Colazo and Ambrose, 2011; Lima et al., 2013),

which contrasts with the 36 to 46% usually observed for lactating cows (Vasconcelos et al., 1999; Bello et al., 2006). This is relevant considering that lack of ovulation to the initial GnRH may result in compromised embryo quality (Cerri et al., 2005) and reduced fertility (Chebel et al., 2006) in cows following TAI.

Another factor likely contributing to reduced fertility of heifers to TAI services is the high proportion that displays estrus before the PGF treatment of the protocol (~20%; Rivera et al., 2004; 2005). More specifically, reduced P/AI likely occurs because heifers that display premature estrus receive TAI too late. To circumvent this issue an intravaginal P4 releasing device has been added to the protocols to minimize the number of heifers that display estrus prematurely (Rivera et al., 2005). The reasoning behind this approach was that elevated P4 levels (such as those naturally found during the luteal phase of the estrous cycle) inhibit endogenous GnRH secretion and thus prevent premature estrous behavior and subsequent ovulation (Kim et al., 2003). Results from experiments with beef cows and dairy heifers demonstrated that including an exogenous source of P4 during synchronization of ovulation suppressed premature estrus expression (Martínez et al., 2002; Rivera et al., 2005) and ovulation which resulted in increased P/AI of TAI services (Martínez et al., 2002). Thereafter, most of the synchronization of ovulation protocols developed and optimized for use in dairy heifers (Rabaglino et al., 2010; Lima et al., 2011; Lima et al., 2013) incorporated a P4 releasing insert (e.g., CIDR and PRID).

In an attempt to further increase P/AI, additional research was dedicated to optimize Cosynch protocols with P4 supplementation for use in beef cattle (Bridges et al., 2008; Whittier et al., 2010) and dairy heifers (Rabaglino et al., 2010; Lima et al., 2011; Lima et al., 2013). Among the many variants evaluated, a protocol that shortened the period of follicular dominance by shortening the interval from initial GnRH to induction of luteolysis with PGF, and extended

proestrus (time from onset of luteal regression to the GnRH-induced LH surge) from 48 or 56 h to 72 h seemed most effective (Bridges et al., 2008; Lima et al., 2011; Lima et al., 2013). This protocol is commonly referred to as the 5-d Cosynch protocol (**5dCP**; GnRH + CIDR insertion, 5 d later CIDR removal + PGF, 24 h later PGF, 72 h later GnRH + TAI). Because the time from GnRH to PGF treatment is shortened, heifers that ovulate in response to the initial GnRH present a newly formed CL and therefore an increased risk for incomplete luteolysis after a single PGF treatment (Rowson et al., 1972; Henricks et al., 1974). Thus, the addition of a second PGF treatment 24 h later. Studies with Holstein heifers published to date evaluating this version of the 5dCP consistently reported P/AI ranging from 55 to 65% for conventional semen (Lima et al., 2013; Kasimanickam et al., 2014; Silva et al., 2015; Karakaya-Bilen et al., 2019) and 51 to 55% for sex-sorted semen (Silva et al., 2015; Karakaya-Bilen et al., 2019). Another important consideration is that 14 to 27% of heifers display estrus between CIDR removal and the scheduled day of TAI (Silva et al., 2015; Macmillan et al., 2017). Although estrus detection after CIDR removal is not required to achieve acceptable fertility (Lima et al., 2013; Karakaya-Bilen et al., 2019), including a short period of AIE may improve overall P/AI relative to submission of all heifers to TAI (Macmillan et al., 2017) because those that express estrus early may have reduced fertility if they receive TAI. Therefore, when synchronizing heifers with the 5dCP dairy farms have the option to either implement blanket use of TAI without the extra labor and cost of estrus detection or include a short period of AIE after CIDR removal that could potentially increase reproductive efficiency of heifers. Regardless of AIE inclusion, and unlike standard Ovsynch, the 5dCP leads to comparable P/AI to that of heifers inseminated to spontaneous or PGF-induced estrus (Silva et al., 2015; Colazo and Mapletoft, 2017), which may result in greater adoption of TAI by dairy producers.

2.3.2. Variations of the 5-d Cosynch protocol

One of the major limitations of 5dCP relative to other protocols is the multiple and inconvenient time intervals that result in extra labor and cost dedicated to heifer handling. Thus, modifications that result in reduced labor and hormonal treatments without a negative effect on fertility are highly desirable and will likely increase acceptance by dairy producers. In this regard, extensive research has been conducted to identify such modifications; however, their effect on P/AI has been difficult to estimate because of the inconsistent results observed. For instance, some studies reported that the removal of the second PGF had no deleterious effect on dairy heifer fertility (Rabaglino et al., 2010; Kasimanickam et al., 2014), whereas others reported that two PGF treatments were necessary to maximize P/AI for both dairy heifers and beef cattle (Kasimanickam et al., 2009; Lima et al., 2013). Similarly, some groups reported that omission of the initial GnRH resulted in either no effect (Colazo and Ambrose, 2011; Lima et al., 2011) or an increase (Macmillan et al., 2017) in P/AI. Others (Lima et al., 2013) reported a 4 to 5 percentage point reduction when removing the GnRH treatment. Differences in semen type used (sexed vs. conventional), sample size, AIE after CIDR removal (yes or no), and other factors may have contributed to the discrepancies observed in P/AI among different studies. Thus, additional research is needed to identify potential modifications of the 5dCP that allow simplification and reduced animal handling without affecting P/AI of TAI services.

From a practical perspective, removal of the initial GnRH is less critical, since CIDR insertion is scheduled for the same day and time. Therefore, removing this step would not reduce the number of heifer-handling events. Conversely, omitting the additional PGF treatment minimizes the number of human interventions and thereby the number of days of the week that

animal handling is required (i.e., from 4 to 3 d). The use of two PGF treatments in the 5dCP is justified by the increased risk of incomplete luteolysis for heifers that ovulate in response to the initial GnRH if the GnRH treatment is included. Nonetheless, because only 25 to 34% of heifers ovulate in response to GnRH (Colazo and Ambrose, 2011; Lima et al., 2013) removal of the additional PGF may affect only a relatively small proportion of animals. Although results for the multiple variants of the 5dCP have not been consistent, it is apparent that those designed to optimize ovarian responses have been more effective. Thus, dairy producers need to decide if the reduction in cost and effort associated with fewer treatment interventions offsets the potential negative economic effect of reducing P/AI in some heifers.

3. Reproductive and economic performance of dairy heifers managed with different reproductive management strategies

3.1. Timing of pregnancy and dairy herd profitability during the rearing period

When selecting a reproductive management strategy for dairy heifers, it is important to consider the potential effect of each method on insemination and conception risk, the two main drivers of reproductive performance in a herd. For dairy heifers, a direct benefit of improved reproductive performance is reducing time to pregnancy, which in turn shortens time to first calving. This is relevant because first calving marks the end of the rearing period and the beginning of heifers inflowing into the milking herd. By definition, the rearing period is financially non-productive. Heifers need to be fed and taken care of without any financial return until they initiate first lactation. Therefore, raising heifers typically represents a large financial investment for most farms, usually accounting for up to 20% of total operating costs (Gabler et al., 2000; Karszes, 2014). Therefore, the choice of reproductive management strategy should be

grounded on maximizing the number of pregnant heifers immediately after they become eligible, so that feed and other costs associated with rearing can be minimized. Another important consideration when selecting a reproductive program is implementation cost, which is driven by the cost of semen, detection of estrus, pregnancy testing, reproductive hormones for synchronization of estrus and ovulation and labor costs associated with program implementation. Ultimately, the economic benefits of improving reproductive performance should offset the cost and effort invested in program implementation.

In general, farms may use strategies that prioritize AIE, TAI or a compromise between the two (i.e., combined programs). For dairy operations that prioritize AIE after spontaneous or PGF-induced estrus, optimizing reproductive performance may depend mostly on implementation of a proactive and consistent estrus detection method that ensures timely insemination of heifers soon after they become eligible for AI (i.e., beginning of the AIP). For these farms, estrus-detection aids (e.g., tail paint, automated estrus-detection systems) are available to either complement or improve estrus detection efficiency (Holman et al., 2011; Silper et al., 2015; Mayo et al., 2019). On the other hand, for farms that either prefer to or may benefit from the use of TAI, most variation in reproductive performance is probably explained by the type of synchronization of ovulation protocol used, compliance with hormonal treatments, and the fertility obtained after TAI services.

Few experiments have compared reproductive and economic performance of dairy heifers managed with strategies that rely primarily on AIE versus more intensive TAI-based reproductive programs. For instance, Stevenson et al. (2008) reported similar time to pregnancy but reduced cost per pregnancy for heifers submitted to AI after induction of estrus with PGF every 14-d as compared with submission of all heifers to TAI. In agreement, Lopes et al. (2013)

reported reduced days to pregnancy and total costs for an AIE-based strategy (PGF treatments every 11 d) relative to an all-TAI strategy. For these experiments, reduced reproductive costs (Stevenson et al., 2008; Lopes et al., 2013) along with reduced feed and other costs associated with shortened time to pregnancy (Lopes et al., 2013) resulted in reduced total costs for the AIE-based strategies. Conversely, more recently, Silva et al. (2015) reported reduced time to pregnancy and cost per pregnancy (-$17/heifer) for a TAI-based than for an AIE-based program. In this experiment, the economic benefit of reduced time to pregnancy (e.g., reduced feed cost) observed for the TAI-based strategy offset the added cost of hormonal treatments and labor associated with program implementation.

Two factors might explain, at least in part, the discrepancy observed between results from Silva et al. (2015) and those from Stevenson et al. (2008) and Lopes et al. (2013). First, heifers managed with TAI-based strategies in Stevenson et al. (2008) and Lopes et al. (2013) were synchronized with either a 7-d Cosynch or a 5-d Cosynch with shortened proestrus (i.e., time between PGF and final GnRH < 72 h) and no initial GnRH, both of which may have led to suboptimal fertility after TAI. As a result, TAI-based strategies in these experiments did not optimize synchrony of ovulation and the endocrine milieu before TAI which led to reduced P/AI relative to the AIE strategies (13 to 25 percentage points). On the other hand, heifers in Silva et al. (2015) received TAI after synchronization with a variant of 5dCP known to maximize P/AI in heifers (i.e., initial GnRH, additional PGF treatment, and extended proestrus). As a result, P/AI for TAI services were similar to that of AIE services.

Another caveat of the experiments presented in Stevenson et al. (2008) and Lopes et al. (2013) was that heifers in the AIE-based strategies became eligible for AI earlier than heifers in the TAI-based strategies. This delay in time to first service imposed by the experimental designs

was substantial and explained part of the delay in time to pregnancy observed for TAI-based programs. In contrast, Silva et al. (2015) matched the beginning of the AIP with the second PGF treatment of the 5dCP. This allowed heifers managed with the all-TAI strategy to receive AI within 1 or 2 days after they became eligible for pregnancy. As a result, the TAI-based program increased the insemination rate and reduced time to pregnancy relative to the AIE-based strategy. Collectively, results from these multiple experiments suggested that matching the day of PGF treatment or CIDR removal with the beginning of the AIP and utilizing protocols that maximize fertility is critical to ensure the success of TAI-based programs for dairy heifers.

A potential disadvantage for the AIE-based strategy evaluated in Silva et al. (2015) was that heifers received the first PGF treatment for induction of estrus 7 days after the beginning of the AIP. This is relevant because if the PGF treatment were given at the beginning of the AIP, a large proportion of heifers would have displayed estrus within 3 to 4 d, hastening insemination and reducing the gap with the all-TAI based strategy. Therefore, it remains to be determined whether an AIE-based strategy that includes a PGF treatment at the beginning of the AIP would result in similar time to pregnancy than an all-TAI program after synchronization with the 5dCP.

Reproductive management programs that combine AIE and TAI can help reduce time to pregnancy through rapid insemination of most heifers through AI after PGF-induced estrus and submission to TAI of heifers not detected in estrus. Such type of program is rarely used for heifers (NAHMS, 2018), but have been widely adopted for managing first AI service in lactating cows (Caraviello et al., 2006). In this regard, the Presynch-Ovsynch (Chebel et al., 2006; Giordano et al., 2016) and Presynch-Cosynch (Stevenson, 2011) protocols are typical examples used for lactating cows. Briefly, these protocols consist of two PGF treatments 14 days apart (Presynch), with the first GnRH of the Ovsynch portion of the protocol given 12 days after the

second PGF treatment of Presynch. In farms with reasonable estrus detection efficiency, up to 50 to 70% of cows may display estrus and become inseminated during the Presynch portion of the protocol (Chebel et al., 2006; Fricke et al., 2014; Giordano et al., 2016), which may reduce the number of cows that complete the protocol and receive TAI. Therefore, these combined programs have the advantage of reducing the number of cows submitted to TAI, reducing the number of hormonal treatments and animal-handling interventions required relative to all-TAI programs. In addition, combined programs may also be more advantageous than all-AIE programs, as delayed insemination is prevented by the inclusion of TAI after the period of AIE. This is particularly relevant for herds with low estrus detection efficiency. To the best of my knowledge, combined programs have not been evaluated for use in dairy heifers. Therefore, it remains to be determined if reproductive management strategies for first service that combine a period of AIE after PGF-induced estrus and TAI for heifers not AIE results in better reproductive and economic performance than all-AIE or all-TAI programs.

3.2. Impact of reduced age at first calving on the economic and productive performance of cows

For replacement heifers, faster generation of pregnancies through efficient and proactive reproductive management not only reduces rearing costs, but also generates income more rapidly via earlier incorporation of heifers into the milking herd. Unfortunately, data from most published research investigating the effect of different reproductive programs on the economic performance of dairy heifers (Stevenson et al., 2008; Lopes et al., 2013; Silva et al., 2015) only accounted for costs accrued during the rearing period. Little or no consideration was given to the potential economic benefit of earlier onset of milk production (i.e., productive life). To incorporate such benefit, individual heifer costs and revenues generated after first calving should

also be monitored and accounted for. In addition, all individual economic parameters should be evaluated for a fixed period after the beginning of the AIP to account for the opportunity cost of delayed lactation.

An approach previously used to assess the economic impact of improved reproductive performance of heifers has been to investigate the association between AFC and heifer profitability. Although many factors can explain variation in AFC within a herd (e.g., nutrition, growth rate), when heifers become eligible for pregnancy at the same age the main determinant of AFC is time to pregnancy. Heifers that conceive earlier after they become eligible for pregnancy calve at a younger age and thereby have reduced rearing cost and achieve earlier positive cash flow in their lifetime. In this regard, Ettema and Santos (2004) conducted an observational study to investigate the association between AFC and the productive and economic performance of Holstein replacement heifers accounting for both, rearing and first lactation (up to 310 d) outcomes. Heifers (n = 1,933) were eligible for AI at the same age range (12 to 13 mo) but were not randomly assigned to groups of insemination at different ages. Rather, heifers were retrospectively assigned to AFC groups after first calving (low = ≤ 23.0 mo; medium = 23.1 to 24.6 mo; high ≥ 24.7) under the assumption that differences in AFC were due to variation in reproductive performance. As expected, rearing costs increased as AFC increased (+$40 and +$108 for medium and high AFC heifers, respectively). Conversely, gross income for first lactation (accounting for milk income and costs generated during lactation) was lowest for the low AFC group, followed by the high and medium groups. Reduced income for the low AFC group was attributed to lesser milk yield during first lactation and a greater incidence of stillbirths. When accounting for both rearing costs and first lactation income, there were no statistically significant differences between groups. Nevertheless, numerical differences of

potential value to dairy farms were observed between the medium and low groups (+$138/heifer in favor of medium AFC group) and the high and medium groups (+$99/heifer in favor of medium AFC group). Thus, although younger AFC was associated with reduced milk yield and first lactation income, extending AFC beyond ~25 mo (i.e., high AFC group) also compromised economic performance. This was because extending AFC beyond 25 mo increased rearing costs without improving first lactation milk production relative to medium AFC. Based on these results, the authors concluded that the greatest economic return was attained when calving occurred between 23 to 24 months of age. A caveat of this study was that in spite of accounting for first lactation performance, the true opportunity cost of delayed lactation was not accounted for in the analysis. Thus, although these results were valuable to provide a basic understanding of the association between dairy heifer economic performance and AFC (and albeit indirectly, of reproductive performance), more research is needed to elucidate the economic implications of earlier onset of productive life in replacement heifers.

In agreement with Ettema and Santos (2004), other research groups have also observed the negative association between younger AFC and first lactation milk yield. For instance, using records from more than one million Italian dairy heifers, Pirlo et al. (2000) observed reduced first lactation milk yield for heifers calving at 22 mo of age relative to those calving at 23 and 24 mo. Other studies have reported that despite reduced first lactation yield, younger AFC may result in greater lifetime milk production. For instance, Lin et al. (1986) conducted a randomized controlled experiment with Canadian Holsteins to evaluate productive performance of heifers with early or late beginning of the AIP (12 and 15 mo of age, respectively). As expected, heifers in the early AI group calved at a younger age (23 vs. 26 mo for early and late, respectively) and had reduced first lactation milk yield than those in the late group. However, when evaluating

performance up to 61 mo of age [including second and third lactation data (Lin et al., 1988)] heifers inseminated in the early group produced more total milk than those in the late group. Similarly, using records from almost 14 million Holstein heifers, Hutchison et al. (2017) observed that despite reduced first lactation milk yield, heifers calving from 21 to 22 mo of age had greater lifetime milk production than those calving at 24 mo. Thus, it appears that the AFC target for a farm must be selected in light of the potential advantages of younger AFC (i.e., reduced rearing costs and extended lifetime productivity) but also considering the possible adverse effects of younger calving age on performance and health (e.g., milk yield and greater stillbirth incidence) during the first lactation. Thus, further research using randomized controlled experiments should explore the identification of AFC targets (and therefore, breeding age) that maximize long-term profitability of heifers.

In summary, results from the few experiments conducted to evaluate reproductive management strategies have been inconclusive regarding their effect on heifer profitability. This is primarily due to the limited data available about the economic implications of delayed onset of first lactation. On the other hand, observational studies have provided valuable findings suggesting that AFC between 22 to 24 mo is associated with increased economic return. Since AFC largely depends on age at conception, reproductive efficiency is key to reduce average AFC with minimal variation and thus avoid delayed AFC in most heifers. Therefore, more research is needed to investigate if reproductive management strategies for heifers that result in high pregnancy rates and reduced AFC will improve farm profitability by reducing rearing costs and the opportunity cost of delayed onset of first lactation.

4. Summary

Raising replacement dairy heifers represents a substantial financial investment for most farms, typically representing up to 20% of total operating costs. Thus, a potential strategy to reduce the burden of heifer rearing cost in dairy farms is to optimize AFC though a proactive and effective reproductive program that hastens pregnancy after the beginning of the AIP. Reducing time to pregnancy is financially attractive because it shortens the nonlactating period of heifers, reducing rearing costs, and favoring earlier positive cash flow via milk production. Only a limited number of studies have evaluated the effect of different management strategies on the reproductive and economic performance of replacement heifers. In addition, the few economic analyses conducted did not incorporate the opportunity cost of delayed lactation.

Thus, the experiment presented in Chapter II of this section had the objective of comparing the insemination dynamics and time to pregnancy for heifers managed with first AI service programs designed to have different insemination rates through the incorporation of synchronization of estrus and ovulation for TAI. We evaluated programs that relied primarily on AIE, TAI, or a combination of both. In Chapter III, a deterministic and stochastic analysis of the profitability of heifers managed with such strategies is presented.

5. References

Akins, M., M. Vanderwerff, M. Hagedorn, P. Hoffman, S. Gunderson, and T. Kohlman. 2015. Economic costs and labor efficiencies associated with raising dairy herd replacements on Wisconsin dairy farms and custom heifer raising operations. University of Wisconsin-Extension, Madison, WI, USA.

Bello, N. M., J. Steibel, and J. Pursley. 2006. Optimizing ovulation to first GnRH improved outcomes to each hormonal injection of Ovsynch in lactating dairy cows. J. Dairy Sci. 89:3413-3424.

Bridges, G., L. Helser, D. Grum, M. Mussard, C. Gasser, and M. Day. 2008. Decreasing the interval between GnRH and PGF2α from 7 to 5 days and lengthening proestrus increases timed-AI pregnancy rates in beef cows. Theriogenology 69:843-851.

Caraviello, D., K. Weigel, P. Fricke, M. Wiltbank, M. Florent, N. Cook, K. Nordlund, N. Zwald, and C. Rawson. 2006. Survey of management practices on reproductive performance of dairy cattle on large US commercial farms. J. Dairy Sci. 89:4723-4735.

Cerri, R., H. Rutigliano, R. Bruno, R. Chebel, and J. Santos. 2005. Effect of artificial insemination (AI) protocol on fertilization and embryo quality in high-producing dairy cows. J. Dairy Sci. 88 (Suppl. 1): 86. (Abstr.)

Chebel, R. C., F. A. Braga, and J. C. Dalton. 2007. Factors affecting reproductive performance of Holstein heifers. Anim. Reprod. Sci. 101:208-224.

Chebel, R. C., J. E. Santos, R. L. Cerri, H. M. Rutigliano, and R. Bruno. 2006. Reproduction in dairy cows following progesterone insert presynchronization and resynchronization protocols. J. Dairy Sci. 89:4205-4219.

Colazo, M. and D. Ambrose. 2011. Neither duration of progesterone insert nor initial GnRH treatment affected pregnancy per timed-insemination in dairy heifers subjected to a Co-synch protocol. Theriogenology 76:578-588.

Colazo, M. G., J. P. Kastelic, H. Davis, M. D. Rutledge, M. F. Martinez, J. A. Small, and R. J. Mapletoft. 2008. Effects of plasma progesterone concentrations on LH release and ovulation in beef cattle given GnRH. Domest. Anim. Endocrinol. 34:109-117.

Colazo, M. G. and R. J. Mapletoft. 2017. Pregnancy per AI in Holstein heifers inseminated with sex-selected or conventional semen after estrus detection or timed-AI. Can. Vet. J. 58:365-70.

Ettema, J. and J. Santos. 2004. Impact of age at calving on lactation, reproduction, health, and income in first-parity Holsteins on commercial farms. J. Dairy Sci. 87:2730-2742.

Ferguson, J. D. and A. Skidmore. 2013. Reproductive performance in a select sample of dairy herds. J. Dairy Sci. 96:1269-1289.

Fogwell, R., W. Reid, C. Thompson, M. Thome, and D. Morrow. 1986. Synchronization of estrus in dairy heifers: a field demonstration. J. Dairy Sci. 69:1665-1672.

Fricke, P., J. Giordano, A. Valenza, G. Lopes, M. Amundson, and P. Carvalho. 2014. Reproductive performance of lactating dairy cows managed for first service using timed artificial insemination with or without detection of estrus using an activity-monitoring system. J. Dairy Sci. 97:2771-2781.

Gabler, M., P. Tozer, and A. J. Heinrichs. 2000. Development of a cost analysis spreadsheet for calculating the costs to raise a replacement dairy heifer. J. Dairy Sci. 83:1104-1109.

Geary, T., J. W. Pas, F. Thrift, and S. Dolezal. 1998. Effects of a timed insemination following synchronization of ovulation using the Ovsynch or CO-Synch protocol in beef cows. Prof. Anim. Sci. 14:217-220.

Giordano, J., P. Fricke, J. Guenther, G. Lopes Jr, M. Herlihy, A. Nascimento, and M. Wiltbank. 2012. Effect of progesterone on magnitude of the luteinizing hormone surge induced by two different doses of gonadotropin-releasing hormone in lactating dairy cows. J. Dairy Sci. 95:3781-3793.

Giordano, J., M. Thomas, G. Catucuamba, M. Curler, R. Wijma, M. Stangaferro, and M. Masello. 2016. Effect of extending the interval from Presynch to initiation of Ovsynch in a Presynch-Ovsynch protocol on fertility of timed artificial insemination services in lactating dairy cows. J. Dairy Sci. 99:746-757.

Hafs, H., J. Manns, and B. Drew. 1975. Onset of oestrus and fertility of dairy heifers and suckled beef cows treated with prostaglandin F2α. Anim. Sci. 21:13-20.

Harsh, S. B., C. A. Wolf, and E. Wittenberg. 2001. Profitability and production efficiency of the crop and livestock enterprises of Michigan dairy operations: 1998 summary and analysis. Agric. Econ Staff Paper No. 01-04. Michigan State Univ., East Lansing.

Heinrichs, A. J., C. Jones, S. Gray, P. Heinrichs, S. Cornelisse, and R. Goodling. 2013. Identifying efficient dairy heifer producers using production costs and data envelopment analysis. J. Dairy Sci. 96:7355-7362.

Helmer, S. and J. Britt. 1985. Mounting behavior as affected by stage of estrous cycle in Holstein heifers. J. Dairy Sci. 68:1290-1296.

Henricks, D., J. Long, J. Hill, and J. Dickey. 1974. The effect of prostaglandin F2α during various stages of the oestrous cycle of beef heifers. Reproduction 41:113-120.

Holman, A., J. Thompson, J. Routly, J. Cameron, D. Jones, D. Grove-White, R. Smith, and H. Dobson. 2011. Comparison of oestrus detection methods in dairy cattle. Vet. Rec. 169:47-47.

Hutchison, J., P. VanRaden, D. Null, J. Cole, and D. Bickhart. 2017. Genomic evaluation of age at first calving. J. Dairy Sci. 100:6853-6861.

Kaim, M., M. Rosenberg, and Y. Folman. 1990. Management of reproduction in dairy heifers based on the synchronization of estrous cycles. Theriogenology 34:537-547.

Karakaya-Bilen, E., E. S. Ribeiro, R. S. Bisinotto, A. Gümen, and J. E. P. Santos. 2019. Effect of presynchronization with prostaglandin F2α before the 5-d timed AI protocol on ovarian responses and pregnancy in dairy heifers. Theriogenology 132:138-143.

Karszes, J. 2014. Dairy Replacement Programs: Costs & Analysis 3rd Quarter 2012. Extension Bulletin 2014-02, Department of Animal Science and Charles H. Dyson School of Applied Economics and Management, Cornell University, Ithaca, NY.

Kasimanickam, R., M. Day, J. Rudolph, J. Hall, and W. Whittier. 2009. Two doses of prostaglandin improve pregnancy rates to timed-AI in a 5-day progesterone-based synchronization protocol in beef cows. Theriogenology 71:762-767.

Kasimanickam, R., P. Firth, G. M. Schuenemann, B. Whitlock, J. Gay, D. Moore, J. Hall, and W. Whittier. 2014. Effect of the first GnRH and two doses of PGF2α in a 5-day progesterone-based CO-Synch protocol on heifer pregnancy. Theriogenology 81:797-804.

Kim, I.-H., G.-H. Suh, and D.-S. Son. 2003. A progesterone-based timed AI protocol more effectively prevents premature estrus and incomplete luteal regression than an Ovsynch protocol in lactating Holstein cows. Theriogenology 60:809-817.

King, G. J. and H. A. Robertson. 1974. A two injection schedule with prostaglandin F2α for the regulation of the ovulatory cycle of cattle. Theriogenology 1:123-128.

Kuhn, M., J. Hutchison, and G. Wiggans. 2006. Characterization of Holstein heifer fertility in the United States. J. Dairy Sci. 89:4907-4920.

Lauderdale, J., B. Seguin, J. Stellflug, J. Chenault, W. Thatcher, C. Vincent, and A. Loyancano. 1974. Fertility of cattle following PGF2α injection. J. Anim. Sci. 38:964-967.

Lima, F., H. Ayres, M. Favoreto, R. S. Bisinotto, L. F. Greco, E. Ribeiro, P. Baruselli, C. Risco, W. Thatcher, and J. Santos. 2011. Effects of gonadotropin-releasing hormone at initiation of the 5-d timed artificial insemination (AI) program and timing of induction of ovulation relative to AI on ovarian dynamics and fertility of dairy heifers. J. Dairy Sci. 94:4997-5004.

Lima, F., E. Ribeiro, R. Bisinotto, L. Greco, N. Martinez, M. Amstalden, W. Thatcher, and J. Santos. 2013. Hormonal manipulations in the 5-day timed artificial insemination protocol to optimize estrous cycle synchrony and fertility in dairy heifers. J. Dairy Sci. 96:7054-7065.

Lin, C., A. McAllister, T. Batra, A. Lee, G. Roy, J. Vesely, J. Wauthy, and K. Winter. 1986. Production and reproduction of early and late bred dairy heifers. J. Dairy Sci. 69:760-768.

Lin, C., A. McAllister, T. Batra, A. Lee, G. Roy, J. Vesely, J. Wauthy, and K. Winter. 1988. Effects of early and late breeding of heifers on multiple lactation performance of dairy cows. J. Dairy Sci. 71:2735-2743.

Lopes, G., C. Johnson, L. Mendonça, P. Silva, J. Moraes, A. Ahmadzadeh, J. Dalton, and R. Chebel. 2013. Evaluation of reproductive and economic outcomes of dairy heifers inseminated at induced estrus or at fixed time after a 5-day or 7-day progesterone insert-based ovulation synchronization protocol. J. Dairy Sci. 96:1612-1622.

Louis, T., H. Hafs, and B. Seguin. 1973. Progesterone, LH, estrus and ovulation after prostaglandin F2α in heifers. Proc. Soc. Exp. Biol. Med. 143:152-155.

Macmillan, K., K. Loree, R. Mapletoft, and M. Colazo. 2017. Optimization of a timed artificial insemination program for reproductive management of heifers in Canadian dairy herds. J. Dairy Sci. 100:4134-4138.

Martínez, M. F., J. P. Kastelic, G. P. Adams, B. Cook, W. O. Olson, and R. J. Mapletoft. 2002. The use of progestins in regimens for fixed-time artificial insemination in beef cattle. Theriogenology 57:1049-1059.

Mayo, L., W. Silvia, D. Ray, B. Jones, A. Stone, I. Tsai, J. Clark, J. Bewley, and G. Heersche Jr. 2019. Automated estrous detection using multiple commercial precision dairy monitoring technologies in synchronized dairy cows. J. Dairy Sci. 102:2645-2656.

NAHMS. 2007. Dairy 2007, Part I: Reference of Dairy Cattle Health and Management Practices in the United States, 2007. Centers for Epidemiology and Animal Health, USDA: Animal and Plant Health Inspection Service: Veterinary Services, Fort Collins, CO.

NAHMS. 2018. Dairy 2014, Part III: Reference of Dairy Cattle Health and Management Practices in the United States, 2014. #696.0218. Centers for Epidemiology and Animal Health, USDA: Animal and Plant Health Inspection Service: Veterinary Services, Fort Collins, CO.

Pirlo, G., F. Miglior, and M. Speroni. 2000. Effect of age at first calving on production traits and on difference between milk yield returns and rearing costs in Italian Holsteins. J. Dairy Sci. 83:603-608.

Pursley, J., M. Mee, and M. Wiltbank. 1995. Synchronization of ovulation in dairy cows using PGF2α and GnRH. Theriogenology 44:915-923.

Pursley, J., M. Wiltbank, J. Stevenson, J. Ottobre, H. Garverick, and L. Anderson. 1997. Pregnancy rates per artificial insemination for cows and heifers inseminated at a synchronized ovulation or synchronized estrus1. J. Dairy Sci. 80:295-300.

Rabaglino, M., C. Risco, M.-J. Thatcher, I. Kim, J. Santos, and W. Thatcher. 2010. Application of one injection of prostaglandin F2α in the five-day Co-Synch+ CIDR protocol for estrous synchronization and resynchronization of dairy heifers. J. Dairy Sci. 93:1050-1058.

Rivera, H., H. Lopez, and P. Fricke. 2004. Fertility of Holstein dairy heifers after synchronization of ovulation and timed AI or AI after removed tail chalk. J. Dairy Sci. 87:2051-2061.

Rivera, H., H. Lopez, and P. Fricke. 2005. Use of intravaginal progesterone-releasing inserts in a synchronization protocol before timed AI and for synchronizing return to estrus in Holstein heifers. J. Dairy Sci. 88:957-968.

Rowson, L., R. Tervit, and A. Brand. 1972. The use of prostaglandins for synchronization of oestrus in cattle. J. Reprod. Fertil. 29:145-145.

Sangsritavong, S., D. Combs, R. Sartori, L. Armentano, and M. Wiltbank. 2002. High feed intake increases liver blood flow and metabolism of progesterone and estradiol-17β in dairy cattle. J. Dairy Sci. 85:2831-2842.

Sartori, R., J. Haughian, R. Shaver, G. Rosa, and M. Wiltbank. 2004. Comparison of ovarian function and circulating steroids in estrous cycles of Holstein heifers and lactating cows. J. Dairy Sci. 87:905-920.

Savio, J., L. Keenan, M. Boland, and J. Roche. 1988. Pattern of growth of dominant follicles during the oestrous cycle of heifers. Reproduction 83:663-671.

Schmitt, E., T. Diaz, M. Drost, and W. Thatcher. 1996. Use of a gonadotropin-releasing hormone agonist or human chorionic gonadotropin for timed insemination in cattle. J. Anim. Sci. 74:1084-1091.

Silper, B., A. Madureira, M. Kaur, T. Burnett, and R. Cerri. 2015. Comparison of estrus characteristics in Holstein heifers by 2 activity monitoring systems. J. Dairy Sci. 98:3158-3165.

Silva, T., F. Lima, W. Thatcher, and J. Santos. 2015. Synchronized ovulation for first insemination improves reproductive performance and reduces cost per pregnancy in dairy heifers. J. Dairy Sci. 98:7810-7822.

Stevenson, J. 2011. Alternative programs to presynchronize estrous cycles in dairy cattle before a timed artificial insemination program1. J. Dairy Sci. 94:205-217.

Stevenson, J., J. Rodrigues, F. Braga, S. Bitente, J. Dalton, J. Santos, and R. Chebel. 2008. Effect of Breeding Protocols and Reproductive Tract Score on Reproductive Performance of Dairy Heifers and Economic Outcome of Breeding Programs1. J. Dairy Sci. 91:3424-3438.

Stevenson, J., M. Schmidt, and E. Call. 1984. Stage of Estrous Cycle, Time of Insemination, and Seasonal Effects on Estrus and Fertility of Holstein Heifers After Prostaglandin F2α1. J. Dairy Sci. 67:1798-1805.

Tanabe, T. and R. Hann. 1984. Synchronized estrus and subsequent conception in dairy heifers treated with prostaglandin F2 α. I. Influence of stage of cycle at treatment. J. Anim. Sci. 58:805-811.

Vasconcelos, J., R. Silcox, G. Rosa, J. Pursley, and M. Wiltbank. 1999. Synchronization rate, size of the ovulatory follicle, and pregnancy rate after synchronization of ovulation beginning on different days of the estrous cycle in lactating dairy cows. Theriogenology 52:1067-1078.

Whittier, W., R. Kasimanickam, J. Currin, H. Schramm, and M. Vlcek. 2010. Effect of timing of second prostaglandin F2α administration in a 5-day, progesterone-based CO-Synch protocol on AI pregnancy rates in beef cows. Theriogenology 74:1002-1009.

Wiltbank, M. C. and J. R. Pursley. 2014. The cow as an induced ovulator: Timed AI after synchronization of ovulation. Theriogenology 81:170-185.

CHAPTER II

REPRODUCTIVE PERFORMANCE OF REPLACEMENT DAIRY HEIFERS SUBMITTED TO FIRST SERVICE WITH PROGRAMS THAT FAVOR INSEMINATION AT DETECTED ESTRUS, TIMED AI, OR A COMBINATION OF BOTH

M. Masello, M. M. Perez, G. E. Granados, M. L. Stangaferro, B. Ceglowski,

M. J. Thomas, and J. O. Giordano

Journal of Dairy Science 2019; 102(2): 1671-1681.

DOI: 10.3168/jds.2018-15200

ABSTRACT

Our objective was to compare the insemination dynamics and time to pregnancy for up to 100 d after the beginning of the artificial insemination period (**AIP**) for heifers managed with first AI service programs that relied primarily on insemination at detected estrus (**AIE**) after $PGF_{2\alpha}$ (**PGF**) treatments, timed AI (**TAI**), or a combination of both. Holstein heifers were randomly assigned to receive first AI service with gender-selected semen after 368 ± 10 d of age with: 1) PGF+AIE (n = 317): AIE after synchronization of estrus with up to three $PGF_{2\alpha}$ treatments every 14-d starting on the first day of the AIP. Heifers not AIE up to 9 d after the third $PGF_{2\alpha}$ received a 5d-Cosynch protocol with progesterone supplementation [GnRH + controlled internal drug release insert (**CIDR**)-5 d-CIDR removal and $PGF_{2\alpha}$-3 d-GnRH and TAI] before TAI. Heifers detected in estrus from CIDR removal and $PGF_{2\alpha}$ until the day before TAI received AIE with no GnRH treatment; 2) PGF+TAI (n = 334): two $PGF_{2\alpha}$ treatments 14 d apart with the second treatment at the beginning of the AIP. Heifers received AIE for up to 9 d

after the second $PGF_{2\alpha}$ treatment. Heifers not AIE received TAI after the 5d-Cosynch protocol; and 3) ALL-TAI (n = 315): TAI after the 5d-Cosynch protocol. Heifers failing to conceive to a previous AI received a subsequent AI with conventional semen at detected estrus or TAI after the 5d-Cosynch protocol. Binomial outcomes were analyzed by logistic regression whereas time to AI and pregnancy were analyzed with Cox's regression in SAS. The hazard of first AI up to 45 d of the AIP was greater for ALL-TAI than for PGF+AIE [hazard ratio (**HR**) = 1.72; 95% CI 1.45 to 2.03] and PGF+TAI (HR = 1.51; 95% CI 1.28 to 1.77), but similar for PGF+AIE and PGF+TAI (HR = 1.14; 95% CI 0.97 to 1.33)]. A greater proportion of heifers received AIE in PGF+AIE (98.7%) than in PGF+TAI (78.5%). Overall, first service pregnancy per AI (P/AI) did not differ (PGF+AIE = 42.0%; PGF+TAI = 47.3%, ALL-TAI = 43.8%). Time to pregnancy was reduced for ALL-TAI compared with PGF+AIE (HR = 1.20, 95% CI 1.02 to 1.42), but was similar to that of PGF+TAI (HR = 1.13, 95% CI 0.96 to 1.33). Time to pregnancy did not differ for PG+AIE and PGF+TAI (HR = 1.07, 95% CI 0.91 to 1.25). Median days to pregnancy were 27, 23, and 21 for heifers in PGF+AIE, PGF+TAI and ALL-TAI, respectively. We concluded that an ALL-TAI program for first service reduced time to pregnancy, albeit a relatively small reduction, when compared with a program that relied primarily on AIE after induction of estrus with $PGF_{2\alpha}$ treatments. The program that combined synchronization of estrus and TAI (PGF+TAI) resulted in similar time to pregnancy than the predominant TAI and predominant AIE programs.

Keywords: insemination at detected estrus, 5d-Cosynch, timed AI, dairy heifers

INTRODUCTION

Reproductive efficiency of dairy heifers is critical for the economics of the heifer enterprise because reducing time to pregnancy reduces the rearing and opportunity costs of replacements (Ettema and Santos, 2004; Lopes et al., 2013; Silva et al., 2015). Traditionally, reproductive management programs for dairy heifers have been based primarily on inseminations after detected estrus (**AIE**) rather than through synchronization of ovulation for timed AI (**TAI**) (NAHMS, 2018). High estrus detection efficiency and P/AI for programs that rely primarily on AIE (Stevenson et al., 2008; Lopes et al., 2013; Colazo and Mapletoft, 2017) and poor P/AI for TAI services with 7 d Ovsynch-like protocols (Schmitt et al., 1996; Pursley et al., 1997) are likely main reasons for the low adoption of TAI in heifers.

Although estrus expression is usually not a major limitation for the success of AIE-based programs for heifers (Stevenson et al., 2008; Lopes et al., 2013; Colazo and Mapletoft, 2017), total reliance on AIE may lead to suboptimal reproductive performance of the heifer herd because of biological variation in timing of estrus expression and poor estrus detection efficiency due to management constraints. Conversely, the implementation of TAI can improve AI submission rates reducing the number of days to first service and the interval between subsequent services. In addition, 5d-Cosynch protocols with progesterone supplementation (5d-Cosynch) are now an alternative to obtain comparable P/AI than for AI services after spontaneous or $PGF_{2\alpha}$-induced estrus (Silva et al., 2015, Colazo and Mapletoft, 2017). Thus, TAI programs including 5d-Cosynch for synchronization of ovulation may be an alternative to reduce time to pregnancy by minimizing days to first service and achieving the same or greater P/AI than with programs based on all-AIE.

Reproductive management programs for first service that combine inseminations after $PGF_{2\alpha}$-induced estrus with Presynch-like protocols (i.e., two $PGF_{2\alpha}$ treatments 14 d apart) and submission to a TAI protocol 10 to 14 after Presynch are a compromise between all-AIE and all-TAI programs. These programs can help reduce time to pregnancy after the end of the voluntary waiting period through rapid insemination of most cows after Presynch and immediate submission to TAI of those cows not detected in estrus. Programs such as Presynch-Ovsynch (Chebel et al., 2006; Fricke et al., 2014; Giordano et al., 2016) and Presynch-Cosynch (Stevenson, 2011; Ribeiro et al., 2012) have been extensively evaluated and are widely used for first service management of lactating dairy cows (Caraviello et al., 2006; Ferguson and Skidmore, 2013). In spite of the fact that these programs may effectively improve reproductive performance of dairy heifers, they have not been evaluated extensively in research and are rarely used for heifers in commercial farms (NAHMS, 2018). Indeed, the combination of high estrus detection efficiency after Presynch, good P/AI for AIE services (Stevenson et al., 2000; Colazo and Mapletoft, 2017), and immediate submission to TAI of heifers not detected in estrus after Presynch may reduce time to pregnancy as effectively as all-TAI programs.

Few experiments evaluated the impact of management strategies that result in different insemination dynamics on time to pregnancy of dairy heifers. For instance, Lopes et al. (2013) reported reduced days to pregnancy for heifers submitted to first service after induction of estrus with $PGF_{2\alpha}$ every 11 d compared with submission to TAI after a 5 or 7-day Cosynch protocol. Greater P/AI and earlier submission to first service due to fewer days until heifer were eligible for AI favored the AIE-based program. Conversely, more recently Silva et al. (2015) reported reduced time to pregnancy for an all-TAI than for an all-AIE program. An equal number of days until heifers were eligible for AI and P/AI for TAI than AIE services favored the TAI program as

heifers were inseminated earlier. Therefore, it remains unclear if all-TAI programs including 5d-Cosynch for synchronization of ovulation for first service reduce time to pregnancy when compared with all-AIE programs for dairy heifers. Moreover, the insemination and pregnancy dynamics of heifer managed with Presynch-Ovsynch or Presynch-Cosynch like protocols has not been extensively explored.

Thus, our objective was to compare the insemination dynamics and time to pregnancy during the artificial insemination period (**AIP**) for heifers managed with first AI service programs that relied primarily on AIE, TAI, or a combination of both. We hypothesized that systematic use of reproductive management programs for dairy heifers that hasten insemination through all-TAI or programs that combine an intensive period of AIE followed by submission to TAI would reduce time to insemination and time to pregnancy when compared with a program that relied primarily on insemination after $PGF_{2\alpha}$-induced estrus.

MATERIALS AND METHODS

All procedures performed with heifers were approved by the Animal Care and Use Committee of Cornell University.

Farms and Heifer Management

Nulliparous Holstein heifers from 2 commercial dairy farms located in New York State were enrolled in this experiment (farm A located in Washington county and farm B located in Wayne county), from November 2015 to February 2017. In both farms, heifers were housed in freestall barns with concrete flooring and self-locking headgates in the feeding lane. Freestall surfaces were covered with mattresses and either sand (farm A) or dried manure bedding (farm

B). Heifers were fed a total mixed ration once a day with unlimited access to feed and water. The diet was formulated to meet or exceed the requirements for a Holstein heifer weighing 450 kg and gaining 0.8 kg/day (Cornell Net Carbohydrate and Protein System; Higgs et al., 2015).

Experimental Treatments

Once a month heifers (n = 966) of >310 d of age were stratified by age in days and then randomly allocated to one of three different treatments by the dairy herd management software (DairyComp305, ValleyAg Software, Tulare, CA). Each individual treatment consisted of a different reproductive management strategy for first AI service with gender-selected semen. Heifers became eligible for insemination (i.e., beginning of the AIP) at 368 ± 10 d of age. Body weight data at 368 ± 10 d of age was available for a subgroup of heifers at each farm. In farm A (n = 27), BW was 394 ± 34 (mean and SD) kg whereas in Farm B (n = 105) it was 383 ± 34 kg.

In farm A, estrus detection for all AI services was performed once a day by a combination of visual observation of behavioral signs of estrus and tail-paint removal whereas, only visual observation was used in farm B. In farm A, heifers were inseminated immediately after detection of estrus whereas in farm B heifers were inseminated 6 to 12 h after observation of behavioral estrus (performed inseminations twice a day).

Heifers in the PGF+AIE (n = 317) treatment received AI at detected estrus (**AIE**) after synchronization of estrus with up to three $PGF_{2\alpha}$ treatments (25 mg of Dinoprost tromethamine given s.c., Lutalyse HighCon, Zoetis, New York, NY) every 14 d starting on the first day of the AIP (Figure 1). Heifers not inseminated at detected estrus for up to 9 d after the third $PGF_{2\alpha}$ treatment received a 5d-Cosynch protocol (5d-Cosynch) for synchronization of ovulation and timed artificial insemination (**TAI**). Thus, TAI for heifers not AIE occurred 45 d after the

beginning of the AIP. The 5d-Cosynch protocol consisted of an initial GnRH treatment i.m. (100 µg of Gonadorelin chloride, Zoetis, New York, NY) plus insertion of a controlled internal drug release insert [**CIDR** (1.38 g of progesterone, Zoetis, New York, NY)]; 5 d later 25 mg of $PGF_{2\alpha}$ s.c. and CIDR removal; and finally a GnRH treatment (100 µg of Gonadorelin) and insemination 3 d after $PGF_{2\alpha}$ and CIDR removal. The use of TAI in the PGF+AIE group was a last resource to ensure insemination of all heifers within a reasonable amount of time after the beginning of the AIP rather than a preferred method of submission for AI in this group. For the PGF+TAI (n = 334) treatment, estrus was synchronized with two $PGF_{2\alpha}$ treatments 14 d apart with the second treatment given at the beginning of the AIP (Figure 1). Heifers not AIE within 9 d of the second $PGF_{2\alpha}$ treatment were enrolled in the 5d-Cosynch protocol to receive TAI. Thus, all heifers received first service within 17 d of the beginning of the AIP. Heifers in the ALL-TAI (n = 315) treatment were submitted to the 5d-Cosynch protocol for synchronization of ovulation and TAI. The beginning of the AIP for this treatment group coincided with the time of $PGF_{2\alpha}$ treatment and CIDR removal (Figure 1). All heifers in this treatment group received first service within 3 d of the beginning of the AIP.

Heifers from the three treatment groups detected in estrus after CIDR removal and the PGF treatment of 5d-Cosynch until the day before TAI received AIE with no GnRH treatment. These inseminations were considered a separate type of AI service for further analyses (i.e., during the synchronization protocol).

Heifers in the three treatment groups failing to conceive to first or subsequent inseminations received AI with conventional semen after detection of estrus or TAI. Re-inseminations at detected estrus occurred any time after a previous insemination. Heifers diagnosed nonpregnant through transrectal ultrasonography 31 ± 3 d after a previous

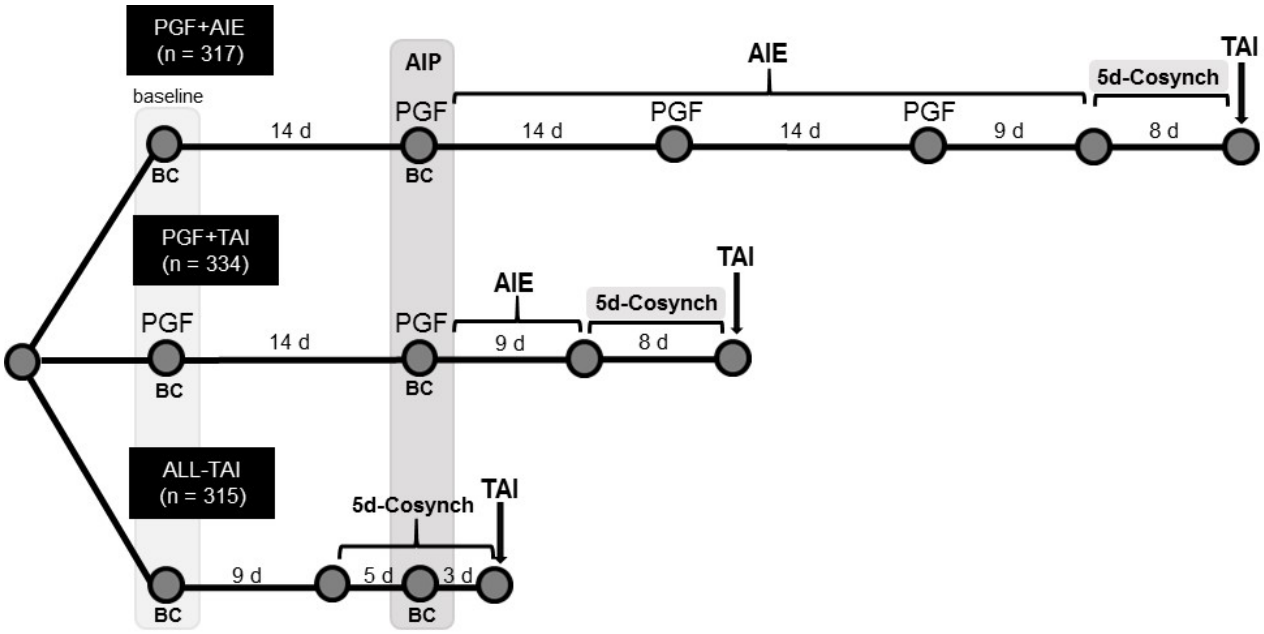


Figure 1. Graphical depiction of experimental procedures. Holstein heifers from 2 commercial farms were randomly assigned to one of three treatments before first AI service: 1) PGF+AIE (n = 317): insemination at detected estrus (AIE) after synchronization of estrus with up to three PGF2α treatments every 14 d starting on the first day of the artificial insemination period (AIP). Heifers not AIE up to 9 d after the third PGF2α treatment received a 5d-Cosynch protocol with progesterone supplementation (5d-Cosynch; GnRH + CIDR- 5d-CIDR removal + PGF2α-3 d-GnRH + TAI) for synchronization of ovulation before TAI, 2) PGF+TAI (n = 334): two PGF2α treatments 14 d apart with the second treatment at the beginning of the AIP. Heifers were AIE for up to 9 d after the second PGF2α treatment. Heifers not AIE received TAI after the 5d-Cosynch protocol, and 3) ALL-TAI (n = 315): TAI after the 5d-Cosynch protocol. In a subgroup of heifers, a blood sample was collected 14 d before the AIP to determine circulating concentrations of progesterone (P4). BC = blood collection.

insemination were enrolled in the 5d-Cosynch protocol for resynchronization of ovulation and TAI two days after nonpregnancy diagnosis. As for first service, heifers from the three treatment groups detected in estrus after CIDR removal and the PGF treatment of 5d-Cosynch until the day before TAI received AIE with no GnRH treatment. These inseminations were considered a separate type of AI service for further analyses (i.e., during the synchronization protocol).

Pregnancy Diagnosis

In both farms, pregnancy diagnosis was conducted by transrectal ultrasonography (Easi-Scan, BCF Technology Ltd., Livingston, UK) of the reproductive tract and ovaries 31 ± 3 d after AI by a veterinarian in Farm A and a technician from the research team in Farm B. Heifers re-inseminated before pregnancy diagnosis were considered not pregnant to the previous AI.

Blood Sample Collection

In a subgroup of heifers from both farms (n = 162), two blood samples were collected 14 d apart to determine circulating concentrations of progesterone (**P4**). Samples were collected 14 d before and at the beginning of the AIP (Figure 1). Blood samples (~8 to 9 mL) were collected by puncture of the caudal vein or artery using evacuated tubes containing sodium heparin (Vacutainer BD, Franklin Lakes, NJ). After collection, samples were placed in crushed ice and transported to the laboratory for further processing. Samples were centrifuged at 2,000 x g for 20 min at 4 ºC. Plasma aliquots were harvested and transferred to Eppendorf vials for storage at -20 ºC until assayed.

Determination of Progesterone Concentrations

A commercial solid-phase, no-extraction radioimmunoassay (ImmuChem Coated Tube, MP Biomedicals, Costa Mesa, CA) previously validated for use in bovine (Garbarino et al., 2004; Skenandore et al., 2017) was used to determine plasma concentrations of progesterone. In order to assess precision of the assay, control samples with high (6.2 ng/mL) and low (0.3 ng/mL) concentrations of P4 were included at the beginning and end of each assay (n = 2 assays). Average detection limit of the assay was 0.1 ng/mL. Average intra-assay CV for the high concentration sample was 9.2%, whereas the interassay CV was 15.0%. For the low-concentration sample the average intraassay CV was 29.2%, whereas the interassay CV was 27.0%. Heifers were considered cyclic if at least one of the two samples collected had circulating P4 ≥ 1 ng/mL.

Statistical Analysis

A sample size calculation was performed using the sample size calculation option of WinPepi version 11.51 (Abramson, 2011). According to the calculations performed, 332 heifers per treatment were needed to detect a hazard ratio of 1.25 for time to pregnancy with an average probability of survival at the end of the observation period of 100 d of 5%, a probability of type I error (alpha) of 5%, and a probability of type II error (beta) of 20%. The smaller number of heifers with data available for analysis for some of the treatments (i.e., as compared to the number of heifers required based on the sample size calculation) was due to losses beyond the control of the researchers after initial enrollment.

Time to first service and time to pregnancy after the beginning of the AIP were analyzed using Cox's proportional hazard regression with the PHREG procedure of SAS (version 9.4, SAS Institute Inc., Cary, NC). Treatment was included as a fixed effect and farm as a random

effect. For the analysis of time to pregnancy, a heifer was considered pregnant if pregnancy was maintained until at least 66 ± 3 d of gestation. Heifers that left the herd due to death or sale were right-censored. Kaplan-Meier survival curves were created to illustrate time to event outcomes using the survival analysis option in MedCalc (version 12.5.0.0; MedCalc Software bvba, Ostend, Belgium).

Binary outcomes (proportion of heifers with P4 ≥ 1 ng/mL, proportion of heifers AIE vs. TAI, P/AI, and proportion of pregnant heifers at the end of the observation period) were analyzed using logistic regression with the GLIMMIX procedure of SAS. Treatment was included in models as a fixed effect and farm as a random effect. When appropriate (i.e., statistically significant differences were detected among treatments), the Least Significant Difference (**LSD**) post hoc mean separation test was used to determine differences between least square means. The proportion of cyclic heifers at the beginning of the AIP was evaluated with the Fisher's exact test using the FREQ procedure of SAS because in one of the treatments 100% of the heifers had a positive outcome. Pregnancy per AI at 31 ± 3 d after AI was evaluated for AI services at detected estrus, TAI services, and both types of AI services combined. For AI services outcomes, the effect of season of insemination (cold vs. warm) was offered to the models. The cold season was defined as the period from September 21 to June 20 and the warm season from June 21 to September 20.

All proportions reported are Least Squares Means (**LSM**) generated using the LSMEANS statement of PROC GLIMMIX of SAS. However, for simplification of interpretation and for descriptive data, some of the proportions are reported as arithmetic means obtained with PROC FREQ of SAS. Variables were considered significant if P ≤ 0.05, whereas *P*-values > 0.05 and ≤ 0.10 were considered a tendency.

RESULTS

Cyclicity Status and Reproductive Outcomes for First AI Service

Among heifers with P4 data available (n = 162), 69% had P4 ≥ 1 ng/mL at baseline (14 days before the beginning of the AIP) and the proportion with P4 ≥ 1 ng/mL did not differ (P = 0.62) among treatments (Table 1). At the beginning of the AIP, the overall proportion of heifers with P4 ≥ 1 ng/mL was 84% and differed ($P < 0.01$) among treatments because fewer heifers in the PGF+AIE treatment had P4 ≥ 1 ng/mL than in the PGF+TAI and ALL-TAI treatments (Table 1). There was no difference ($P = 0.20$) in the proportion of cyclic heifers (i.e., at least one of the samples with P4 ≥ 1 ng/mL) by the beginning of the AIP (Table 1).

For first service, the total proportion of heifers that received AIE was greater ($P < 0.01$) for the PGF+AIE (98.7%) than for the PGF+TAI (78.5%) treatment. For the PGF+AIE treatment (arithmetic mean reported), 78.5% (248/317) of the heifers received AIE after the first $PGF_{2\alpha}$, 17.0% (54/317) after the second, and 3.2% (10/317) after the third $PGF_{2\alpha}$ treatment. Thus, 1.3% of the heifers in the PGF+AIE treatment received TAI. The proportion of heifers in the PGF+AIE treatment that were AIE after a single $PGF_{2\alpha}$ treatment was similar ($P = 0.49$) to that of heifers AIE after two $PGF_{2\alpha}$ treatments in the PGF+TAI treatment (PGF+AIE = 76.1%; PGF+TAI = 78.5%).

The pattern of insemination and interval from the beginning of the AIP to first service was affected ($P < 0.01$; Figure 2) by treatment. The hazard of first AI up to 45 d of the AIP was greater for the ALL-TAI than for the PGF+AIE [hazard ratio (HR) = 1.72; 95% CI 1.45 to 2.03] and PGF+TAI (HR = 1.51; 95% CI 1.28 to 1.77) treatments, but similar for the PGF+AIE and

Table 1. Cyclicity status before the artificial insemination period for heifers enrolled in the experiment.

Item	Treatment[1]			
	PGF+AIE	PGF+TAI	ALL-TAI	*P*-value
	%, (n)			
P4[2] ≥ 1 ng/mL at baseline[3]	64.3 (56)	73.1 (52)	69.2 (52)	0.62
P4 ≥ 1 ng/mL at beginning of AIP	67.3[a] (55)	88.7[b] (53)	96.2[b] (53)	< 0.01
Cyclic[4] at beginning of AIP	92.9 (56)	94.3 (53)	100 (53)	0.20

[a,b] Different superscripts within a row indicate significant differences (P ≤ 0.05).
[1]Treatment: at 368 ±10 d of age, heifers received first AI after: 1) PGF+AIE: insemination at detected estrus (AIE) after $PGF_{2\alpha}$ treatments 14 d apart (up to 3) starting at the beginning of the AIP. Heifers not AIE within 9 d of the third $PGF_{2\alpha}$ treatment were enrolled in a 5d-Cosynch protocol [5d-Cosynch = controlled internal drug release insert (CIDR) + GnRH-5 d-CIDR-out + $PGF_{2\alpha}$-3 d-GnRH + TAI], 2) PGF+TAI: AIE after the second of two $PGF_{2\alpha}$ treatments 14 d apart. Heifers not AIE within 9 d after the second $PGF_{2\alpha}$ were enrolled in 5d-Cosynch and 3) ALL-TAI: timed AI after a 5d-Cosynch protocol. In a subgroup of heifers from all treatment groups (n = 162) two blood samples were collected 14 d apart to determine circulating concentrations of progesterone.
[2]P4 = progesterone.
[3]Sample collected 14 d before the artificial insemination period (AIP).
[4]P4 ≥ 1 ng/mL in at least one of the two samples collected.

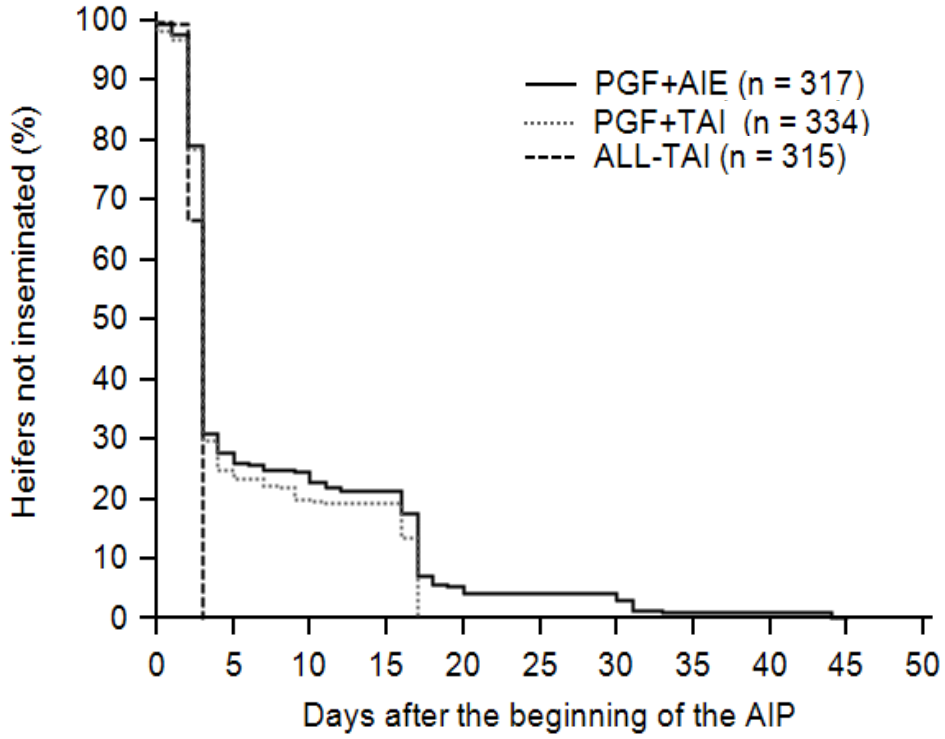


Figure 2. Kaplan-Meier survival curves for time to insemination during the artificial insemination period (AIP). The hazard of AI was greater ($P < 0.01$) for the ALL-TAI than the PGF+AIE [hazard ratio (HR) = 1.72; 95% CI 1.45 to 2.03] and the PGF+TAI (HR = 1.51; 95% CI 1.28 to 1.77) treatments but, similar for the PGF+AIE and PGF+TAI treatments (HR = 1.14; 95% CI 0.97 to 1.33). Mean days to first AI were 6.9 ± 0.46, 5.7 ± 0.31 and 2.7 ± 0.03 for the PGF+AIE, PGF+TAI, and ALL-TAI treatments, respectively.

PGF+TAI treatments (HR = 1.14; 95% CI 0.97 to 1.33). Mean days to first AI were, 6.9 ± 0.46, 5.7 ± 0.31 and 2.7 ± 0.03 for the PGF+AIE, PGF+TAI and ALL-TAI treatments, respectively.

For all first AI services combined, P/AI 31 ± 3 d after AI did not differ between treatments ($P = 0.38$; Table 2), and it was similar ($P = 0.21$) for heifers that received AIE after PGF-synchronized estrus (46.1%; n = 582), AIE during 5d-Cosynch (45.7%; n = 127), or a TAI (40.1%; n = 257) service. In contrast, P/AI for all AIE services combined (46.0%; n = 709) tended ($P = 0.10$) to be greater than TAI services (40.1%; n = 257). Overall, P/AI was not affected ($P = 0.22$) by season of AI (cold = 43.1%; warm = 47.2%). For AIE services synchronized with $PGF_{2\alpha}$, P/AI for the PGF+TAI treatment was greater ($P = 0.03$; Table 2) than for PGF+AIE (52.9% and 44.0%, respectively). In addition, P/AI for heifers AIE in the PGF+TAI treatment (i.e., after two $PGF_{2\alpha}$) tended ($P = 0.06$) to be greater than for heifers AIE after the first $PGF_{2\alpha}$ treatment in the PGF+AIE treatment (PGF+TAI = 53.6% vs. PGF+AIE = 45.2%). In contrast, P/AI for heifers in the PGF+AIE treatment receiving one $PGF_{2\alpha}$ treatment were similar ($P = 0.97$) to that of heifers receiving two or three $PGF_{2\alpha}$ treatments (1 $PGF_{2\alpha}$ = 42.4%; 2 or 3 $PGF_{2\alpha}$ = 42.2%). No difference in P/AI was detected for AIE services during the 5d-Cosynch protocol ($P = 0.86$; Table 2) or for TAI services ($P = 0.13$; Table 2) between the ALL-TAI and PGF+TAI treatments. Out of all first AI services in the ALL-TAI treatment, 33% (104/315) occurred during the protocol (majority between the time of CIDR removal and the scheduled TAI) and P/AI were similar ($P = 0.43$) for AIE (46.5%) and TAI services (41.8%).

Reproductive Outcomes for Second and Greater AI Services

The proportion of heifers AIE before resynchronization of ovulation with the 5d-Cosynch protocol for second and greater services tended ($P = 0.06$) to differ among treatments [PGF+AIE

Table 2. Pregnancy per AI (P/AI) 31 ± 3 d after first AI service and after second or greater AI services combined.

Item	Treatment[1]			*P*-value
	PGF+AIE	PGF+TAI	ALL-TAI	
	%, (n)			
Overall P/AI for first AI service[2]	42.0 (317)	47.3 (334)	43.8 (315)	0.38
P/AI for AIE after $PGF_{2\alpha}$[3]	44.0 (313)	52.9 (269)	-	0.03
P/AI for AIE during 5d-Cosynch[4]	-	45.0 (20)	47.1 (104)	0.86
P/AI for TAI[5]	-	29.0 (45)	41.4 (211)	0.13
Overall P/AI for second and greater AI services[6]	58.8 (311)	55.7 (318)	57.6 (311)	0.72
P/AI for AIE after AI[7]	59.1 (154)	58.5 (188)	60.3 (189)	0.94
P/AI for AIE during 5d-Cosynch	62.0 (45)	60.0 (30)	66.9 (21)	0.83
P/AI for TAI	53.6 (112)	47.1 (100)	47.7 (101)	0.58

[1]Treatment: at 368 ±10 d of age, heifers received first AI after: 1) PGF+AIE: insemination at detected estrus (AIE) after $PGF_{2\alpha}$ treatments 14 d apart (up to 3) starting at the beginning of the AIP. Heifers not AIE within 9 d of the third $PGF_{2\alpha}$ treatment were enrolled in a 5d-Cosynch protocol (5d-Cosynch = CIDR + GnRH-5 d-CIDR-out + $PGF_{2\alpha}$-3 d-GnRH + TAI), 2) PGF+TAI: AIE after the second of two $PGF_{2\alpha}$ treatments 14 d apart. Heifers not AIE within 9 d after the second $PGF_{2\alpha}$ were enrolled in 5d-Cosynch and 3) ALL-TAI: timed AI after a 5d-Cosynch protocol.
[2]Gender-selected semen was used for all first AI services.
[3]Heifers inseminated at detected estrus after $PGF_{2\alpha}$-induced estrus.
[4]Heifers inseminated anytime during the 5d-Cosynch protocol. Heifers did not receive GnRH at insemination.
[5]TAI = timed AI. P/AI not included for the PGF+AIE group because n = 4.
[6]Conventional semen was used for all second and greater AI services.
[7]Heifers inseminated at detected estrus after a previous AI service and before initiation of resynchronization with 5d-Cosynch.

(59.7%), PGF+TAI (66.6%) and ALL-TAI (69.7%)]. Similarly, the proportion of heifers AIE during the 5d-Cosynch protocol differed ($P = 0.02$) among treatments [PGF+AIE (11.2%), PGF+TAI (7.8%) and ALL-TAI (5.7%)]. Overall, P/AI for all second and greater AI services combined was not different among treatments ($P = 0.72$; Table 2) and seasons ($P = 0.60$). Further, treatment did not affect P/AI for heifers that received AIE before ($P = 0.94$) and during resynchronization with 5d-Cosynch ($P = 0.83$), or TAI ($P = 0.58$; Table 2).

Type of insemination affected overall P/AI ($P = 0.02$) because P/AI for AIE services before (59.3%; n =531) and during resynchronization with 5d-Cosynch (65.6%; n = 96) were greater than after TAI services (51.4%; n = 313). Moreover, P/AI for all AIE combined (60.3%; n = 627) was greater than for TAI services (51.4%; n = 313). Heifers (all treatment groups) that received AIE between CIDR removal and the expected TAI day (P/AI = 62.3%; n = 96) had greater ($P = 0.01$) P/AI than heifers inseminated on the scheduled day of TAI (P/AI = 46.9%; n = 313).

Time to Pregnancy and Non-pregnant Heifers at the end of the Artificial Insemination Period

The hazard of pregnancy for up to 100 d of AIP was greater for the ALL-TAI than the PGF+AIE treatment (HR 1.20, 95% CI 1.02 to 1.42; Figure 3), but was similar for the PGF+AIE and PGF+TAI (HR 1.07, 95% CI 0.91 to 1.25) treatments, and for the ALL-TAI and PGF+TAI treatments (HR 1.13, 95% CI 0.96 to 1.33). Median days from the beginning of the AIP to pregnancy were 27 (95% CI 23 to 36), 23 (95% CI 17 to 26), and 21 (95% CI 10 to 23) for heifers in the PGF+AIE, PGF+TAI, and ALL-TAI treatment, respectively. Furthermore, mean (±SE) days from the beginning of the AIP to pregnancy were 36 ± 1.8, 32 ± 1.8 and 28 ± 1.7, for heifers in the PGF+AIE, PGF+TAI and ALL-TAI treatment, respectively. Overall, the

proportion of heifers not pregnant the end of the AIP (includes heifers sold and dead during the 100 d) did not differ ($P = 0.97$) for the PGF+AIE (7.0%), PGF+TAI (6.5%) and ALL-TAI (6.8%) treatments.

DISCUSSION

In the present experiment, we evaluated the reproductive performance of dairy heifers managed with reproductive management strategies that differed on the method of submission to first service. We compared a program that relied primarily on AI after detection of estrus versus one that relied primarily on TAI. An intermediate program designed to reduce days to first service through a combination of AIE and TAI was also included. In support of our hypothesis, the program that relied primarily on TAI reduced time to pregnancy when compared with the program using predominant AIE after induction of estrus with $PGF_{2\alpha}$ treatments. The reduction of time to pregnancy, which was of a small magnitude, was primarily due to earlier insemination because P/AI was similar for AIE and TAI services in the PGF+AIE and ALL-TAI groups. Conversely, the ALL-TAI program did not reduce time to pregnancy when compared with the program that combined synchronization of estrus and TAI. A high proportion of heifers AIE within a short time frame after the beginning of the AIP and high P/AI for these AIE services for the combined approach reduced differences in time to pregnancy between groups. For the PGF+TAI treatment, immediate submission to a TAI protocol (i.e., within 9 d) rather than extending the period of estrus detection was also important to reduce time to pregnancy. Collectively, our data suggests that ALL-TAI programs for first AI service may be more effective to increase the pregnancy rate of heifers than programs that rely primarily on AIE services after systematic synchronization of estrus with $PGF_{2\alpha}$. Conversely, ALL-TAI programs

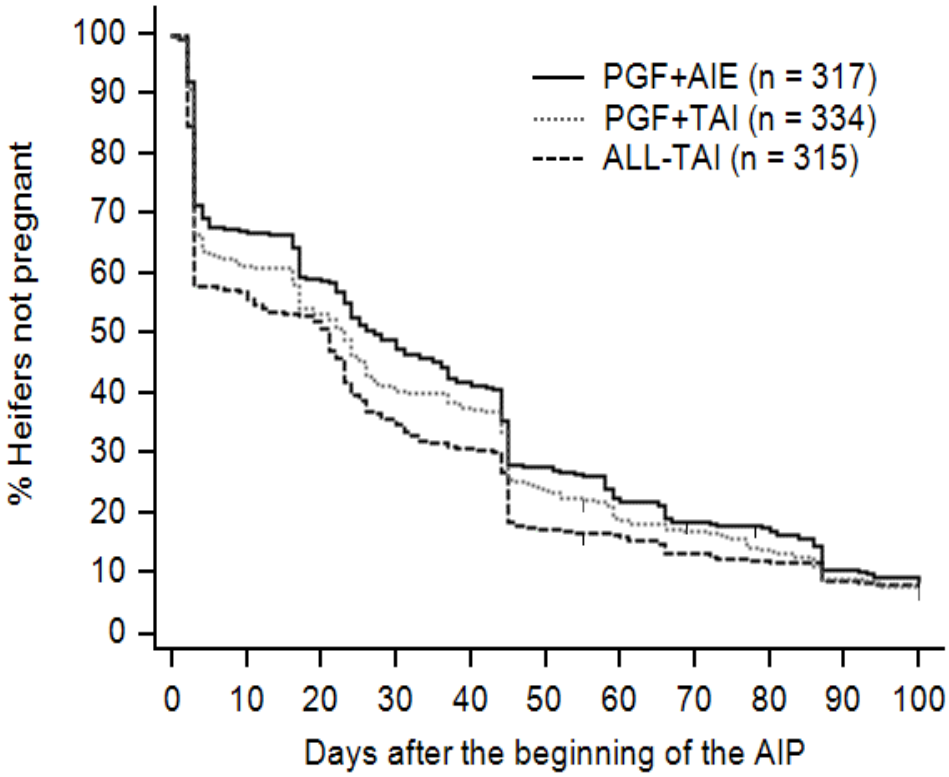


Figure 3. Kaplan-Meier survival curves for time to pregnancy up to 100 d after the beginning of the artificial insemination period (AIP). The hazard of pregnancy was greater for the ALL-TAI than the PGF+AIE group (HR = 1.20; 95% CI 1.02 to 1.42) but it was similar for the ALL-TAI and PGF+TAI (HR = 1.13; 95% CI 0.96 to 1.32) and the PGF+AIE and PGF+TAI groups (HR = 1.07; 95% CI 0.91 to 1.25). Median days to pregnancy were 27 (95% CI 23 to 36) for the PGF+AIE group, 23 for the PGF+TAI group (95% CI 17 to 26) and 21 for the ALL-TAI group (95% CI 10 to 23). Further, mean (±SE) days from the beginning of the AIP to pregnancy were 36 ± 1.8, 32 ± 1.8 and 28 ± 1.7 for heifers in the PGF+AIE, PGF+TAI, and ALL-TAI treatment, respectively. Downward vertical lines on survival curves depict right censoring of heifers due to herd exit (i.e., sale or death).

may not be more effective than a combined approach (i.e., AIE plus TAI) when estrus detection efficiency immediately after the beginning of the AIP is high as in our experiment (e.g., ~80% of heifers detected in estrus). Thus, programs that combine AIE and TAI may be an effective strategy to attain similar reproductive performance than ALL-TAI programs for farms that prefer to maximize inseminations at detected estrus rather than TAI. These combined programs, however, may not be more effective for reducing time to pregnancy than programs that result in high estrus detection efficiency after PGF-induced estrus every 14 d.

Few experiments compared insemination and pregnancy dynamics of dairy heifers managed with strategies that rely primarily on TAI versus less intensive AIE-based reproductive programs. In agreement with our findings, Silva et al. (2015) reported that heifers submitted to first service with a TAI-based program (5d-Cosynch with two $PGF_{2\alpha}$ treatments) were inseminated earlier and had greater pregnancy rate when compared with heifers managed with a program that relied primarily on AIE after spontaneous and $PGF_{2\alpha}$-induced estrus. In contrast, Lopes et al. (2013) reported that heifers receiving TAI-based programs (5d or 7d-Cosynch with one $PGF_{2\alpha}$ treatment) had similar (5d-Cosynch) or more (7d-Cosynch) mean days to first AI but delayed time to pregnancy when compared with all AIE after induction of estrus with $PGF_{2\alpha}$ every 11 d. A key aspect of the experiment reported in Lopes et al. (2013) was that heifers in the AIE treatment became eligible for insemination 7.5 or 9.5 d earlier than heifers in the 5d- and 7d-Cosynch programs, respectively. This delay on time to first service imposed by the experimental design was substantial and explained part of the delay on time to pregnancy for the TAI programs. In contrast, in the current experiment and that of Silva et al. (2015) the TAI programs resulted in earlier pregnancy because all heifers were inseminated within either 3 or 2 d of the beginning of the AIP, respectively. Unlike the current experiment and that of Silva et al.

(2015), Lopes et al. (2013) also reported a significant reduction in P/AI (16.3 and 25.4 percentage points for 5d and 7d-Cosynch with a single $PGF_{2\alpha}$ treatment and no initial GnRH) for heifers that received TAI compared with AIE. Thus, matching the beginning of the AIP with the day of TAI or the day of induction of luteolysis and P4 device removal, and obtaining similar P/AI for TAI than AIE services seems critical to benefit from TAI programs for dairy heifers.

Implementation of TAI at or near the beginning of the AIP also contributes to reduce time to pregnancy because return to estrus is tightly synchronized reducing overall timing of re-insemination at detected estrus. Indeed, the greater proportion (statistical tendency) of heifers that received AIE for second and greater AI services for the ALL-TAI group in our experiment may have been, at least in part, the result of tighter synchrony of estrus after previous inseminations.

We included two $PGF_{2\alpha}$ treatments to synchronize estrus in the PGF+TAI treatment group because synchronization of estrus programs with two $PGF_{2\alpha}$ treatments 11 to 14 d apart (i.e., Presynch) ensure that most cyclic heifers or cows have a responsive CL to $PGF_{2\alpha}$ at the time of the second treatment (King and Robertson, 1974; Stevenson et al., 2000). Ultimately, we expected to hasten overall timing of insemination by inducing a greater proportion of heifers to display estrus immediately after the beginning of the AIP. The similar proportion of heifers AIE after the first $PGF_{2\alpha}$ of the PGF+AIE treatment group and the second $PGF_{2\alpha}$ of the PGF+TAI treatment, however, did not support our reasoning. It is unlikely that the lack of difference was due to poor estrus detection because it was conducted equally for both groups, $PGF_{2\alpha}$ treatments were given on the same days of the week and time by the same personnel, and heifers were managed and housed in the same pens. In addition, the proportion of heifers AIE in the PGF+TAI group (78.5%) was similar to that reported in a previous experiment for dairy heifers

detected in estrus after two $PGF_{2\alpha}$ treatments 14 d apart (Colazo and Mapletoft, 2017). Most likely, having a vast majority of heifers cyclic when they became eligible for insemination in the PGF+AIE group resulted in a good response to $PGF_{2\alpha}$ and spontaneous estrus which led to the lack of difference in proportion of heifers that received AIE.

The greater P/AI for AIE services in the PGF+TAI than the PGF+AIE treatment was unexpected but may be explained, at least in part, by the occurrence of more estrus events before first service (i.e., due to first $PGF_{2\alpha}$ treatment), improved synchrony of ovulation, or both. In lactating dairy cows it has been shown that more estrus events before first service increase first service P/AI (Thatcher and Wilcox, 1973; Butler and Smith, 1989; Darwash et al., 1997). In addition, Stevenson and Phatak (2005) reported greater P/AI in lactating dairy cows inseminated at detected estrus after two than after a single $PGF_{2\alpha}$ treatment before the end of the voluntary waiting period. Unlike our experiment, in which heifers were of the approximate same age when eligible for first service, DIM at first service may have affected results for P/AI because cows that received two $PGF_{2\alpha}$ treatments were inseminated at later DIM which has been shown to increase P/AI in lactating dairy cows (Stangaferro et al., 2018). Additional research with dairy heifers is needed to confirm if induction of estrus with two $PGF_{2\alpha}$ treatments leads to greater P/AI than insemination at detected estrus after a single $PGF_{2\alpha}$ treatment. This is relevant because any potential increase in P/AI must offset the burden (i.e., additional handling) and costs (i.e., labor and $PGF_{2\alpha}$) of the additional $PGF_{2\alpha}$ treatment.

In the current study, first service inseminations with gender-selected semen at detected estrus during the 5d-Cosynch protocol led to similar P/AI than inseminations after $PGF_{2\alpha}$-induced estrus or TAI. On the other hand, for second and subsequent AI services with conventional semen, inseminations during the protocol resulted in similar P/AI than after

spontaneous estrus but greater than after TAI. Thus, immediate insemination without GnRH treatment of heifers that display estrus before the scheduled TAI seems a reasonable strategy to maximize overall P/AI with synchronization of ovulation programs. Obtaining similar or greater P/AI with TAI programs than other methods of submission to insemination is critical to benefit from predominant TAI programs that hasten insemination immediately after the beginning of the AIP. Otherwise, delayed pregnancy may be observed (Lopes et al., 2013).

The choice of a synchronization of ovulation protocol for TAI in commercial dairy operations depends primarily on the balance between optimizing P/AI and the constraints of protocol implementation. In our experiment, including GnRH at CIDR insertion and a single $PGF_{2\alpha}$ treatment in the 5d-Cosynch protocol was primarily due to the simplicity of giving GnRH at CIDR insertion and the logistical difficulty of adding a second $PGF_{2\alpha}$ treatment. Although the protocol used may not have optimized the physiological response of heifers to the protocol due to a greater risk for incomplete luteolysis in heifers that ovulated in response to GnRH (Lima et al., 2013), the effect on P/AI is difficult to estimate because results from previous experiments have been inconclusive. Some experiments reported that two $PGF_{2\alpha}$ treatments were necessary to maximize P/AI when the initial GnRH treatment was given to dairy heifers (Kasimanickam et al., 2009; Lima et al., 2013) whereas others reported no differences in P/AI for both dairy and beef heifers that received GnRH and a single $PGF_{2\alpha}$ treatment (Rabaglino et al., 2010; Kasimanickam et al., 2014).

Semen type (i.e., gender-selected vs. conventional) plays a major role on defining P/AI (Chebel et al., 2010), possibly contributing to differences observed in P/AI and pregnancy dynamics among different studies. The ratio of P/AI for gender-selected to conventional semen that we observed (78% and 76% of that obtained with conventional semen for AIE and TAI

services, respectively) is similar to that previously reported for commercial dairy farms in the U.S. (Norman et al., 2010) and for heifers randomized to insemination for first service with gender-selected or conventional semen (Chebel et al., 2010). Therefore, results of the current experiment for P/AI and pregnancy dynamics should be interpreted within the context of type of semen used. Our results may better reflect the conditions of commercial farms that use gender-selected semen for first service. Conversely, the expected performance of the treatments evaluated on farms that use conventional semen only may differ substantially as greater P/AI for first service may dramatically change the pregnancy dynamics.

Finally, an important consideration for the use of the intensive programs for synchronization of estrus and TAI (e.g., ALL-TAI and PGF+TAI in our experiment) for dairy heifers is their impact on the profitability of the heifer enterprise. Reducing time to pregnancy shortens the non-lactating period of a dairy animal which, in turn, results in fewer days on feed during the rearing period and favors an earlier positive cash flow via milk production. Therefore, for intensive programs including TAI to be more profitable than primarily AIE programs, the expected reduction in time to pregnancy must offset the associated additional labor and treatment costs. In this regard, previous economic comparisons of more intensive TAI and AIE programs suggested that differences in costs and revenues were strongly associated with the observed reproductive performance but also depended on the type of inputs and the methods used to quantify the benefit of earlier pregnancy (Lopes et al., 2013; Silva et al., 2015). The economic effect of managing heifers with the reproductive management strategies compared in our experiment warrants further investigation and will be reported in a separate manuscript.

CONCLUSION

Data from the present experiment demonstrated that a reproductive management strategy for dairy heifers designed to submit heifers to TAI at or near the beginning of the AIP resulted in reduced time to first AI and reduced time to pregnancy when compared with a strategy that resulted in almost all inseminations after induction of estrus with $PGF_{2\alpha}$ treatments every 14 d. Nonetheless, results of our study support the notion that in herds with effective estrus detection programs, the combination of AIE and TAI may result in similar reproductive performance than with programs that rely mostly on TAI. Thus, dairy farms have the option to either select a strategy that reduces days to first service through TAI or a combined strategy that attempts to maximize AIE but includes a TAI protocol immediately after completion of a short period of estrus detection. Additional research is needed to determine the impact of the different reproductive management strategies evaluated on the economic performance of the dairy heifer enterprise.

ACKNOWLEDGMENTS

This research was financially supported through a grant (AOR 15-020) from the New York Farm Viability Institute (Syracuse, NY) to J. O. Giordano. We would like to thank the two commercial dairy farms that participated of this research for their support and the use of their cows and facilities. We would also like to extend our gratitude to Zoetis for providing the hormonal products used to synchronize estrus and ovulation.

REFERENCES

Abramson, J. H. 2011. WINPEPI updated: Computer programs for epidemiologists, and their teaching potential. Epidemiol. Perspect. Innov. 8:1.

Butler, W. and R. Smith. 1989. Interrelationships between energy balance and postpartum reproductive function in dairy cattle. J. Dairy Sci. 72:767-783.

Caraviello, D., K. Weigel, P. Fricke, M. Wiltbank, M. Florent, N. Cook, K. Nordlund, N. Zwald, and C. Rawson. 2006. Survey of management practices on reproductive performance of dairy cattle on large US commercial farms. J. Dairy Sci. 89:4723-4735.

Chebel, R., F. Guagnini, J. Santos, J. Fetrow, and J. Lima. 2010. Sex-sorted semen for dairy heifers: Effects on reproductive and lactational performances. J. Dairy Sci. 93:2496-2507.

Chebel, R. C., J. E. Santos, R. L. Cerri, H. M. Rutigliano, and R. Bruno. 2006. Reproduction in dairy cows following progesterone insert presynchronization and resynchronization protocols. J. Dairy Sci. 89:4205-4219.

Colazo, M. G. and R. J. Mapletoft. 2017. Pregnancy per AI in Holstein heifers inseminated with sex-selected or conventional semen after estrus detection or timed-AI. Can. Vet. J. 58:365.

Darwash, A., G. Lamming, and J. Wooliams. 1997. The phenotypic association between the interval to post-partum ovulation and traditional measures of fertility in dairy cattle. Animal Sci. 65:9-16.

Ettema, J. and J. Santos. 2004. Impact of age at calving on lactation, reproduction, health, and income in first-parity Holsteins on commercial farms. J. Dairy Sci. 87:2730-2742.

Ferguson, J. D. and A. Skidmore. 2013. Reproductive performance in a select sample of dairy herds. J. Dairy Sci. 96:1269-1289.

Fricke, P., J. Giordano, A. Valenza, G. Lopes, M. Amundson, and P. Carvalho. 2014. Reproductive performance of lactating dairy cows managed for first service using timed artificial insemination with or without detection of estrus using an activity-monitoring system. J. Dairy Sci. 97:2771-2781.

Garbarino, E. J., J. Hernandez, J. Shearer, C. Risco, and W. Thatcher. 2004. Effect of lameness on ovarian activity in postpartum Holstein cows. J. Dairy Sci. 87:4123-4131.

Giordano, J., M. Thomas, G. Catucuamba, M. Curler, R. Wijma, M. Stangaferro, and M. Masello. 2016. Effect of extending the interval from Presynch to initiation of Ovsynch in a Presynch-Ovsynch protocol on fertility of timed artificial insemination services in lactating dairy cows. J. Dairy Sci. 99:746-757.

Higgs, R. J., L. E. Chase, D. A. Ross, and M. E. Van Amburgh. 2015. Updating the Cornell Net Carbohydrate and Protein System feed library and analyzing model sensitivity to feed inputs. J. Dairy Sci. 98: 6340-6360.

Kasimanickam, R., M. Day, J. Rudolph, J. Hall, and W. Whittier. 2009. Two doses of prostaglandin improve pregnancy rates to timed-AI in a 5-day progesterone-based synchronization protocol in beef cows. Theriogenology 71:762-767.

Kasimanickam, R., P. Firth, G. M. Schuenemann, B. Whitlock, J. Gay, D. Moore, J. Hall, and W. Whittier. 2014. Effect of the first GnRH and two doses of PGF2α in a 5-day progesterone-based CO-Synch protocol on heifer pregnancy. Theriogenology 81:797-804.

King, G. J. and H. A. Robertson. 1974. A two injection schedule with prostaglandin F2α for the regulation of the ovulatory cycle of cattle. Theriogenology 1:123-128.

Lima, F., E. Ribeiro, R. Bisinotto, L. Greco, N. Martinez, M. Amstalden, W. Thatcher, and J. Santos. 2013. Hormonal manipulations in the 5-day timed artificial insemination protocol to optimize estrous cycle synchrony and fertility in dairy heifers. J. Dairy Sci. 96:7054-7065.

Lopes, G., Jr., C. Johnson, L. Mendonça, P. Silva, J. Moraes, A. Ahmadzadeh, J. Dalton, and R. Chebel. 2013. Evaluation of reproductive and economic outcomes of dairy heifers inseminated at induced estrus or at fixed time after a 5-day or 7-day progesterone insert-based ovulation synchronization protocol. J. Dairy Sci. 96:1612-1622.

NAHMS. 2018. Dairy 2014, Part III: Reference of Dairy Cattle Health and Management Practices in the United States, 2014. #696.0218. Centers for Epidemiology and Animal Health, USDA: Animal and Plant Health Inspection Service: Veterinary Services, Fort Collins, CO.

Norman, H., J. Hutchison, and R. Miller. 2010. Use of sexed semen and its effect on conception rate, calf sex, dystocia, and stillbirth of Holsteins in the United States. J. Dairy Sci. 93:3880-3890.

Pursley, J., M. Wiltbank, J. Stevenson, J. Ottobre, H. Garverick, and L. Anderson. 1997. Pregnancy rates per artificial insemination for cows and heifers inseminated at a synchronized ovulation or synchronized estrus1. J. Dairy Sci. 80:295-300.

Rabaglino, M., C. Risco, M.-J. Thatcher, I. Kim, J. Santos, and W. Thatcher. 2010. Application of one injection of prostaglandin F2α in the five-day Co-Synch+ CIDR protocol for estrous synchronization and resynchronization of dairy heifers. J. Dairy Sci. 93:1050-1058.

Ribeiro, E., A. Monteiro, F. Lima, H. Ayres, R. Bisinotto, M. Favoreto, L. Greco, R. Marsola, W. Thatcher, and J. Santos. 2012. Effects of presynchronization and length of proestrus on fertility of grazing dairy cows subjected to a 5-day timed artificial insemination protocol. J. Dairy Sci. 95:2513-2522.

Schmitt, E., T. Diaz, M. Drost, and W. Thatcher. 1996. Use of a gonadotropin-releasing hormone agonist or human chorionic gonadotropin for timed insemination in cattle. J. Anim. Sci. 74:1084-1091.

Silva, T., F. Lima, W. Thatcher, and J. Santos. 2015. Synchronized ovulation for first insemination improves reproductive performance and reduces cost per pregnancy in dairy heifers. J. Dairy Sci. 98:7810-7822.

Skenandore, C., A. Pineda, J. Bahr, A. Newell-Fugate, and F. Cardoso. 2017. Evaluation of a commercially available radioimmunoassay and enzyme immunoassay for the analysis of progesterone and estradiol and the comparison of two extraction efficiency methods. Domest. Anim. Endocrinol. 60:61-66.

Stangaferro, M., R. Wijma, M. Masello, M. J. Thomas, and J. Giordano. 2018. Extending the duration of the voluntary waiting period from 60 to 88 days in cows that received timed artificial

insemination after the Double-Ovsynch protocol affected the reproductive performance, herd exit dynamics, and lactation performance of dairy cows. J. Dairy Sci. 101:717-735.

Stevenson, J. 2011. Alternative programs to presynchronize estrous cycles in dairy cattle before a timed artificial insemination program1. J. Dairy Sci. 94:205-217.

Stevenson, J. and A. Phatak. 2005. Inseminations at estrus induced by presynchronization before application of synchronized estrus and ovulation. J. Dairy Sci. 88:399-405.

Stevenson, J., J. Rodrigues, F. Braga, S. Bitente, J. Dalton, J. Santos, and R. Chebel. 2008. Effect of Breeding Protocols and Reproductive Tract Score on Reproductive Performance of Dairy Heifers and Economic Outcome of Breeding Programs1. J. Dairy Sci. 91:3424-3438.

Stevenson, J., J. Smith, and D. Hawkins. 2000. Reproductive Outcomes for Dairy Heifers Treated with Combinations of Prostaglandin F2α, Norgestomet, and Gonadotropin-Releasing Hormone1. J. Dairy Sci. 83:2008-2015.

Thatcher, W. and C. Wilcox. 1973. Postpartum Estrus as an Indicator of Reproductive Status in the Dairy Cow1. J. Dairy Sci. 56:608-610.

CHAPTER III

EFFECT OF REPRODUCTIVE MANAGEMENT PROGRAMS FOR FIRST SERVICE ON REPLACEMENT DAIRY HEIFER ECONOMICS

M. Masello, M. M. Perez, G. E. Granados, M. L. Stangaferro, B. Ceglowski,

M. J. Thomas, and J. O. Giordano

Submitted for review to the Journal of Dairy Science

ABSTRACT

Our objective was to evaluate cash flow for dairy heifers managed for first service with programs that relied primarily on insemination at detected estrus (**AIE**), timed AI (**TAI**), or a combination of both. Holstein heifers from 2 commercial farms were randomized to receive first service with sexed-semen after the beginning of the AI period (**AIP**) at 12 mo of age with 1 of 3 treatments: 1) **PGF+AIE** (n=317): AIE after $PGF_{2\alpha}$ injections every 14 d (up to 3) starting at the beginning of the AIP. Heifers not AIE 9 d after the third $PGF_{2\alpha}$ were enrolled in the 5d-Cosynch (**5d-CP**) protocol, 2) **ALL-TAI** (n=315): TAI after ovulation synchronization with the 5dCP protocol, and 3) **PGF+TAI** (n=334): AIE after 2 $PGF_{2\alpha}$ injections 14 d apart (second $PGF_{2\alpha}$ at beginning of AIP). If not AIE 9 d after the second $PGF_{2\alpha}$, the 5dCP protocol was used for TAI. After first service heifers were AIE or received TAI after the 5dCP with conventional semen. Individual heifer cash flow (**CF**) for up to a 15-mo period (Day 0 = beginning of AIP) was calculated using reproductive cost (rearing only), feed cost (rearing only), income over feed cost (lactation only), calf value, operating cost, and with or without replacement cost. A stochastic analysis with Monte Carlo simulation was used to estimate differences in CF for a range of

market values for inputs and outputs. Time to pregnancy for up to the end of the AIP at 100 d was analyzed by Cox's proportional regression, binary data with logistic regression, and continuous outcomes by ANOVA. Time to pregnancy (HR and 95% CI) was reduced for the ALL-TAI versus the PGF+AIE treatment (1.20; 1.02-1.42) but it was similar for ALL-TAI and PGF+TAI (1.13; 0.95-1.33) and the PGF+AIE and PGF+TAI treatments (1.07; 0.91-1.25). The proportion of heifers not pregnant by 100 d did not differ (PGF+AIE=7.0%; PGF+TAI=6.5%; ALL-TAI=6.8%). When including replacement cost, CF ($/slot per 15 mo) differences were $51 and $42 in favor of the PGF+TAI and ALL-TAI compared with the PGF+AIE treatment, and $9 in favor of the PGF+TAI compared with the ALL-TAI treatment but did not differ statistically. Excluding heifers that were replaced to evaluate the effect of timing of pregnancy differences only, the difference in CF between the PGF+AIE with the PGF+TAI and ALL-TAI treatment was the same (i.e., $15) and favored the programs that used more TAI, but also did not differ statistically. Stochastic simulation results were in line with those of the deterministic analysis confirming the benefit of the programs that used more TAI. We concluded that submission of heifers for first service with TAI only or TAI in combination with AIE generated numerical differences in CF of potential value to commercial dairy farms. Reduced rearing cost and increased revenue during lactation increased CF under fixed (not statistically significant) or simulated variable market conditions.

Keywords: cash flow, pregnancy, synchronization, dairy heifer

INTRODUCTION

Raising replacement heifers is a critical task for dairy operations as it provides the future revenue-generating units of a herd. Nevertheless, the heifer rearing period (**RP**) is financially non-productive and therefore a large investment for most dairy farms. Estimations of heifer rearing cost indicated that the heifer enterprise might represent as much as 15 to 20 % of total operating expenses (Harsh et al., 2001), with no financial return until heifers enter the milking herd. Therefore, management strategies designed to reduce the cost of the heifer-raising program may have a pronounced effect on the overall profitability of dairy farms. Of particular interest are approaches aimed at optimizing age at first calving (**AFC**) to reduce days on feed and thus, raising cost (Ettema and Santos, 2004). In addition, earlier incorporation of heifers to the milking herd shortens the time required for a dairy cow to attain positive lifetime cash flow (Ettema and Santos, 2004).

As management strategies aimed at reducing the time required for heifers to reach breeding size are limited and gestation length is fixed, one of the few management opportunities to optimize AFC is reducing time to pregnancy after the beginning of the artificial insemination period (**AIP**). This might be accomplished by implementing proactive reproductive management programs that maximize the insemination and conception risk after heifers reached the appropriate size to become pregnant. In this regard, Masello et al. (2019) reported that a first service program designed to increase the insemination risk by using TAI for all heifers at the beginning of the AIP reduced time to pregnancy when compared with a program that relied on insemination after induction of estrus with up to three $PGF_{2\alpha}$ treatments every 14 d. In addition, a program that combined AI at detected estrus (**AIE**) for 9 d after two $PGF_{2\alpha}$ treatments 14 d apart and TAI for heifers not AIE resulted in similar time to pregnancy than the ALL-TAI program.

Although results from this experiment suggested that programs that reduce days to first service with ALL-TAI or a combination of AIE and TAI might benefit heifer reproductive performance, ultimately the choice of program for most commercial farms should be based on economic outcomes for the heifer enterprise. In particular, because there are multiple potential interactions among factors that affect cash flow of reproductive management programs. For example, programs for first service that rely more on synchronization of estrus and TAI might be more expensive to implement due to the increased labor and cost for hormonal treatments but may improve cash flow of the heifer enterprise by reducing raising cost and earlier generation of revenue during lactation.

Thus, the primary objective of this study was to compare cash flow and parameters of economic performance for heifers enrolled in the experiment reported in Masello et al. (2019). Specifically, our objective was to analyze individual heifer cash flow for a fixed period of time (i.e., 15 mo) after the beginning of the AIP. A secondary objective was estimating variation in cash flow under different input pricing scenarios through stochastic Monte Carlo simulation. We hypothesized that systematic use of reproductive management programs that hastened insemination through more aggressive use of TAI would be more expensive to implement but would increase accumulated cash flow relative to a program that relied primarily on AIE after $PGF_{2\alpha}$-induced estrus.

MATERIALS AND METHODS

All procedures performed with heifers were approved by the Animal Care and Use Committee of Cornell University.

Farms and Heifer Management

Information about farms, animals, and experimental procedures are described in detail in Masello et al. (2019). Briefly, nulliparous Holstein heifers from 2 commercial dairy farms located in New York State were enrolled in this experiment (farm A located in Washington county and farm B located in Wayne county), from November 2015 to February 2017. In both farms, heifers were housed in freestall barns with concrete flooring and self-locking headgates in the feeding lane. Freestall surfaces were covered with mattresses and either sand (farm A) or dried manure bedding (farm B). Heifers were fed a total mixed ration once a day with unlimited access to feed and water.

Experimental Treatments

Once a month, heifers (n = 966) of >310 d of age were stratified by age in days and then randomly allocated to one of three treatments by the dairy herd management software (DairyComp305, ValleyAg Software, Tulare, CA). Treatments consisted of different reproductive management strategies for first AI service with gender-selected semen. Heifers became eligible for insemination [i.e., beginning of the artificial insemination period (**AIP**)] at 368 ± 10 d of age.

In farm A, estrus detection for all AI services was performed once a day by a combination of visual observation of behavioral signs of estrus and tail-paint removal whereas, only visual observation was used in farm B. In farm A, heifers were inseminated immediately after detection of estrus whereas in farm B heifers were inseminated 6 to 12 h after observation of behavioral estrus (performed inseminations twice a day).

Heifers in the PGF+AIE (n = 317) treatment received AIE after synchronization of estrus with up to three $PGF_{2\alpha}$ treatments every 14 d starting on the first day of the AIP. Heifers not AIE for up to 9 d after the third $PGF_{2\alpha}$ treatment received a 5d-Cosynch protocol (5d-Cosynch) for synchronization of ovulation and timed artificial insemination (**TAI**). Thus, TAI for heifers not AIE occurred 45 d after the beginning of the AIP. The 5d-Cosynch protocol consisted of an initial GnRH treatment i.m. plus insertion of a controlled internal drug release insert (**CIDR**); 5 d later 25 mg of $PGF_{2\alpha}$ s.c. and CIDR removal; and finally, a GnRH treatment and insemination 3 d after $PGF_{2\alpha}$ and CIDR removal. The use of TAI in the PGF+AIE group was a last resource to ensure insemination of all heifers within a reasonable amount of time after the beginning of the AIP rather than a preferred method of submission for AI in this group. For the PGF+TAI (n = 334) treatment, estrus was synchronized with two $PGF_{2\alpha}$ treatments 14 d apart with the second treatment given at the beginning of the AIP. Heifers not AIE within 9 d of the second $PGF_{2\alpha}$ treatment were enrolled in the 5d-Cosynch protocol to receive TAI. Thus, all heifers received first service within 17 d of the beginning of the AIP. Heifers in the ALL-TAI (n = 315) treatment were submitted to the 5d-Cosynch protocol for synchronization of ovulation and TAI. The beginning of the AIP for this treatment group coincided with the time of $PGF_{2\alpha}$ treatment and CIDR removal. All heifers in this treatment group received first service within 3 d of the beginning of the AIP.

Heifers from the three treatment groups detected in estrus after CIDR removal and the $PGF_{2\alpha}$ treatment of 5d-Cosynch until the day before TAI received AIE with no GnRH treatment.

Heifers in the three treatment groups failing to conceive to first or subsequent inseminations received AI with conventional semen after detection of estrus or TAI. Re-inseminations at detected estrus occurred any time after a previous insemination. Heifers

diagnosed nonpregnant through transrectal ultrasonography 31 ± 3 d after a previous insemination were enrolled two days after nonpregnancy diagnosis in the 5d-Cosynch protocol for resynchronization of ovulation and TAI. As for first service, all heifers detected in estrus after CIDR removal and the $PGF_{2\alpha}$ treatment of 5d-Cosynch until the day before TAI received AIE with no GnRH treatment.

Seventeen heifers were removed from the experiment because after experiencing pregnancy loss were moved to a pen where natural service occurred, but individual services were not recorded.

Calculation of Economic Outcomes

In order to estimate cash flow per slot (i.e., unit of space at the dairy occupied by each heifer at the beginning of the AIP) for the 15-mo experimental period, all expenses during the rearing period (reproductive cost, feed cost, replacement cost, and other operating expenses), were estimated for each heifer enrolled for 15 mo after initiation of the AIP. In addition, for heifers that became pregnant and calved during the 15-mo period, expenses (feed cost, replacement cost, and other operating expenses) and revenues (milk sales and calf value) were also estimated for the lactation period. Therefore, for heifers that calved within 15-mo after the beginning of the AIP, two different periods of time were evaluated for economic outcomes: (1) the rearing period (**RP**), defined as the time from the beginning of the AIP until first calving or herd exit due to death or sale, and (2) the first lactation period (**FLP**), defined as the time from first calving until completion of a total of 15-mo after the initiation of the AIP for individual heifers. The choice of 15 mo to evaluate cash flow was to provide most heifers enough time to

complete the rearing period and generate data for their first lactation before pregnancy could have a substantial effect on milk production (i.e., assuming some heifers could become pregnant at 50 DIM). A total of 15 mo provided heifers that became pregnant on the first day of the AIP a maximum of 174 DIM in their first lactation whereas heifers that became pregnant on the last day of the AIP could have had 74 DIM assuming a gestation length of ~280 d and 30.4 d per month. In addition, to better represent the herd replacement dynamics under conditions of constant herd size and a stable in- and out-flow of lactating cows, we assumed that slots initially occupied by a heifer in the experiment would be occupied at all times during the 15-mo period. Thus, if a heifer left the herd during the RP or the FLP, a virgin heifer or a recently calved first lactation cow replaced the heifer or cow that left, respectively. In both cases, replacements were randomly selected from the pool of heifers and cows enrolled in the experiment so that a heifer or lactating cow from the same farm and treatment was used to replace the one that left the herd. Therefore, each slot for the heifer or cow that left the herd was occupied until the end of the 15-mo period by heifers or cows that could better represent the conditions of each farm and treatment. A particular slot may have been occupied by more than two different heifers or cows if the replacement heifer or cow also left the herd during the 15-mo study period.

Although the analysis of interest was cash flow including the effect of the replacement dynamics, a second analysis was conducted to estimate cash flow without the effect of the replacement dynamics. This was done to isolate the effect of timing of pregnancy because replacement cost could have profound effects on overall cash flow.

Thus, cash flow was estimated for each heifer or each slot for 15 mo by subtracting the total RP cost from the total FLP profit. Total cash flow during the RP and FLP were calculated using the following equations:

Total RP cash flow = Repro + FC + Replac + OE

Total FLP cash flow = IOFC + Calf Value - Replac - OE

where Repro = cost of implementing reproductive management program, FC = feed cost, Replac = replacement cost, OE = operating expenses, and IOFC = income over feed cost.

Price selection for the different inputs and outputs included in the deterministic economic analyses represent the economic conditions and market values for the New York State dairy industry during the experimental period (i.e., November 2015 to June 2018).

Reproductive Cost. Cost incurred during the RP was calculated for each slot and was the aggregation of the costs associated with all AI services (i.e., hormonal treatment for synchronization of estrus and ovulation, semen for AI, and labor for hormonal treatments), pregnancy testing, and detection of estrus during the 15-mo period. Reproductive cost generated during the FLP, if any, were not included in the analysis. For hormonal treatments, cost of a dose of GnRH, $PGF_{2\alpha}$, and each CIDR device were set at \$1.72, \$2.21, and \$11.92, respectively. Reproductive hormones costs were obtained from the local veterinary clinic that provided products to these farms during the experiment. Labor cost for application of hormonal treatment and insertion of intravaginal progesterone releasing devices was set at \$15 per hour. Thus, assuming that an operator can give 60 injections per hour, average labor cost for injections was set at \$0.25 per injection, whereas the cost of applying an intravaginal progesterone releasing device was set at \$0.75 per device assuming application of 20 CIDR devices per hour. The cost of a unit of sexed-sorted (\$25) and of conventional (\$12) semen was estimated based on the

blend price of semen units of different genetic merit and for the volume of sales representative of the size of the farms in the current experiment. Labor cost associated with each insemination was set at $1, assuming 15 inseminations per hour. Pregnancy testing was set at $2.75 per examination based on a cost of $110 per hour and 40 heifers examined per hour based on data provided by a bovine practitioner involved with the research. Estrus detection cost was calculated based on a total of 1.25 h of detection per day at $15 per hour of labor divided by the average daily number of heifers to be observed, which resulted on an average of $0.028/heifer per day of rearing.

Feed Cost. For both the RP and the FLP, feed cost was calculated based on the total DMI for each individual slot multiplied by the cost of each kg of DM. Feed cost was set at $0.17 per kg of DM from the beginning of the AIP until the beginning of the close-up period (i.e., ≥ 259 days in gestation) for which feed cost was set at $0.22 per kg of DM. On the other hand, feed cost was set at $0.29 per kg during the lactation period.

DMI Estimation. Daily DMI for individual heifers was estimated using (NRC, 2001) equations. During the rearing period, DMI was estimated as a function of body weight (**BW**), as

$$\text{DMI (kg/d)} = BW^{0.75} \times \{[(0.2435 \times NE_m) - (0.0466 \times NE_m^2) - \text{SubFact}]/\text{DivFact}\}$$

where NE_m = net energy of diet for maintenance (Mcal/kg), SubFact = adjustment for effects of age above or below 12 months, and DivFact = dietary concentration adjustment factor above or below 1 NE_m. Because the age of all heifers enrolled in the experiment was at least 12 mo, the

adjustment for the effect of age (SubFact) was set at 0.0869. In addition, because the NE_m of the diets offered to heifers from both farms was greater than 1 (i.e., 1.54 Mcal/kg), the adjustment for dietary concentration (DivFact) was set at 1.54 (i.e., equivalent to NE_m of the diet). Additionally, because intake decreases immediately prior to calving, an adjustment factor (DMI_factor) for days pregnant was calculated and multiplied by the initial equation as follows:

if days pregnant > 210 and < 259 then

$$\text{DMI\_factor} = \{1 + [(210 - \text{days pregnant}) \times 0.0025]\}$$

if days pregnant ≥ 259 then

$$\text{DMI\_factor} = \{[1.71 - (0.69 \times \exp(0.35 \times (\text{days pregnant} - 280)))]/100\} \times \text{BW}$$

During lactation, DMI was estimated as a function of fat corrected milk (**FCM**), BW, and week of lactation (DIM/7), as

$$\text{DMI (kg/d)} = 0.372 \times \text{FCM} + 0.0968 \times \text{BW}^{0.75} \times \{1 - \exp[-0.192 \times (\text{DIM}/7 + 3.67]\}$$

Body Weight Estimation. During the rearing period, BW was modeled daily for every heifer using the Gompertz growth function as previously described in Winsor (1932):

$$\text{BW (kg)} = A \times \exp(-\exp(B - C \times \text{age})$$

where age = heifer age in days, A = asymptotic weight or maximum growth response, B = scale parameter related to initial weight, and C = growth rate. Body weight data at different time points during the rearing period for a subgroup of heifers (n = 572), BW of mature (i.e., third and fourth lactation) cows (n = 30), and BW at first calving were only available for farm B (n = 489). To generate the curve, all body weight data was combined (n = 1,308) and plotted against age in days. The Gompertz growth function was then fitted using the Calculate option of the CurveExpert software (version 2.6.5; Hyams, 2011). Parameters A, B and C for the resulting equation were 695, 0.90, and 0.004, respectively.

During lactation, BW was modeled daily for every cow using the Korver function as previously described in (Van Arendonk, 1985):

$$\text{BW (kg)} = A \times \{1 - [1 - (B/A)^{1/3}] \times \exp(-C \times \text{age})\}^3 - (P1/P2) \times \text{DIM} \times \exp(1 - \text{DIM}/P2)$$

where age = cow age in days, A = mature live weight (kg), B = birth weight (kg), C = growth rate, P1 = maximum decrease in live weight during lactation, and P2 = time during lactation with minimum live weight. Data used for average mature live weight (720 kg) and birth weight (39 kg) were retrieved from both farms. Parameters C, P1, and P2 were 0.004, 30, and 60, respectively, based on (Kalantari et al., 2010).

Calf Value. All heifers that initiated a first lactation during the 15-mo period generated a one-time revenue when the calf was born. Calf value was estimated based on the number of calves born and the market value based on sex of the calf. The market value for newborn calves was set

at $109 for female calves, and $97 for male calves (Progressive Dairyman, 2019). Calf value was set at $0 for heifers with calves born dead (stillbirths).

Replacement Cost. For each heifer that left the herd due to death or sale, the replacement cost was equal to the market value of a replacement heifer minus the salvage value of the heifer sold for beef ($0 if the heifer left the herd due to death), and the value of the calf born from the replacement. The salvage value for heifers sold was calculated based on their estimated BW at the time of sale times the beef price. During the RP, the value of the calf born from a replacement virgin heifer was $0 if the replacement did not calve during the replacement period. In contrast, if the replacement heifer was able to conceive and calve during the replacement period, then the value of the calf born was set at the average calf value obtained in the current experiment ($95/calf), which accounts for male, female, stillborn and twin calves. During the FLP, in contrast, calf value was set to $95 in all cases based on the assumption that all replacements entering the herd in their first lactation contributed with a newborn calf. For replacement cost calculations during the RP, the market value of a virgin heifer was set at $700, based on the average value of lean and heavy replacement heifers during the study period (Progressive Dairyman, 2019). In addition, the beef price for heifers sold during the rearing period was set at $2.70 per kg (USDA National Agricultural Statistic Service, 2019). In contrast, for replacement cost during the FLP, the market value of a springer heifer (i.e., heifer close to calving) was set at $1,565 (USDA Economic Research Service, 2019) and the value of beef for cows sold was set at $1.50 (USDA National Agricultural Statistic Service, 2019).

Milk Income Over Feed cost. Daily milk income over feed cost (**IOFC**) was estimated for all heifers that started first lactation during the 15-mo period by subtracting feed cost from milk revenue (i.e., milk volume kg × milk price per kg). A slot's IOFC was set to $0 when heifers and their corresponding replacement (if present) did not calve within the 15-mo period. Milk price was the average monthly price reported by the Dairy Market Watch report (Cornell Cooperative Extension of Chautauqua County, 2018) from September 2016 (first heifer initiated first lactation period) to June 2018 (last heifer completed 15-mo experimental period). The weighted average accounting for milk class (class I to IV) usage was $0.36 per kg (i.e., $16.5 per 100 lbs).

Daily Milk and FCM Production Estimation. The MilkBot model (Ehrlich, 2013) was used to estimate daily milk production for each heifer that calved during the study period (i.e., within 15 mo after the beginning of the AIP). Details about MilkBot parameters and daily milk yield calculations have been previously described (Ehrlich, 2013). Briefly, monthly milk data was retrieved from the dairy herd management software to estimate individual lactation curves based on 4 parameters: scale, ramp, offset, and decay. For heifers that calved but left the herd before the second monthly milk test (i.e., either no milk data available or only available for the first test, n = 45), daily milk production was calculated based on the average of each parameter corresponding to the same farm and experimental treatment as the heifer that left the herd.

In order to estimate daily DMI during lactation, FCM was calculated based on daily milk weight (**DMW**; kg/d) estimated by the MilkBot and monthly test fat percentage as follows:

$$\text{FCM (kg/d)} = 0.4 \times \text{DMW} + 0.15$$

$$\times \text{Fat \%} \times \text{DMW}$$

where DMW = daily milk weight (kg/d) estimated by the MilkBot model and Fat % = fat percentage reported in the monthly milk tests. Because daily milk fat percentage data were not available, fat percentage for a specific monthly test was repeated for a 30-d period (i.e., value obtained from first test was used from 1 to 30 DIM, from 2nd test was used from 31 to 60 DIM, and so on).

Other Operating Expenses. Other operating expenses not accounted for by reproductive cost, feeding cost, and replacement cost were set at $0.85 per day per heifer for the RP (Karszes, 2014) and $3.10 per day per cow during the FLP (Karszes et al., 2017, 2018). During the RP, the calculation of other operating expenses included the following items: hired labor, bedding, veterinary services, machinery operation and ownership, and building and operation ownership. During the FLP, other operating expenses included hired labor, professional nutritional services, machine repairs, rent and lease, fuel, bedding, milking supplies, utilities, and other professional fees. Other operating expenses were calculated for each heifer based on the individual heifer daily expense (rearing = $0.85; first lactation = $3.10) multiplied by the number of days in the RP or in FLP during the 15-mo after the initiation of the AIP.

Stochastic Analysis

Differences between treatments in cash flow for the 15-mo period (ALL-TAI vs. PGF+AIE, ALL-TAI vs. PGF+TAI, and PGF+TAI vs. PGF+AIE) under varying input and output values were estimated by stochastic Monte Carlo simulation models using @Risk software (version 7.5, Palisade Corp., Ithaca, NY). Simulations were run and recorded for 10,000

iterations with replacement. To build the models, reproduction and production outcomes as well as herd exit dynamics observed for the experiment were used as fixed inputs (Supplemental Table S1). Thus, variation for every iteration of the simulation was introduced for hormone cost (GnRH, $PGF_{2\alpha}$, and CIDR devices), semen price (sexed and conventional semen), milk price, feed cost, beef price (for culled heifers and cows), price of a replacement heifer or cow, price of a newborn calf (female and male), and fixed cost. Historical data was available from 2010 to 2019 for milk price (USDA Agricultural Marketing Service, 2019), beef price of sold heifers and cows (USDA National Agricultural Statistic Service, 2019), price for replacement heifers (Progressive Dairyman, 2019) and cows (USDA Economic Research Service, 2019), newborn calves (Progressive Dairyman, 2019), and feed cost of lactation period (Gould, 2017). For these variables, distributions used in the simulations were fitted using the BestFit function of @Risk and are presented in Supplemental Table S2. This function selects the best-fitting distribution based on the lowest value for the Akaike information criterion. Stochasticity for the rest of the inputs (i.e., variables without historical data such as hormone and semen cost, rearing period feed cost, and other operating expenses) was generated using a pert distribution with the average price calculated for this study as the most likely value and a 15% reduction or increment as minimum and maximum values, respectively. For instance, the cost of a CIDR device (including labor) was $12.7 per device (most likely), with minimum and maximum values of $10.8 and $14.6, respectively. Values for the parameters used for each distribution are presented in Supplemental Table S2.

Statistical Analysis

Binary outcomes [proportion of heifers AIE; pregnancies per AI (**P/AI**); proportion of heifers that left the herd, calved, had stillbirths, had a calving ease >2, and had a female calf] were analyzed using logistic regression with the GLIMMIX procedure of SAS (version 9.4, SAS Institute Inc., Cary, NC) fitting a binomial distribution. Continuous quantitative outcomes [RP and FLP duration, AFC, BW at 1st calving, DMI, milk yield, incomes (milk income, calf value, IOFC), expenses (reproductive cost, replacement cost, feed cost, and other operating expenses), and cash flow] were analyzed by ANOVA using PROC MIXED of SAS. Time to pregnancy after the beginning of the AIP was analyzed using Cox's proportional hazard regression with the PHREG procedure of SAS. For the analysis of time to pregnancy, a heifer was considered pregnant if pregnancy was maintained until at least 66 ± 3 d of gestation. Heifers that left the herd due to death or sale were right-censored.

For all outcomes, the effect of experimental treatment was included in all models as a fixed effect whereas farm was included as a random effect. In addition, for AI service outcomes (proportion AIE and P/AI) the effect of season of insemination (cold vs. warm) was offered to the models and retained if significant (data not reported). The cold season was defined as the period from September 21 to June 20 and the warm season from June 21 to September 20. Assumptions of normality of residuals and homoscedasticity for linear regression models were tested by evaluating normal probability plots (Q-Q plot) and plotting residuals versus predicted values. For logistic regression models, goodness of fit was evaluated using the ratio between the Pearson statistic and its degrees of freedom. In all cases, model fit was deemed acceptable based on a ratio of approximately one.

The effect of treatment on milk yield measured in monthly milk tests was evaluated with models that included treatment, monthly milk test number, and the treatment by test number interaction as fixed effects, whereas cows within treatment was included as a random effect.

All proportions and quantitative (± SEM) outcomes reported are Least Squares Means (**LSM**) generated using the LSMEANS statement of PROC GLIMMIX and with the LSMEANS option of PROC MIXED of SAS, respectively. When appropriate, the Least Significant Difference (**LSD**) post hoc mean separation test was used to determine differences between LSM.

Variables were considered significant if $P \leq 0.05$, whereas P-values > 0.05 and ≤ 0.10 were considered a tendency.

RESULTS

Reproductive Performance and Herd Exit Dynamics during the Rearing Period

For first service, the total proportion of heifers that received AIE was greater ($P < 0.01$; Table 1) for the PGF+AIE treatment than for the PGF+TAI treatment. For the PGF+AIE treatment (arithmetic mean reported), 78.5% (248/317) of the heifers received AIE after the first $PGF_{2\alpha}$, 17.0% (54/317) after the second, and 3.2% (10/317) after the third $PGF_{2\alpha}$ treatment.

For all first AI services combined, P/AI 31 ± 3 d after AI did not differ between treatments ($P = 0.38$; Table 1), and was similar ($P = 0.21$) for heifers that received AIE after $PGF_{2\alpha}$-synchronized estrus (46.1%; n = 582), AIE during the 5-d Cosynch (45.7%; n = 127), or completion of the TAI protocol (40.1%; n = 257). Similarly, overall P/AI for all second and greater AI services combined was not different among treatments ($P = 0.72$; Table 1).

The hazard of pregnancy for up to 100 d after the beginning of the AIP was greater for the ALL-TAI than the PGF+AIE treatment (Table 1) but was similar for the PGF+AIE and PGF+TAI treatments, and for the ALL-TAI and PGF+TAI treatments. Conversely, the proportion of heifers not pregnant by 100 d after the beginning of the AIP was not different (P = 0.97; Table 1) among treatments.

Overall, the total proportion of heifers that left the herd during the RP did not differ (P = 0.33; Table 1), because treatment did not affect the proportion of heifers sold (P = 0.23) or that died (P = 0.23) during the RP.

The length of the RP (i.e., time from the beginning of the AIP until calving or herd exit), was greatest for the PGF+AIE, intermediate for the PGF+TAI, and least for the ALL-TAI treatment, when only heifers that completed the 15-mo experimental period (P = 0.01) were included in the analysis (Table 1). In contrast, when analyzed per slot (i.e., including replacement dynamics) RP length was not different (P = 0.35; Table 1) among treatments.

Table 1. Effect of first-service management strategy on reproductive performance during the rearing period.

	Treatment[1]			
Item	PGF+AIE	PGF+TAI	ALL-TAI	*P*-value
Heifers AIE 1st service [% (no.)]	98.7 (317)	78.5 (334)	–	<0.01
P/AI 1st service [% (no.)]	42.0 (317)	47.3 (334)	43.8 (315)	0.38
P/AI 2nd and greater AI [% (no.)]	58.8 (311)	55.7 (318)	57.6 (311)	0.72
Time to pregnancy				
Hazard ratio (95% CI)	Referent[2]	1.07 (0.91-1.25)	1.20 (1.02-1.42)	0.08
Mean (d)	36 ± 1.8	32 ± 1.8	28 ± 1.7	–
Median (d)	27 (23-36)	23 (17-26)	21 (10-23)	–
NP at 100 d [% (no.)]	7.0 (317)	6.5 (334)	6.8 (315)	0.97
Left the herd [% (no.)]	4.7 (313)	5.4 (330)	7.4 (306)	0.33
Sold [% (no.)]	4.4 (313)	5.1 (330)	7.4 (306)	0.23
Died [% (no.)][3]	0.3 (313)	0.3 (330)	0.0 (306)	0.23
RP length (d)				
Not culled during RP[4]	309 ± 6[a]	304 ± 6[ab]	300 ± 6[b]	0.01
Per slot	315 ± 7	311 ± 7	310 ± 7	0.35

[a,b] Different superscripts within a row indicate significant differences ($P \leq 0.05$).
[1]Treatment: at 368 ±10 d of age, heifers received first AI after: 1) PGF+AIE: insemination at detected estrus (AIE) after $PGF_{2\alpha}$ treatments 14 d apart (up to 3) starting at the beginning of the AIP. Heifers not AIE within 9 d of the third $PGF_{2\alpha}$ treatment were enrolled in a 5d-Cosynch protocol [5d-Cosynch = controlled internal drug release insert (CIDR) + GnRH-5 d-CIDR-out + $PGF_{2\alpha}$-3 d-GnRH + TAI], 2) PGF+TAI: AIE after the second of two $PGF_{2\alpha}$ treatments 14 d apart. Heifers not AIE within 9 d after the second $PGF_{2\alpha}$ were enrolled in 5d-Cosynch and 3) ALL-TAI: timed AI after a 5d-Cosynch protocol.
[2]Time to pregnancy did not differ for ALL-TAI and PGF+TAI (Hazard Ratio = 1.13, 95% CI = 0.96 to 1.33).
[3]The proportion of heifers that died was evaluated with the Fisher's exact test using FREQ procedure of SAS because in the ALL-TAI treatment 0% of heifers had a positive outcome.
[4]Heifers that were sold or died during the rearing period were removed from the analysis.

Economic Outcomes for the Rearing Period

The effect of treatments on economic outcomes during the RP is presented in Table 2. Reproductive cost was greater ($P < 0.001$) for the ALL-TAI than for the PGF+AIE and PGF+TAI treatments. In contrast, no difference was observed for DMI ($P = 0.23$), feed cost ($P = 0.23$), replacement cost ($P = 0.14$), and other operating expenses ($P = 0.35$). When only heifers that completed the 15-mo experimental period were included in the analysis (i.e., not culled only), reproductive cost was also greater ($P < 0.001$) for the ALL-TAI than the PGF+AIE and PGF+TAI treatments. In addition, for these heifers treatment also affected DMI ($P = 0.01$), feed cost ($P = 0.01$) and other operating expenses ($P = 0.01$), as all were greatest for PGF+AIE, intermediate for PGF+TAI, and lowest for the ALL-TAI treatment.

Calving Features, Herd Exit Dynamics, and Milk Yield

Overall, the proportion of heifers that calved within 15-mo after the beginning of the AIP was similar ($P = 0.34$; Table 3). In contrast, AFC differed ($P = 0.01$) among treatments because it was greater for the PGF+AIE than for the ALL-TAI treatment, but similar for the PGF+AIE and PGF+TAI treatments (Table 3).

For heifers with data for BW at first calving available (n = 489), BW averaged 560 kg and did not differ ($P = 0.42$) between treatments (Table 3). Similarly, among heifers with calving ease data available (n = 797), the proportion with a calving ease score > 2 did not differ ($P = 0.23$) between treatments (Table 3). In addition, no difference between treatments was observed for the proportion of stillbirths ($P = 0.21$), proportion of female calves born ($P = 0.70$), and for the proportion of heifers that left the herd due to sale or death ($P = 0.53$).

Table 2. Effect of first-service management strategy on expenses during the rearing period.

	Treatment[1]					
Item (per slot)	PGF+AIE (n =313)	PGF+TAI (n = 330)	Diff[2]	ALL-TAI (n = 306)	Diff[3]	*P*-value
All heifers						
Hormonal treatment, $	12.1 ± 4.7[a]	15.8 ± 4.7[b]	3.7	26.9 ± 4.7[c]	15	<0.001
Semen and AI, $	40.8 ± 1.1	40.2 ± 1.1	-0.6	41.6 ± 1.1	0.8	0.69
Pregnancy diagnosis, $	7.1 ± 0.7	6.9 ± 0.7	-0.2	7.1 ± 0.7	-0.0	0.60
Detection of estrus, $	8.7 ± 0.2	8.6 ± 0.2	-0.1	8.6 ± 0.2	-0.1	0.35
Total reproductive cost, $	68.8 ± 6.0[a]	71.5 ± 6.0[a]	2.7	84.1 ± 6.0[b]	15	<0.001
DMI, kg	3,592 ± 87	3,536 ± 87	-56	3,518 ± 87	-74	0.26
Feed cost, $	618 ± 15	608 ± 15	-10	605 ± 15	-13	0.23
Replacement cost, $	-27.7 ± 12	-35.9 ± 12	-8.2	-55.7 ± 12	-28	0.14
Other operating expenses, $	268 ± 6	264 ± 6	-4.0	264 ± 6	-4.0	0.35
Not culled						
Hormonal treatment, $	11.0 ± 4.2[a]	14.4 ± 4.1[b]	3.4	24.2 ± 4.2[c]	13	<0.001
Semen and AI, $	37.8 ± 0.8	37.5 ± 0.8	-0.3	37.9 ± 0.8	0.1	0.95
Pregnancy diagnosis, $	6.7 ± 0.6	6.5 ± 0.6	-0.2	6.5 ± 0.6	-0.2	0.28
Detection of estrus, $	8.6 ± 0.2	8.4 ± 0.2	-0.2	8.3 ± 0.2	-0.3	0.01
Total reproductive cost, $	64.2 ± 5.0[a]	66.8 ± 5.0[a]	2.6	76.9 ± 5.0[b]	13	<0.001
DMI, kg	3,526 ± 78[a]	3,462 ± 77[ab]	-64	3,409 ± 78[b]	-117	0.01
Feed cost, $	607 ± 13[a]	595 ± 13[ab]	-12	586 ± 13[b]	-21	0.01
Replacement cost, $	–	–	–	–	–	–
Other operating expenses, $	263 ± 5	259 ± 5	-4.0	255 ± 5	-8.0	0.01

[a,b] Different superscripts within a row indicate significant differences ($P \leq 0.05$).
[1]Treatment: at 368 ±10 d of age, heifers received first AI after: 1) PGF+AIE: insemination at detected estrus (AIE) after $PGF_{2\alpha}$ treatments 14 d apart (up to 3) starting at the beginning of the AIP. Heifers not AIE within 9 d of the third $PGF_{2\alpha}$ treatment were enrolled in a 5d-Cosynch protocol [5d-Cosynch = controlled internal drug release insert (CIDR) + GnRH-5 d-CIDR-out + $PGF_{2\alpha}$-3 d-GnRH + TAI], 2) PGF+TAI: AIE after the second of two $PGF_{2\alpha}$ treatments 14 d apart. Heifers not AIE within 9 d after the second $PGF_{2\alpha}$ were enrolled in 5d-Cosynch and 3) ALL-TAI: timed AI after a 5d-Cosynch protocol.
[2]Difference between PGF+TAI and PGF+AIE
[3]Difference between ALL-TAI and PGF+AIE
[4]Heifers that were sold or died during the rearing period were removed from the analysis.

Table 3. Effect of first-service management strategy on calving features and herd exit dynamics during the first lactation.

	Treatment[1]			
Item	PGF+AIE	PGF+TAI	ALL-TAI	*P*-value
Calved [% (no.)]	95.0 (313)	94.4 (330)	92.4 (306)	0.34
AFC (mo)	22.3 ± 0.1[a]	22.1 ± 0.1[ab]	22.0 ± 0.1[b]	0.01
Weight at 1st calving (kg)	565 ± 4	559 ± 5	557 ± 5	0.42
Calving ease > 2	4.4 (264)	5.8 (281)	8.1 (250)	0.23
Stillbirths [% (no.)]	10.6 (297)	7.2 (310)	11.1 (282)	0.21
Female calves [% (no.)]	68.0 (297)	68.4 (310)	65.4 (280)	0.70
Left the herd [% (no.)]	8.8 (297)	6.6 (311)	8.6 (282)	0.53
Sold [% (no.)]	7.2 (297)	5.6 (311)	7.6 (282)	0.60
Died [% (no.)]	1.7 (287)	1.0 (311)	1.1 (282)	0.70
FLP length (d)				
Not culled[2]	148 ± 6	152 ± 6	155 ± 6	0.07
Per slot	141 ± 7	145 ± 6	146 ± 7	0.35

[a,b] Different superscripts within a row indicate significant differences ($P \leq 0.05$).

[1]Treatment: at 368 ±10 d of age, heifers received first AI after: 1) PGF+AIE: insemination at detected estrus (AIE) after $PGF_{2\alpha}$ treatments 14 d apart (up to 3) starting at the beginning of the AIP. Heifers not AIE within 9 d of the third $PGF_{2\alpha}$ treatment were enrolled in a 5d-Cosynch protocol [5d-Cosynch = controlled internal drug release insert (CIDR) + GnRH-5 d-CIDR-out + $PGF_{2\alpha}$-3 d-GnRH + TAI], 2) PGF+TAI: AIE after the second of two $PGF_{2\alpha}$ treatments 14 d apart. Heifers not AIE within 9 d after the second $PGF_{2\alpha}$ were enrolled in 5d-Cosynch and 3) ALL-TAI: timed AI after a 5d-Cosynch protocol.

[2]Heifers that were sold or died during the rearing period and first lactation period were removed from the analysis. When only heifers that completed the 15-mo period were included in the analysis, overall FLP length tended ($P = 0.07$) to differ between treatments. Conversely, when analyzed per slot FLP length did not differ ($P = 0.35$) between treatments (Table 3).

The effect of treatments on milk yield during the first 10 monthly milk tests was evaluated for all heifers that calved and initiated their first lactation (Figure 1). We observed an effect of test number ($P < 0.001$); however, we did not observe an effect of treatment ($P = 0.63$) or a treatment by test number interaction ($P = 0.16$).

First Lactation Period Economic Outcomes

The effect of treatments on economic and productive outcomes during the FLP is presented in Table 4. When all heifers were included in the analysis, no differences ($P > 0.10$) were observed for DMI, feed cost, milk yield, milk income, IOFC, replacement cost, and other operating expenses. Conversely, we observed a tendency ($P = 0.08$) for a treatment effect on calf value, whereby cows in PGF+TAI treatment had the greatest calf value, followed by the PGF+AIE treatment and least for the ALL-TAI treatment (Table 4). When only heifers that completed the 15-mo experimental period were included in the analysis, no differences ($P > 0.10$) were observed for DMI, feed cost, milk yield, milk income, IOFC, and replacement cost. In contrast, other operating expenses tended ($P = 0.07$) to differ between treatments, whereby expenses were greatest in ALL-TAI, intermediate in PGF+TAI and lowest in PGF+AIE (Table 4). Similarly, there was a tendency ($P = 0.08$) for a treatment effect on calf value, whereby ALL-TAI heifers had the lowest calf value, followed by the PGF+AIE and greatest for PGF+TAI heifers.

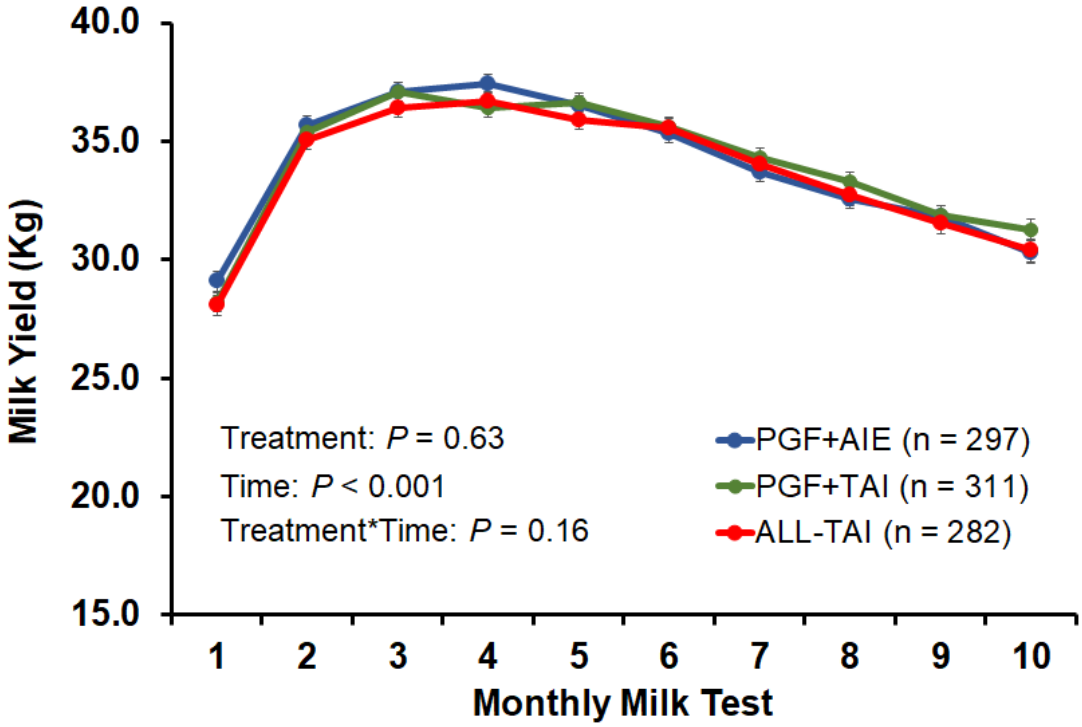


Figure 1. Milk yield (kg) based on monthly test date data for heifers that calved within the 15-mo study period. All values are presented as LSM ± SEM.

Table 4. Effect of first-service management strategy on productive parameters, revenues and expenses during the first lactation.

	Treatments[1]					
Item (per slot)	PGF+AIE (n =313)	PGF+TAI (n = 330)	Diff[2]	ALL-TAI (n = 306)	Diff[3]	*P*-value
All heifers						
DMI, kg	3,240 ± 164	3,341 ± 163	101	3,354 ± 164	114	0.39
Feed cost, $	940 ± 47	969 ± 48	29	973 ± 48	33	0.39
Milk, kg	4,858 ± 200	4,996 ± 198	138	5,008 ± 200	150	0.51
Milk income, $	1,749 ± 72	1,799 ± 71	50	1,803 ± 72	54	0.51
IOFC, $	809 ± 26	829 ± 25	20	830 ± 26	21	0.67
Replacement cost, $	69.5 ± 15.3	48.1 ± 14.9	-21	58.7 ± 15.4	-11	0.50
Other operating expenses, $	436 ± 21	449 ± 21	13	452 ± 21	16	0.35
Calf value, $	89.1 ± 2.2	92.4 ± 2.1	3.3	85.6 ± 2.2	-3.5	0.08
Not culled[4]						
DMI, kg	3,426 ± 152	3,511 ± 152	85	3,586 ± 153	160	0.12
Feed cost, $	994 ± 44	1,018 ± 44	24	1,039 ± 44	45	0.12
Milk, kg	5,143 ± 181	5,252 ± 179	109	5,360 ± 182	217	0.22
Milk income, $	1,851 ± 65	1,891 ± 64	40	1,930 ± 65	79	0.22
IOFC, $	858 ± 22	872 ± 22	14	889 ± 23	31	0.42
Replacement cost, $	–	–	–	–	–	–
Other operating expenses, $	457 ± 20	470 ± 20	13	480 ± 20	23	0.07
Calf value, $	94.2 ± 2.2	98.2 ± 2.2	4.0	92.4 ± 2.2	-1.8	0.08

[a,b] Different superscripts within a row indicate significant differences ($P \leq 0.05$).

[1]Treatment: at 368 ±10 d of age, heifers received first AI after: 1) PGF+AIE: insemination at detected estrus (AIE) after $PGF_{2\alpha}$ treatments 14 d apart (up to 3) starting at the beginning of the AIP. Heifers not AIE within 9 d of the third $PGF_{2\alpha}$ treatment were enrolled in a 5d-Cosynch protocol [5d-Cosynch = controlled internal drug release insert (CIDR) + GnRH-5 d-CIDR-out + $PGF_{2\alpha}$-3 d-GnRH + TAI], 2) PGF+TAI: AIE after the second of two $PGF_{2\alpha}$ treatments 14 d apart. Heifers not AIE within 9 d after the second $PGF_{2\alpha}$ were enrolled in 5d-Cosynch and 3) ALL-TAI: timed AI after a 5d-Cosynch protocol.

[2]Difference between PGF+TAI and PGF+AIE.

[3]Difference between ALL-TAI and PGF+AIE.

[4]Heifers that were sold or died during the rearing and first lactation period were removed from the analysis

Economic Outcomes for 15-mo After the Beginning of the AIP

The effect of treatments on total cash flow for the RP, FLP, and the accumulated cash flow for the 15-mo period is presented in Table 5. When all heifers were included in the analysis, we observed a tendency ($P = 0.08$) for a treatment effect on total RP cost, which was lowest for ALL-TAI, intermediate for PGF+TAI, and highest for the PGF+AIE treatment. In contrast, no treatment effect was observed on total FLP profit ($P = 0.92$) and on cash flow for the 15-mo period ($P = 0.22$). When only heifers that completed the 15-mo experimental period were included in the analysis, no differences ($P > 0.10$) were observed for total RP cost, total FLP profit, and cash flow for the 15-mo period (Table 5).

Stochastic Analysis

Differences in cash flow for the 15-mo period after the beginning of the AIP for all heifers included in the study under diverse pricing scenarios are presented in Figure 2. Differences in cash flow between the PGF+TAI and PGF+AIE treatments were 100% of the time positive values (i.e., in favor of the PGF+TAI treatment). The mean difference after 10,000 iterations was \$53.9/slot per 15 mo (95% CI: \$29.1 to \$84.1), and it ranged from \$10.1 to \$128. Milk price was the main contributor to the total variance, explaining ~51% of the total variation in cash flow differences. Greater milk price favored the PGF+TAI treatment. Similarly, differences in cash flow between the ALL-TAI and PGF+AIE ranged from a minimum of \$23.4/slot to a maximum of \$128/slot per 15 mo, with a positive average (i.e., in favor of the ALL-TAI treatment) of \$48.8/slot (95% CI: \$17.2 to \$84.1) with milk price as the main contributor of the total variance (~40%). Greater milk price favored the PGF+TAI treatment. Finally, results for the differences in cash flow between the ALL-TAI and PGF+TAI treatments

Table 5. Effect of first-service management strategy on revenues and expenses accumulated during 15 mo after the beginning of the AIP.

	Treatments[1]					
Item ($/slot)	PGF+AIE (n =313)	PGF+TAI (n = 330)	Diff[2]	ALL-TAI (n = 306)	Diff[3]	*P*-value
All heifers						
Total RP cost	928 ± 18	909 ± 18	-19	898 ± 18	-30	0.08
Total FLP profit	393 ± 20	424 ± 19	31	405 ± 20	12	0.48
Cash flow 15-mo	-534 ± 33	-483 ± 32	51	-492 ± 33	42	0.22
Not culled[4]						
Total RP cost	934 ± 23	921 ± 23	-13	918 ± 23	-16	0.36
Total FLP profit	496 ± 13	501 ± 12	5.0	503 ± 13	7.0	0.92
Cash flow 15-mo	-436 ± 26	-421 ± 26	15	-421 ± 27	15	0.80

[a,b] Different superscripts within a row indicate significant differences ($P \leq 0.05$).
[1]Treatment: at 368 ±10 d of age, heifers received first AI after: 1) PGF+AIE: insemination at detected estrus (AIE) after $PGF_{2\alpha}$ treatments 14 d apart (up to 3) starting at the beginning of the AIP. Heifers not AIE within 9 d of the third $PGF_{2\alpha}$ treatment were enrolled in a 5d-Cosynch protocol [5d-Cosynch = controlled internal drug release insert (CIDR) + GnRH-5 d-CIDR-out + $PGF_{2\alpha}$-3 d-GnRH + TAI], 2) PGF+TAI: AIE after the second of two $PGF_{2\alpha}$ treatments 14 d apart. Heifers not AIE within 9 d after the second $PGF_{2\alpha}$ were enrolled in 5d-Cosynch and 3) ALL-TAI: timed AI after a 5d-Cosynch protocol.
[2]Difference between PGF+TAI and PGF+AIE.
[3]Difference between ALL-TAI and PGF+AIE.
[4]Heifers that were sold or died during the rearing and first lactation period were removed from the analysis.

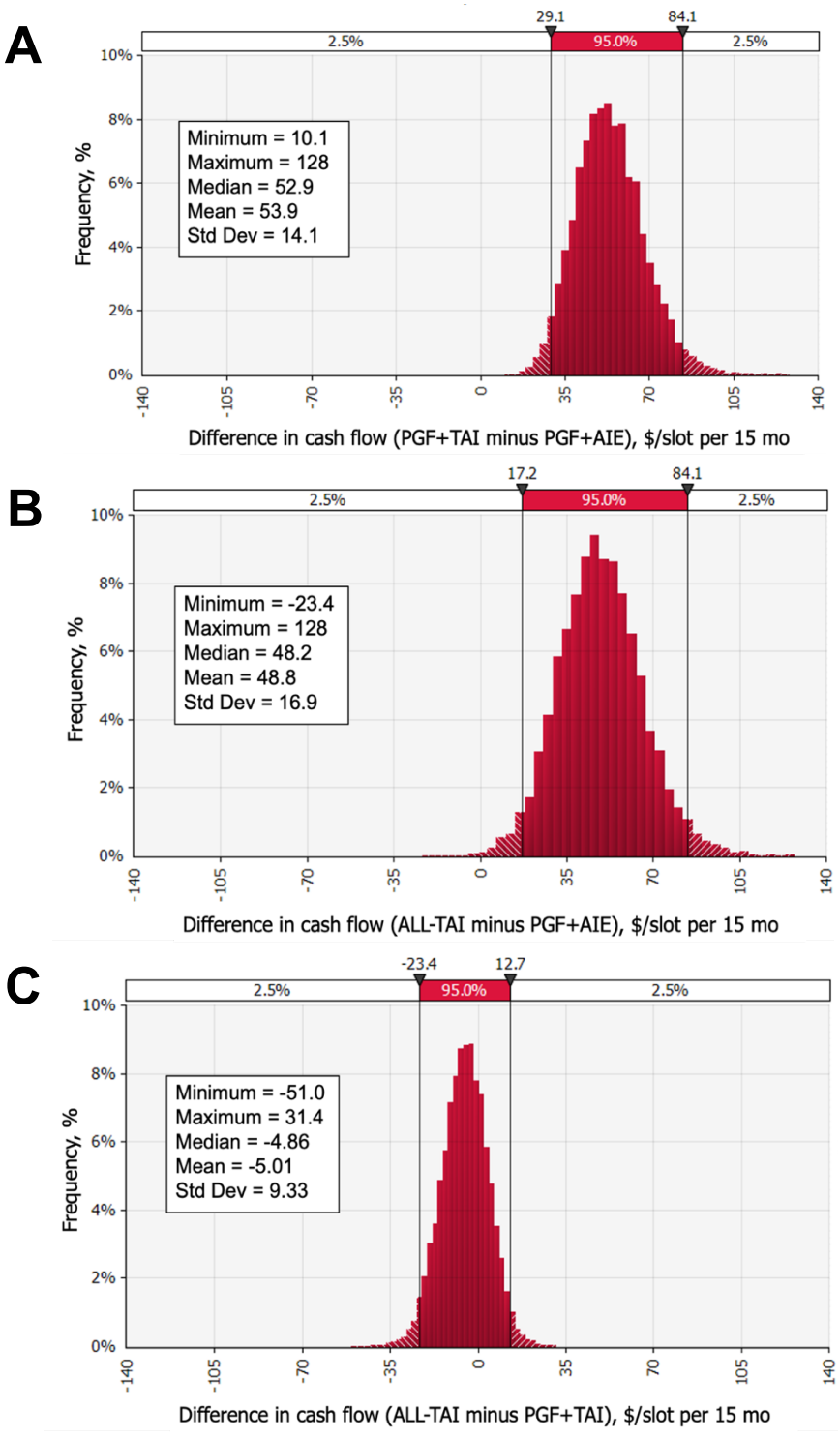


Figure 2. Relative frequency distribution of the difference in cash flow ($/slot per 15 mo) between the (A) PGF+TAI and PGF+AIE, (B) ALL-TAI and PGF+AIE, and (C) ALL-TAI and PGF+TAI after 10,000 iterations of simulation with stochasticity for economic input values.

had a mean of $-5.01/slot per 15 mo (95% CI: $-23.4 to $12.7) and a minimum and a maximum of $-51.0 and $31.4, respectively. In this case, the contribution to variance was led by the cost of a new replacement heifer (29%) closely followed by the cost of a new replacement cow (24%), and both combined represented ~55% of the total variation in cash flow differences.

DISCUSSION

Our study examined economic outcomes for replacement Holstein heifers managed with three reproductive management programs that varied in method of submission to first service from almost exclusive use of AIE to exclusive use of TAI. We aimed to provide an all-encompassing comparison that reflected to the best extent possible the conditions of commercial dairy farms. Thus, the main outcome of interest was cash flow per slot for a fixed period of time (i.e., 15 mo after the beginning of the AIP) accounting for the multiple interactions among reproductive performance, productivity, and replacement dynamics as previously reported for lactating dairy cows by our group (Stangaferro et al., 2018; Stangaferro et al., 2019) and others (De Vries, 2004; 2006; Gobikrushanth et al., 2014). The evaluation of cash flow without the effect of the replacement dynamics was explored to isolate the effect of timing of pregnancy because replacement cost can have profound effects on overall cash flow.

The greatest effect of the treatments evaluated on reproductive performance was shifting timing of pregnancy through different insemination risks as the overall first service fertility was similar regardless of the method of submission to AI. Time to pregnancy was reduced for the programs that relied more on TAI with greatest hazard of pregnancy for ALL-TAI, intermediate for PGF+TAI, and lowest for the PGF+AIE program. In spite of these differences, the proportion of heifers pregnant at the end of the observation period and calving was not affected.

A direct consequence of earlier pregnancy for the programs that relied more on TAI was a modest difference in RP length, and consequently AFC which reduced RP cost and numerically increased cash flow during the FLP. Collectively, these effects resulted in numerical differences in overall cash flow per slot for the 15-mo period that would be of potential value to commercial dairy farms. Nevertheless, from a strict statistical perspective, our hypothesis of improved cash flow for the programs that incorporated more aggressive use of TAI was not supported. Minor monetary differences and compensation among multiple parameters coupled with large variation among heifers likely explained our observations. The dramatic reduction in cash flow differences among treatments for the analysis including only heifers not culled revealed a smaller contribution of differences in time to pregnancy. Moreover, the different magnitude of change in the differences between treatments illustrated the complexity of the effect of replacement cost on cash flow of dairy animals, especially when accounting for the replacement dynamics during rearing and first lactation. Such prominent role of replacement cost on cash flow highlights the need to account for culling practices and potential cost at the time of selecting or comparing reproductive management programs for replacements. In summary, in spite of the lack of statistical significance and based on the accumulated trends for the rearing and lactation periods accounting or not for heifers that exited the herd, total cash flow trended (in order of magnitude) in favor of the PGF+TAI and ALL-TAI relative to the PGF+AIE program. Thus, under similar conditions than those of our study and assuming that results are repeatable, both programs incorporating systematic use of TAI early after the beginning of the AIP would be expected to improve cash flow of the heifer enterprise as compared with programs similar to PGF+AIE which relied primarily on AIE for submission to the first service.

In support of our other hypothesis, reproductive cost was greater for the ALL-TAI than for the PGF+AIE and PGF+TAI programs. This difference was largely explained by greater hormonal treatment cost, which accounted for ~94% of the difference in total reproductive cost relative to the PGF+AIE program. These results were expected because previous studies evaluating reproductive management for heifers consistently reported increased hormonal treatment cost for ALL-TAI relative to AIE-based programs (Stevenson et al., 2008; Lopes et al., 2013; Silva et al., 2015). On the other hand, the cost of semen and pregnancy diagnosis had a negligible effect on overall reproductive cost (<$1/slot). Such small effect was likely explained by the overall similar fertility observed across treatments not only for first AI service (sexed semen) but also for second and subsequent AI services (conventional semen). Interestingly, although more heifers received TAI in the PGF+TAI than in the PGF+AIE program, the additional cost of synchronization of ovulation did not translate into greater overall reproductive cost. This was because the minor increment in hormone cost was compensated by the accumulation of minor savings in the other items that contributed to reproductive cost, which was numerically greater for the PGF+AIE program.

Despite greater reproductive cost, total cost accrued during the RP tended to be lower for ALL-TAI relative to the PGF+AIE program. Rearing cost for PGF+TAI was intermediate but resembled more closely that of the ALL-TAI treatment. These results were expected, because reduced time to pregnancy for ALL-TAI heifers resulted in lower AFC and consequently a numerical reduction in expenses directly affected by RP duration (i.e., feed cost and other operating expenses). This reduction, however, accounted only for ~28% of the total difference in total rearing cost. The main contribution to rearing cost differences was for replacement cost (~46% of the difference) which favored the ALL-TAI program (statistical tendency).

Paradoxically, this was the result of a positive cash flow for heifers that were sold because a heifer sold after the beginning of the AIP was heavier than the heifer that replaced it. Conversely, when removing the effect of replacement dynamics (i.e., culled heifers not included in the analysis) the reduction in RP expense was mostly because of reduced feed cost of ALL-TAI heifers, which accounted for ~50% of the difference with PGF+AIE heifers. A substantial proportion of this gain, however, was offset by greater hormonal treatment cost, to the extent that no significant differences in total RP cost were observed. In summary, most economic items trended in favor of the PGF+TAI and ALL-TAI relative to the PGF+AIE program. Nonetheless, the differences in timing of pregnancy between treatments were of insufficient magnitude to have a large impact on RP expenses when other expenses not directly associated with RP duration (i.e., replacement and reproductive cost) were accounted for. Of note, reproductive cost was offset regardless of the method to estimate cash flow indicating that as long as timing of pregnancy is reduced, the additional investment in synchronization of ovulation for TAI seemed justified. On the other hand, given the relevance of replacement cost on cash flow differences, it seems reasonable to suggest considering the potential effect of culling practices and market value of animals (i.e., sold and bought) at the time of evaluating reproductive management strategies for heifers.

Regardless of the type of analysis (i.e., all heifers enrolled vs. not culled only), most economic differences during first lactation presented similar trends as those observed for the RP. Reduced AFC for the ALL-TAI treatment translated into numerically greater IOFC. This gain, however, was offset to the extent of resulting in no statistically significant differences for the FLP by factors not directly affected by lactation length (i.e., calf value and replacement cost) and by the greater cost of maintaining lactating cows than heifers (i.e., other operating expenses).

The observed unfavorable differences (statistical tendency) for calf value for the ALL-TAI treatment were in line with the slight numerical reduction in the number of born female calves and with the slight increment in the number of stillbirths as compared with the PGF+AIE treatment. Nevertheless, the contribution of calf value to overall FLP profit was inconsequential (i.e., $3/slot). Similar than for the RP, outcomes for the FLP for the PGF+TAI program were mostly intermediate. The tendency for greater calf value and the numerically lower replacement cost resulted in numerically greater FLP profit relative to ALL-TAI. Collectively, we observed that cash flow during the FLP followed expected trends (i.e., greater for ALL-TAI and PGF+TAI than for PGF+AIE), but minor monetary differences and compensation among items not directly affected by the timing of first calving likely resulted in lack of significant differences on total FLP cash flow.

Taken together, data from the RP and FLP suggested that the magnitude of the differences observed in timing of pregnancy and consequently on AFC were of sufficient magnitude to generate numerical differences for cash flow over the 15-mo period that would be of economic value to commercial dairy farms but were of insufficient magnitude to be statistically significant. The latter can be explained by the magnitude of the difference in reproductive performance and the effect of large heifer-to-heifer variation on overall cash flow differences. The small magnitude of the differences in time to pregnancy between treatments was the result of the high insemination risk for the PGF+AIE treatment because a high proportion of heifers received AIE within a short time after the beginning of the AIP (i.e., 78% AIE after the first $PGF_{2\alpha}$ treatment in PGF+AIE) and had similar P/AI than heifers in the other treatments. Our results are in agreement with those of Ribeiro et al. (2012) who in a simulation study reported that the economic benefits of incorporating programs that relied mostly on TAI for managing

reproduction of replacement heifers were inconsequential when estrus detection efficiency was at least 70%. In contrast, Silva et al. (2015) reported reduced rearing cost for heifers managed with an ALL-TAI (5-d Cosynch with initial GnRH and two $PGF_{2\alpha}$ treatments) program compared with a predominant AIE-based program, even when a high proportion of heifers were AIE (>80%) relatively early after the beginning of the AIP. Differences in insemination risk between experiments, and consequently timing of pregnancy imposed by different experimental designs likely explain the contrasts in results observed for our experiment and that of Silva et al. (2015). On the other hand, in spite of the reasonable number of heifers included in each treatment in our study, substantial heifer-to-heifer variation contributed to the lack of statistically significant differences in cash flow per 15 mo. This was a reflection of the major variation in cash flow per slot (range $-2,776 to $470; data not shown) driven primarily by large differences in replacement cost and milk income between slots. This is in agreement with recent studies that included a similar comprehensive approach to calculate cash flow of dairy cows managed with different reproductive management programs (Stangaferro et al., 2018; Stangaferro et al., 2019).

Stochastic analysis with Monte Carlo simulation is a useful tool to estimate trends and the expected magnitude of differences in cash flow for different management strategies when multiple inputs and outputs and their variation across time might affect cash flow (McArt et al., 2014; McArt and Oetzel, 2015). In this case, we estimated a range of differences in cash flow for the programs compared under a wide range of market conditions. We observed that the PGF+TAI and ALL-TAI treatments were economically favorable relative to the PGF+AIE program under all for PGF+TAI or almost all scenarios for ALL-TAI (i.e., > 98%) suggesting that under a wide range of conditions caused by market fluctuations the programs incorporating more aggressive use of TAI would be beneficial to the economics of the heifer enterprise. Based

on the range of values and shape of the distribution for cash flow differences, it was apparent that the PGF+TAI treatment would lead to greater and more consistent positive differences compared with the PGF+AIE treatment than the ALL-TAI treatment. Conversely, implementing either the PGF+TAI or ALL-TAI treatments would likely be neutral because values for their cash flow differences were almost equally distributed around zero. Collectively, the results of the stochastic and deterministic analysis suggested that the selection of reproductive management programs for replacement heifers should be made in light of the known or expected insemination risk for AIE or TAI-based programs, the effect of culling practices, and current and future prices for milk and replacements. In addition, the cost of hormonal treatments seemed less relevant because its effect on overall cash flow differences was small compared with the value of earlier pregnancy.

CONCLUSION

We concluded that reproductive management programs that used TAI in combination with AIE or TAI as the primary method to submit heifers for first service generated differences in cash flow of potential value to commercial dairy farms. Reduced rearing cost and more revenue generated during the first lactation increased cash flow for up to 15-mo after heifers became eligible for pregnancy under fixed or simulated variable market conditions. Greater reproductive cost due to use of TAI were offset by the positive effect of earlier pregnancy on cash flow of replacements heifers.

ACKNOWLEDGMENT

This research was financially supported through a grant (AOR 15-020) from the New York Farm Viability Institute (Syracuse, NY) to J. O. Giordano. We would like to thank the two commercial dairy farms that participated in this research for their support and the use of their cows and facilities. We would also like to extend our gratitude to Zoetis for providing the hormonal products used to synchronize estrus and ovulation. Finally, we thank Jim Ehrlich from DairySights LLC (Argyle, NY) for his calculations of daily milk production using the MilkBot model.

REFERENCES

Cornell Cooperative Extension of Chautauqua County. 2018. Dairy Market Watch. Accessed Aug. 8, 2018. http://chautauqua.cce.cornell.edu/resources/

De Vries, A. 2004. Economics of delayed replacement when cow performance is seasonal. J. Dairy Sci. 87:2947-2958.

De Vries, A. 2006. Economic value of pregnancy in dairy cattle. J. Dairy Sci. 89:3876-3885.

Ehrlich, J. L. 2013. Quantifying inter-group variability in lactation curve shape and magnitude with the MilkBot® lactation model. PeerJ 1:e54.

Ettema, J. and J. Santos. 2004. Impact of age at calving on lactation, reproduction, health, and income in first-parity Holsteins on commercial farms. J. Dairy Sci. 87:2730-2742.

Gobikrushanth, M., A. De Vries, J. Santos, C. Risco, and K. Galvão. 2014. Effect of delayed breeding during the summer on profitability of dairy cows. J. Dairy Sci. 97:4236-4246.

Gould, B. 2017. Understanding dairy markets. National average dairy feed costs. Accessed Dec. 1, 2019. http://future.aae.wisc.edu/data/monthly_values/by_area/3002.

Harsh, S. B., C. A. Wolf, and E. Wittenberg. 2001. Profitability and production efficiency of the crop and livestock enterprises of Michigan dairy operations: 1998 summary and analysis. Agric. Econ. Staff Paper No. 01-04. Michigan State Univ., East Lansing.

Hyams, D. G. 2011. CurveExpert Professional: documentation. Release 2.6.5. Accessed Aug. 12, 2018. https://docs.curveexpert.net/curveexpert/pro/_static/CurveExpertPro.pdf

Kalantari, A., H. Mehrabani-Yeganeh, M. Moradi, A. Sanders, and A. De Vries. 2010. Determining the optimum replacement policy for Holstein dairy herds in Iran. J. Dairy Sci. 93:2262-2270.

Karszes, J. 2014. Dairy Replacement Programs: Costs & Analysis 3rd Quarter 2012. Extension Bulletin 2014-02, Department of Animal Science and Charles H. Dyson School of Applied Economics and Management, Cornell University, Ithaca, NY.

Karszes, J., A. Christman, A. Howlett, W. A. Knoblauch. 2018. Dairy farm management business summary, New York State, 2017. Charles H. Dyson School of Applied Economics and Management, Cornell University, Ithaca, NY.

Karszes, J., W. A., Knoblauch, C. Dyamond. 2017. Dairy farm management business summary, New York State, 2016. Charles H. Dyson School of Applied Economics and Management, Cornell University, Ithaca, NY.

Lopes, G., C. Johnson, L. Mendonça, P. Silva, J. Moraes, A. Ahmadzadeh, J. Dalton, and R. Chebel. 2013. Evaluation of reproductive and economic outcomes of dairy heifers inseminated at induced estrus or at fixed time after a 5-day or 7-day progesterone insert-based ovulation synchronization protocol. J. Dairy Sci. 96:1612-1622.

Masello, M., M. Perez, G. Granados, M. Stangaferro, B. Ceglowski, M. Thomas, and J. Giordano. 2019. Reproductive performance of replacement dairy heifers submitted to first service with programs that favor insemination at detected estrus, timed artificial insemination, or a combination of both. J. Dairy Sci. 102:1671-1681.

McArt, J., D. Nydam, G. Oetzel, and C. Guard. 2014. An economic analysis of hyperketonemia testing and propylene glycol treatment strategies in early lactation dairy cattle. Prev. Vet. Med. 117:170-179.

McArt, J. and G. Oetzel. 2015. A stochastic estimate of the economic impact of oral calcium supplementation in postparturient dairy cows. J. Dairy Sci. 98:7408-7418.

NRC. 2001. Nutrient requirements of dairy cattle: 2001. National Academies Press.

Progressive Dairyman. 2019. Market Watch: Cattle. Accessed several times from 2018 to 2019. Accessed Aug. 30, 2019. https://www.progressivedairy.com/news/organizations/market-watch-cattle.

Ribeiro, E., K. Galvão, W. Thatcher, and J. Santos. 2012. Economic aspects of applying reproductive technologies to dairy herds. Anim. Reprod. 9:370-387.

Silva, T., F. Lima, W. Thatcher, and J. Santos. 2015. Synchronized ovulation for first insemination improves reproductive performance and reduces cost per pregnancy in dairy heifers. J. Dairy Sci. 98:7810-7822.

Stangaferro, M., R. Wijma, and J. Giordano. 2019. Profitability of dairy cows submitted to the first service with the Presynch-Ovsynch or Double-Ovsynch protocol and different duration of the voluntary waiting period. J. Dairy Sci. 102:4546-4562.

Stangaferro, M., R. Wijma, M. Masello, M. J. Thomas, and J. Giordano. 2018. Economic performance of lactating dairy cows submitted for first service timed artificial insemination after a voluntary waiting period of 60 or 88 days. J. Dairy Sci. 101:7500-7516.

Stevenson, J., J. Rodrigues, F. Braga, S. Bitente, J. Dalton, J. Santos, and R. Chebel. 2008. Effect of Breeding Protocols and Reproductive Tract Score on Reproductive Performance of Dairy Heifers and Economic Outcome of Breeding Programs1. J. Dairy Sci. 91:3424-3438.

USDA Agricultural Marketing Service. 2019. Milk marketing order statistics. Individual milk order prices. Accessed Dec. 1, 2019. https://www.ams.usda.gov/resources/marketing-order-statistics/uniform-price

USDA Economic Research Service. 2019. US quarterly milk production and related data. Accessed several times from 2018 to 2019. Accessed Dec. 1, 2019. https://www.ers.usda.gov/data-products/dairy-data/

USDA National Agricultural Statistics Service. 2019. Agricultural Prices. Accessed several times from 2018 to 2019. Accessed Dec. 1, 2019. https://www.nass.usda.gov/

Van Arendonk, J. 1985. A model to estimate the performance, revenues and costs of dairy cows under different production and price situations. Agric. Syst. 16:157-189.

Winsor, C. P. 1932. The Gompertz curve as a growth curve. Proc. Natl. Acad. Sci. USA 18:1-8.

Supplemental Table S1. Values (arithmetic means) for inputs used to estimate cash flow for 15 mo after the beginning of the artificial insemination period.

Item	PGF+AIE	PGF+TAI	ALL-TAI
Number of GnRH doses per slot (n)	0.9	1.1	2.6
Number of $PGF_{2\alpha}$ doses per slot (n)	1.8	2.7	1.5
Number of CIDR devices per slot (n)	0.5	0.6	1.5
Number of 1st services per slot (n)	1.1	1.1	1.1
Number of 2nd and greater services per slot (n)	1.1	1.0	1.1
Pregnancy testing cost ($/slot)	7.2	7.2	7.0
Estrus detection cost ($/slot)	8.8	8.6	8.6
Days rearing (d)	317	312	311
DMI rearing period (kg/slot)	3,468	3,415	3,401
DMI rearing period close-up (kg/slot)	140	133	130
Body weight of heifers sold (kg)	507	499	495
Exit the herd during rearing (%)			
Sold (%)	3.5	4.2	5.9
Died (%)	0.3	0.3	0.0
Heifers calved (%)	95.0	94.0	92.0
Replacement heifers calved (%)	1.0	1.5	2.6
Number of live female calves born (n)	181	198	162
Number of live male calves born (n)	85	94	90
Days in lactation (d)	139	144	145
Milk production (kg/slot)	4,827	4,972	4,980
DMI lactation period (kg/d)	3,212	3,320	3,330
Body weight of cows sold (kg)	596	590	596
Exit the herd during lactation (%)			
Sold (%)	7.3	6.1	7.8
Died (%)	1.9	0.9	1.0

Supplemental Table S2. Stochastic inputs used to estimate cash flow for 15 mo after the beginning of the artificial insemination period.

Item	Distribution	Measure/parameters
GnRH cost* ($/dose)	Pert	min = 1.67; most likely = 1.97; max = 2.27
$PGF_{2\alpha}$ cost* ($/dose)	Pert	min = 2.09; most likely = 2.46; max = 2.83
CIDR cost* ($/device)	Pert	min = 10.8; most likely = 12.7; max = 14.6
Sexed semen cost* ($/dose)	Pert	min = 22.1; most likely = 26.0; max = 29.9
Conventional semen cost* ($/dose)	Pert	min = 11.1; most likely = 13.0; max = 15.0
Feed cost – rearing period ($/kg)	Pert	min = 0.14; most likely = 0.17; max = 0.20
Feed cost – rearing period close-up ($/kg)	Pert	min = 0.19; most likely = 0.22; max = 0.25
Feed cost – lactation period ($/kg)	Uniform	min = 0.21; max = 0.44
Milk price ($/kg)	Gamma	$\alpha = 3.42$; $\beta = 0.04$
Replacement heifer ($/head)	Extreme value	$\alpha = 553$; $\beta = 165$
Replacement cow ($/head)	Triangular	min = 1,082; most likely = 1,330; max = 2,234
Beef price for sold heifers ($/kg)	Extreme value	$\alpha = 2.62$; $\beta = 0.26$
Beef price for sold cows ($/kg)	Extreme value	$\alpha = 1.53$; $\beta = 0.21$
Female calf ($/newborn)	Triangular	min = 25; most likely = 25; max = 275
Male calf ($/newborn)	Exponential	$\beta = 72.0$
Other operating costs rearing ($/d)	Pert	min = 0.72; most likely = 0.85; max = 0.98
Other operating costs lactation ($/d)	Pert	min = 2.6; most likely = 3.1; max = 3.6

*Includes labor costs for hormone administration and insemination.

SECTION II

DEVELOPMENT AND INTEGRATION OF NEW TECHNOLOGIES FOR CATTLE REPRODUCTIVE MANAGEMENT

CHAPTER IV

DETERMINATION OF REPRODUCTIVE STATUS AND IMPLEMENTATION OF TIMED AI IN CATTLE

1. General introduction

One of the main goals of cattle operations is to provide nutrient-dense foods (i.e., dairy and meat) to the global human population, which is expected to increase to 10 billion by the year 2050 (FAO, 2017). Although impressive gains in cattle productive efficiency have been made in the past five decades (Capper et al., 2009), a doubling of the food supply is required by the year 2050 to meet expected population demands (Global Agricultural Productivity Report, 2017). Thus, finding effective means to increase productivity and sustainability of cattle operations to meet the growing demand for animal products is a major challenge faced by humanity. In this regard, cattle reproductive efficiency is a major factor that determines the productivity and consequently the sustainability of both dairy and meat enterprises (De Vries, 2006; Rodgers et al., 2012).

Reproductive efficiency of lactating dairy cows is primarily driven by timing of pregnancy after calving and the proportion of cows that becomes pregnant during lactation.

Thus, effective reproductive management programs in farms that use artificial insemination (**AI**) must ensure timely insemination after the end of the voluntary waiting period, minimize the interval between inseminations after a failed AI service, and maximize fertility to AI while striving to simplify cow management. To achieve these critical goals multiple management practices are used by farms, including determination of the reproductive physiological status of cows (i.e., pregnancy, anovulation, estrus) and synchronization of ovulation for timed artificial insemination (**TAI**).

An approach used to determine the reproductive status of females is direct or indirect estimation of hormone concentrations at a single or multiple time points in bodily fluids such as blood (Matsas et al., 1992; Nakami et al., 2017), plasma or serum (Breukelman et al., 2012; Giordano et al., 2012a), milk (Nebel, 1988; Waldmann and Raud, 2016; Bruinjé and Ambrose, 2019), and saliva (Gao et al., 1988; Kanchev et al., 1988). Females can then be classified in a specific reproductive status group (e.g., non-pregnant vs. pregnant, anovular vs. cyclic) based on the known pattern or hormone levels associated with a specific reproductive physiological status of practical interest. For example, in cattle progesterone (**P4**) produced by a functional corpus luteum (**CL**) on the ovaries during the estrous cycle and pregnancy is useful to identify non-pregnant cows, confirm estrus and cyclicity, and classify cows based on their expected response to hormonal treatments.

Another important practice used to optimize reproductive performance, simplify management, and increase profitability by many cattle farms is synchronization of ovulation for TAI. As described in Chapter I of this dissertation, synchronization of ovulation protocols consist of a combination of two or more sequential hormonal treatments to control follicular dynamics, luteal regression, and ovulation. By synchronizing ovulation, cows can be inseminated

by appointment regardless of estrus expression, which facilitates timely insemination of cows eligible for pregnancy. In addition, more sophisticated synchronization of ovulation protocols can also be used to increase the likelihood of conception to TAI (Moreira et al., 2001; Souza et al., 2008; Giordano et al., 2012b).

As the average herd size continues to rise (Von Keyserlingk et al., 2013; Barkema et al., 2015), along with a growing interest to improve animal welfare (Barkema et al., 2015), implementation of both traditional and novel reproductive management practices has become increasingly more challenging. Indeed, current methods to determine cow reproductive status require the expertise of trained technicians or veterinarians that are often unavailable for farms, and if available may not be affordable for some operations. Similarly, a major barrier for the adoption of TAI is that many farms lack the personnel or facilities to properly implement complex TAI protocols that may require up to 7 injections per cow. In addition, because cows need to be restrained multiple times for reproductive status determination, and to receive hormonal treatments, implementation of currently available methods for reproductive status determination and synchronization protocols for submission to AI can negatively affect animal well-being and disrupt cow natural behaviors (e.g., feeding and resting when desired).

Therefore, innovative technologies to increase reproductive efficiency while optimizing labor resources and animal welfare might help address many of the current challenges associated with the implementation of common reproductive management practices. This chapter aims to describe current knowledge about the development and potential incorporation of such technologies in reproductive management of cattle operations.

2. Determination of functional corpus luteum presence as a tool to monitor reproductive status of cattle

Although several methods are available to determine the reproductive status of cows, this can be either fully or partially accomplished by determining the presence or absence of a functional CL on the ovaries based on palpation, visualization, or determination of circulating concentrations of P4.

The CL is a transient endocrine gland that forms in the ovary after ovulation (i.e., day 0 of the estrous cycle) from cells that previously constituted an ovarian follicle. One of the main functions of the CL is to produce the steroid hormone P4, which not only regulates gonadotropin secretion during the estrous cycle, but also has a vital role in the establishment and maintenance of pregnancy in most mammalian species, including ruminants (Bazer et al., 2009). Once formed, the CL grows gradually during the first half of the estrous cycle, reaching a plateau (~4.5 cm^2) by day 8 after ovulation (Sartori et al., 2004; Herzog et al., 2010). Similarly, P4 production and secretion by the CL increases gradually and achieves maximum systemic levels around day 12 to 16 after ovulation (Henricks et al., 1970; Sartori et al., 2004). Once in circulation, P4 is metabolized by the liver (Parr et al., 1993) and excreted in urine and feces. Interestingly, lactating cows appear to have a higher rate of P4 metabolism than nulliparous heifers, presumably due to increased liver blood flow arising from increased feed intake during lactation (Vasconcelos et al., 2003; Wiltbank et al., 2006; Wiltbank et al., 2014). Consequently, maximum P4 levels during the estrous cycle tend to be lesser for lactating cows than for heifers (5 and 7 ng/mL for cows and heifers, respectively; Sartori et al., 2004).

In the absence of pregnancy, luteal regression is triggered ~16 to 19 days after ovulation by PGF produced by the endometrium (McCracken et al., 1999). Typically, luteal regression

comprises 2 distinct processes; (1) a rapid decline in P4 synthesis and secretion resulting in a reduction in circulating concentrations to less than 1 ng/mL within 24 to 48 hours [functional luteolysis; (Mann and Lamming, 2006; Ginther et al., 2009; Ginther et al., 2010)], and (2) physical disappearance of functional luteal tissue from the ovary [structural luteolysis; (Niswender et al., 2000)]. After completion of luteolysis, a series of well-orchestrated hormone-release and ovarian follicle growth patterns culminate with estrus expression, ovulation, and the formation of a new CL. Therefore, during the bovine estrous cycle, periods of functional CL presence and absence alternate because of the cyclic formation and lysis of the CL. Conversely, when pregnancy is successfully established, the events that trigger luteolysis are abolished, allowing the CL to continue producing P4 until a few days before parturition.

Because of these distinct patterns of secretion, circulating P4 can be used as a marker for the presence or absence of a functional CL and therefore as a tool to monitor reproductive status of cows. In this regard, 1 ng/mL is a commonly used P4 cutoff to estimate the presence of a putative functional CL using bovine serum or plasma (Ginther et al., 2010; Stevenson, 2019). However, a more stringent cutoff of 0.5 ng/mL is also commonly used because P4 levels above this threshold at the time of TAI have been associated with negative reproductive outcomes in lactating dairy cows (Bisinotto et al., 2010; Giordano et al., 2013). Moreover, P4 can also be detected and measured in cow milk because P4 levels parallel those of blood, albeit slightly more concentrated (Heap et al., 1973; Hoffmann et al., 1976).

As nonpregnant cows have different P4 profiles than pregnant cows (Giordano et al., 2012a), a potential use of P4 testing in reproductive management is the identification of nonpregnant cows post insemination. More specifically, testing could be implemented after AI at the time nonpregnant cows are expected to have low P4 before they return to estrus and pregnant

cows are expected to have elevated circulating P4 concentrations (~18 to 24 d after a previous AI). In this regard, a myriad of research studies have shown that determination of plasma or milk P4 concentrations at 21 to 24 days after AI can be 91 to 100% accurate in identifying nonpregnant animals (Wishart, 1975; Heap et al., 1976; Pope et al., 1976; Pennington et al., 1985). More recently, Wilsdorf et al. (2016) reported that ~20% of nonpregnant cows could be identified as early as 7 d after AI with a false positive rate of <5%. From a management standpoint, the main advantage of using P4 testing for early nonpregnancy diagnosis is that it allows earlier reinsemination of nonpregnant animals, which may improve reproductive and economic performance of the herd (Giordano et al., 2013). A caveat of P4 testing for determination of pregnancy status of cows is that low P4 18 to 24 d post insemination is an accurate indication of nonpregnancy but elevated P4 concentration is not an accurate indicator of pregnancy (Nebel, 1988). This is because some nonpregnant cows may continue to have P4 concentrations above the cutoff used to determine the presence of a functional CL beyond the time of expected CL regression after insemination (Giordano et al., 2012a; Wijma et al., 2016b; Ricci et al., 2017). As cows can be incorrectly classified as pregnant when they are not, the use of P4 testing at 18 to 24 days post insemination is useful for the identification of nonpregnant rather than pregnant cows.

Monitoring functional CL presence can also serve as a practical decision-making tool for the assignment of cows to resynchronization strategies at the time of nonpregnancy diagnosis (**NPD**). Reproductive management programs for second and greater AI typically consist of a period of reinsemination to spontaneous estrus (**AIE**) followed by implementation of TAI protocols initiated at the time of or 7 d prior NPD for cows not AIE. Traditionally, all non-pregnant cows were submitted to a single TAI protocol regardless of their ovarian physiological

status. In contrast, recently developed reproductive management strategies for non-pregnant cows include assignment of cows to hormonal treatments tailored to subgroups of cows with the same ovarian status at the time of NPD (McArt et al., 2010; Giordano et al., 2015; Wijma et al., 2018; Masello et al., 2020). Most commonly, these new strategies consist of assignment of cows to different hormonal treatments based on the presence or absence of a CL as well as presence and size of ovarian follicles. For instance, some resynchronization programs are designed to promote AIE after NPD through a PGF treatment to promote estrus expression only to cows with functional CL present. Conversely, cows not expected to respond to PGF because they lack a functional CL are assigned to protocols designed to maximize P/AI of these cows (Giordano et al., 2015; Masello et al., 2020). Other strategies take advantage of the normal ovarian dynamics of cows after a previous insemination to assign a subgroup of cows to treatments that shorten the re-insemination interval and other cows to treatments that optimize fertility of TAI services (Wijma et al., 2017; Wijma et al., 2018).

Other potential uses of P4 testing in cattle include the determination of the pubertal status of beef (Imwalle et al., 1998; Cooke and Arthington, 2009) and dairy (Lammers et al., 1999; Rius et al., 2005) heifers, confirm luteal regression and ovulation in synchronization of ovulation protocols (Barletta et al., 2018), determination of resumption of postpartum ovarian cyclicity in beef (Peters and Riley, 1982; Wheaton and Lamb, 2007) and dairy (Galvão et al., 2010; Stangaferro et al., 2018) cows, and confirmation of estrus (Friggens et al., 2008).

In summary, methods for accurate determination of CL presence and functionality are valuable for decision-making in dairy and beef cattle production. In both types of operations, consistent and proactive use of such methods would enable effective monitoring of reproductive status to apply suitable reproductive interventions that maximize reproductive performance of

the herd or simplify management. Ideally, such methods should be inexpensive and easy to conduct under field conditions so they can be easily incorporated into reproductive management programs.

3. Current methods for determination of a functional corpus luteum

Although P4 quantification is the gold standard to evaluate luteal function, accurate detection and quantification of P4 in bodily fluids of cattle is not easily accomplished on-farm. Most methods available typically require shipment of samples to off-farm facilities for analysis with laboratory assays such as radioimmunoassay (**RIA**; Gowan and Etches, 1979) or enzyme-linked immunosorbent assay (**ELISA**; Arnstadt and Cleere, 1981). Unfortunately, for most commercial farms, shipment of milk or blood samples is not practical or cost-effective. In addition, the time interval between sample collection and availability of results (usually days) impedes the rapid execution of management interventions to optimize reproductive management and performance.

To circumvent the use of off-farm laboratory assays, novel technologies have been recently developed for on-farm P4 quantification. For instance, a fully automated real-time P4 analyzer (Herd-Navigator, DeLaval International, Tumba, Sweden) has recently become available for commercial farms. Briefly, an automatic in line sampler collects a representative milk sample from cows of interest during a milking session. Samples are then quantitatively assessed for P4 using a lateral-flow immunoassay technique (Yu and Maeda, 2017). Thereafter, the system generates individual P4 profiles and assigns cows into one of three categories: nonpregnant non-cyclic, nonpregnant cyclic, or potentially pregnant (Yu and Maeda, 2017). In addition, for cyclic cows an estrus alert can be generated using advanced proprietary algorithms that detect when P4 declines below a pre-defined threshold. Using this automated system, Bruinjé and Ambrose

(2019) reported high sensitivity (>95%), specificity (>94%), and overall accuracy (>90%) for diagnosing pregnancy between 41 and 54 days after AI. Despite these encouraging results and the potential benefits of this system for many dairy operations, this type of technology may not be affordable for the average farm. Moreover, because this system relies on milk samples, it may not be a viable option for herds with non-lactating animals such as beef or nulliparous dairy heifers.

Because of the difficulties associated with on-farm quantification of P4, diagnosis of functional CL presence under field conditions has traditionally been performed by palpation of the ovaries per rectum (transrectal palpation; **TP**) and transrectal ultrasonography (**TUS**). The main advantage of these methods is that they provide immediate cow-side results, enabling prompt interventions. However, because ovarian structures can be directly visualized rather than palpated, TUS presents advantages over TP. For example, TUS can more accurately estimate the size and number of corpora lutea as well as certain CL features (e.g., presence of a cavity) that are not as easily detectable through TP (Whitfield, 2018). Nonetheless, a disadvantage of both TP and TUS is their limited ability to predict CL functionality. Indeed, numerous studies have shown that when using a dichotomized P4 concentration value as the reference method (e.g., functional CL; P4 ≥ 1 ng/mL, nonfunctional CL; P4 < 1 ng/mL), the overall accuracy for diagnosing a functional CL was in the range of 57 to 77% for TP (Mortimer et al., 1983; Pathiraja et al., 1986; Bicalho et al., 2008) and 75 to 78% for TUS (Bicalho et al., 2008; Sauls-Hiesterman et al., 2020). In most cases, the main reason for low accuracy was low specificity (i.e., true negative rate), which ranged from 38 to 70% (Mortimer et al., 1983; Bicalho et al., 2008; Sauls-Hiesterman et al., 2020). At least in part, the poor ability to correctly identify true negatives can be explained by the asynchrony between functional and structural luteolysis

(Herzog et al., 2010). More specifically, cessation of P4 production and secretion by the CL typically occurs earlier than the physical disappearance of the CL from the ovary (Herzog et al., 2010). For this reason, in some cows a CL may be visualized by TUS or palpated by TP but is no longer functional. As a result, some cows may be inaccurately classified in groups of CL functionality (i.e., functional vs not functional). To overcome this issue, CL diameter thresholds have been used to estimate the functionality of the CL (Bicalho et al., 2008; McArt et al., 2010; Giordano et al., 2015). For example, Bicalho et al. (2008) reported that using a CL diameter cutoff of >22 mm improved specificity (from 38 to 83%) and overall accuracy (from 78 to 85%) relative to strict visualization of luteal tissue. Similarly, others have shown substantial inter-rater agreement between TUS and P4 concentration when a CL diameter cutoff of >20 mm was used to define functionality of the visualized CL (Giordano et al., 2015).

Although estimation of CL size by TUS is a reasonable tool to determine functional CL presence, a caveat for on-farm implementation is the need for trained technicians or veterinarians to conduct the examination of the ovaries. This may be problematic for farms that cannot afford routine visits from veterinarians or the purchase of ultrasound equipment. In addition, shortage of veterinary services is a well-documented issue in numerous regions of the US that precludes effective incorporation of these methods into reproductive management of many farms. Indeed, the National Institute of Food and Agriculture (USDA, 2020) has reported that 37 out of 50 states in the US have at least one area where veterinary services are limited or inexistent. Therefore, inexpensive technologies that sidestep the need for qualified personnel for determination of CL presence on-farm may be beneficial to some cattle operations.

4. Lateral Flow Immunoassays

4.1. Potential advantages relative to current methods

Lateral flow immunoassay (**LFIA**) test strips are point-of-care diagnostic tools developed in the 1980s. These tools were originally designed to diagnose human pregnancy through the qualitative assessment of human chorionic gonadotropin (hCG) in urine (Valanis and Perlman, 1982). Thereafter, LFIA have gained widespread use in other applications such as human clinical diagnostics, veterinary diagnostics, and food safety (Tisone and O'Farrell, 2009). In part, their widespread adoption can be explained by their low-cost and ease of implementation under resource-poor or non-laboratory settings (Posthuma-Trumpie et al., 2009; Sajid et al., 2015). Indeed, LFIA can be performed without the need for trained personnel or expensive equipment, do not require sample shipment and processing, and results are obtained faster than by laboratory assays such as RIA or ELISA. In addition, LFIA systems offer extensive versatility as multiple biomatrices can be used for testing. Analytes can be quantified in urine, milk, plasma, whole blood, among others (Posthuma-Trumpie et al., 2009). Moreover, albeit dependent on the type and format, LFIA may also offer other advantages including no need for sample pre-processing, small sample volume, and prolonged test strip shelf life under numerous environmental conditions (Sajid et al., 2015).

These attributes make LFIA diagnostics attractive for on-farm testing, particularly for farms located in areas where transporting samples to specialized laboratories could be expensive and time-consuming, and where veterinary support might be difficult to obtain. Moreover, since implementation of this type of technology requires a relatively small investment, it can be easily adopted by farms, which are often financially limited to capitalize on new technologies. In addition, because these tests can accommodate different sample biomatrices, sampling method (i.e., type of bodily fluid collected for analysis) can be selected based on the cow's physiological

status (e.g., milk for lactating and whole blood for nonlactating animals) or according to the resources available for sample collection.

Thus, a LFIA design capable of accurately determining the presence of a functional CL in cow bodily fluids has the potential to circumvent many of the limitations associated with the current implementation of traditional methods in both dairy and beef cattle operations. Attempts to develop such test have been previously reported and will be discussed in detail later in this chapter.

4.2. Structure, function, and considerations for LFIA development

A typical LFIA comprises a prefabricated, disposable test strip containing in dry form all necessary reagents for the detection of a given analyte. Briefly, a liquid sample is added to one end of the test strip which then migrates by capillary action along a fibrous membrane. Next, analytes in the sample react with specific areas of the test strip embedded with pre-dispensed reagents. This interaction results in the formation of immunocomplexes visualized as colored lines on the strip. The quantity of a specific analyte can then be estimated based on the signal intensity of the developed lines.

Materials commonly used to assemble LFIA test strips include; (1) a sample pad, typically made of cellulose fiber (2) a glass fiber conjugate pad that stores labelled anti-analyte antibody (i.e., primary antibody), (3) a nitrocellulose membrane card with printed test and control lines, and (4) a cellulose fiber absorbent pad at the end of the strip to capture any waste from the assay and to prevent backflow of the sample (Posthuma-Trumpie et al., 2009; Sajid et al., 2015). For

proper function of the assay, nitrocellulose membrane and pads need to overlap to ensure continuous sample flow across the strip.

Numerous formats have been described for LFIA (Sajid et al., 2015). The selection of a suitable format typically depends on the features of the analyte of interest. When the target analyte is of low molecular weight (i.e., hapten), such as P4 (~314 Kda), the format is restricted to the competitive design (Posthuma-Trumpie et al., 2009). This is because other formats, such as the "sandwich" format, are not feasible for small analytes that cannot bind to two antibodies simultaneously. In a typical competitive LFIA layout, an antigen-protein conjugate (same antigen as the target analyte) is immobilized at the test line, whereas a secondary antibody (i.e., antibody against the primary antibody) is immobilized at the control line. Once applied onto the sample pad, the sample migrates to the conjugate pad where the analyte binds to the labelled anti-analyte antibody stored in the pad. This interaction will determine the availability of labelled antibody to bind with the antigen-protein conjugate at the test line. Therefore, free analyte in the sample and immobilized analyte on the test strip "compete" for a limited number of antibody binding sites. Any excess of labelled antibody will continue to flow and will be captured by the secondary antibody immobilized at the control line.

Thus, for samples with high concentration of target analyte most of the labelled antibody binding sites will be already occupied when it reaches the test line, resulting in the development of a weak colorimetric signal. In contrast, such samples will show a strong control line signal, indicating that most labelled antibody was not captured by the test line but was captured by the secondary antibody. Conversely, samples with low analyte levels will develop an intense colorimetric signal at the test line (due to large amount of labelled antibody with free binding sites reaching the test line) and consequently a weak signal at the control line. The intensity of

these lines can be estimated subjectively based on visual observation (Waldmann and Raud, 2016; Nakami et al., 2017) or objectively with a scanner and imaging software (Laitinen and Vuento, 1996; Posthuma-Trumpie et al., 2008; Samsonova et al., 2015) or a specialized LFIA reader device (Lu et al., 2017; Srinivasan et al., 2018). With the latter methods, a calibration curve may be constructed to provide semiquantitative or quantitative results (Samsonova et al., 2015; Lu et al., 2017; Srinivasan et al., 2018).

Selecting a suitable nitrocellulose membrane is a critical step in the development of a competitive LFIA. Commercially available nitrocellulose membranes often differ in pore size (Millipore, 2013), which can directly affect the capillary flow rate of a liquid across the test strip (Posthuma-Trumpie et al., 2009). This is relevant because LFIA are dynamic systems; the formation of immunocomplexes at the test and control lines is contingent to the period of time reagents are in close proximity, which is ultimately defined by the capillary flow rate (Millipore, 2013). Therefore, if the aim is to maximize the analytical sensitivity of the assay (i.e., lowest concentration that can be detected; Dohoo et al., 2014), then the membrane with the slowest capillary rate should be selected. Conversely, if the main goal is to provide fast results regardless of analytical sensitivity, then a membrane with faster capillary flow may be more adequate.

Another important factor to consider when developing LFIA is the selection of a reporter label. For decades, the most commonly used label for LFIA has been colloidal gold nanoparticles (Posthuma-Trumpie et al., 2009; Sajid et al., 2015). The main advantage of this type of label is that it generates a direct colorimetric signal; no reader or special equipment are needed to visualize the test and control lines. However, analytical sensitivity can significantly be improved when brighter labels that do require a specialized reader, such as fluorescent dyes, are used as reporters (Gong et al., 2017). In addition, fluorescent dyes may also offer advantages over other

common LFIA labels, such as horseradish peroxidase (**HRP**), which requires post-assay staining and therefore adds an extra step and complexity to the process (Samsonova et al., 2015). Thus, selection of a reporter label should be made in light of the desired analytical sensitivity, feasibility of using a reader to obtain results, and overall complexity of the assay.

4.3. Progesterone lateral flow immunoassays

The first attempt to develop a competitive LFIA for the quantification of P4 was done by Laitinen and Vuento (1996). Their LFIA design comprised a monoclonal anti-P4 antibody immobilized at the "detection zone" (i.e., test line), and a P4-protein conjugate labelled with gold nanoparticles in the "label loading area" (equivalent to the conjugate pad). Therefore, in this competitive layout, free P4 in the sample (milk) and labelled P4-protein conjugate competed for binding sites on the antibody at the test line (note that this competitive layout is slightly different to that described in section 4.2 of this chapter). Using RIA as the reference method, the authors concluded that the detection limit for the developed LFIA was 5 ng/mL if results were visually examined. However, the limit of detection was significantly improved (2 ng/mL) when a chromatography scanner was used to objectively interpret and quantify the results.

Posthuma-Trumpie et al. (2008) developed a P4-LFIA with a different competitive layout using a primary antibody along with a secondary antibody labelled with colloidal carbon. In this design, a P4-protein conjugate was immobilized at the test line. Similar to Laitinen and Vuento (1996), a digital scanner and software were used to interpret and quantify the results. The authors reported that the highest analytical sensitivity (0.6 ng/mL) was achieved when using a high concentration of the P4-protein conjugate coupled with a high dilution of the antibody. Although

these results were encouraging, the assay was developed using spiked buffer samples and did not work when using other biomatrices such as milk samples.

More recently, Samsonova et al. (2015) developed a competitive LFIA capable of detecting as low as 0.8 ng/mL of P4 in whole milk samples. The assay comprised P4 labeled with HRP at the sample pad (conjugate pad was not included in this design), polyclonal anti-P4 antibody at the test line, and anti-HRP antibody at the control line. To run the assay, a mixture of labelled P4-protein and sample were first added onto the sample pad. Ten minutes later, test strips were placed into tubes containing 2 mL of substrate solution for 5 minutes to generate blue-colored test and control lines. After staining, test strips were dried for an additional 15 minutes at room temperature. Once dried, strips were scanned, and the intensity of the lines was measured using an imaging software. Quantitative results were obtained by constructing a calibration curve using milk samples with known concentrations of P4. The authors reported overall good correlation ($r = 0.97$; $n = 46$) between the LFIA and the reference method (ELISA).

Collectively, data from these and other (Sananikone et al., 2004; Safronova et al., 2012) studies suggest that LFIAs coupled with a scanner to display and objectively quantify results may be a viable option to assess P4 concentrations in bovine samples. Unfortunately, most of the commercially available LFIA systems for P4 do not include a portable scanner or reader. Instead, a color chart for comparison is usually included so that strips can be classified into different P4 scores (i.e., low, medium and high) based on the apparent color intensity of the test line (Nakami et al., 2017). Therefore, on-farm implementation of LFIA currently depends on the subjective interpretation of assay outcome by the user, which, in turn, may result in an inaccurate diagnosis. Indeed, recent studies evaluating commercially available P4-LFIA for use in milk (Waldmann

and Raud, 2016) or whole blood (Nakami et al., 2017) have reported human error in the interpretation of the results, particularity for samples with medium concentrations of P4.

Although previous research has demonstrated the feasibility of quantifying P4 with LFIA, to the best of my knowledge, none of the published reports to date have evaluated the ability of LFIAs to predict the presence (P4 ≥ 1 ng/mL) or absence (P4 < 1 ng/mL) of a functional CL, which may have more value for on-farm decision-making. Hence, further research is required to develop and validate easy-to-use LFIA systems capable of accurately determining the presence of a functional CL and that incorporate a portable outcome reader to facilitate future adoption by cattle operations.

5. Automated hormone delivery for implementation of timed AI protocols

5.1. Potential of automated hormone delivery in cattle operations

Since the advent of Ovsynch in the 1990s, synchronization of ovulation protocols for TAI have been widely adopted by both beef (Lamb et al., 2010) and dairy (NAHMS, 2018) cattle operations. In part, this is because TAI protocols offer the unique advantage of enabling insemination of cows by appointment regardless of estrus expression while achieving similar or greater fertility than by AI at detected estrus (Pursley et al., 1997; Santos et al., 2017). However, a major caveat of implementing TAI programs is the need to administer multiple hormonal treatments, usually in the form of intramuscular injections. Hence, implementation of TAI typically requires significant human intervention and cow handling, which not only represents a cost burden for many farms but also can negatively affect cow time budgets and well-being.

Over the past few decades, the average size of dairy herds has steadily increased in the US and around the world (Von Keyserlingk et al., 2013; Barkema et al., 2015). Concomitantly, the availability of qualified labor for dairy farms has decreased significantly (Mottram, 2016). These two factors combined with the increased interest in reducing disruption of cow time budgets (Barkema et al., 2015; Ventura et al., 2016) has resulted in the development of novel technologies for automation of farm activities such as milking, estrus detection, health monitoring, and feeding management (Sørensen et al., 2016; Stangaferro et al., 2016; Jorgensen et al., 2017; Reith and Hoy, 2018). Nevertheless, administration of reproductive hormones for TAI continues to be an intensive and time-consuming task because technologies that automate hormone delivery have not yet been developed. This is particularly relevant as labor constraints and animal handling issues are exacerbated and synchronization of ovulation protocols for TAI continue to evolve to include numerous, inconveniently timed treatments in an effort to maximize fertility (Souza et al., 2008; Bartolome et al., 2009; Santos et al., 2017). Thus, a potential strategy to reduce the burden of implementing synchronization of ovulation protocols is to develop an all-encompassing automated delivery device for releasing all hormones of interest in the sequence, pattern, and dose required to synchronize ovulation. Such device must be placed in an easily accessible body part or cavity to facilitate implementation and re-use. In this regard, the vaginal route offers unique benefits and will be described in more detail later in this chapter.

Automation of hormone delivery has the potential to positively affect herd reproductive management in numerous ways. For example, it may reduce labor needs for synchronization of ovulation. Automating synchronization of ovulation may allow farms with major labor constraints to implement TAI protocols whereas farms with access to sufficient labor resources may have the opportunity to re-allocate labor resources to other tasks (e.g., health monitoring,

feeding, business decision-making). This is particularly relevant for large herds, for which handling cows for synchronization of ovulation may require the time and effort of several workers at a time. Moreover, it may also be beneficial for smaller farms that often lack adequate facilities, cannot afford enough qualified personnel, or existing personnel have limited time to administer hormonal treatments on a regular basis. Thus, automation of hormone delivery has the potential to optimize the use of labor resources by reducing the number of people required to synchronize ovulation, which may enhance productivity and profitability of farms.

Another potential benefit of automating hormone delivery is the improvement of animal handling, well-being, and performance by optimizing cow time budgets and reducing human interventions. In most herds, cows receive hormonal treatments while restrained in self-locking head-gates or in palpation rails (NAHMS, 2018), both of which may limit the ability of cows to rest and feed when desired. Indeed, it has been well-documented that cows may present disrupted natural behaviors after being restrained for prolonged periods of time (Bolinger et al., 1997; Cooper et al., 2008). Hence, by reducing the number of times cows need to be restrained to receive treatments, automated hormone delivery has the potential to improve animal well-being and the public perception of animal care on cattle operations. Indeed, while up to 7 animal-handling events are typically required with the injection methods presently used, only two cow handlings would be necessary to synchronize ovulation with an automated delivery device (i.e., one to insert and one to remove the device).

Finally, automated hormone administration may also enhance protocol compliance by facilitating timely and accurate delivery of hormonal treatments for synchronization of ovulation for TAI. This is relevant considering that human and technical errors are one of the main reasons for impaired protocol efficacy (Stevenson and Phatak, 2005; Galvão et al., 2013). Furthermore,

automation may enable the unrestrained delivery of hormone doses with varying frequencies and target volumes that can better mimic cow physiology and hormone patterns. Ultimately, automation may lead to the development of novel synchronization protocols to maximize fertility after TAI.

5.2. The vagina as an alternative route for hormone delivery in cattle

5.2.1 Vaginal anatomy and physiology in bovine females

The vagina is a fibromuscular tubular organ that connects the uterus to the external genitalia of mammalian females, and its primary function is to serve as a copulatory organ and as the birth canal during parturition (Senger, 2003). It rests horizontally and in a collapsed state inside the pelvic cavity proximal to the urinary bladder (Dyce et al., 2009). In adult bovine females, the length of the vagina typically varies from 25 to 30 cm, however, it is capable of great expansion in both length and diameter (Dyce et al., 2009). The main blood supply to the vagina originates from the vaginal artery (branch of the internal iliac artery) and from the uterine artery. Its venous drainage, on the other hand, is through a rich venous plexus that drains into the vaginal vein and the accessory vaginal vein, both of which eventually drain into the internal iliac vein (Dyce et al., 2009).

The vaginal wall comprises 4 distinct concentric layers (Senger, 2003): (1) serosa (outer), (2) muscularis; double layer of smooth muscle, (3) submucosa; houses blood vessels, nerves and lymphatics, and (4) mucosa (inner); secretory layer of epithelium. The histology of the mucosal epithelium differs depending on the vaginal region (i.e., cranial vs. caudal) and across different stages of the estrous cycle. Near the cervix (cranial region), the vaginal epithelium is usually

columnar and capable of secreting large quantities of mucus, whereas in the vestibule (caudal region) the vaginal epithelium is stratified and less secretory in nature (Senger, 2003).

Interestingly, the histological structure of the cranial vagina is the one that varies most during the estrous cycle. In this regard, Cole (1930) and Miroud and Noakes (1991) described in detail the cyclic histological changes of the cranial vaginal mucosa by inspecting vaginas from slaughtered animals (Cole, 1930) or by performing sequential vaginal biopsies (Miroud and Noakes, 1991). During the luteal phase, the vaginal epithelium comprised numerous (2 to 4) layers of low columnar flattened cells with limited mucus-secretion capacity. On the other hand, during estrus, the epithelium was reduced to a single layer of tall columnar, very active, mucus-secreting cells. Despite comprising a single layer, the large size of these cells conferred the epithelium with substantial thickness. Because of the increased secretory capacity of the vaginal epithelium, bovine estrus is characterized by a copious production and discharge of vaginal mucus, presumed to be essential for sperm transport (Rutllant et al., 2005; Rathbone and Burke, 2013).

Other physical properties of the vaginal fluid such as pH and viscosity may also undergo cyclic changes. Indeed, during estrus, vaginal mucus is typically low in viscosity and slightly acidic (~6.5), whereas during the luteal phase mucus becomes thicker (i.e., greater viscosity) and pH becomes closer to neutrality [~6.9; (Roark and Herman, 1950; Schilling and Zust, 1968)].

5.2.2. Considerations for intravaginal delivery and device design

Among the different body cavities available for hormone delivery in cattle, the vagina offers unique benefits. For example, hormone delivery systems can be easily and quickly

introduced and removed and can remain in the vagina for prolonged periods (weeks). In addition, a rich blood supply and high permeability to a wide range of molecules facilitates transfer of hormones to the blood circulation for distribution to target tissues (Richardson and Illum, 1992; Hussain and Ahsan, 2005; Rathbone and Burke, 2013). Despite these benefits, factors inherent to the vaginal environment, physicochemical properties of the hormone of interest, and nature of the delivery system itself may negatively affect hormone delivery, and thereby deserve special consideration when designing intravaginal (**IVG**) devices. For example, a key aspect for the design of IVG delivery systems is to understand the process of vaginal hormone absorption and the potential effect of the multiple factors that may affect absorption. This is relevant because eliciting the desired biological response of reproductive hormones depends upon adequate and efficient absorption into the systemic circulation.

Once the hormone of interest is successfully released from the delivery system, hormone transport across the vaginal wall may occur via 3 different routes: (1) transcellular; concentration-driven diffusion through the lipid continuum of cell membranes, (2) intercellular; diffusion between cells via tight junctions, and (3) receptor-mediated transport (Richardson and Illum, 1992). The type and feasibility of transport across the vaginal epithelium for a given hormone is highly contingent on the lipophilicity and hydrophilicity of the molecule. Using rabbits, Corbo et al. (1990) showed that permeability of the vaginal mucosa decreased as hydrophilicity of progestins increased. In addition, permeability to P4, a lipophilic molecule, was greater than that for mannitol, a highly hydrophilic compound. Molecular weight also seems to affect absorption, since smaller molecules are more readily absorbed than larger ones (Hussain and Ahsan, 2005). Therefore, it appears that small, lipophilic molecules are more easily absorbed than larger, hydrophilic molecules. Although lipophilicity of hormones intended for IVG

delivery is important for transport across the vaginal mucosa, some level of hydrophilicity may also be important to facilitate diffusion across the vaginal fluid (highly aqueous in nature) in order to reach the vaginal wall for absorption (Woolfson et al., 2000; Hussain and Ahsan, 2005).

Physiological factors inherent to the vaginal environment, such as the amount and viscosity of vaginal fluid, also play a pivotal role in absorption of molecules. For instance, the presence of exceedingly viscous fluid may obstruct vaginal absorption, whereas copious amounts of mucus, such as those observed during estrus, may result in loss of dosage via vaginal discharges (Richardson and Illum, 1992; Hussain and Ahsan, 2005). In addition, as many hormones are weak electrolytes, changes in vaginal pH may also alter absorption by modifying their degree of ionization (Richardson and Illum, 1992; Hussain and Ahsan, 2005).

The nature of the delivery system employed also plays an important role in accomplishing successful IVG delivery in farm animals. In this regard, Rathbone et al. (1997) and Rathbone et al. (2000) provide an overview of important considerations for the development of IVG delivery systems. For example, size (i.e., length and width) needs to be minimized to ensure ease of device insertion and removal, avoid unnecessary irritation of the vaginal mucosa, and to reduce the risk of device expulsion by the animal. In addition, IVG devices typically require a specialized retention mechanism (usually in the form of "wings") that enables high retention rates (at least 95%) while exerting minimal pressure on the vaginal wall. Finally, it is important to consider that the use of medical grade silicone rubber or other "skin-safe" material may reduce the amount of copious "cloudy" discharge typically observed as an irritation response when inserting extraneous devices into the vagina of livestock.

5.2.3. Previous efforts to develop an automated intravaginal hormone delivery device

Intravaginal devices have been used in cattle since the early 1970s when the first IVG inserts for sustained P4 release were developed and commercialized. In general, these inserts consist of a silicone matrix impregnated with P4, allowing gradual and constant release of P4 into the vaginal cavity through passive diffusion. Although this type of technology has been widely adopted by farmers and is regularly used in TAI protocols, administration of other hormones of interest (e.g., PGF, GnRH, FSH, LH, eCG, estradiol) would require controlled release of fixed amounts rather than sustained, uncontrolled release of variable amounts of hormone. This is because successful synchronization requires the release of treatments at pre-defined time intervals at a rate and amount that elicits the desired physiological effect (e.g., ovulation for GnRH and luteal regression for PGF).

Thus, an all-encompassing automated IVG device that enables precise control of the amount, rate, and time of hormone release would facilitate automation of synchronization of ovulation in cattle. In addition, such system should incorporate multiple compartments or reservoirs, so that various hormones can be delivered from a single device. In this regard, the Intelligent Breeding Device (**IBD**) developed and commercialized in New Zealand in the late 1990s satisfied most of these requirements (Rathbone et al., 1997; Abdullah, 2000; Rathbone et al., 2001; Ismail, 2007). This electronically-controlled device comprised an outer plastic housing, four hormone reservoirs [one large reservoir (~5 mL) at the base of the device and three smaller reservoirs (~0.5 mL each) at the head of the device], a printed circuit board, a battery, and an 'umbrella' retention mechanism. The system was designed to allow continuous and uniform P4 release over a 10-d period, along with pulsatile or single doses of either PGF or estradiol. The timing and amount of delivery were controlled by the pre-programmed circuit board, which in

turn, controlled a switch mechanism operated by a solenoid. In the "on" setting, each hormone was released from the reservoir via positive pressure generated by a spring and plunger. Although the IBD device included most of the desired features for an automated IVG delivery system, issues with retention rates and in vivo delivery (Rathbone et al., 1997; Abdullah, 2000) precluded further commercialization of the product and is therefore no longer available for cattle producers.

More recently, two studies from the same laboratory (Cross et al., 2004; Künnemeyer et al., 2004) reported an automated controlled-release IVG device similar to the IBD. This design, however, included an additional feature; an antenna that allowed two-way wireless communication with external sources. This feature enabled the user to perform remote control and monitoring of hormone delivery. Controlled release was accomplished using gas-cell technology. Briefly, a pre-programmed microcontroller induced gas production by the cell, which in turn propelled a syringe piston that released the hormone out of the reservoir. Moreover, the device was equipped with temperature and activity sensors that enabled real-time estrus expression monitoring. Despite these attractive features, the authors reported a reduction in dosage accuracy and failures with wireless communication when testing the devices in vivo. In addition, devices included a single hormone reservoir (~40 mL), which would have precluded its use for synchronization of ovulation.

Thus, although previous efforts were made to develop electronically controlled IVG hormone delivery devices, design and functionality limitations precluded their widespread use. Due to the relevance of TAI for cattle operations and the current constrains of TAI program implementation, further research is required to develop and validate effective IVG devices capable of automatically delivering multiple hormonal treatments at different rates and amounts.

Such devices would enable automation of synchronization of ovulation which, in turn, has the potential to simplify herd management and improve animal well-being relative to the needle-injection methods presently used.

5.2.4. Intravaginal administration of prostaglandin-F2α

Successful automation of synchronization of ovulation through an IVG delivery device is only feasible if hormonal treatments can elicit the desired physiological response when given intravaginally. In this regard, although sustained release of P4 through non-automated IVG devices has been extensively studied and is frequently used in TAI protocols (Macmillan and Peterson, 1993; Chebel et al., 2006), data for the feasibility of the IVG route for other hormones of interest such as PGF have been sparse.

Prostaglandin-F2α is a lipid signaling molecule synthesized by the uterus and other tissues (Harizi et al., 2008) with the primary function of triggering luteal regression in cattle and other ruminants. The main precursor of uterine PGF is arachidonic acid, a derivative of the hydrolysis of membrane phospholipids by the action of phospholipase A (Coffman and Pinto, 2016). Arachidonic acid is then converted to PGF by the action of cyclooxygenase and PGF synthase enzymes (Goff, 2004; Coffman and Pinto, 2016). Once in circulation PGF is rapidly metabolized (< 9 minutes) after one or two passages through the lungs (Davis et al., 1980; Shrestha et al., 2012).

Since the first PGF commercial product became available in 1979, the administration of exogenous PGF to synchronize estrus and ovulation has become widely adopted by both beef and dairy cattle operations. In the US, there are two main PGF analogues currently available;

Dinoprost and Cloprostenol. The chemical structure of Dinoprost is similar to that of the naturally occurring PGF molecule (Moreira and Hammon, 2012; Coffman and Pinto, 2016) and therefore has a half-life of 7 to 8 minutes (Kimball et al., 1976). On the other hand, Cloprostenol has an additional benzyl chlorine ring at position 17 of the PGF molecule structure (De Rensis et al., 2012) which reduces the rate of metabolism and therefore extends half-life in circulation [~3 h (Reeves, 1978; Bourne et al., 1980)]. Moreover, due to this structural modification, Cloprostenol has been demonstrated to have a greater affinity for the PGF receptor (Moreira and Hammon, 2012) and consequently greater potency than Dinoprost. Indeed, while 25 mg of Dinoprost are needed to induce complete luteal regression in cattle, only 0.5 mg of Cloprostenol are required to obtain the same response (Dukes et al., 1974). Despite these differences, it is still unclear whether Cloprostenol improves luteal regression rates in cattle because data available from the few experiments conducted have been inconsistent (Stevenson and Phatak, 2010; Martins et al., 2011; Pursley et al., 2012).

A few studies have been conducted to evaluate the performance of IVG administration of PGF in cattle and most had some major experimental design limitations. For example, Heinonen et al. (1996) conducted an experiment using non-lactating Zebu cattle to compare the efficacy of a low IVG dose (175 μg) versus a standard intramuscular (**IM**) dose (500 μg) of Cloprostenol for induction of estrus expression. Estrus detection was performed for 12 days and cows not detected in estrus received a second PGF dose. Overall, estrus response did not differ between IVG (62.5%) and IM (60.6%) treatments. A limitation of this study was that, because CL presence and P4 concentration dynamics after treatments were not determined, the efficacy of IVG-PGF to induce luteal regression could not be estimated. In addition, the ability to detect significant

differences in estrus expression risk between treatments may have been compromised due to the limited number of cows used in the experiment (~35 per group).

In another randomized controlled experiment conducted by Zdunczyk et al. (1994), the feasibility of IVG-PGF to induce luteal regression was evaluated. Briefly, cows (n = 22) with an active CL (P4 > 2 ng/mL) were randomly assigned to either IVG or IM treatment with 500 μg of Cloprostenol. The authors reported a similar pattern of decline in P4 levels for IM and IVG-treated cows, with both groups having mean P4 below 1 ng/mL by 32 h after treatments. A caveat of this experiment was that P4 profiles were evaluated for a short time interval (36 h). This is relevant because P4 levels at the approximate time points where GnRH and TAI take place during synchronization of ovulation protocols (~56 and 72 h after PGF, respectively) may be of more value to evaluate the efficacy of IVG-PGF.

Using heifers in the diestrus phase (10 to 14 d after estrus) of the estrous cycle, Louis et al. (1973) evaluated P4 profiles up to 72 h after IM or IVG administration of PGF. Interestingly, the decline in blood P4 for heifers treated intravaginally (n = 6) with Dinoprost (30 mg) was slightly delayed relative to that of heifers treated intramuscularly (n = 5) with the same analogue at the same stage of the estrous cycle. These differences, however, were not statistically significant. More recently, Wijma et al. (2016a) conducted a series of experiments to evaluate circulating P4 profiles and to determine the feasibility of inducing complete luteal regression after IVG treatment with PGF. In a first experiment, cows received different doses (25, 50, or 125 mg) and number (1 or 2) of Dinoprost treatments. A positive (25 mg Dinoprost IM) and a negative (saline IVG) control group were also included. Their results demonstrated that delivering two-25 mg doses of Dinoprost 12 h apart was the most effective strategy to induce complete luteal regression in lactating dairy cows. Indeed, cows treated with this regimen had a

P4 profile similar to that of cows treated intramuscularly. Moreover, this regimen was superior to administering a larger dose, or a similar dose given as a single treatment.

Thus, while data from the few experiments conducted (Louis et al., 1973; Zdunczyk et al., 1994; Wijma et al., 2016a) suggested that it may be feasible to induce luteal regression through IVG delivery of PGF, an additional PGF dose seems to be required to obtain similar P4 profiles to that of cows receiving a single IM treatment. A major caveat of all these experiments; however, was the limited number of cows used (~10 per treatment group), which precluded determining differences in luteal regression risk between treatments. Hence, future research should include larger numbers of cows to evaluate the proportion of cows undergoing complete luteal regression (i.e., P4 < 1 ng/mL) and thereby confirm whether IVG delivery could be used as an alternative route of PGF administration in cattle.

6. Summary

This section summarized some of the current challenges associated with the implementation of common reproductive management practices used in beef and dairy operations. In addition, it summarized current research regarding innovative technologies to address current challenges in cattle reproductive management.

As the average herd size continues to rise along with the growing interest to improve animal handling and welfare, implementation of traditional reproductive management practices has become increasingly more challenging. Indeed, current methods to determine cow reproductive status require the expertise of trained technicians or veterinarians that are often unavailable for farms. Similarly, a major challenge for implementation of current TAI protocols is the need to

administer multiple intramuscular injections, which not only requires significant human intervention and cow manipulation but may also disrupt cow natural behaviors. Despite previous efforts, novel technologies to circumvent these issues are limited or unavailable, and if available, they may not be affordable or practical for many cattle operations.

Thus, the main objective of the study presented in Chapter V was to develop a novel portable platform to determine reproductive status of cattle based on circulating concentrations of P4 in plasma. On the other hand, the main goal of experiments presented in Chapters VI and Chapter VII was to develop and validate an electronically controlled IVG delivery device to automate synchronization of ovulation. More specifically, the experiment presented in Chapter VI was designed to test the efficacy of the vaginal route for induction of luteal regression with PGF, and Chapter VII describes the development and validation of a prototype IVG device for automated hormone delivery.

7. References

Abdullah, P. 2000. Synchronization of follicular development, oestrus and ovulation using oestradiol benzoate and progesterone in dairy cattle: a thesis presented in partial fulfillment of the requirements for the degree of Master of Philosophy (Veterinary Clinical Science) at Massey University. Massey University.

Arnstadt, K. and W. Cleere. 1981. Enzyme-immunoassay for determination of progesterone in milk from cows. Reproduction 62:173-180.

Barkema, H. W., M. Von Keyserlingk, J. Kastelic, T. Lam, C. Luby, J.-P. Roy, S. LeBlanc, G. Keefe, and D. Kelton. 2015. Invited review: Changes in the dairy industry affecting dairy cattle health and welfare. J. Dairy Sci. 98:7426-7445.

Barletta, R., P. Carvalho, V. Santos, L. Melo, C. Consentini, A. Netto, and P. Fricke. 2018. Effect of dose and timing of prostaglandin F2α treatments during a Resynch protocol on luteal regression and fertility to timed artificial insemination in lactating Holstein cows. J. Dairy Sci. 101:1730-1736.

Bartolome, J., J. Van Leeuwen, M. Thieme, O. Sa'Filho, P. Melendez, L. Archbald, and W. Thatcher. 2009. Synchronization and resynchronization of inseminations in lactating dairy cows with the CIDR insert and the Ovsynch protocol. Theriogenology 72:869-878.

Bazer, F. W., G. Wu, T. E. Spencer, G. A. Johnson, R. C. Burghardt, and K. Bayless. 2009. Novel pathways for implantation and establishment and maintenance of pregnancy in mammals. MHR: Basic science of reproductive medicine 16:135-152.

Bicalho, R., K. Galvão, C. Guard, and J. Santos. 2008. Optimizing the accuracy of detecting a functional corpus luteum in dairy cows. Theriogenology 70:199-207.

Bisinotto, R., E. Ribeiro, L. Martins, R. Marsola, L. Greco, M. Favoreto, C. Risco, W. Thatcher, and J. Santos. 2010. Effect of interval between induction of ovulation and artificial insemination (AI) and supplemental progesterone for resynchronization on fertility of dairy cows subjected to a 5-d timed AI program. J. Dairy Sci. 93:5798-5808.

Bolinger, D., J. Albright, J. Morrow-Tesch, S. Kenyon, and M. Cunningham. 1997. The effects of restraint using self-locking stanchions on dairy cows in relation to behavior, feed intake, physiological parameters, health, and milk yield. J. Dairy Sci. 80:2411-2417.

Bourne, G., S. Moss, P. Phillips, and B. Shuker. 1980. The metabolic fate of the synthetic prostaglandin cloprostenol ('Estrumate') in the cow: Use of ion cluster techniques to facilitate metabolite identification. Biomed. Mass Spectrom. 7:226-230.

Breukelman, S., Z. Perényi, M. Taverne, H. Jonker, G. Van der Weijden, P. Vos, L. de Ruigh, S. Dieleman, J.-F. Beckers, and O. Szenci. 2012. Characterisation of pregnancy losses after embryo transfer by measuring plasma progesterone and bovine pregnancy-associated glycoprotein-1 concentrations. Vet. J. 194:71-76.

Bruinjé, T. and D. Ambrose. 2019. Validation of an automated in-line milk progesterone analysis system to diagnose pregnancy in dairy cattle. J. Dairy Sci. 102:3615-3621.

Capper, J. L., R. A. Cady, and D. Bauman. 2009. The environmental impact of dairy production: 1944 compared with 2007. J. Anim. Sci. 87:2160-2167.

Chebel, R. C., J. E. Santos, R. L. Cerri, H. M. Rutigliano, and R. Bruno. 2006. Reproduction in dairy cows following progesterone insert presynchronization and resynchronization protocols. J. Dairy Sci. 89:4205-4219.

Coffman, E. A. and C. R. Pinto. 2016. A Review on the Use of Prostaglandin F2α for Controlling the Estrous Cycle in Mares. J. Equine Vet. Sci. 40:34-40.

Cole, H. 1930. A Study of the Mucosa of the Genital Tract of the Cow, with Special Reference to the Cyclic Changes. Am. J. Anat. 46:261-290.

Cooke, R. and J. Arthington. 2009. Plasma progesterone concentrations as puberty criteria for Brahman-crossbred heifers. Livest. Sci. 123:101-105.

Cooper, M., D. Arney, and C. J. Phillips. 2008. The effect of temporary deprivation of lying and feeding on the behaviour and production of lactating dairy cows. Animal 2:275-283.

Corbo, D. C., J. C. Liu, and Y. W. Chienx. 1990. Characterization of the barrier properties of mucosal membranes. J. Pharm. Sci. 79:202-206.

Cross, P. S., R. Künnemeyer, C. R. Bunt, D. A. Carnegie, and M. J. Rathbone. 2004. Control, communication and monitoring of intravaginal drug delivery in dairy cows. Int. J. Pharm. 282:35-44.

Davis, A., I. Fleet, F. Harrison, and F. M. Walker. 1980. Pulmonary metabolism of prostaglandin F2a in the conscious non-pregnant ewe and sow. J. Physiol.

De Rensis, F., R. Saleri, P. Tummaruk, M. Techakumphu, and R. Kirkwood. 2012. Prostaglandin F2α and control of reproduction in female swine: a review. Theriogenology 77:1-11.

De Vries, A. 2006. Economic value of pregnancy in dairy cattle. J. Dairy Sci. 89:3876-3885.

Dohoo, I., W. Martin, and H. Stryhn. 2014. Veterinary Epidemiologic Research. 2nd ed. Friesens Manitoba Canada.

Dukes, M., W. Russell, and A. Walpole. 1974. Potent luteolytic agents related to prostaglandin F 2α. Nature 250:330-331.

Dyce, K. M., W. O. Sack, and C. J. G. Wensing. 2009. Textbook of veterinary anatomy-E-Book. Elsevier Health Sciences.

FAO 2017. The future of food and agriculture–Trends and challenges. Food and Agriculture Organisation Rome. Accessed April 2020. http://www.fao.org/3/a-i6583e.pdf

Friggens, N., M. Bjerring, C. Ridder, S. Højsgaard, and T. Larsen. 2008. Improved detection of reproductive status in dairy cows using milk progesterone measurements. Reprod. Domest. Anim. 43:113-121.

Galvão, K., P. Federico, A. De Vries, and G. M. Schuenemann. 2013. Economic comparison of reproductive programs for dairy herds using estrus detection, timed artificial insemination, or a combination. J. Dairy Sci. 96:2681-2693.

Galvão, K., M. Frajblat, W. Butler, S. Brittin, C. Guard, and R. Gilbert. 2010. Effect of early postpartum ovulation on fertility in dairy cows. Reprod. Domest. Anim. 45:e207-e211.

Gao, Y., R. Short, and T. Fletcher. 1988. Progesterone concentrations in plasma, saliva and milk of cows in different reproductive states. Br. Vet. J. 144:262-268.

Global Harvest Initiative, 2017. 2017. Global Agricultural Productivity Report. Global Harvest Initiative. Washington, D. C. Accessed April 2020. https://www.globalharvestinitiative.org/wp-content/uploads/2017/10/GHI_2017-GAP-Report_FINAL.pdf

Ginther, O., R. Araujo, M. Palhao, B. Rodrigues, and M. Beg. 2009. Necessity of sequential pulses of prostaglandin F2alpha for complete physiologic luteolysis in cattle. Biol. Reprod. 80:641-648.

Ginther, O., H. Shrestha, M. Fuenzalida, A. Shahiduzzaman, M. Hannan, and M. Beg. 2010. Intrapulse temporality between pulses of a metabolite of prostaglandin F2α and circulating concentrations of progesterone before, during, and after spontaneous luteolysis in heifers. Theriogenology 74:1179-1186.

Giordano, J., P. Fricke, and V. Cabrera. 2013. Economics of resynchronization strategies including chemical tests to identify nonpregnant cows. J. Dairy Sci. 96:949-961.

Giordano, J., J. Guenther, G. Lopes Jr, and P. Fricke. 2012a. Changes in serum pregnancy-associated glycoprotein, pregnancy-specific protein B, and progesterone concentrations before and after induction of pregnancy loss in lactating dairy cows. J. Dairy Sci. 95:683-697.

Giordano, J., M. Stangaferro, R. Wijma, W. Chandler, and R. Watters. 2015. Reproductive performance of dairy cows managed with a program aimed at increasing insemination of cows in estrus based on increased physical activity and fertility of timed artificial inseminations. J. Dairy Sci. 98:2488-2501.

Giordano, J., M. Wiltbank, J. Guenther, R. Pawlisch, S. Bas, A. Cunha, and P. Fricke. 2012b. Increased fertility in lactating dairy cows resynchronized with Double-Ovsynch compared with Ovsynch initiated 32 d after timed artificial insemination. J. Dairy Sci. 95:639-653.

Goff, A. K. 2004. Steroid hormone modulation of prostaglandin secretion in the ruminant endometrium during the estrous cycle. Biol. Reprod. 71:11-16.

Gong, X., J. Cai, B. Zhang, Q. Zhao, J. Piao, W. Peng, W. Gao, D. Zhou, M. Zhao, and J. Chang. 2017. A review of fluorescent signal-based lateral flow immunochromatographic strips. J. Mater. Chem. B 5:5079-5091.

Gowan, E. and R. Etches. 1979. A solid-phase radioimmunoassay for progesterone and its application to pregnancy diagnosis in the cow. Theriogenology 12:327-343.

Harizi, H., J.-B. Corcuff, and N. Gualde. 2008. Arachidonic-acid-derived eicosanoids: roles in biology and immunopathology. Trends Mol. Med. 14:461-469.

Heap, R., M. Gwyn, J. Laing, and D. Walters. 1973. Pregnancy diagnosis in cows; changes in milk progesterone concentration during the oestrous cycle and pregnancy measured by a rapid radioimmunoassay. J. Agri. Sci. 81:151-157.

Heap, R., R. Holdsworth, J. Gadsby, J. Laing, and D. Walters. 1976. Pregnancy diagnosis in the cow from milk progesterone concentration. Br. Vet. J. 132:445-464.

Heinonen, K., T. Shiferaw, and M. Heinonen. 1996. Oestrous synchronisation in Ethiopian highland zebu cattle by means of intravaginal cloprostenole administration. Trop. Anim. Health Prod. 28:121-125.

Henricks, D., J. Dickey, and G. Niswender. 1970. Serum luteinizing hormone and plasma progesterone levels during the estrous cycle and early pregnancy in cows. Biol. Reprod. 2:346-351.

Herzog, K., M. Brockhan-Lüdemann, M. Kaske, N. Beindorff, V. Paul, H. Niemann, and H. Bollwein. 2010. Luteal blood flow is a more appropriate indicator for luteal function during the bovine estrous cycle than luteal size. Theriogenology 73:691-697.

Hoffmann, B., W. Schmidt, O. Günzler, and R. Hamburger. 1976. Milk progesterone as a parameter for fertility control in cattle; methodological approaches and present status of application in Germany. Br. Vet. J. 132:469-476.

Hussain, A. and F. Ahsan. 2005. The vagina as a route for systemic drug delivery. J. Control. Release 103:301-313.

Imwalle, D., D. Patterson, and K. Schillo. 1998. Effects of melengestrol acetate on onset of puberty, follicular growth, and patterns of luteinizing hormone secretion in beef heifers. Biol. Reprod. 58:1432-1436.

Ismail, A. A. 2007. Preformulation and Formulation of Steroids and Assessment of an Electronically Modulated Intravaginal Device for Induced Calving or Oestrous synchronization of Cattle. Doctoral Dissertation, The University of Waikato.

Jorgensen, M., A. Adams-Progar, A. de Passille, J. Rushen, S. Godden, H. Chester-Jones, and M. Endres. 2017. Factors associated with dairy calf health in automated feeding systems in the Upper Midwest United States. J. Dairy Sci. 100:5675-5686.

Kanchev, L., C. P. Marinova, and B. Stankov. 1988. Bovine salivary progesterone: Application to the assessment of ovarian function and early pregnancy diagnosis. Anim. Reprod. Sci. 17:1-8.

Kimball, F., J. Lauderdale, N. Nelson, and R. Jackson. 1976. Comparison of luteolytic effectiveness of several prostaglandin analogs in heifers and relative binding affinity for bovine luteal prostaglandin binding sites. Prostaglandins 12:985-995.

Künnemeyer, R., P. Cross, C. Bunt, D. Carnegie, and M. Rathbone. 2004. Electronically controlled, intravaginal drug delivery. Proceedings of the Institution of Mechanical Engineers, Part B: J. Engin. Manuf. 218:1409-1415.

Laitinen, M. P. and M. Vuento. 1996. Immunochromatographic assay for quantitation of milk progesterone. Acta Chem. Scand. 50:141-145.

Lamb, G., C. Dahlen, J. Larson, G. Marquezini, and J. Stevenson. 2010. Control of the estrous cycle to improve fertility for fixed-time artificial insemination in beef cattle: a review. J. Anim. Sci. 88:E181-E192.

Lammers, B., A. J. Heinrichs, and R. Kensinger. 1999. The Effects of Accelerated Growth Rates and Estrogen Implants in Prepubertal Holstein Heifers on Estimates of Mammary Development and Subsequent Reproduction and Milk Production1. J. Dairy Sci. 82:1753-1764.

Louis, T., H. Hafs, and B. Seguin. 1973. Progesterone, LH, estrus and ovulation after prostaglandin F2α in heifers. Proc. Soc. Exp. Biol. Med. 143:152-155.

Lu, Z., D. O'Dell, B. Srinivasan, E. Rey, R. Wang, S. Vemulapati, S. Mehta, and D. Erickson. 2017. Rapid diagnostic testing platform for iron and vitamin A deficiency. Proc. Natl. Acad. Sci. 114:13513-13518.

Macmillan, K. and A. Peterson. 1993. A new intravaginal progesterone releasing device for cattle (CIDR-B) for oestrous synchronisation, increasing pregnancy rates and the treatment of post-partum anoestrus. Anim. Reprod. Sci. 33:1-25.

Mann, G. and G. Lamming. 2006. Timing of prostaglandin F2α release episodes and oxytocin receptor development during luteolysis in the cow. Anim. Reprod. Sci. 93:328-336.

Martins, J., R. Policelli, L. Neuder, W. Raphael, and J. Pursley. 2011. Effects of cloprostenol sodium at final prostaglandin F2α of Ovsynch on complete luteolysis and pregnancy per artificial insemination in lactating dairy cows. J. Dairy Sci. 94:2815-2824.

Masello, M., B. Ceglowski, M. Thomas, and J. Giordano. 2020. A reproductive management program aimed at increasing reinsemination of nonpregnant dairy cows at detected estrus resulted in similar reproductive performance compared with a program that favored timed artificial insemination. J. Dairy Sci. 103(4): 3719-3729.

Matsas, D., R. Nebel, and K. Pelzer. 1992. Evaluation of an on-farm blood progesterone test for predicting the day of parturition in cattle. Theriogenology 37:859-868.

McArt, J., L. Caixeta, V. Machado, C. Guard, K. Galvao, O. Sá Filho, and R. Bicalho. 2010. Ovsynch versus Ultrasynch: reproductive efficacy of a dairy cattle synchronization protocol incorporating corpus luteum function. J. Dairy Sci. 93:2525-2532.

McCracken, J. A., E. E. Custer, and J. C. Lamsa. 1999. Luteolysis: a neuroendocrine-mediated event. Physiol. Rev. 79:263-323.

Millipore, E. 2013. Rapid Lateral Flow Test Strips: Considerations for Product Development. EMD Millipore Corporation: Billerica, MA, USA.

Miroud, K. and D. Noakes. 1991. Histological changes in the vaginal mucosa of the cow during the oestrous cycle, after ovariectomy and following exogenous oestradiol benzoate and progesterone treatment. Br. Vet. J. 147:469-477.

Moreira, F. and D. Hammon. 2012. Lutalyse® and cloprostenol: Clearing up misconceptions: Technical bulletin. Zoetis, 12p.

Moreira, F., C. Orlandi, C. Risco, R. Mattos, F. Lopes, and W. Thatcher. 2001. Effects of presynchronization and bovine somatotropin on pregnancy rates to a timed artificial insemination protocol in lactating dairy cows. J. Dairy Sci. 84:1646-1659.

Mortimer, R., J. Olson, E. Huffman, P. Farin, L. Ball, and B. Abbitt. 1983. Serum progesterone concentration in pyometritic and normal postpartum dairy cows. Theriogenology 19:647-653.

Mottram, T. 2016. Animal board invited review: precision livestock farming for dairy cows with a focus on oestrus detection. Animal 10:1575-1584.

NAHMS. 2018. Dairy 2014, Part III: Reference of Dairy Cattle Health and Management Practices in the United States, 2014. #696.0218. Centers for Epidemiology and Animal Health, USDA: Animal and Plant Health Inspection Service: Veterinary Services, Fort Collins, CO.

Nakami, W., V. Tsuma, K. Milkey, M. Dickerson, M. Wong, H. Mutembei, J. Muthee, O. Odipo, and W. Ngetich. 2017. Lateral flow immunoassay for whole blood progesterone detection as a tool for assessment of reproductive status in cattle. Inter. J. Vet. Sci. 6:19-25.

Nebel, R. 1988. On-farm milk progesterone tests. J. Dairy Sci. 71:1682-1690.

Niswender, G. D., J. L. Juengel, P. J. Silva, M. K. Rollyson, and E. W. McIntush. 2000. Mechanisms controlling the function and life span of the corpus luteum. Physiol. Rev. 80:1-29.

Parr, R., I. Davis, M. Miles, and T. Squires. 1993. Liver blood flow and metabolic clearance rate of progesterone in sheep. Res. Vet. Sci. 55:311-316.

Pathiraja, N., E. Oyedipe, A. Voh Jr, and P. Dawuda. 1986. Accuracy of rectal palpation in the diagnosis of corpora lutea in Zebu cows. Br. Vet. J. 142:467-471.

Pennington, J., L. Schultz, and W. Hoffman. 1985. Comparison of Pregnancy Diagnosis by Milk Progesterone on Day 21 and Day 24 Postbreeding: Field Study in Dairy Cattle1. J. Dairy Sci. 68:2740-2745.

Peters, A. and G. Riley. 1982. Milk progesterone profiles and factors affecting post partum ovarian activity in beef cows. Anim. Sci. 34:145-153.

Pope, G., I. Majzlik, P. Ball, and J. Leaver. 1976. Use of progesterone concentrations in plasma and milk in the diagnosis of pregnancy in domestic cattle. Br. Vet. J. 132:497-506.

Posthuma-Trumpie, G. A., J. Korf, and A. van Amerongen. 2008. Development of a competitive lateral flow immunoassay for progesterone: influence of coating conjugates and buffer components. Anal. Bioanal. Chem. 392:1215-1223.

Posthuma-Trumpie, G. A., J. Korf, and A. van Amerongen. 2009. Lateral flow (immuno) assay: its strengths, weaknesses, opportunities and threats. A literature survey. Anal. Bioanal. Chem. 393:569-582.

Pursley, J., J. Martins, C. Wright, and N. Stewart. 2012. Compared to dinoprost tromethamine, cloprostenol sodium increased rates of estrus detection, conception and pregnancy in lactating dairy cows on a large commercial dairy. Theriogenology 78:823-829.

Pursley, J., M. Wiltbank, J. Stevenson, J. Ottobre, H. Garverick, and L. Anderson. 1997. Pregnancy rates per artificial insemination for cows and heifers inseminated at a synchronized ovulation or synchronized estrus1. J. Dairy Sci. 80:295-300.

Rathbone, M. J. and C. R. Burke. 2013. Controlled Release Intravaginal Veterinary Drug Delivery. Pages 247-270 in Long Acting Animal Health Drug Products. Springer.

Rathbone, M. J., C. R. Burke, C. R. Ogle, C. R. Bunt, S. Burggraaf, and K. L. Macmillan. 2000. Design and development of controlled release intravaginal veterinary drug delivery systems. Pages 173-200 in Controlled Release Veterinary Drug Delivery. Elsevier.

Rathbone, M. J., J. E. Kinder, K. Fike, F. Kojima, D. Clopton, C. R. Ogle, and C. R. Bunt. 2001. Recent advances in bovine reproductive endocrinology and physiology and their impact on drug delivery system design for the control of the estrous cycle in cattle. Adv. Drug Deliv. Rev. 50:277-320.

Rathbone, M. J., K. L. Macmillan, C. R. Bunt, and S. Burggraaf. 1997. Conceptual and commercially available intravaginal veterinary drug delivery systems. Adv. Drug Deliv. Rev. 28:363-392.

Reeves, P. R. 1978. Distribution, elimination, and residue studies in the cow with the synthetic prostaglandin estrumate. J. Agric. Food Chem. 26:152-155.

Reith, S. and S. Hoy. 2018. Behavioral signs of estrus and the potential of fully automated systems for detection of estrus in dairy cattle. Animal 12:398-407.

Ricci, A., P. Carvalho, M. Amundson, and P. Fricke. 2017. Characterization of luteal dynamics in lactating Holstein cows for 32 days after synchronization of ovulation and timed artificial insemination. J. Dairy Sci. 100:9851-9860.

Richardson, J. L. and L. Illum. 1992. (D) Routes of delivery: Case studies:(8) The vaginal route of peptide and protein drug delivery. Adv. Drug Deliv. Rev. 8:341-366.

Rius, A., E. Connor, A. Capuco, P. Kendall, T. Auchtung-Montgomery, and G. Dahl. 2005. Long-day photoperiod that enhances puberty does not limit body growth in Holstein heifers. J. Dairy Sci. 88:4356-4365.

Roark, D. B. and H. A. Herman. 1950. Physiological and histological phenomena of the bovine estrual cycle with special reference to vaginal-cervical secretions. Missouri Agric. Exp. Stn. Bull. 455.

Rodgers, J. C., S. Bird, J. Larson, N. DiLorenzo, C. Dahlen, A. DiCostanzo, and G. Lamb. 2012. An economic evaluation of estrous synchronization and timed artificial insemination in suckled beef cows. J. Anim. Sci. 90:4055-4062.

Rutllant, J., M. López-Béjar, and F. López-Gatius. 2005. Ultrastructural and rheological properties of bovine vaginal fluid and its relation to sperm motility and fertilization: a review. Reprod. Domest. Anim. 40:79-86.

Safronova, V., J. Samsonova, V. Grigorenko, and A. Osipov. 2012. Lateral flow immunoassay for progesterone detection. Moscow Univ. Chem. Bull. 67:241-248.

Sajid, M., A.-N. Kawde, and M. Daud. 2015. Designs, formats and applications of lateral flow assay: A literature review. J. Saudi Chem. Soc. 19:689-705.

Samsonova, J., V. Safronova, and A. Osipov. 2015. Pretreatment-free lateral flow enzyme immunoassay for progesterone detection in whole cows' milk. Talanta 132:685-689.

Sananikone, K., M. Delwiche, R. BonDurant, and C. Munro. 2004. Quantitative lateral flow immunoassay for measuring progesterone in bovine milk. Transactions of the ASAE 47:1357.

Santos, V., P. Carvalho, C. Maia, B. Carneiro, A. Valenza, and P. Fricke. 2017. Fertility of lactating Holstein cows submitted to a Double-Ovsynch protocol and timed artificial insemination versus artificial insemination after synchronization of estrus at a similar day in milk range. J. Dairy Sci. 100:8507-8517.

Sartori, R., J. Haughian, R. Shaver, G. Rosa, and M. Wiltbank. 2004. Comparison of ovarian function and circulating steroids in estrous cycles of Holstein heifers and lactating cows. J. Dairy Sci. 87:905-920.

Sauls-Hiesterman, J., B. Voelz, and J. Stevenson. 2020. A shortened resynchronization treatment for dairy cows after a nonpregnancy diagnosis. Theriogenology 141:105-112.

Schilling, E. and J. Zust. 1968. Diagnosis of oestrus and ovulation in cows by pH-measurements intra vaginam and by apparent viscosity of vaginal mucus. Reproduction 15:307-311.

Senger, P. L. 2003. Pathways to pregnancy and parturition. 2nd ed. Current Conceptions, Inc. Pullman, WA.

Shrestha, H. K., M. A. Beg, R. R. Burnette, and O. Ginther. 2012. Plasma clearance and half-life of prostaglandin F2alpha: a comparison between mares and heifers. Biol. Reprod. 87:18, 11-16.

Sørensen, L. P., M. Bjerring, and P. Løvendahl. 2016. Monitoring individual cow udder health in automated milking systems using online somatic cell counts. J. Dairy Sci. 99:608-620.

Souza, A., H. Ayres, R. Ferreira, and M. Wiltbank. 2008. A new presynchronization system (Double-Ovsynch) increases fertility at first postpartum timed AI in lactating dairy cows. Theriogenology 70:208-215.

Srinivasan, B., D. O'Dell, J. L. Finkelstein, S. Lee, D. Erickson, and S. Mehta. 2018. ironPhone: mobile device-coupled point-of-care diagnostics for assessment of iron status by quantification of serum ferritin. Biosens. Bioelectron. 99:115-121.

Stangaferro, M., R. Wijma, L. Caixeta, M. Al-Abri, and J. Giordano. 2016. Use of rumination and activity monitoring for the identification of dairy cows with health disorders: Part I. Metabolic and digestive disorders. J. Dairy Sci. 99:7395-7410.

Stangaferro, M., R. Wijma, M. Masello, M. J. Thomas, and J. Giordano. 2018. Extending the duration of the voluntary waiting period from 60 to 88 days in cows that received timed artificial insemination after the Double-Ovsynch protocol affected the reproductive performance, herd exit dynamics, and lactation performance of dairy cows. J. Dairy Sci. 101:717-735.

Stevenson, J. 2019. Spatial relationships of ovarian follicles and luteal structures in dairy cows subjected to ovulation synchronization: Progesterone and risks for luteolysis, ovulation, and pregnancy. J. Dairy Sci. 102:5686-5698.

Stevenson, J. and A. Phatak. 2005. Inseminations at estrus induced by presynchronization before application of synchronized estrus and ovulation. J. Dairy Sci. 88:399-405.

Stevenson, J. and A. Phatak. 2010. Rates of luteolysis and pregnancy in dairy cows after treatment with cloprostenol or dinoprost. Theriogenology 73:1127-1138.

Tisone, T. C. and B. O'Farrell. 2009. Manufacturing the next generation of highly sensitive and reproducible lateral flow immunoassay. Pages 1-26 in Lateral Flow Immunoassay. Springer.

USDA National Institute of Food and Agriculture. 2020. Veterinary Services Shortages Situations. Accessed April 2020. https://nifa.usda.gov/vmlrp-map?state=All&field_status_value=All&field_vsgp_status_value=All&fy%5Bvalue%5D%5Byear%5D=2020&=Apply

Valanis, B. G. and C. S. Perlman. 1982. Home pregnancy testing kits: prevalence of use, false-negative rates, and compliance with instructions. Am. J. Public Health 72:1034-1036.

Vasconcelos, J. L. M., S. Sangsritavong, S.-J. Tsai, and M. Wiltbank. 2003. Acute reduction in serum progesterone concentrations after feed intake in dairy cows. Theriogenology 60:795-807.

Ventura, B. A., M. A. Von Keyserlingk, H. Wittman, and D. M. Weary. 2016. What difference does a visit make? Changes in animal welfare perceptions after interested citizens tour a dairy farm. PLoS One 11.

Von Keyserlingk, M., N. Martin, E. Kebreab, K. Knowlton, R. Grant, M. Stephenson, C. Sniffen, J. Harner Iii, A. Wright, and S. Smith. 2013. Invited review: Sustainability of the US dairy industry. J. Dairy Sci. 96:5405-5425.

Waldmann, A. and A. Raud. 2016. Comparison of a lateral flow milk progesterone test with enzyme immunoassay as an aid for reproductive status determination in cows. Vet. Rec. vetrec-2015-103605.

Wheaton, J. and G. Lamb. 2007. Induction of cyclicity in postpartum anestrous beef cows using progesterone, GnRH and estradiol cypionate (ECP). Anim. Reprod. Sci. 102:208-216.

Whitfield, L. 2018. Ovarian ultrasound for fertility management in dairy cattle. Livestock 23:154-160.

Wijma, R., M. Pérez, M. Masello, M. Stangaferro, and J. Giordano. 2018. A resynchronization of ovulation program based on ovarian structures present at nonpregnancy diagnosis reduced time to pregnancy in lactating dairy cows. J. Dairy Sci. 101:1697-1707.

Wijma, R., M. Stangaferro, M. Masello, G. Granados, and J. Giordano. 2017. Resynchronization of ovulation protocols for dairy cows including or not including gonadotropin-releasing hormone to induce a new follicular wave: Effects on re-insemination pattern, ovarian responses, and pregnancy outcomes. J. Dairy Sci. 100:7613-7625.

Wijma, R., M. L. Stangaferro, and J. O. Giordano. 2016a. Circulating progesterone dynamics after intravaginal instillation of prostaglandin-F2α to lactating dairy cows. Theriogenology 85:1660-1668.

Wijma, R., M. L. Stangaferro, M. M. Kamat, S. Vasudevan, T. L. Ott, and J. O. Giordano. 2016b. Embryo mortality around the period of maintenance of the corpus luteum causes alterations to the ovarian function of lactating dairy cows. Biol. Reprod. 95:112, 111-114.

Wilsdorf, L., D. Keisler, S. Poock, W. Lamberson, R. Escalante, and M. Lucy. 2016. Test for nonpregnancy in dairy cows based on plasma progesterone concentrations before and after timed artificial insemination. J. Dairy Sci. 99:5858-5865.

Wiltbank, M., H. Lopez, R. Sartori, S. Sangsritavong, and A. Gümen. 2006. Changes in reproductive physiology of lactating dairy cows due to elevated steroid metabolism. Theriogenology 65:17-29.

Wiltbank, M., A. Souza, P. Carvalho, A. Cunha, J. Giordano, P. Fricke, G. Baez, and M. Diskin. 2014. Physiological and practical effects of progesterone on reproduction in dairy cattle. Animal 8:70-81.

Wishart, D. 1975. Early pregnancy diagnosis in cattle. The Veterinary record 96:34-38.

Woolfson, A. D., R. K. Malcolm, and R. Gallagher. 2000. Drug delivery by the intravaginal route. Crit. Rev. Ther. Drug Carr. Syst. 17; 509-555.

Yu, G.-M. and T. Maeda. 2017. Inline progesterone monitoring in the dairy industry. Trends Biotechnol. 35:579-582.

Zdunczyk, S., P. Andresen, T. Janowski, and A. Chmielewski. 1994. The effectiveness of intravaginal administration of estrumate for cows in silent heat in relation to the progesterone concentration in the blood. DTW. Deutsche tierarztliche Wochenschrift 101:440-441.

CHAPTER IV

A LATERAL FLOW-BASED PORTABLE PLATFORM FOR DETERMINATION OF REPRODUCTIVE STATUS OF CATTLE

M. Masello, Z. Lu, D. Erickson, J. Gavalchin, and J. O. Giordano

Journal of Dairy Science 2020; 103(5): 4743-4753.
DOI: 10.3168/jds.2019-17517

ABSTRACT

Our objective was to develop and validate a tool integrating a disposable fluorescence-based lateral flow immunoassay (**LFIA**) coupled with a portable imaging device for estimating circulating plasma concentrations of progesterone (**P4**). First, we developed and optimized a competitive LFIA test strip to measure P4 in bovine plasma. The LFIA design included a sample pad, a conjugate pad that stores R-Phycoerythrin-anti-P4 conjugates, a glass-fiber spacer pad, a nitrocellulose membrane with printed test and control lines, and a cellulose-fiber absorbent pad. To perform a test, 20 μL of plasma and 50 μL of running buffer were added on the sample pad. After 3 min, 45 μL of running buffer were added to initiate sample flow. After allowing 15 min to stabilize the colorimetric signal, strips were introduced in an LFIA portable reader wirelessly linked to a laptop to determine P4 concentration based on test-to-control-line signal (**T/C** ratio). In a series of experiments (n = 6), the ability of the LFIA to differentiate plasma samples with ≥ 1 or < 1 ng/mL of P4 was evaluated. For each experiment, a calibration curve was constructed using plasma with known concentrations of P4 (0.1 to 3.7 ng/mL; n = 5). The resulting linear equation was then used to determine a T/C ratio cutoff to differentiate samples with ≥ 1 or < 1 ng/mL of P4. In addition, to evaluate the ability of the platform to assign samples to P4 concentrations groups without a calibration curve for individual batches, we performed a

receiver operating characteristic analysis to identify a single cutoff value for T/C ratio that could potentially be used for all batches. Overall, calibration curves showed a linear relationship between T/C ratio and P4 levels [mean coefficient of determination (R2) = 0.74; range 0.42 to 0.99]. Next, plasma samples from lactating dairy cows (n = 58) were tested in triplicate to determine the ability of the LFIA system to differentiate plasma samples with ≥ 1 or < 1 ng/mL of P4 using a radioimmunoassay for P4 as reference test. Overall, the LFIA assay correctly classified 90% of the samples, with 97% sensitivity, 83% specificity, 85% positive predictive value and 96% negative predictive value. Agreement between the tests was substantial (kappa = 0.79; 95% CI 0.64 to 0.95). When using a single cutoff value for T/C ratio selected by receiver operating characteristic analysis, sensitivity and specificity to determine CL presence were 97 (95% CI 82 to 99) and 79% (95% CI 60 to 92), respectively. These data suggest that the developed portable LFIA system can accurately differentiate plasma samples with ≥ 1 or < 1 ng/mL of P4.

Keywords: lateral flow immunoassay, progesterone concentration determination, reproductive status, cattle

INTRODUCTION

Reproductive management of cattle is critical to the profitability and sustainability of both dairy (Giordano et al., 2012) and beef (White et al., 2015) cattle operations. Therefore, multiple management practices are used by farms to optimize reproductive performance and simplify management of females. Among others, an important practice is the determination of the reproductive status of cows including estrus, pregnancy, non-pregnancy, and anovulation. In addition, for some commercial farms determination of the reproductive status of cows also includes defining the presence or absence of a functional corpus luteum (**CL**) at the time of non-pregnancy diagnosis or during synchronization of ovulation protocols for timed AI. Although several methods and technologies are available to determine the reproductive status of cows, this can be either fully or partially accomplished through estimation of circulating concentrations of the steroid hormone progesterone (**P4**) produced by the CL. Determination of circulating concentrations of P4 can be used to confirm estrus (Friggens et al., 2008), as a method for non-pregnancy diagnosis (Waldmann and Raud, 2016; Wilsdorf et al., 2016), determine resumption and cessation of ovarian cyclicity (Galvão et al., 2010), confirm luteal regression and ovulation in synchronization of ovulation protocols (Barletta et al., 2018), and assign cows to hormonal treatments for synchronization of ovulation (McArt et al., 2010; Wijma et al., 2018). Unfortunately, decision-making based on accurate detection and quantification of P4 in bodily fluids (i.e., blood or milk) of cattle is not easily accomplished or cost effective for most commercial farms. Sophisticated equipment to conduct P4 testing based on assays in laboratory or farm settings are available (Skenandore et al., 2017; Adriaens et al., 2018; Bruinjé and Ambrose, 2019) but are not easily accessible to all farms due to cost, logistics of implementation, or both. Conversely, low-cost systems are available but they may not be sufficiently accurate or

practical for their intended use (Posthuma-Trumpie et al., 2009; Waldmann and Raud, 2016). Thus, on-farm determination of the reproductive status of cows such as non-pregnancy, anovulation, and presence of a functional CL on the ovaries generally depends on veterinarian or farm personnel-administered exams, either by transrectal palpation or ultrasonography of the reproductive organs. A disadvantage of these methods includes requiring the expertise of highly qualified technicians and expensive equipment, which may problematic for farms located in areas where veterinary support is scarce or for farms that are unable to cover the costs of these exams. In addition, some of the methods available and widely used (i.e., transrectal palpation; NAHMS, 2018) may be less accurate than desired. For example, the reported accuracy of rectal palpation to determine the presence of a functional CL ranged from 57 to 70% (Bicalho et al., 2008).

Lateral flow immunoassays (**LFIA**) are gaining widespread use as rapid, point-of-care diagnostic tools for medical and veterinary applications (Tisone and O'Farrell, 2009; Sajid et al., 2015). Generally, LFIA tests are inexpensive and easy to conduct, which makes them an appealing tool for use in reproductive management of cattle. Attempts to develop LFIA tests for P4 determination have been previously reported (Waldmann and Raud, 2016; Xu et al., 2016; Nakami et al., 2017), but these have drawbacks that seem to preclude their widespread use. For instance, most of the developed LFIA for P4 depend on the subjective interpretation of assay results by the user, which may lead to improper interpretation of results (Waldmann and Raud, 2016). In addition, some of the previously developed LFIA systems (Xu et al., 2016; Nakami et al., 2017) were not designed to differentiate samples within a narrow range of P4 concentrations, in particular around 1 ng/mL which is the most commonly used cutoff to classify cows in relevant reproductive statuses (Ginther et al., 2010; Stevenson, 2019).

Thus, our primary objective was to develop a system integrating a disposable fluoresence-based LFIA combined with a fluorescence portable imaging device to estimate circulating concentrations of P4. Once developed, our objective was to validate our platform and its accuracy for differentiation of bovine plasma samples with ≥ 1 or < 1 ng/mL of P4. This cutoff was selected because it is commonly used in research and in practice to estimate the presence of a putative functional CL in cattle (Ginther et al., 2010; Stevenson, 2019), which can be used as the sole method or as an aid to determine the reproductive status of cattle. In terms of performance, we aimed to achieve at least 90% accuracy for differentiation of cows with ≥ 1 or < 1 ng/mL of P4, and substantial interrater agreement (i.e., kappa value of 0.61 to 0.80) with a radioimmunoassay (RIA) for P4 for classification of plasma samples in groups of P4 ≥ 1 or < 1 ng/mL.

MATERIALS AND METHODS

Reagents and Materials

R-phycoerythrin (**RPE**) fluorophore and reagents (LL-modifier and LL-quencher) used for antibody-RPE conjugate preparation were purchased from Innova Biosciences Ltd. (Lightning-Link Conjugation Kit, Cambridge, UK). Synthetic P4 conjugated to bovine serum albumin (**P4-BSA**) was purchased from Aviva Systems Biology (San Diego, CA). Mouse monoclonal antibodies to P4 (**P4Ab**) were purchased from Hytest Ltd. (Turku, Finland). Goat anti-mouse antibody, amine-free phosphate buffer saline (PBS) buffer at 0.01 M pH 7.4, Tween 20, bovine serum albumin (BSA), borate buffer, and sucrose were acquired from Sigma-Aldrich (St. Louis, MO). Glass fiber conjugate and spacer pads (300 x 5 mm), High Flow Plus 180 membrane cards (300 x 60 mm), and cellulose fiber pads for sample and absorbent pads (300 x 20 cm) were acquired from EMD-Millipore (Billerica, MA, USA).

Progesterone LFIA Architecture and Principle

The P4 LFIA test strip was designed to perform a competitive type immunoassay. Briefly, our design (Figure 1A) includes a sample pad, a conjugate pad that stores the RPE-P4Ab conjugates, a spacer pad, a nitrocellulose membrane with printed test (P4-BSA) and control lines (anti-mouse Ab), and an absorbent pad at the end of the strip to collect any waste from the assay. The sample and conjugate pads are designed to collect and incubate the sample with the RPE-P4Ab conjugates, respectively. On the other hand, the spacer pad is designed to prevent the sample and RPE-P4Ab mixture from flowing through the nitrocellulose membrane before sample flow is initiated. During incubation, the sample rehydrated the RPE-P4Ab conjugates which then interacted with the P4 present in the sample. This competitive interaction determined the availability of RPE-P4Ab conjugates to bind with P4-BSA and anti-mouse IgG immobilized at the test and control lines, respectively.

Antibody-RPE Conjugate Pad Preparation

Monoclonal anti-P4 antibodies were conjugated with the RPE fluorophore by following the protocol provided in the Lightning-Link conjugation kit. First, in order to achieve a 1:1 mAb:RPE conjugation molar ratio (recommended by manufacturer), 10 µg of P4Ab were diluted to 1 mg/mL in 1x PBS before mixing it with 1 µL of LL-modifier reagent. Next, 10 µg of RPE were re-suspended in the solution and incubated for 3 h in a light-free environment at room temperature (20 to 25 ºC). During incubation, the P4Ab attached to the surface of RPE via

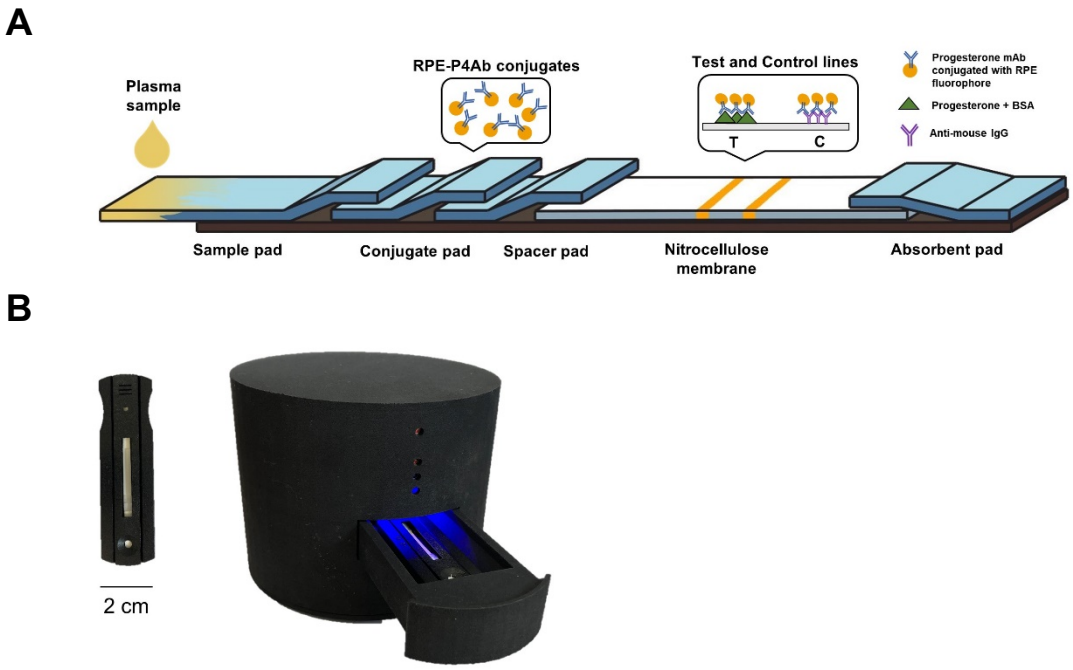


Figure 1. (A) Architecture of the lateral flow immunoassay (LFIA) test strip for progesterone (P4). The LFIA design includes a sample pad, a conjugate pad that stores R-phycoerythrin-anti-P4 conjugates (RPE-P4Ab), a spacer pad, a nitrocellulose membrane with printed test (P4+BSA) and control (anti-mouse antibody) lines, and an absorbent pad at the end of the strip to collect any waste from the assay. (B) Lateral-flow immunoassay test strip housed in a plastic cassette and the TIDBIT reader.

covalent bonding. After incubation, 1 µL of LL-Quencher reagent was added to the conjugates which were then stored at 4 ºC in the dark until use. Before application into the conjugate pad, conjugates were mixed and diluted 400 times in conjugate buffer (2 mM borate buffer with 5% sucrose). On each test strip, 3 µL of the RPE-P4Ab conjugates were applied.

Progesterone LFIA Strip Assembly

High Flow Plus 180 membrane cards were used as the base layer of the LFIA test strips. The membrane cards have a 2 mm clear polyester film backing that contain a nitrocellulose membrane and the adhesive sections to attach the sample, conjugate, spacer and absorbent pads. We chose the membrane with a slow capillary flow rate (45 seconds/cm) to maximize the reaction time between the RPE-P4Ab conjugates and the analytes immobilized on the nitrocellulose membrane. For the test and control lines, P4-BSA and anti-mouse IgG were diluted to 1.5 mg/mL and 0.3 mg/mL, respectively, in 1x PBS and dispensed onto the nitrocellulose membrane using an automated Lateral Flow Reagent Dispenser (ClaremontBio Solutions, Upland, CA) and a dual syringe pump (Legato 200, ClaremontBio Solutions, Upland, CA). The dual syringe pump operated at a flow rate of 6.4 µL/min to ensure 1 mm of line width. Subsequently, membrane cards were dried in an incubator for a minimum of 8 h at 37 ºC. Once membranes were dried, they were stored in a humidity-controlled (< 10% relative humidity) chamber at room temperature until use. The final assay was assembled by first applying the spacer pad onto the adhesive part of the nitrocellulose membrane card, followed by the conjugate and sample pads. Next, a cellulose fiber pad was attached to the other end of the nitrocellulose membrane to serve as the absorbent pad. To ensure a continuous sample flow path across the test strip, all pads were laminated with a 2 mm overlap. The final step was to cut the membrane card

into individual 4 mm strips using a paper trimmer (Dahle North America, Inc.), which were then sealed and stored with silica gel desiccants at 4 ºC in the dark until use.

LFIA Operation Protocol

To perform a test, first 20 µL of bovine plasma and 50 µL of running buffer (1x TBS with 1% BSA, 1.5% Tween 20, and 0.1% sodium azide) were added on the sample pad and strips were placed in a light-free environment for 3 minutes. Then 45 µL of running buffer was added to the sample pad to initiate flow. After allowing 15 minutes for the fluorescent signal to develop, the test strip was inserted into the TIDBIT (Figure 1B), a portable fluorescence reader previously described in Lu et al. (2017). Briefly, the TIDBIT consists of an integrated optical sensor platform designed to detect and capture the fluorescent signal displayed on the test strips. It is equipped with six blue LED covered by band-pass optical filters centered at the excitation wavelength required for the RPE fluorophore (i.e., ~488 nm). Once developed, the fluorescent signal is captured by a 5-megapixel 1080p HD CMOS camera (Raspberry Pi, Cambridge, UK) fitted with a focusing lens. In order to interpret and display the results, output images generated by the TIDBIT are wirelessly transferred to a standard laptop. After each test, results are stored in both the TIDBIT reader and the laptop, and all previous results could be accessed at any time.

Output images generated by the reader were then analyzed to determine test-to-control-line signal (**T/C**) ratio. Intensity of the test and control lines were quantified by using the Plot Profile option of the image processing software, ImageJ (Schneider et al., 2012). Briefly, the Plot Profile option displays a two-dimensional graph of the intensities of pixels along a rectangular section, where the x-axis represents the horizontal distance through the selection and the y-axis

the vertically averaged pixel intensity. Once the plots were generated, the Straight Line selection tool was used to measure peak intensities corresponding to the test and control line regions.

Assay Performance in Bovine Plasma Samples

Bovine plasma samples (n = 58) were used to evaluate the ability of the LFIA test strips to differentiate samples with ≥ 1 or < 1 ng/mL of P4. Stored plasma samples obtained from previous work (Masello et al., 2019) were used to evaluate the ability of the LFIA system to differentiate samples with ≥ 1 or < 1 ng/mL of P4. Briefly, blood samples (~8 to 9 mL) were collected from lactating Holstein cows by puncture of the caudal vein or artery using evacuated tubes containing EDTA. After collection, samples were placed in crushed ice and transported to the laboratory for further processing. Samples were centrifuged at 2,000 x g for 20 min at 4ºC. Plasma samples were harvested and transferred to Eppendorf vials for storage at -20ºC until assayed. A commercial solid-phase, no-extraction RIA (Immuchem Coated Tube, MP Biomedicals, Costa Mesa, CA) for P4 was used as the reference test and to classify samples based on a 1 ng/mL of P4 cutoff. Six batches of test strips were manufactured to perform six independent experiments (i.e., one batch per experiment). For each experiment, a calibration curve was constructed using five plasma samples with known concentrations of P4 (0.1 to 3.7 ng/mL). For calibration sample selection, we ensured that at least one sample was within each one of the following P4 ranges: < 0.1 ng/mL (limit of detection of the RIA), 0.1 to 1.0 ng/mL, 1.1 to 1.4 ng/mL, and 1.5 to 4.0 ng/mL. The range of P4 concentrations in the calibration curve is representative of the range observed for lactating Holstein cows during the estrous cycle and pregnancy (Hankele et al., 2020). Samples with > 4 ng/mL of P4 were not included because the lesser practical value of accurate quantification of P4 concentrations when circulating

concentrations of P4 are > 4 ng/mL. To generate a linear calibration curve, each calibration sample was tested in triplicate and the resulting average T/C ratio was plotted against P4 concentrations from the reference test. The linear regression equation was then used to determine the T/C ratio cutoff to differentiate plasma samples with ≥ 1 or < 1 ng/mL of P4 (i.e., the T/C ratio corresponding to 1 ng/mL of P4). This T/C ratio was used to evaluate assay performance by serving as a cutoff value for the classification of the 58 bovine plasma samples (~10 for each experiment). To have an equal number of positive and negative samples, stock plasma samples were first classified as negative (< 1 ng/mL) or positive (≥ 1 ng/mL). Within each category, we then randomly selected 5 samples. All samples were tested in triplicate and in random order. Experiments were conducted by a single technician who was blinded to P4 concentrations.

Statistical Analyses

The main outcome of interest for this study was to evaluate the ability of our system based on the LFIA test strips to correctly differentiate plasma samples with ≥ 1 or < 1 ng/mL of P4. To evaluate assay performance, we created a 2 x 2 frequency table based on the following criteria:

- ***True positive*** = positive LFIA test strip outcome (i.e., T/C ratio lower than the estimated threshold for P4 ≥ 1 ng/mL) and P4 ≥ 1 ng/mL by RIA.
- ***False positive*** = positive LFIA test strip outcome and P4 < 1 ng/mL.
- ***True negative*** **=** negative LFIA test strip outcome (i.e., T/C ratio greater than the estimated threshold for P4 < 1 ng/mL) and P4 < 1 ng/mL by RIA.
- ***False negative*** **=** negative LFIA test strip outcome and P4 ≥ 1 ng/mL.

This frequency table was then used to estimate the overall sensitivity (**Se**), specificity (**Sp**), positive predictive value (**PPV**), negative predictive value (**NPV**), and accuracy of the LFIA test strips. Confidence intervals and tests for these statistics were computed using the "exact binomial" option of the PROC FREQ of SAS (version 9.4, SAS Institute Inc., Cary, NC). For each statistic, an exact test was performed under the null hypothesis that the proportion equals 0.5.

To calculate the coefficient of variation (**CV**) for each sample, the standard deviation was divided by the mean T/C ratio corresponding to the 3 replicates of each one of the samples.

The level of agreement between LFIA test trips and RIA (reference test) to differentiate plasma samples with ≥ 1 or < 1 ng/mL of P4 was determined through calculation of the kappa value for interrater agreement obtained with FREQ procedure of SAS under the null hypothesis that kappa = 0. A kappa of 1 indicates a perfect agreement whereas a kappa of 0 indicates no agreement (Landis and Koch, 1977). Linear models for calibration curves were generated with PROC REG of SAS including T/C ratio as dependent variable and known concentrations of P4 as independent variable.

To examine the ability of the current LFIA-based system to differentiate plasma samples with ≥ 1 or < 1 ng/mL of P4 without a calibration curve for individual batches, we performed receiver operating characteristic (**ROC**) analysis using the ROC curve option in MedCalc (version 12.5.0.0; MedCalc Software bvba, Ostend, Belgium) to identify a single cutoff value for T/C ratio that could potentially be used for all batches. For these analyses, data did not include results from the calibration samples resulting in a total of 58 data points to generate the ROC curves.

The reference test for all analyses performed was the dichotomized P4 concentration determined by RIA using a P4 concentration cutoff of 1 ng/mL. This cutoff was used because this is a common circulating P4 concentration value used in research or in practice to estimate the presence of a putative functional CL in cattle (Ginther et al., 2010; Stevenson, 2019).

For all analyses, variables were considered significant if $P \leq 0.05$, whereas P-values > 0.05 and ≤ 0.10 were considered a tendency.

RESULTS AND DISCUSSION

In the present study, we developed and evaluated a novel platform to estimate circulating P4 concentrations in bovine plasma. The platform consists of a fluorescent-based LFIA test strip, combined with a portable outcome reader that is wirelessly linked to a standard laptop or smart device. The design of the developed LFIA represents a competitive-type assay, where free P4 in the sample and immobilized P4 in the test strip compete for a limited number of antibody binding sites. We selected this format because the small size of the P4 molecule (~314 Da) precludes binding to more than one antibody simultaneously (i.e., hapten molecule). As expected of our competitive-type architecture, the T/C ratios for samples with low P4 levels were greater than that of samples with high P4 levels (Figures 2A to C). In Figure 2A, we present a set of typical fluorescence images acquired by the portable reader from bovine plasma samples with low, medium, and high P4 levels. Because of the competitive design, the colorimetric signal of the test line decreased as P4 concentration increased, whereas the colorimetric signal of the control line increased as P4 concentration increased. This is further illustrated in Figure 2B, where we present the profile plots of the test and control line regions created with the image processing software. These plots were used to measure the peak values of the test and control

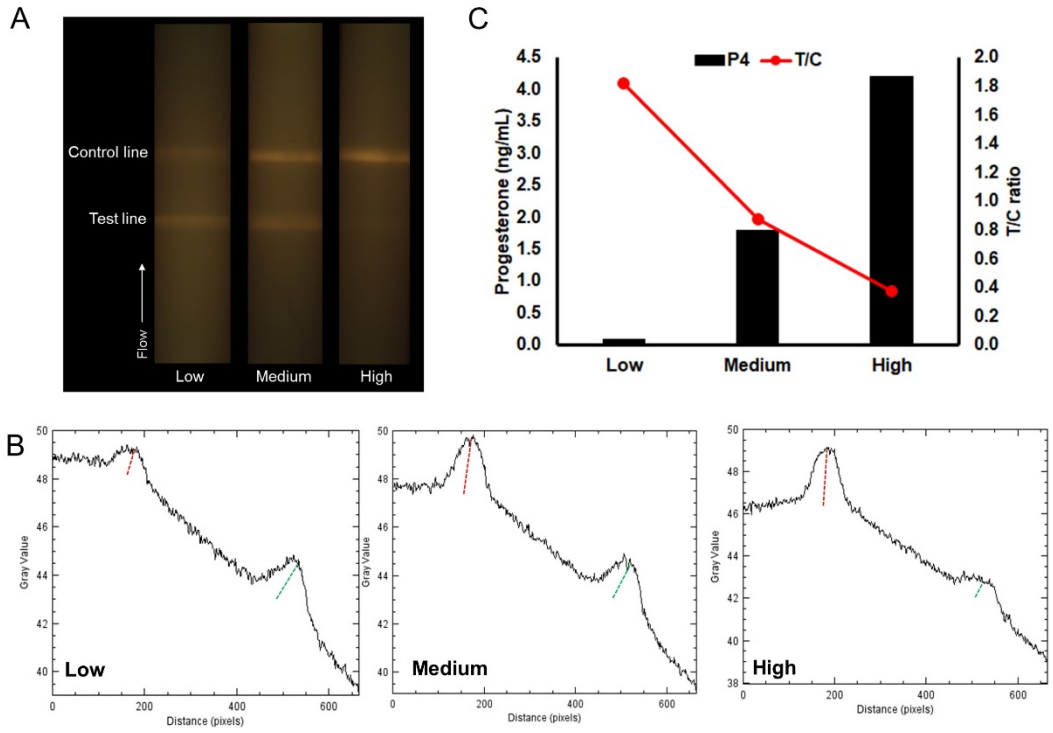


Figure 2. (A) Set of typical fluorescence images acquired by the portable reader from bovine plasma samples with low, medium, and high progesterone (P4) levels. Because of the competitive design, the colorimetric signal of the test line decreased as P4 concentration increased, whereas the colorimetric signal of the control line increased as P4 concentration increased. (B) Profile plots of the test and control line regions created with the image processing software. Green dashed line corresponds to the test line region whereas the red dashed line corresponds to the control line region. (C) Graph depicting changes in average test-to-control-line-ratio (red solid line) with increasing levels of P4 (blacks bars). As expected from a competitive LFIA design, test-to-control ratios decreased as P4 levels increased.

lines in order to calculate T/C ratios. As expected from a competitive LFIA design, T/C ratios decreased as P4 levels increased (Figure 2C).

The use of calibration curves in LFIA systems for the quantitative prediction of the concentration of an analyte has been previously reported (Lu et al., 2017; Srinivasan et al., 2018). In the present study, however, we created calibration curves to estimate a dichotomized rather than a quantitative P4 concentration in order to determine the ability of the LFIA system to differentiate bovine plasma samples with ≥ 1 or < 1 ng/mL of P4 as this may have be the most obvious practical value. Because of the known high variation in performance between different batches of LFIA strips (Sajid et al., 2015), we constructed a calibration curve for each experiment (i.e., one for each batch). Features and measures of variability for the calibration curves for each experiment are presented in Table 1, and representative examples of the curves are illustrated in Figure 3 (all other curves are presented in Supplementary Figure 1). Four out of 6 experiments showed a significant effect ($P < 0.05$) or a trend ($P = 0.06$) for the linear relationship between average T/C ratio and P4 concentration (average $R^2 = 0.88$; range 0.74 to 0.99). In contrast, calibration test results for experiments 3 and 6 did not show a statistically significant linear trend, and both curves had a suboptimal R^2 (experiment 3 = 0.53; experiment 6 = 0.42). The lack of a linear trend and the resulting low R^2 for these two experiments can be explained, at least in part, by the presence of outliers (*i.e.,* observations with an absolute value of studentized residual greater than 3). Indeed, when outliers were removed (one sample for each experiment), we observed a statistically significant ($P < 0.05$) linear trend for both experiments and an increase in the R^2 (experiment 3 = 0.94; experiment 6 = 0.98). In both cases, the samples removed corresponded to those within the range of 0.1 to 1.0 ng/mL (experiment 3 = 0.6 ng/mL; experiment 6 = 0.8 ng/mL), which might indicate that our current LFIA design is less accurate

Table 1. Calibration curves features and measures of variability.

Experiment[1] no.	No. of Samples	P4 range (ng/mL)	CV (%)[2]	Linear Model R^2	*P*-value
1	5	0.1 – 2.0	15.9	0.96	< 0.01
2	5	0.1 – 2.0	6.3	0.99	< 0.01
3	5	0.1 – 2.0	11.1	0.53	0.16
4	5	0.1 – 3.7	13.3	0.81	0.04
5	5	0.1 – 3.7	10.1	0.74	0.06
6	5	0.1 – 3.2	10.0	0.42	0.24

[1]Experiment: six batches of test strips were manufactured to perform six independent experiments (i.e., one batch per experiment). For each experiment, a calibration curve was constructed using five plasma samples with known concentrations of progesterone.
[2]CV = average intra-assay coefficient of variation.

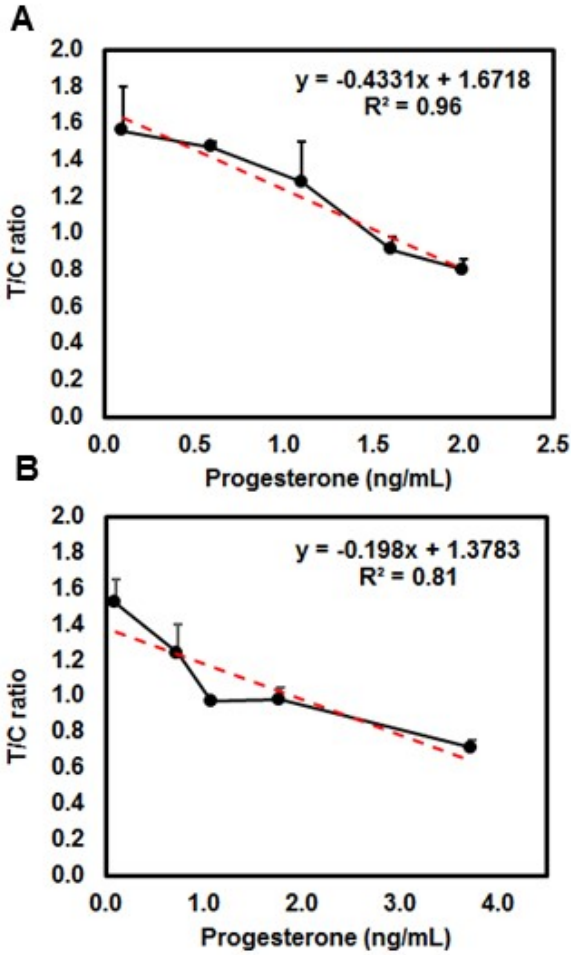


Figure 3. Representative calibration curves (experiments 1 and 4) showing the average T/C ratios of the colorimetric signals at various (n = 5) standard P4 concentrations in bovine plasma. Dotted red line shows linear curve fit. Error bars shown are standard error, n = 3.

within that particular range of P4 concentrations. From a practical perspective, this is somewhat problematic because differentiating samples in the range of P4 concentrations around 1 ng/mL is likely the most valuable. The cutoff commonly used to determine the presence or absence of a functional CL is 1 ng/mL, and based on recent research there may be interest on differentiating samples in the range of 0.3 to 0.5 ng/mL (Brusveen et al., 2009). Using the latter cutoffs at the time of the GnRH treatment for induction of ovulation before timed AI was better than the traditional 1 ng/mL cutoff to separate cows with different likelihood of pregnancy after timed AI (Brusveen et al., 2009).

As expected, our platform was able to accurately differentiate plasma samples with ≥ 1 or < 1 ng/mL of P4 (Table 2). We observed high sensitivity ($P < 0.0001$; Table 2) which resulted in a low number of false negatives and a high ($P < 0.0001$) negative predictive value. Similarly, albeit of a lower magnitude, specificity and positive predictive value for the LFIA strips were also high (Table 2; $P < 0.01$). Average intra-assay CV for the 58 samples was 16.0% (range; 0.5 to 42.8). Collectively, the high sensitivity and specificity of the LFIA-based system resulted in an overall high accuracy (90%; $P < 0.0001$) for classification of plasma samples in groups with ≥ 1 or < 1 ng/mL of P4. Removing the outliers from the calibration curves in experiments 3 (n = 1) and 6 (n = 1) increased the T/C ratio cutoff value required to differentiate plasma samples with ≥ 1 or < 1 ng/mL of P4, increasing the number of false negatives. Although removing outliers resulted in no major change in specificity (83%, 95% CI 64 to 94), this change resulted in a reduction in sensitivity from 97% (95% CI 82 to 99) to 93% (95% CI 77 to 99), which reduced overall accuracy to 88% (95% CI 77 to 95). Thus, removing outliers from calibration curves dramatically increased the R^2 of the linear models but did not improve overall accuracy of the assay. In addition, the kappa statistic with ($P < 0.0001$; kappa = 0.79, 95% CI 0.64 to 0.95) and

Table 2. Outcomes of performance for the ability of the LFIA system to differentiate plasma samples with ≥ 1 or < 1 ng/mL of progesterone.

Parameter[1]	Value	95% CI	*P*-value
Sensitivity, % (n/n)	97 (28/29)	82, 99	< 0.0001
Specificity, % (n/n)	83 (24/29)	64, 94	< 0.01
PPV[2], % (n/n)	85 (28/33)	68, 95	< 0.0001
NPV[3], % (n/n)	96 (24/25)	80, 99	< 0.0001
Accuracy, % (n/n)	90 (52/58)	79, 96	< 0.0001
Kappa value	0.79	0.64, 0.95	< 0.0001

[1] Six batches of test strips were manufactured to perform six independent experiments (i.e., one batch per experiment). To calculate each parameter (and respective confidence interval), data of all experiments combined (n = 58 samples) were used. Confidence intervals and exact tests for sensitivity, specificity, PPV, NPV, and accuracy were computed under the null hypothesis that the proportion equals 0.5. For the kappa statistic, the null hypothesis was set to kappa = 0 (no agreement).
[2] PPV = positive predictive value.
[3] NPV = negative predictive value.

without outliers ($P < 0.0001$; kappa = 0.76, 95% CI 0.59 to 0.93) was very similar and in both cases represented substantial agreement (Watson and Petrie, 2010) suggesting that, for future experiments, removing one outlier from a calibration curve might not be necessary in order to improve performance of the LFIA.

The reason for the greater number of false positives than false negatives when implementing the current LFIA design is unclear at the moment. One possible explanation may be cross-reactivity of the antibodies with analytes other than P4. Indeed, manufacturers of the anti-P4 antibody used in the current LFIA report 25% cross-reactivity with 11-Hydroxyprogesterone and 10% towards 5-alpha-pregnane-3,20 dione, both of which are P4 metabolites. In addition, all samples that led to false positive outcomes had P4 levels in the range of 0.2 to 0.8 ng/mL, whereas all samples with P4 below this range (n = 18) were accurately classified as negatives. These results suggest that perhaps our current LFIA design is less accurate to correctly classify samples when P4 levels are in the range of 0.1 to 1 ng/mL, in particular those with P4 levels in the range of 0.2 to 0.8 ng/mL. The practical relevance of inaccurately classifying cows as having ≥ 1 or < 1 ng/mL of P4 when they have such circulating P4 levels may depend on the specific use of the assay results. For example, it may be less problematic when the assay is used to assign cows to synchronization of ovulation protocols (i.e., treatment the cow receives may not be optimal but result in acceptable fertility) than when using it to confirm estrus or luteal regression at the end of a timed AI program. The consequences of false positive outcomes are greater for the latter situations (i.e., decide not to inseminate a cow when the cow is truly in estrus or assume failure to regress a CL before timed AI). Given the flexibility of the tool developed, these issues may be circumvented by adapting assay outcomes to its intended use. For example, different T/C ratios could be used to classify cows in groups of

circulating P4 levels if the tool is used to confirm estrus or assign cows to synchronization of ovulation protocols based on the presence of a functional CL. A caveat of using circulating P4 concentrations as the sole method to determine the presence of a functional CL is assuming that circulating concentrations of P4 ≥ 1 ng/mL are always indicative of the presence of a functional CL. It is well known that some cows may have circulating concentrations of P4 ≥ 1 ng/mL due to the presence of a luteal or a partially luteinized follicular cyst capable of producing sufficient P4 to rise circulating concentrations above the cutoff (Giordano et al., 2016). Thus, further research is necessary to determine the practical implications of misclassifying cows in CL functionality groups based on circulating P4. Although this is uncertain at the moment, we speculate that the effect of misclassifying cows on herd reproductive performance and economics will depend on the type of reproductive management decision made based on results from the LFIA system.

To examine the diagnostic ability of the current LFIA to differentiate plasma samples with ≥ 1 or < 1 ng/mL of P4 without a calibration curve for individual batches, we performed ROC analyses to identify a single cutoff value for T/C ratio that could potentially be used for all batches. To generate the ROC curves, we used the traditional cutoff value (i.e., 1 ng/mL) for P4 concentration for determination of the presence of a functional CL (Ginther et al., 2010). Although there may be physiological conditions under which this cutoff is not necessarily associated with the presence of a functional CL, we used this value because it is commonly used in research and in practice. The optimal cutoff T/C ratio selected based on the ROC curve constructed with the 1 ng/mL cutoff for P4 resulted in a sensitivity and specificity of 96.5% (95% CI 82.2 to 99.9) and 79.3% (95% CI 60.3 to 92.0), respectively (Figure 4). The T/C ratio cutoff at which sensitivity and specificity were optimized was ≤ 1.35. We also examined the area under the curve (**AUC**), as a summary measure of the overall accuracy of a diagnostic test

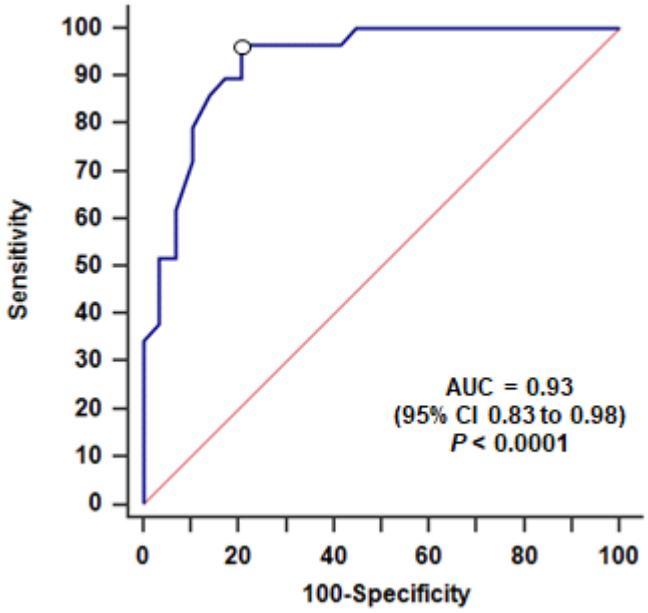


Figure 4. Receiver operating characteristic curve for T/C ratio of the LFIA strips and plasma progesterone concentration ≥ 1 ng/mL or < 1 ng/mL as the reference test. The white circle in the left corner of the curve illustrates the optimal sensitivity (96.5%; 95% CI 82.2 to 99.9) and specificity (79.3%; 95% CI 60.3 to 92.0) determined by the ROC analysis. The cutoff T/C ratio value at this point was ≤ 1.35. The area under the curve was 0.93 (P < 0.0001; 95% CI 0.83 to 0.98).

(Šimundić, 2009; Watson and Petrie, 2010). In this case, the AUC indicates the probability that a randomly selected sample obtained from a group of samples with P4 concentration ≥ 1 ng/mL would have a higher predicted probability of being classified as high P4 than a randomly chosen sample from a group of samples with P4 < 1 ng/mL (Watson and Petrie, 2010). For example, when AUC = 1, the test is perfectly accurate, meaning that samples with unknown concentrations of P4 would be always correctly assigned to high or low P4 groups based on the cutoff of interest. Conversely, if the AUC is equal to 0.5 the test is no better than chance alone at discriminating between the two outcomes (Watson and Petrie, 2010), meaning that samples with unknown concentrations of P4 would have a 50% chance of being incorrectly assigned to the high or low P4 group. In the present study, the AUC was 0.93 ($P < 0.0001$; 95% CI 0.83 to 0.98), which is generally classified as of excellent diagnostic accuracy (Šimundić, 2009). Although the specificity for the ROC to differentiate plasma samples with ≥ 1 or < 1 ng/mL of P4 was lower than that obtained with calibration curves, using a single cutoff value reduces overall complexity of the system for on-farm implementation. In addition, our current LFIA assembly is done manually with one batch at a time, significantly increasing variation between batches. Future automated assembly at a commercial scale may significantly reduce variation across batches, which can potentially improve the performance of the LFIA using a single T/C ratio cutoff when compared to that obtained with calibration curves.

Overall, results from the ROC analyses showed that sensitivity increased, and specificity decreased as the cutoff T/C ratio increased (Table 3). In practice, different thresholds for T/C ratio can be selected to either favor sensitivity or specificity, depending on the intended application of the LFIA system results. For instance, if the intent is to maximize early reinsemination of nonpregnant cows after a previous AI, probably a higher cutoff is more

Table 3. Sensitivity and specificity for different T/C ratio cutoff points obtained by ROC analysis to differentiate plasma samples with ≥ 1 or < 1 ng/mL of progesterone with the LFIA system.

T/C ratio cutoff[1]	Sensitivity (%)[3]	95 % CI	Specificity (%)[3]	95 % CI
< 0.37	0	0 – 12	100	88 – 100
≤ 0.77	34	18 – 54	100	88 – 100
≤ 0.81	38	21 – 58	97	82 – 100
≤ 0.90	52	33 – 71	97	82 – 100
≤ 0.92	52	33 – 71	93	77 – 99
≤ 1.02	62	42 – 79	93	77 – 99
≤ 1.05	72	53 – 87	90	73 – 98
≤ 1.08	79	60 – 92	90	73 – 98
≤ 1.09	86	68 – 96	86	68 – 96
≤ 1.25	90	73 – 98	83	64 – 94
≤ 1.26	90	73 – 98	79	60 – 92
≤ 1.35[2]	97	82 – 100	79	60 – 92
≤ 1.42	97	82 – 100	59	39 – 77
≤ 1.45	100	88 – 100	55	36 – 74
≤ 2.52	100	88 – 100	0	0 – 12

[1]Test-to-control-line ratio.
[2] Optimal cutoff indicated by the receiver operating characteristic curve analyses.
[3] A radioimmunoassay for progesterone was used as the reference test.

appropriate because we would increase sensitivity and thus reduce the misclassification of cows as nonpregnant when they are truly pregnant. However, if the intent is to assign cows to specific hormonal treatments, a lower cutoff may be more appropriate because we would increase specificity and thus avoid misclassification of cows as having a functional CL present when in fact they lack a functional CL. This is relevant because a very well documented problem with cows in the Ovsynch protocol for synchronization of ovulation is that cows that lack a functional CL at the time of the $PGF_{2\alpha}$ treatment will have reduced fertility when compared with cows with a CL (Giordano et al., 2016). These cows can instead be re-assigned to another synchronization of ovulation protocol designed to increase fertility of cows lacking a functional CL (Wijma et al., 2018). Additional research is needed to confirm that values for sensitivity and specificity can be customized for different applications by modifying the T/C ratio cutoff.

Because of the asynchrony in timing of structural and functional luteolysis (*i.e.*, physical disappearance of the CL vs. the cessation of P4 production by luteal cells and metabolism of P4), traditional methods for on-farm determination of functional CL presence (i.e., transrectal palpation and transrectal ultrasonography) usually result in high sensitivity but low specificity. This is due, at least in part, to the fact that luteal tissue can be palpated (rectal palpation) or visualized (transrectal ultrasonography) for days after P4 production ceases (Herzog et al., 2010). As a result, cows are misclassified as having a functional CL when in fact P4 is already below 1 ng/mL (*i.e.*, false positives). For instance, Kelton et al. (1991) reported that the sensitivity and specificity of transrectal palpation for diagnosing a functional CL were 82.6% and 52.6%, respectively. Similarly, Bicalho et al. (2008) and Sauls-Hiesterman et al. (2020) reported that the sensitivity and specificity for transrectal ultrasonography were approximately 90% and 40%, respectively. Because our LFIA system predicts the presence of a functional CL based on P4

levels rather than rectal palpation of the ovaries or visual observation of luteal tissue by ultrasonography, the number of false positives may be reduced, which may explain the substantially greater specificity compared with reported values for some of the traditional methods to detect the presence of a functional CL. In contrast, our results for specificity, overall accuracy, and interrater agreement are in the same range than previously reported in studies in which determination of a CL by transrectal ultrasonography was optimized by using a cutoff CL diameter rather than strict visualization of luteal tissue (Bicalho et al., 2008; McArt et al., 2010). A caveat of the current LFIA system is that it cannot differentiate the source of P4. Therefore, it will not be possible to differentiate a functional CL from other ovarian structures that may be present on the ovaries and produce P4 such as luteal or follicular cysts with a partially luteinized wall.

Unlike the traditional methods, our LFIA portable system provides the additional advantage of not requiring expensive equipment or expertise to diagnose CL presence. In addition, if our system ever becomes immediate and cow-side (i.e., no need for sample processing and includes fully automated image processing), then it could potentially be more beneficial than traditional methods because it will not need expert personnel to diagnose CL presence. As for traditional methods; however, our current LFIA system also presents a number of limitations that need to be addressed. For example, the need to use plasma rather than whole blood and the total time from sample dispensing until results become available (~18 min). Thus, future work will focus on further optimizing the performance of our current LFIA design for determination of P4 concentrations in whole blood samples and strategies to reduce time from sample collection until results become available. In addition, algorithms for automated image processing and determination of T/C ratios need to be developed and validated.

CONCLUSION

We developed and demonstrated a simple and novel method for estimating concentrations of P4 in bovine plasma samples from lactating dairy cows. The system consisting of a disposable fluoresence-based LFIA combined with a fluorescence portable imaging device presented high accuracy for differentiation of plasma samples with ≥ 1 or < 1 ng/mL of P4. Additional improvements of this assay system may lead to the development of a rapid, low-cost, cow-side tool for determination of the reproductive status of cattle based on estimation of circulating concentrations of P4.

ACKNOWLEDGMENT

This work was supported by the USDA National Institute of Food and Agriculture, AFRI Foundational program area priority Nanotechnology for Agricultural and Food Systems project number 2016-08814. Any opinions, findings, conclusions, or recommendations expressed in this publication are those of the authors and do not necessarily reflect the view of the National Institute of Food and Agriculture (NIFA) or the United States Department of Agriculture (USDA). This research work was also partially supported by the Cornell Initiative for Digital Agriculture (CIDA). The authors thank Manuel Masello for his assistance designing some of the figures presented in the current manuscript.

REFERENCES

Adriaens, I., W. Saeys, T. Huybrechts, C. Lamberigts, L. François, K. Geerinckx, J. Leroy, B. De Ketelaere, and B. Aernouts. 2018. A novel system for on-farm fertility monitoring based on milk progesterone. J. Dairy Sci. 101:8369-8382.

Barletta, R., P. Carvalho, V. Santos, L. Melo, C. Consentini, A. Netto, and P. Fricke. 2018. Effect of dose and timing of prostaglandin F2α treatments during a Resynch protocol on luteal regression and fertility to timed artificial insemination in lactating Holstein cows. J. Dairy Sci. 101:1730-1736.

Bicalho, R., K. Galvão, C. Guard, and J. Santos. 2008. Optimizing the accuracy of detecting a functional corpus luteum in dairy cows. Theriogenology 70:199-207.

Bruinjé, T. and D. Ambrose. 2019. Validation of an automated in-line milk progesterone analysis system to diagnose pregnancy in dairy cattle. J. Dairy Sci. 102:3615-3621.

Brusveen, D., A. Souza, and M. Wiltbank. 2009. Effects of additional prostaglandin F2α and estradiol-17β during Ovsynch in lactating dairy cows. J. Dairy Sci. 92:1412-1422.

Friggens, N., M. Bjerring, C. Ridder, S. Højsgaard, and T. Larsen. 2008. Improved detection of reproductive status in dairy cows using milk progesterone measurements. Reproduction in Domestic Animals 43:113-121.

Galvão, K., M. Frajblat, W. Butler, S. Brittin, C. Guard, and R. Gilbert. 2010. Effect of early postpartum ovulation on fertility in dairy cows. Reprod. Dom. Anim. 45: 207-211.

Ginther, O., H. Shrestha, M. Fuenzalida, A. Shahiduzzaman, M. Hannan, and M. Beg. 2010. Intrapulse temporality between pulses of a metabolite of prostaglandin F2α and circulating concentrations of progesterone before, during, and after spontaneous luteolysis in heifers. Theriogenology 74:1179-1186.

Giordano, J., A. Kalantari, P. Fricke, M. Wiltbank, and V. Cabrera. 2012. A daily herd Markov-chain model to study the reproductive and economic impact of reproductive programs combining timed artificial insemination and estrus detection. J. Dairy Sci. 95:5442-5460.

Giordano, J., M. Thomas, G. Catucuamba, M. Curler, M. Masello, M. Stangaferro, and R. Wijma. 2016. Reproductive management strategies to improve the fertility of cows with a suboptimal response to resynchronization of ovulation. J. Dairy Sci. 99:2967-2978.

Hankele, A. K., K. Rehm, J. Bérard, G. Schuler, L. Bigler, and S. E. Ulbrich. 2020. Progestogen profiling in plasma during the estrous cycle in cattle using an LC-MS based approach. Theriogenology 142:376-383.

Herzog, K., M. Brockhan-Lüdemann, M. Kaske, N. Beindorff, V. Paul, H. Niemann, and H. Bollwein. 2010. Luteal blood flow is a more appropriate indicator for luteal function during the bovine estrous cycle than luteal size. Theriogenology 73:691-697.

Kelton, D. F., K. E. Leslie, W. G. Etherington, B. N. Bonnett, and J. S. Walton. 1991. Accuracy of rectal palpation and of a rapid milk progesterone enzyme-immunoassay for determining the presence of a functional corpus luteum in subestrous dairy cows. Can. Vet. J. 32:286-291.

Landis, J. R. and G. G. Koch. 1977. The measurement of observer agreement for categorical data. Biometrics 33:159-174.

Lu, Z., D. O'Dell, B. Srinivasan, E. Rey, R. Wang, S. Vemulapati, S. Mehta, and D. Erickson. 2017. Rapid diagnostic testing platform for iron and vitamin A deficiency. Proc. Natl. Acad. Sci. 114:13513-13518.

Masello, M., M. Scarbolo, M. V. Schneck, M. M. Perez, E. M. Schillkowsky, E. M. Sitko, F. Sosa Hernandez, E. M. Cabrera, R. M. Rossi, and J. O Giordano. 2019. Intravaginal instillation of prostaglandin F2α was as effective as intramuscular injection for induction of luteal regression in lactating dairy cows. J. Dairy Sci. 103: https://doi.org/10.3168/jds.2019-17589. In press.

McArt, J., L. Caixeta, V. Machado, C. Guard, K. Galvao, O. Sá Filho, and R. Bicalho. 2010. Ovsynch versus Ultrasynch: reproductive efficacy of a dairy cattle synchronization protocol incorporating corpus luteum function. J. Dairy Sci. 93:2525-2532.

NAHMS.2018. Dairy 2014, Part III: Reference of Dairy Cattle Health and Management Practices in the United States, 2014. #696.0218. Centers for Epidemiology and Animal Health, USDA, Animal and Plant Health Inspection Service, Veterinary Services, Fort Collins, CO.

Nakami, W., V. Tsuma, K. Milkey, M. Dickerson, M. Wong, H. Mutembei, J. Muthee, O. Odipo, and W. Ngetich. 2017. Lateral flow immunoassay for whole blood progesterone detection as a tool for assessment of reproductive status in cattle. Inter. J. Vet. Sci. 6:19-25.

Posthuma-Trumpie, G. A., A. van Amerongen, J. Korf, and W. J. van Berkel. 2009. Perspectives for on-site monitoring of progesterone. Trends Biotechnol. 27:652-660.

Sajid, M., A.-N. Kawde, and M. Daud. 2015. Designs, formats and applications of lateral flow assay: A literature review. J. Saudi Chem. Soc. 19:689-705.

Sauls-Hiesterman, J., B. Voelz, and J. Stevenson. 2020. A shortened resynchronization treatment for dairy cows after a nonpregnancy diagnosis. Theriogenology 141:105-112.

Schneider, C. A., W. S. Rasband, and K. W. Eliceiri. 2012. NIH Image to ImageJ: 25 years of image analysis. Nature methods 9:671.

Šimundić, A.-M. 2009. Measures of diagnostic accuracy: basic definitions. EJIFCC 19:203-211.

Skenandore, C., A. Pineda, J. Bahr, A. Newell-Fugate, and F. Cardoso. 2017. Evaluation of a commercially available radioimmunoassay and enzyme immunoassay for the analysis of progesterone and estradiol and the comparison of two extraction efficiency methods. Domest. Anim. Endocrinol. 60:61-66.

Srinivasan, B., D. O'Dell, J. L. Finkelstein, S. Lee, D. Erickson, and S. Mehta. 2018. ironPhone: mobile device-coupled point-of-care diagnostics for assessment of iron status by quantification of serum ferritin. Biosens. Bioelectron. 99:115-121.

Stevenson, J. 2019. Spatial relationships of ovarian follicles and luteal structures in dairy cows subjected to ovulation synchronization: Progesterone and risks for luteolysis, ovulation, and pregnancy. J. Dairy Sci. 102:5686-5698.

Tisone, T. C. and B. O'Farrell. 2009. Manufacturing the next generation of highly sensitive and reproducible lateral flow immunoassay. Pages 131- 156 in Lateral Flow Immunoassay. Humana Press.

Waldmann, A. and A. Raud. 2016. Comparison of a lateral flow milk progesterone test with enzyme immunoassay as an aid for reproductive status determination in cows. Vet. Rec. 178: 260.

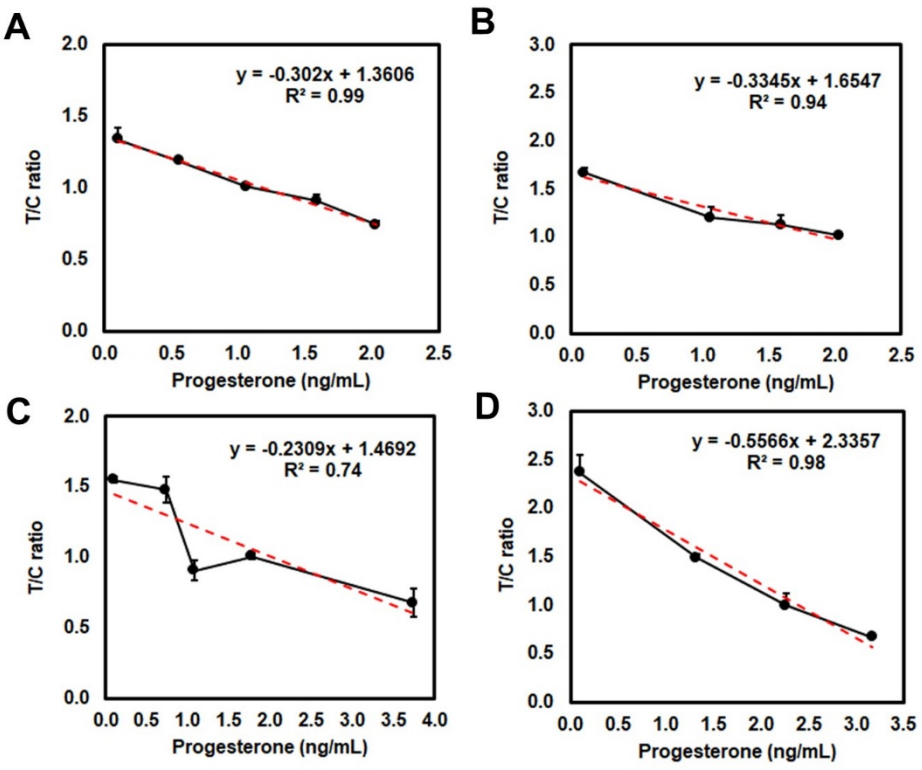
Watson, P. and A. Petrie. 2010. Method agreement analysis: a review of correct methodology. Theriogenology 73:1167-1179.

White, R., M. Brady, J. Capper, J. McNamara, and K. Johnson. 2015. Cow–calf reproductive, genetic, and nutritional management to improve the sustainability of whole beef production systems. J. Anim. Sci. 93:3197-3211.

Wijma, R., M. Pérez, M. Masello, M. Stangaferro, and J. Giordano. 2018. A resynchronization of ovulation program based on ovarian structures present at nonpregnancy diagnosis reduced time to pregnancy in lactating dairy cows. J. Dairy Sci. 101:1697-1707.

Wilsdorf, L., D. Keisler, S. Poock, W. Lamberson, R. Escalante, and M. Lucy. 2016. Test for nonpregnancy in dairy cows based on plasma progesterone concentrations before and after timed artificial insemination. J. Dairy Sci. 99:5858-5865.

Xu, C., W. Yang, C. Xia, L. Wu, and H. Zhang. 2016. Development of a competitive lateral flow immunoassay for progesterone in dairy cows' milk. Med. Weter. 72:494-497.



Supplemental Figure 1. Calibration curves from experiments 2, 3, 5, and 6 showing the average T/C ratios of the colorimetric signals at various standard P4 concentrations in bovine plasma. Dotted red line shows linear curve fit. Error bars shown are standard error, n = 3.

CHAPTER V

INTRAVAGINAL INSTILLATION OF PROSTAGLANDIN-F2α WAS AS EFFECTIVE AS INTRAMUSCULAR INJECTION FOR INDUCTION OF LUTEAL REGRESSION IN LACTATING DAIRY COWS

M. Masello, M. Scarbolo, M.V. Schneck, M. M. Perez, E. M. Schillkowsky, E. M. Sitko,

F. Sosa Hernandez, E.M. Cabrera, R.M. Rossi, and J. O. Giordano.

Journal of Dairy Science 2020; 103(3): 2743-2755.

DOI: 10.3168/jds.2019-17589

ABSTRACT

Our objectives were to test the efficacy of intravaginal (**IVG**) administration of $PGF_{2\alpha}$ (**PGF**) to induce corpus luteum (**CL**) regression, compare circulating progesterone (**P4**) profiles in cows receiving IVG versus intramuscular treatment with PGF, and evaluate reproductive outcomes. Lactating Holstein cows were synchronized using a Double-Ovsynch (**DO**) protocol (GnRH, 7 d later $PGF_{2\alpha}$, 3 d later GnRH, 7 d later GnRH, 7 d later $PGF_{2\alpha}$, 1 d later $PGF_{2\alpha,}$ 32 h later GnRH, 16 to 20 h TAI) to receive timed AI (**TAI**) at 67 ± 3 DIM. Seven days after the first GnRH treatment (time 0), cows with at least one visible CL ≥15 mm were blocked by parity and randomly assigned to a treatment that consisted of i.m. injection (**IM-PGF**; n = 201) or IVG instillation (**IVG-PGF**; n = 201) of PGF. Cows in IM-PGF received a single 25 mg dose of PGF (Dinoprost tromethamine) intramuscular. Cows in IVG-PGF received two 25 mg doses of PGF 12 h apart delivered through a catheter in the cranial portion of the vagina. Blood samples were collected at 0, 12, 48 and 72 h after treatment. Ovulation to the first GnRH of DO was

determined through transrectal ultrasonography. Only cows with P4 ≥1 ng/mL (functional CL) at time 0 (IM-PGF = 169; IVG-PGF = 179) were included in the analyses. Binary and quantitative data were analyzed by logistic regression and ANOVA with repeated measures, respectively. Results are presented as least squares means (**LSM**). Concentrations of P4 and the proportion of cows with a new CL at time 0 did not differ. Overall, the proportion of cows with CL regression using 1 ng/mL (IM-PGF = 89.0%, IVG-PGF = 86.7%) or 0.5 ng/mL (IM-PGF = 82.2%, IVG-PGF = 82.1%) of P4 as cutoff did not differ. Concentrations of P4 were affected by treatment, time and the treatment by time interaction. Cows in IVG-PGF had greater mean P4 at 12 h than cows in IM-PGF. Mean P4 did not differ at 48 or 72 h after treatment. The proportion of cows with estrus recorded within three days of treatment (IM-PGF = 45.4%; IVG-PGF = 48.9%), ovulation risk after treatment (IM-PGF = 88.5%; IVG-PGF = 85.1%) and P/AI after TAI (IM-PGF = 51.5%; IVG-PGF = 57.8%) did not differ. We concluded that two intravaginal doses of 25 mg of PGF 12 h apart were as effective as a single 25 mg intramuscular dose of $PGF_{2\alpha}$ for inducing luteal regression in lactating dairy cattle.

Keywords: intravaginal, prostaglandin-$F_{2\alpha}$, luteal regression, cattle

INTRODUCTION

Effective reproductive management programs for cattle optimize reproductive performance through timely and successful insemination of cows after they become eligible for pregnancy. In this regard, timed AI (**TAI**) after synchronization of ovulation is a technology essential for reproductive success in many dairy and beef cattle operations in North America and around the world (Lamb et al., 2010; Wiltbank and Pursley, 2014). Synchronization of ovulation

protocols consist of a combination of two or more hormonal treatments administered in a sequence to manipulate follicular wave dynamics, corpus luteum (**CL**) regression, and ovulation (Pursley et al., 1995; Pursley et al., 1997). Ultimately, timing of ovulation is synchronized within a narrow time frame facilitating insemination of groups of cows by appointment regardless of estrus expression and detection (Pursley et al., 1995; Vasconcelos et al., 1999; Cartmill et al., 2001).

A wide variety of simple protocols ranging in type, number, and sequence of hormonal treatments are available and used by cattle operations with the main purpose of ensuring immediate insemination after the end of the voluntary waiting period or after a failed AI service (Chebel et al., 2006; Wijma et al., 2018). More sophisticated protocols including up to six or seven hormonal treatments at inconvenient intervals, and some including sustained hormone release implants can also be used to increase the fertility of dairy (Souza et al., 2008; Bartolome et al., 2009) and beef females (Bridges et al., 2008; Kasimanickam et al., 2012). Regardless of their purpose and complexity, a common drawback of all synchronization of ovulation protocols is the need to administer multiple hormonal treatments. Such treatments are most commonly given in the form of i.m. or s.c. injections and in some cases through implantation of sustained hormone release intravaginal devices. In all cases cows that are synchronized need to be located or sorted in a pen and restrained in self-locking head gates, stalls, or palpation rails to receive injections and/or implants. These interventions can be labor intensive, costly, and affect cow natural behaviors. In addition, human and technical errors can reduce compliance impairing protocol efficacy (Stevenson and Phatak, 2005; Galvão et al., 2013). Thus, automation of synchronization of ovulation treatments may help reduce the burden of implementing synchronization of ovulation protocols, reducing labor needs, improving cow welfare by

decreasing cow manipulation, and potentially improving reproductive performance by improving compliance and facilitating the development of novel protocols that optimize the physiological response to hormonal treatments.

A potential strategy to automate synchronization of ovulation is through electronically-controlled hormone delivery devices inserted in body cavities such as the vagina. Successful synchronization of ovulation by automated intravaginal hormone delivery devices is only feasible if hormones used in synchronization protocols elicit the physiological response of interest when given intravaginally. In this regard, Wijma et al. (2016) reported that intravaginal (**IVG**) delivery of two doses of 25 mg of Dinoprost (i.e., natural form of $PGF_{2\alpha}$) 12 h apart successfully induced complete luteal regression and resulted in a circulating progesterone (**P4**) profile similar to that of lactating dairy cows that received a single 25 mg i.m. dose of PGF2α. Circulating concentrations of P4 at 48, 60, and 72 h after treatment did not differ for cows that received IVG or i.m. $PGF_{2\alpha}$, and a similar proportion of cows in both groups had complete CL regression by 60 and 72 h after treatment. Wijma et al. (2016) also reported that delivering two 25 mg doses of Dinoprost 12 h apart was the most effective regimen to induce complete CL regression compared with smaller or larger doses (i.e., 25 and 125 mg) or a similar dose (i.e., 50 mg) delivered as a single treatment. Two 25 mg doses of $PGF_{2\alpha}$ 12 h apart resulted in a P4 concentration dynamics similar to that of cows that received a single 25 mg i.m. dose of Dinoprost making this dose and treatment frequency for IVG delivery a reasonable choice for future experiments to evaluate CL regression and synchronization of ovulation. A caveat of the experiments reported in Wijma et al. (2016) was the limited number of cows used because objectives were to provide proof of concept for IVG delivery of PGF2α and identify a feasible $PGF_{2\alpha}$ dose and treatment frequency for successful induction of CL regression. Thus, it was not

possible to determine if IVG delivery of $PGF_{2\alpha}$ would be as effective as i.m. treatment for induction of complete CL regression and whether or not it could be used as an alternative route of $PGF_{2\alpha}$ administration in cattle.

Therefore, the primary objectives of the current experiment were to evaluate CL regression and the P4 concentration profile of dairy cows treated with $PGF_{2\alpha}$ by IVG instillation or i.m. injection. A secondary objective was to evaluate reproductive outcomes that depend on completion of CL regression. We hypothesized that the proportion of cows with complete CL regression and the P4 concentration profile would be similar for cows that received IVG and i.m. treatment with $PGF_{2\alpha}$. We also hypothesized that the proportion of cows detected in estrus and ovulation after treatment with GnRH would be similar for cows that received IVG or i.m. treatment with $PGF_{2\alpha}$ during the presynchronization portion of a fertility protocol for TAI.

MATERIALS AND METHODS

All procedures performed with cows were approved by the Animal Care and Use Committee of Cornell University.

Cows and General Management

Lactating Holstein Cows (n = 402) from the dairy unit of the Cornell University Ruminant Center (Harford, NY, USA) were enrolled in this experiment conducted from September 2017 to November 2018. Cows were housed in freestall barns, with concrete flooring, self-locking headgates, and fans and sprinklers in the feedline. During the experimental period, the average number of milking cows was 594 (range 538 to 629) with average daily milk yield per cow of 42 kg/d (range 39 to 50). Data for cow numbers and milk yield were retrieved from

the dairy herd management software (DairyComp305, ValleyAg Software, Tulare, CA) using the MONITOR command. Cows were milked thrice daily at ~8 h intervals and were fed a TMR once a day with ad libitum access to feed and water. The diet was formulated to meet or exceed the nutrient requirements of lactating dairy cows producing 48 kg/d with 3.8% fat and 3.1% true protein as determined by the Cornell Net Carbohydrate and Protein System (Higgs et al., 2015).

Experimental Treatments

This experiment was conducted as a randomized complete block design using parity group (*i.e.,* primiparous vs. multiparous) as blocking factor. Every week, lactating nonpregnant cows at 40 ± 3 DIM were enrolled in the Double-Ovsynch (**DO**) protocol (GnRH, 7 d later $PGF_{2\alpha}$, 3 d later GnRH, 7 d later GnRH, 7 d later $PGF_{2\alpha}$, 1 d later $PGF_{2\alpha,}$ 32 h later GnRH, 16 to 20 h TAI) to receive first AI service at 67 ± 3 DIM. At the time of first $PGF_{2\alpha}$ treatment of the DO protocol, transrectal ultrasonography (**TUS**) of the ovaries was performed using a portable ultrasound machine with a 7.5 MHz attached linear probe (Ibex Pro; E.I Medical Imaging, Loveland, CO). Cows with at least one visible corpus luteum (**CL**) ≥ 15 mm in diameter were blocked by parity and randomly allocated to the intramuscular $PGF_{2\alpha}$ (**IM-PGF**; n = 201) or the intravaginal $PGF_{2\alpha}$ (**IVG-PGF**; n = 201) treatment groups. Cows without a CL ≥ 15 mm in diameter were excluded from the experiment. Immediately after randomization, cows in the IM-PGF treatment received 25 mg of $PGF_{2\alpha}$ (Dinoprost tromethamine, Lutalyse HighCon, Zoetis, New York, NY) as a 2 mL intramuscular injection in the semimembranosus or semitendinosus muscle. The number of injections and dose used for the IM-PGF treatment correspond to the regimen recommended and approved for synchronization of ovulation in cattle. Cows in the IVG-PGF treatment received two intravaginal treatments of 25 mg of $PGF_{2\alpha}$ (2 mL of Lutalyse

HighCon) 12 h apart. Cows from both groups received treatments while restrained in self-locking headgates. Before application of the IVG treatments, the vulva and perineal area were manually cleaned using paper towels. Thereafter, the vulvar labia were manually opened and a uterine infusion catheter (44.5 cm long by 0.5 cm of external diameter) attached to a plastic syringe was carefully inserted into the vagina until the vaginal fornix was reached. Once in the cranial portion of the vagina the catheter was pulled backwards 1 to 2 cm and 2 mL of $PGF_{2\alpha}$ were released at a similar rate as the i.m. $PGF_{2\alpha}$ treatment (i.e., as a bolus). After application of treatments cows remained standing for a minimum of 30 minutes.

Cows in both experimental groups completed the DO protocol to receive first AI service.

Evaluation of Ovarian Structures and Responses

Transrectal ultrasonography of the ovaries was performed in all cows at the time of the first GnRH treatment (**G1**) of the DO protocol, 7 d later at the time of application of experimental treatments (*i.e.,* first $PGF_{2\alpha}$ treatment of DO), and at the second (**G2**) and third (**G3**) GnRH treatments of the DO protocol (Figure 1). At each TUS examination, the position and diameter of all ovarian structures (*i.e.,* follicles and corpora lutea) ≥ 8 mm in diameter were recorded on an ovarian map. The diameter of the visualized structures was estimated by using the average of two perpendicular measurements recorded on a single image of the apparent maximal diameter of the structure. All measurements were performed using the internal digital calipers of the ultrasound machine. Luteal volume was calculated as $V = 4/3 \times \pi \times r^3$, where the radius (r) was calculated as 0.5 × CL diameter. For a CL with a cavity (anechoic area surrounded by echoic tissue), the volume of the cavity was calculated and subtracted from the total CL volume.

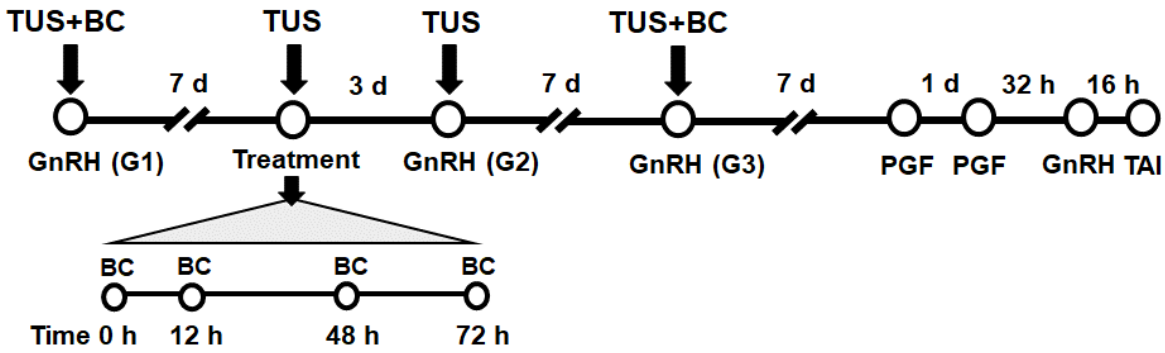


Figure 1. Graphical depiction of experimental procedures. Lactating Holstein cows were synchronized using a Double-Ovsynch (DO) protocol (GnRH, 7 d later PGF2α, 3 d later GnRH, 7 d later GnRH, 7 d later PGF2α, 1 d later PGF2α, 32 h later GnRH, 16 to 20 h TAI) to receive timed AI at 67 ± 3 DIM. Seven days after the first GnRH (G1) treatment, cows with at least one visible corpus luteum (CL) ≥15 mm were blocked by parity and randomly assigned to the IM-PGF (n = 201) or the IVG-PGF (n = 201) treatment. Cows in IM-PGF received 25 mg of PGF2α (Dinoprost tromethamine) intramuscular. Cows in IVG-PGF received two 25 mg doses of PGF 12 h apart delivered through a catheter in the cranial portion of the vagina. After application of treatments, cows in both experimental groups completed the DO protocol to receive first AI service. PGF = PGF2α, TUS = transrectal ultrasonography, BC = Blood collection.

A cow was considered to have ovulated in response to GnRH when one or more follicles ≥ 10 mm were recorded at the time of GnRH treatment and the presence of a new CL was confirmed in the same location 7 d later.

Blood Sample Collection and Determination of Progesterone Concentrations

Blood samples (~8 to 9 mL) were collected by puncture of the caudal vein or artery using evacuated tubes (Vacutainer BD, Franklin Lakes, NJ) with ethylenediaminetetraacetic acid (*i.e.,* EDTA). Samples were placed in crushed ice within 5 min of collection and transported to the laboratory for further processing. Samples were centrifuged at 2,000 × *g* for 20 min at 4 ºC. Plasma aliquots were harvested and transferred to Eppendorf vials for storage at −20 ºC until assayed.

A schematic representation of the blood-sample collection schedule is presented in Figure 1. The first blood sample was collected from all cows enrolled at G1 and 7 d later coincident with the experimental treatments (time 0). At this point, plasma concentrations of P4 were used to confirm the presence of a functional CL (i.e., P4 ≥ 1 ng/mL) in all cows that received the experimental treatments. After application of treatments, blood samples were collected at 12, 48 and 72 h to evaluate circulating P4 concentration dynamics. The 72 h time-point coincided with the G2 treatment of the DO protocol. At G1 and G3, dichotomized P4 concentrations were used to determine the percentage of cows with a functional CL, whereas P4 concentrations at G2 (*i.e.,* 72 h after application of treatments) were used to asses luteal regression after the $PGF_{2\alpha}$ treatments. A cow was considered to have complete CL regression if P4 was ≥ 1 ng/mL at time 0 and P4 < 1 ng/mL 72 h after application of treatments. We further examined luteal regression outcomes using 0.5 ng/mL of P4 as cutoff value because P4 levels above this threshold at the

time of TAI have been associated with negative reproductive outcomes in lactating dairy cows (Bisinotto et al., 2010; Giordano et al., 2013).

All plasma samples were assayed for P4 using a commercial solid-phase, no-extraction RIA (ImmuChem Coated Tube, MP Biomedicals, Costa Mesa, CA), previously validated for use in the bovine (Garbarino et al., 2004; Skenandore et al., 2017). To assess precision of the assay, control samples with high (5.1 ng/mL) and low (0.3 ng/mL) concentrations of P4 were included at the beginning and end of each assay (n = 19). Average detection limit of the assay was 0.1 ng/mL. The average intra-assay coefficient of variation (**CV**) for the high concentration sample was 8.2%, whereas the inter-assay CV was 16.5%. For the low-concentration sample, the average intra-assay CV was 21.4%, whereas the inter-assay CV was 36.7%.

Body Condition Score, Body Weight, and Evaluation of Reproductive Tract Health

For all cows, body condition score (**BCS**) and reproductive tract health status were evaluated and recorded at the time of enrollment (G1). For BCS, a scale of 1 (emaciated) to 5 (fat) with increments of 0.25 was used (Edmonson et al., 1989). Data for BCS was dichotomized using a threshold BCS of 2.75 units (high ≥2.75, low <2.75). To determine reproductive tract health, vaginal discharge was examined using a Metricheck device (Simcro, Hamilton, New Zealand), and scored in a 0 to 5 scale (0 = no discharge, 1 = clear mucus, 2 = clear mucus with flecks of pus, 3 mucopurulent but <50% pus, 4 = mucopurulent with >50% pus, and 5 = foul-smelling discharge) as previously described by McDougall et al. (2007). Based on previous work from our group (Stangaferro et al., 2018), purulent vaginal discharge (**PVD**) was defined as a Metricheck score ≥ 2. In addition, body weight (**BW**) was recorded at G1 from a subgroup of cows in both treatment groups (IM-PGF = 101; IVG-PGF = 106).

Detection of Estrus

All cows were fitted with a neck-mounted electronic rumination and activity monitoring tag (Heat and Rumination System, SCR Dairy by Allflex, Netanya, Israel) approximately 10 d before application of experimental treatments to provide enough time (*i.e.,* at least 5 d) for individual cow baseline data collection. Data generated from individual tags was transferred to the system software (Dataflow; SCR dairy) installed on the on-farm computer every 20 min via antennas located in the barn. The number of cows reaching the heat index score (**HI**; 0 to 100 arbitrary units) threshold set by the activity-monitoring system software (i.e., HI > 35) was recorded for up to 3 d after application of treatments. For each cow, the HI was generated based on activity, rumination time, and estrus length.

Pregnancy Diagnosis

In cows from both treatment groups pregnancy diagnosis was conducted by TUS 32 ± 3 d after first service if the cow was not previously reinseminated at detected estrus. A positive pregnancy outcome was based on the presence of an amount of anechoic uterine fluid and an embryo size consistent with the expected stage of pregnancy. The presence of a heartbeat in the embryo was also used as confirmation of embryo viability. Reconfirmation of pregnancy was performed 60 ± 3 d after AI by TUS in all cows confirmed pregnant at the initial examination unless the cow was reinseminated at detected estrus or left the herd. A cow was considered to have undergone pregnancy loss when confirmed pregnant at the initial examination and nonpregnant at the time of reconfirmation, or if reinsemianted at detected estrus after confirmation of pregnancy.

Statistical Analysis

A sample size calculation was performed using WinPepi software (Abramson, 2011) to assess the non-inferiority of the IVG-PGF treatment compared with the IVG-IM treatment. Based on an expected proportion of cows undergoing luteal regression of 80% and a non-inferiority margin of 10%, a total of 167 cows per treatment were needed for a 1-sided non-inferiority test with a probability of type I error (alpha) of 5%, and a probability of type II error (beta) of 20%.

Binary outcomes (*i.e.,* proportion of cows with P4 ≥ 1 ng/mL, luteal regression after application of experimental treatments, proportion of cows detected in estrus, ovulatory response to G1 and G2, P/AI, and pregnancy loss) were analyzed using logistic regression with the GLIMMIX procedure of SAS (version 9.4, SAS Institute Inc., Cary, NC) fitting a binomial distribution. Continuous quantitative outcomes were analyzed by ANOVA with (progesterone concentration dynamics) or without (follicle size, CL volume and heat index score) repeated measurements using PROC MIXED of SAS.

For all analyses, only cows with a functional CL present at time 0 (*i.e.,* at the time of application of experimental treatments) were included (IM-PGF = 169, IVG-PGF = 179).

Because of the known effect of CL maturity on $PGF_{2\alpha}$-induced CL regression in lactating dairy cows (Momont and Seguin, 1984; Giordano et al., 2012; Stevenson, 2016), the effect of experimental treatments on luteal regression and P4 concentration dynamics was evaluated for all cows regardless of the type of CL present at treatment and within each one of three groups of cows created based on the expected level of CL maturity at the time of application of experimental treatments. Briefly, the CL maturity groups were created as follows: (1) New CL

only (**NCL**) = no visible CL at G1 with newly formed CL 7 d later, (2) Old CL only (**OCL**) = same CL visualized on ovarian maps at G1 and 7 d later, and (3) Old and new CL (**O+NCL**) = at least one CL visualized on ovarian maps at both the time of G1 and 7 d later, and at least one newly formed CL at the time of treatment.

The effect of experimental treatment (IM-PGF vs. IVG-PGF), parity (primiparous vs. multiparous), and the treatment by parity interaction were offered as explanatory variables to all models, whereas logistic regression models for luteal regression, estrus expression, and P/AI also included the effect of BCS, BW, and PVD at enrollment (G1). In addition, for outcomes related to luteal regression, estrus expression, and ovulatory response, the effects of total CL volume and P4 at time 0 were also offered to models as categorical covariates. In both cases, variables were dichotomized using a threshold corresponding to the median value within parity group generated with PROC UNIVARIATE of SAS [CL volume (primiparous = 7,821 mm^3, multiparous = 9,278 mm^3); P4 at time 0 (primiparous = 4.3 ng/mL, multiparous = 3.7 ng/mL)]. Further, the effect of season (cool vs. warm) was offered to the logistic regression models for proportion of cows detected in estrus (season of treatment) and P/AI (season of AI). The cold season was defined as the period from September 21 to June 20 and the warm season from June 21 to September 20.

The effect of experimental treatments on P4 concentration profiles (*i.e.,* P4 at 0, 12, 48, and 72 h after treatment) was evaluated with models that included treatment, time, parity, and the treatment-by-time interaction as fixed effects, whereas cow within treatment was included as a random effect. In addition, trial-by-parity and time-by-parity interactions were offered to the models. In all cases, cow within treatment was the subject of repeated measures analysis using a spatial power covariance structure to adjust for the varying time intervals at which blood was collected.

In all cases, the final model for outcomes of interest was selected by backward elimination of explanatory variables with $P > 0.10$ and determination of the lowest value for the Akaike Information Criterion (**AIC**). Treatment and parity were forced in all models. Body weight, BCS, PVD, CL volume and P4 at time 0 were removed from the luteal regression models and season, BW and PVD from the estrus expression and P/AI models because $P > 0.10$.

Assumptions of normality of residuals and homoscedasticity for linear regression models were tested by evaluating normal probability plots (Q-Q plot) and plotting residuals versus predicted values. For logistic regression models, goodness of fit was evaluated using the ratio between the Pearson statistic and its degrees of freedom. In all cases, model fit was deemed acceptable based on a ratio of approximately one.

All proportions reported are least squares means generated using the LSMEANS statement of PROC GLIMMIX of SAS. Quantitative outcomes are reported as LSM ± SEM obtained with the LSMEANS option of PROC MIXED of SAS. When appropriate, the least significant difference (**LSD**) post-hoc mean separation test was used to determine differences between LSM. All explanatory variables and their interactions were considered significant if $P \leq 0.05$, whereas $P > 0.05$ and ≤ 0.10 was considered a tendency.

RESULTS

Physiological Parameters and Ovarian Status before Application of Experimental Treatments

At G1, the proportion of cows with BCS < 2.75 was similar ($P = 0.64$) for cows in both treatments [IM-PGF = 20.0% (n = 168); IVG-PGF = 18.2% (n = 179)] but was affected ($P < 0.001$) by parity because more multiparous than primiparous cows had BCS < 2.75 (primiparous = 9.8%; multiparous = 33.9%). Likewise, body weight was similar ($P = 0.73$) for both treatment

groups (IM-PGF = 654 ± 7 kg; IVG-PGF = 651 ± 7 kg) but was greater ($P < 0.001$) for multiparous (710 ± 6 kg) than primiparous (594 ± 9 kg) cows. In addition, the proportion of cows with PVD was similar ($P = 0.20$) for both treatments [IM-PGF = 43.0% (n = 167); IVG-PGF = 36.2% (n = 179)], and for primiparous and multiparous cows ($P = 0.37$). In all cases, no interaction was observed between treatment and parity ($P > 0.10$).

The proportion of cows with P4 ≥ 1 ng/mL at G1 was similar for cows in the IM-PGF and IVG-PGF treatments ($P = 0.34$; Table 1) and for primiparous and multiparous cows ($P = 0.91$; primiparous = 52.4%, multiparous = 51.8%). Similarly, the proportion of cows ovulating in response to G1 did not differ by treatment ($P = 0.81$; Table 1) or parity [$P = 0.12$; primiparous = 78.4%, multiparous = 70.2%), and there was no treatment by parity interaction ($P = 0.51$). Total CL volume tissue present at time 0 was similar ($P = 0.81$; Table 1) for the IM-PGF and IVG-PGF treatments, but was greater ($P < 0.01$) for multiparous (11,051 ± 332 mm^3) than for primiparous (8,883 ± 516 mm^3) cows and for O+NCL (13,336 ± 518 mm^3) than for OCL (8,399 ± 552 mm^3) and NCL (8,167 ± 437 mm^3) cows. Similarly, circulating concentration of P4 at time 0 was similar ($P = 0.71$) for cows in the IM-PGF (4.7 ± 0.2 ng/mL) and IVG-PGF treatments (4.8 ± 0.2 ng/mL), but was greater ($P = 0.003$) for primiparous (5.1 ± 0.2 ng/mL) than for multiparous (4.3 ± 0.1 ng/mL) cows. In addition, CL maturity group also affected ($P < 0.001$) P4 concentration at time 0 because it was greater for N+OCL than for NCL and OCL cows, and for OCL than for NCL cows (NCL = 3.1 ± 0.2 ng/mL; OCL = 5.2 ± 0.2 ng/mL; O+NCL = 5.8 ± 0.2 ng/mL).

The distribution of cows based on CL maturity group at time 0 for each treatment group is presented in Table 1. At time 0, there was a similar proportion of cows in the NCL ($P = 0.77$),

Table 1. Physiological parameters and ovarian status before application of experimental treatments.

Item	Treatment[1]		
	IM-PGF	IVG-PGF	*P*-value
Cows with P4 ≥ 1 ng/mL at G1[2] [% (no.)]	49.5 (168)	54.6 (178)	0.34
Ovulation to G1 [% (no.)]	74.0 (168)	75.1 (179)	0.81
CL volume at time 0 (mm^3)	10,032 ± 422	9,902 ± 409	0.81
CL maturity[3] at time 0 [% (no.)]			
Cows with new CL only (NCL)	44.7 (168)	43.1 (179)	0.77
Cows with old CL only (OCL)	26.0 (168)	24.9 (179)	0.81
Cows with old and new CL (O+NCL)	29.0 (168)	31.7 (179)	0.58

[1]Treatment: Lactating Holstein cows were synchronized using a Double-Ovsynch (DO) protocol to receive timed AI at 67 ± 3 DIM. Seven days after the first GnRH (G1) treatment, cows with at least one visible corpus luteum (CL) ≥15 mm were blocked by parity and randomly assigned to the IM-PGF or the IVG-PGF treatment. Cows in IM-PGF received 25 mg of $PGF_{2\alpha}$ intramuscular. Cows in IVG-PGF received two 25 mg doses of $PGF_{2\alpha}$ 12 h apart delivered through a catheter in the cranial portion of the vagina. All proportions and quantitative outcomes are reported as least squares means (LSM).
[2] P4 = progesterone, G1 = first GnRH of Double-Ovsynch protocol. Two cows were eliminated from the analysis because of missing data for P4 concentration at G1.
[3] Corpus luteum (CL) maturity groups were created as follows: (1) New CL only = no visible CL at G1 with a newly formed CL 7 d later, (2) Old CL only = same CL visualized at G1 and 7 d later, and (3) Old and new CL = at least one CL visualized at both G1 and 7 d later, and at least one newly formed CL at time 0. One cow from the IM-PGF was eliminated from the analysis because of missing data for CL maturity at time 0.

OCL ($P = 0.81$), and O+NCL ($P = 0.58$) group in both treatments. For all CL maturity groups, no effect of parity ($P > 0.10$) or treatment by parity interaction ($P > 0.10$) was observed.

Luteal Regression

Overall, the proportion of cows with complete luteal regression at 72 h after application of treatments using the P4 < 1.0 ng/mL cutoff was similar ($P = 0.48$; Figure 2A) for the IM-PGF and IVG-PGF treatments, but tended ($P = 0.06$) to be greater for primiparous (91.3%) than multiparous (83.4%) cows. Similarly, the proportion of cows with P4 < 0.5 ng/mL at 72 h was similar ($P = 0.98$, Figure 2A) for the IM-PGF and IVG-PGF treatments and was not affected by parity [$P = 0.32$; primiparous = 84.3%; multiparous = 79.7%]. In both cases, no treatment by parity interaction was observed ($P > 0.10$). For cows in the IVG-PGF treatment, PVD did not affect luteal regression risk when using the 1.0 ng/mL ($P = 0.32$; PVD = 84.8% vs. no PVD = 89.5%) or 0.5 ng/mL circulating P4 level cutoff ($P = 0.39$; PVD = 80.0% vs. no PVD = 84.8%).

For cows in the IM-PGF treatment, CL maturity group affected ($P = 0.04$) the proportion of cows with P4 < 1 ng/mL 72 h after treatment whereby it was greater for cows in the O+NCL than the NCL group [NCL = 81.0% (n = 73); OCL = 90.6% (n = 47); O+NCL = 96.3% (n = 48)]. In contrast, for the IVG-PGF treatment the proportion of cows with P4 < 1 ng/mL did not differ ($P = 0.28$) by CL maturity group [NCL = 89.7% (n = 75), OCL = 91.3% (n = 48), O+NCL = 82.3% (n = 56)]. For cows in the NCL group, there was no effect of treatment ($P = 0.23$; Figure 2B) for CL regression using P4 < 1.0 ng/mL as cutoff but, more ($P = 0.03$) primiparous (93.9%) than multiparous (78.3%) cows had complete CL regression. For the OCL group, the proportion of cows with P4 < 1 ng/mL was not affected by treatment ($P = 0.76$; Figure 2B) or parity ($P = 0.72$; primiparous = 86.3%, multiparous = 89.1%). In contrast, for the O+NCL group treatment

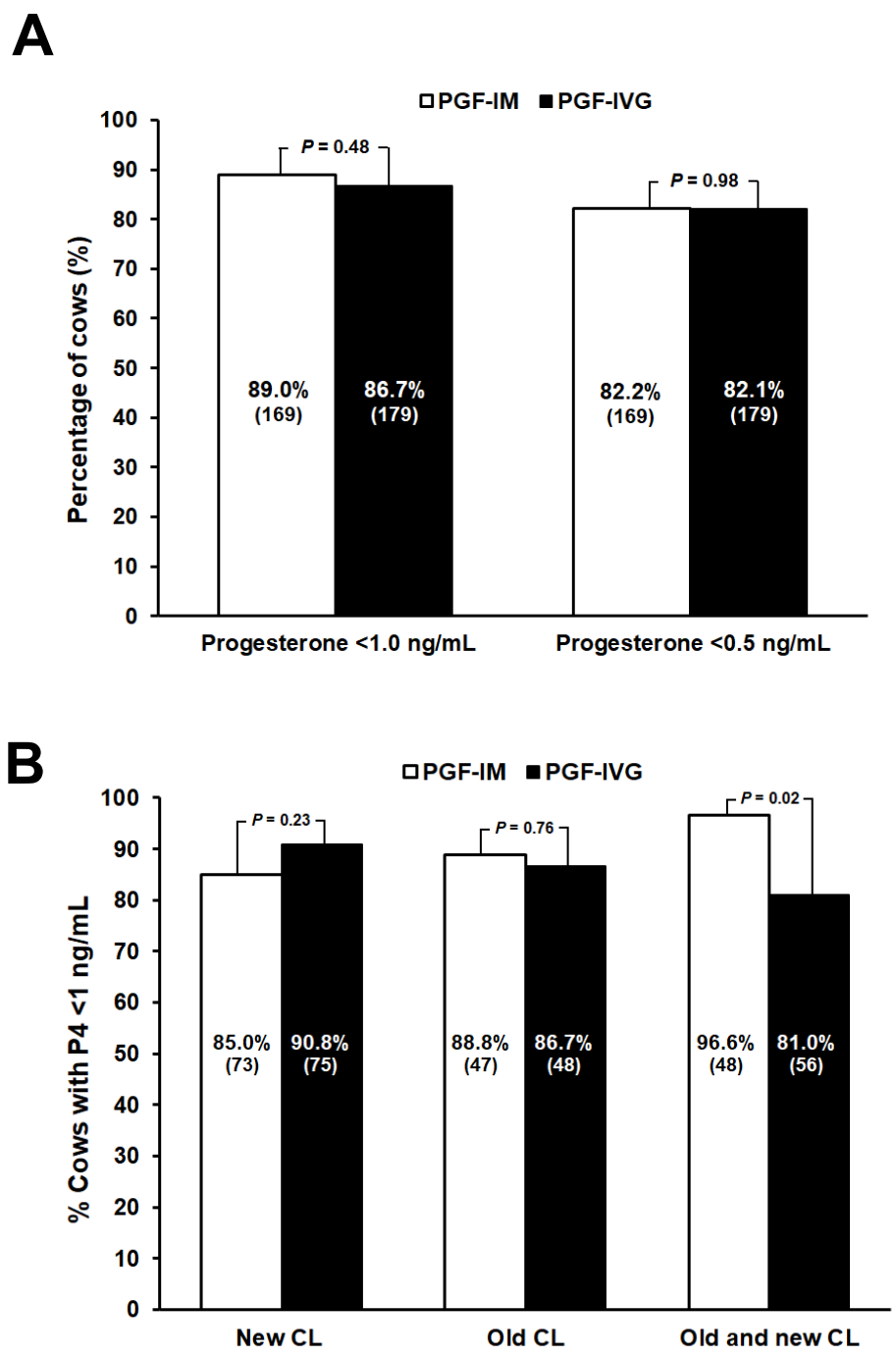


Figure 2. (A) Proportion of cows with < 1 ng/mL and < 0.5 ng/mL of progesterone (P4) at 72 h after application of experimental treatments. (B) Proportion of cows with P4 < 1 ng/mL 72 after treatment based on CL maturity group at time 0. The CL maturity groups were created as follows: (1) New CL only (NCL) = no visible CL at G1 with a newly formed CL 7 d later, (2) Old CL only (OCL) = same CL visualized at G1 and 7 d later, and (3) Old and new CL (O+NCL) = at least one CL visualized at both G1 and 7 d later, and at least one newly formed CL at time 0. Only cows with a functional CL at time 0 were included in the analyses (IM-PGF = 169; IVG-PGF = 179).

affected ($P = 0.02$; Figure 2B) the proportion of cows with P4 < 1 ng/mL because it was greater for the IM-PGF than the IVG-PGF treatment, but was similar ($P = 0.32$) for primiparous (94.0%) and multiparous (88.5%) cows. In all cases, no interaction between treatment and parity was observed ($P > 0.10$).

Circulating Progesterone Concentration Dynamics

The effect of treatment on circulating P4 concentration profiles are presented in Figures 3 and 4. For all cows combined (Figure 3A), there was an effect of treatment ($P = 0.01$), time ($P < 0.001$), and a treatment by time interaction ($P < 0.001$) on circulating concentrations of P4 for up to 72 h after treatment. At 12 h, cows in the IM-PGF treatment had lesser P4 concentrations than cows in the IVG-PGF treatment. No differences were observed at 48 and 72 h after treatment. In addition, parity tended ($P = 0.09$) to affect P4 concentrations and an interaction between parity and time ($P = 0.01$) was observed, whereby primiparous cows had greater circulating P4 concentrations at 0 and 12 h after treatment than multiparous cows but were similar at 48 and 72 h after treatment. No interaction was observed between treatment and parity.

For only those cows with complete luteal regression only (Figure 3B), an interaction was observed between treatment and time ($P < 0.001$), whereby cows in the IM-PGF treatment had lesser ($P < 0.001$) P4 concentrations at 12 h after treatment but, no differences were observed at 48 and 72 h. A parity by time interaction ($P = 0.02$) was also observed because primiparous cows had greater P4 concentrations at 0 and 12 h after treatment than multiparous cows, whereas no differences were observed at 48 and 72 h after treatment. When only cows without complete luteal regression were analyzed (Figure 3C), an interaction ($P < 0.001$) between treatment and time was also observed, whereby cows in the IM-PGF had lesser P4 concentrations at 0 and 12 h

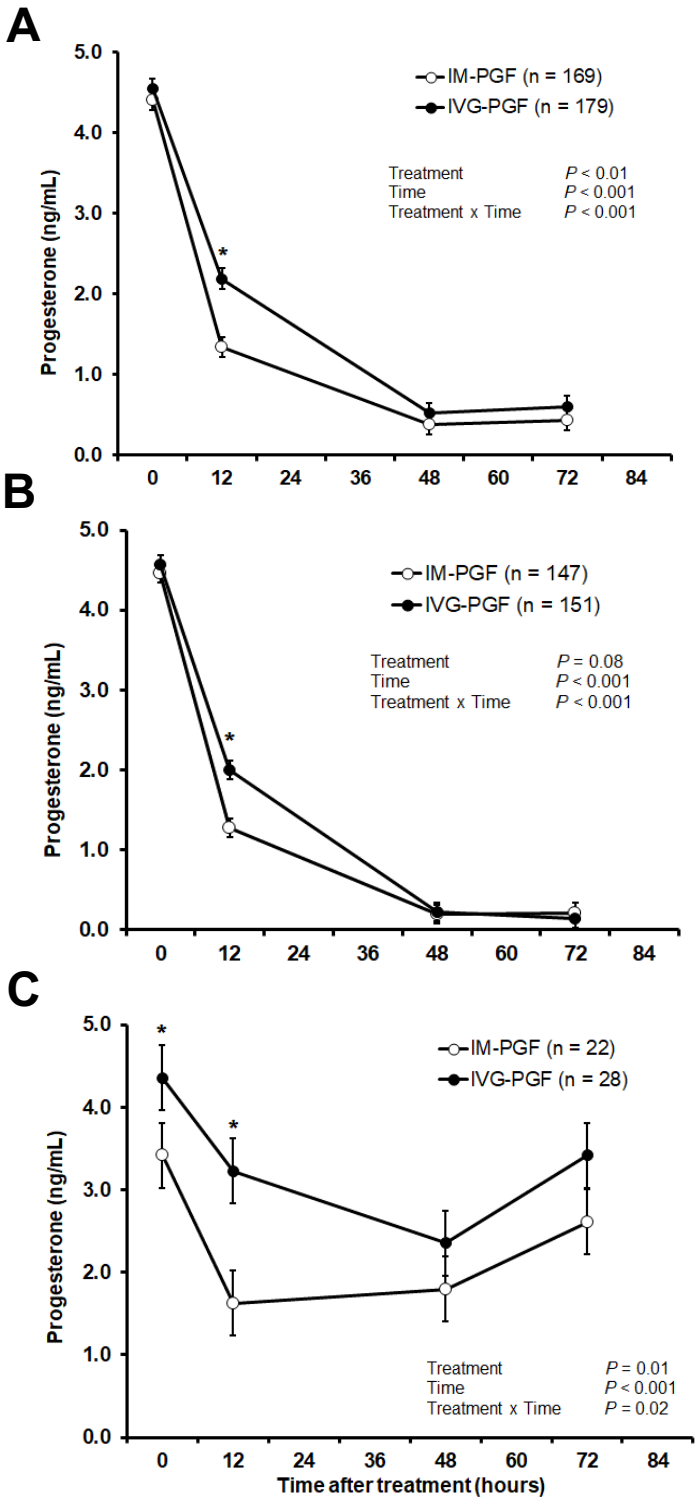


Figure 3. Circulating concentrations of progesterone (P4) from 0 to 72 h after application of treatments for all cows (A), cows with complete luteal regression (P4 < 1 ng/mL at 72 h) only (B), and cows with incomplete luteal regression (P4 ≥ 1 ng/mL at 72 h) only (C). Only cows with a functional CL at time 0 were included in the analyses (IM-PGF = 169; IVG-PGF = 179). Values are presented as LSM ± SEM. Within each time point, an asterisk (*) represents differences (P ≤ 0.05) for pairwise comparisons between treatments.

after treatment when compared to cows in the IM-IVG treatment, whereas no differences were observed 48 and 72 h after treatment. For cows without complete luteal regression, no effects of parity or parity by time interaction were observed.

The effect of treatments on circulating concentration P4 profiles within each CL maturity group are presented in Figure 4. For cows in the OCL group (Figure 4A), we observed a tendency ($P = 0.08$) for a treatment effect; however, we did not observe a treatment by time interaction ($P = 0.32$). Similarly, we observed an effect of parity ($P = 0.02$) but no effects of the parity by time ($P = 0.72$) or parity by treatment ($P = 0.76$) interactions. In contrast, for the NCL group (Figure 4B), there was a treatment by time interaction ($P < 0.001$) for circulating concentrations of P4, whereby cows in the IM-PGF treatment had lesser P4 concentrations at 12 h than the IVG-PGF treatment, but no difference was observed at 48 and 72 h after treatment. For this group of cows, no effects of parity ($P = 0.81$) or treatment by parity interaction ($P = 0.66$) were observed. Likewise, for the O+NCL group we also observed an interaction ($P < 0.001$) between treatment and time. In this group of cows, P4 concentrations were lesser for the IM-PGF than for the IVG-PGF treatment at 12 h, but were similar at 48 and 72 h after treatment. No effect of parity ($P = 0.15$) or treatment by parity interaction ($P = 0.26$) was observed.

Estrus Expression and Ovarian Responses after Treatment

Within three days of the application of treatments, the proportion of cows that displayed estrus as determined by increased physical activity was similar ($P = 0.54$; Table 2) for the IM-PGF and IVG-PGF treatments (45.4% vs. 48.9%, respectively), but tended ($P = 0.06$) to be greater for primiparous (52.3%) than for multiparous cows (41.1%). In addition, BCS ($P < 0.01$) and P4 concentration at the time of treatment ($P < 0.001$) also affected the proportion of cows

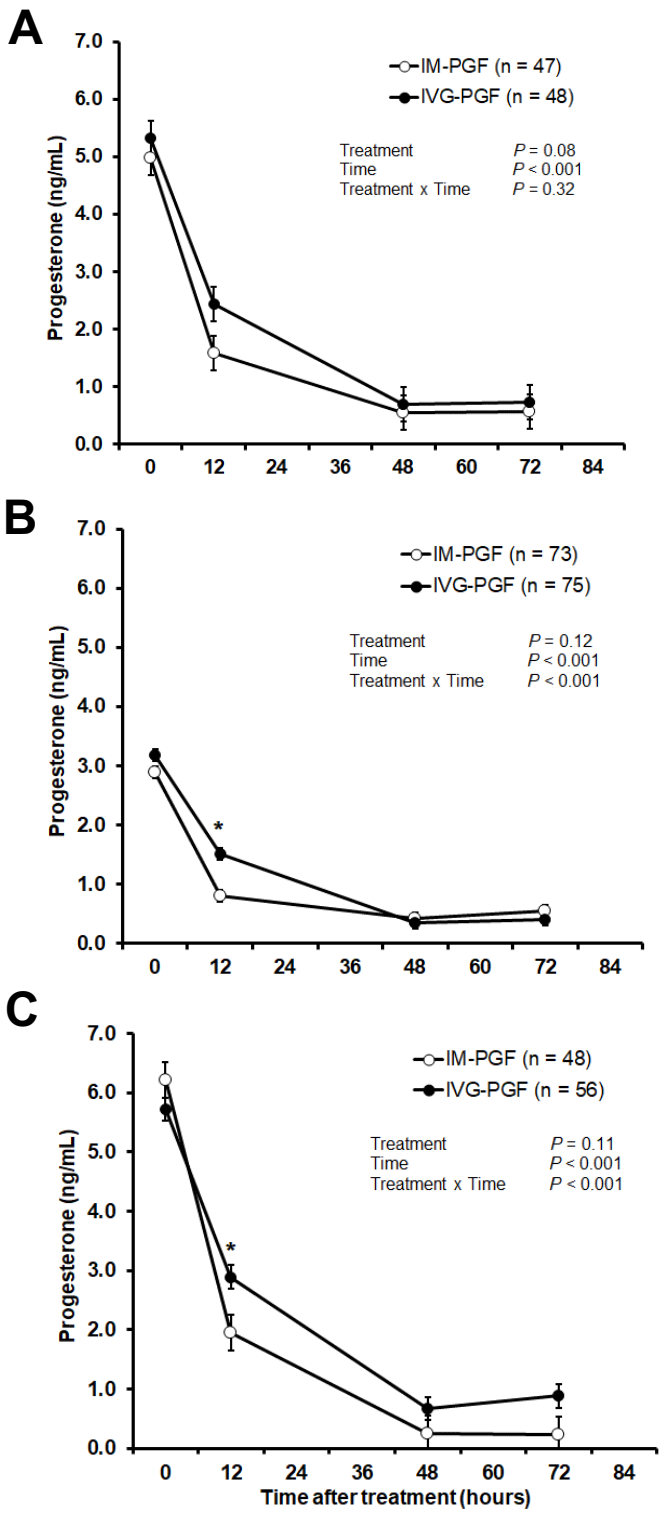


Figure 4. Circulating concentrations of progesterone (P4) from 0 to 72 h after application of treatments for cows with an old CL only (A), new CL only (B), and old and new CL (C) at time 0. Only cows with a functional CL at time 0 were included in the analyses (IM-PGF = 169; IVG-PGF = 179). Values are presented as LSM ± SEM. Within each time point, an asterisk (*) represents differences ($P \leq 0.05$) for pairwise comparisons between treatments.

Table 2. Estrus expression and ovarian responses after application of experimental treatments.

	Treatment[1]		
Item	IM-PGF	IVG-PGF	*P*-value
Cows with detected estrus[2] [% (no.)]	45.4 (166)	48.9 (174)	0.54
Ovulation after G2[3] [% (no.)]	88.5 (166)	85.1 (176)	0.35
Size of the ovulatory follicle (mm)	16.5 ± 0.3	16.5 ± 0.3	0.99
Cows with P4 ≥ 1 ng/mL at G3[4] [% (no.)]	92.7 (167)	91.5 (176)	0.66
CL[5] volume at G3 (mm^3)	7,625 ± 324	7,624 ± 317	0.99

[1] Treatment: Lactating Holstein cows were synchronized using a Double-Ovsynch (DO) protocol to receive timed AI at 67 ± 3 DIM. Seven days after the first GnRH (G1) treatment, cows with at least one visible corpus luteum (CL) ≥15 mm were blocked by parity and randomly assigned to the IM-PGF or the IVG-PGF treatment. Cows in IM-PGF received 25 mg of $PGF_{2\alpha}$ intramuscular. Cows in IVG-PGF received two 25 mg doses of $PGF_{2\alpha}$ 12 h apart delivered through a catheter in the cranial portion of the vagina. All proportions and quantitative outcomes are reported as least squares means (LSM).

[2] Three cows from the IM-PGF treatment and 5 cows from the IVG-PGF were not included in the analysis because of collar malfunction or misplacement during data collection.

[3] G2 = second GnRH treatment of the Double-Ovsynch protocol. Three cows from the IM-PGF treatment and 3 cows from the IVG-PGF treatment exited the herd before confirmation of ovulation.

[4] G3 = third GnRH treatment of the Double-Ovsynch protocol.

[5] CL = corpus luteum.

displaying estrus, because it was greater for cows with BCS ≥ 2.75 (57.3%) than for cows with BCS < 2.75 (37.3%), and lesser for cows with high (34.8%) than low P4 (59.9%) at the time of treatment.

Among cows that displayed estrus within 3 days after treatment (IM-PGF = 78, IVG-PGF = 89), the proportion of cows detected in estrus on day 1 and 2 combined was 40.1% (67/167; arithmetic mean presented) and was not affected by treatment ($P = 0.92$; IM-PGF = 31.8% vs. IVG-PGF = 32.7%), but was greater ($P < 0.001$) for cows with low P4 (58.7%) than with high P4 (13.8%) at the time of treatment. The rest of the cows were detected in estrus on day 3. Further, for cows that displayed estrus the average heat index score was greater ($P < 0.001$) for those in the IVG-PGF (86.5 ± 1.0) than in the IM-PGF treatment (81.1 ± 1.0). In addition, average heat index score was also affected by parity ($P < 0.0001$; primiparous = 86.5 ± 1.2; multiparous = 81.1 ± 0.9) and CL volume at the time of treatment ($P = 0.03$; low = 85.3 ± 1.0; high = 82.3 ± 1.0). Similarly, treatment ($P = 0.001$) and parity ($P = 0.004$) affected the proportion of cows with HI > 80 because it was greater for the IVG-PGF (86%; n = 89) than the IM-PGF treatment (64.1%; n = 78), and for primiparous (86%) relative to multiparous (64.0%) cows.

Overall, the total proportion of cows that ovulated after G2 was similar ($P = 0.35$; Table 2) for the IM-PGF (88.5%) and the IVG-PGF (85.1%) treatments, and for primiparous (87.1%) and multiparous (86.6%) cows. In contrast, CL volume at the time of treatment with $PGF_{2\alpha}$ affected ($P < 0.01$) the proportion of cows ovulating because it was lesser for cows with high (81.6%) than for cows with low (90.8%) CL volume. Among cows that ovulated in response to G2 (IM-PGF = 146, IVG-PGF = 148), ovulatory follicle size was similar ($P = 0.99$; Table 2) for

cows in both treatments, but was greater ($P < 0.001$) for multiparous (17.0 ± 0.2 mm) than primiparous (15.9 ± 0.3 mm) cows. In addition, P4 concentration at the time of $PGF_{2\alpha}$ treatment also affected ovulatory follicle size because it was greater ($P < 0.001$) for cows with low (17.0 ± 0.3 mm) than with high (16.0 ± 0.3 mm) P4 levels.

The proportion of cows with P4 ≥ 1 ng/mL at G3 (i.e., seven days after treatment with GnRH) was not affected by treatment ($P = 0.66$; Table 2), and was similar for primiparous (93.2%) and multiparous (90.9%) cows. Further, CL volume at G3 (cows with CL only) was similar for cows in both treatments ($P = 0.99$; Table 2), but was greater ($P = 0.004$) for multiparous (8,318 ± 258 mm^3) than for primiparous cows (6,931 ± 397 mm^3). In addition, P4 at the time of $PGF_{2\alpha}$ treatment also affected CL volume at G3 because it was greater ($P = 0.01$) for cows with low (8,164 ± 325 mm^3) than with high (7,084 ± 316 mm^3) P4 levels.

Pregnancies per AI and Pregnancy Loss

Forty-one treated cows (IM-PGF = 22, IVG-PGF = 19) were not included in the P/AI analyses because either did not complete the DO protocol (due to farm management decisions beyond control of the researchers) or left the herd before pregnancy diagnosis. Overall, P/AI at 32 ± 3 d after AI was not affected ($P = 0.29$) by treatment [IM-PGF = 51.5% (n = 147), IVG-PGF = 57.8% (n = 160)], but was greater ($P = 0.01$) for primiparous (64.1%, n = 86) than for multiparous (44.9%, n = 221) cows and tended ($P = 0.08$) to be greater for cows with BCS ≥ 2.75 (60.5%; n = 226) than for cows with BCS < 2.75 (48.7%; n = 81). Likewise, P/AI at 60 ± 3 d was similar ($P = 0.28$) for cows in the IM-PGF (47.2%; n = 145) and IVG-PGF (53.1%; n = 160) treatments, but greater ($P = 0.01$) for primiparous (59.5%, n = 86) than for multiparous

(40.9%, n = 219) cows, and for cows with BCS ≥ 2.75 (57.2%; n = 224) than for cows with BCS < 2.75 (43.1%; n = 81).

Pregnancy loss for cows diagnosed pregnant at initial examination was similar ($P = 0.80$) for both treatments [IM-PGF = 5.4% (n = 71); IVG-PGF = 6.3% (n = 90)], and was not affected ($P = 0.66$) by parity [primiparous = 5.0% (n = 59); multiparous = 6.8% (n = 102)].

DISCUSSION

In support of our main hypothesis, the proportion of cows with complete CL regression was similar for cows that received the IVG or i.m. $PGF_{2\alpha}$ treatment. Our data supported the hypothesis when using the traditional P4 concentration cutoff for complete CL regression of 1 ng/mL as well as when using a more stringent cutoff of 0.5 ng/mL. Conversely, our hypothesis that the circulating P4 profile would be similar for both treatments was partially supported because of the greater circulating levels of P4 for the IVG-treated group at 12 h and similar levels at 48 and 72 h after treatment. Moreover, the lack of difference for proportion of cows detected in estrus and with ovulation after treatment with GnRH supported our secondary hypothesis. The similar P/AI of cows in both treatments also supported our secondary hypothesis. Nevertheless, results should be interpreted with caution because the effect of lack of luteal regression or a lesser reduction in circulating P4 after induction of luteolysis during the presynchronization part of a fertility program like DO has not been documented. Indeed, based on the limited number of cows with incomplete luteal regression in our experiment it seems as this may not be as relevant because P/AI was similar for cows with complete and incomplete luteal regression after treatment (data not shown). Collectively, our current results provide additional evidence of the efficacy of two IVG doses of 25 mg of the natural form of $PGF_{2\alpha}$ (i.e.,

Dinoprost) to cause complete CL regression in lactating dairy cows. In addition, results indicated that no differences for important reproductive outcomes (i.e., estrus expression and ovulation in response to GnRH) dependent on completion of CL regression should be expected for cows that receive $PGF_{2\alpha}$ through the IVG or i.m. route of administration. Overall, our current results are in agreement with the few experiments conducted with a limited number of cows to evaluate the effect of IVG instillation of $PGF_{2\alpha}$ on CL regression and other reproductive outcomes (Zdunczyk et al., 1994; Heinonen et al., 1996; Wijma et al., 2016).

Interestingly, we observed a delay in P4 decline after IVG treatment with $PGF_{2\alpha}$ both in cows that had complete CL regression and cows in which regression was induced but the CL did not fully regress (i.e., presented a rebound in circulating concentrations of P4 after 12 h of treatment). This phenomenon, which would likely be more relevant for cows that have complete CL regression and if cows receive IVG $PGF_{2\alpha}$ for induction of luteal regression before TAI (Martins et al., 2011), was not observed by Wijma et al. (2016) for cows that received the same treatment. Multiple factors could explain the observed delay in P4 decline; however, delayed or reduced absorption of $PGF_{2\alpha}$ from the vagina and potentially both factors combined are the most reasonable explanations. Regardless of the cause for the delay in P4 decline, it is plausible to suggest that the success of the IVG treatment was due to the second $PGF_{2\alpha}$ application which likely provided the additional $PGF_{2\alpha}$ required to complete luteal regression and result in a similar rate of P4 decline for both treatments after 12 h. Indeed, Wijma et al. (2016) reported that most cows with incomplete CL regression after a single IVG treatment with $PGF_{2\alpha}$ had a decline in circulating P4 within 6 h of treatment followed by a rebound in P4 levels between 12 and 18 h after treatment. These observations led to the addition of the second 25 mg dose of $PGF_{2\alpha}$ within 12 h of the initial treatment which, in turn, resulted in complete CL regression in all cows. In the

current experiment using the same treatment regimen but with a much larger number of cows, we still observed lack of complete CL regression in some cows in spite of an initial decline in circulating P4 levels within 12 h of treatment. This was not surprising because lack of complete CL regression in cows that receive $PGF_{2\alpha}$ seven days after treatment with GnRH is common and has been extensively reported for cows that received i.m. $PGF_{2\alpha}$ (Brusveen et al., 2009; Martins et al., 2011; Giordano et al., 2012). In addition, each 25 mg dose of $PGF_{2\alpha}$ was given in a volume of 5 mL in Wijma et al. (2016) and 2 mL in the current experiment. Different volumes of $PGF_{2\alpha}$ solution used to provide the same dose of active compound may explain the discrepancy between experiments. Future research should explore the use of more than two sequential IVG treatments with $PGF_{2\alpha}$, different volumes of administration, or both to further improve luteal regression rates and mimic the P4 profiles observed after i.m. treatment with $PGF_{2\alpha}$ in cattle. In the event that automated IVG hormone delivery devices are developed, the frequency, rate, and volume of $PGF_{2\alpha}$ delivery could be easily adjusted to optimize CL regression rates and P4 profiles.

The relevance and practical implications of the apparent delay in circulating P4 decline for cows in the IVG-treated group is unclear at the moment. Based on the results for estrus expression, ovulation after GnRH treatment, and P/AI it does not seem that this delay in P4 reduction in circulation could have major negative consequences when treatment is applied at the first $PGF_{2\alpha}$ of the DO protocol. Our results for estrus expression are in agreement with Zdunczyk et al. (1994) who reported a similar proportion of *Bos indicus* cows detected in estrus for up to 10 d after i.m. or IVG treatment with the synthetic analogue of $PGF_{2\alpha}$ Cloprostenol. A caveat of the current experiment is that treatment with GnRH 72 h after induction of CL regression may have precluded expression of estrus in some cows in spite of having had complete CL regression. In addition, P/AI was not evaluated for the ovulation immediately after induction of luteal

regression with the IVG treatment but rather after a synchronized ovulation with the Ovsynch-56 protocol after pre-synchronization of the estrous cycle. Thus, even though our current results for estrus expression, ovulation after GnRH treatment, and P/AI are encouraging they should be interpreted with caution and further research should be conducted to evaluate these important reproductive outcomes. More time should be provided for cows to display estrus after IVG treatment with $PGF_{2\alpha}$ and AI should be performed after a synchronized ovulation in cows in which CL regression was induced by IVG delivery of $PGF_{2\alpha}$.

In an attempt to elucidate potential reasons for CL regression failures in IVG-treated cows and better explain differences in circulating P4 profiles between treatments, we evaluated CL regression risk and explored P4 profiles of cows grouped based on the level of maturity of their corpora lutea. Cows were grouped based on CL maturity levels as determined by timing of their formation in relationship to the $PGF_{2\alpha}$ treatment. Based on previous observations for CL regression and P4 profiles in cows with corpora lutea of different levels of maturity treated with i.m. $PGF_{2\alpha}$ (Giordano et al., 2012; Stevenson, 2016; Stevenson et al., 2018), we expected that cows with corpora lutea formed after the G1 of DO (i.e., <7 d before $PGF_{2\alpha}$ treatment) and treated with $PGF_{2\alpha}$ would be less likely to present complete luteal regression and P4 levels would not decline to the same levels than for cows with corpora lutea formed before the G1 of DO (i.e., >7 d old at time of $PGF_{2\alpha}$ treatment). Moreover, for cows with a combination of corpora lutea formed before and after the G1 of DO (i.e., at least one CL >7 d old and at least one CL <7 d old) we expected similar luteal regression rates and P4 profiles than for cows with corpora lutea formed before G1 of DO. If the level of CL maturity affected the response to $PGF_{2\alpha}$, strategies to improve the response to IVG treatment could be identified. Results were as expected for the IM-PGF treatment providing additional evidence of compromised luteal regression in cows that

receive an i.m. luteolytic dose of $PGF_{2\alpha}$ 7 d after ovulating in response to a GnRH treatment. Contrary to our expectations, the similar CL regression risk for cows in the IVG-PGF treatment with different type of corpora lutea suggested that the relationship between level of CL maturity and CL regression success may not be the same than for i.m.-treated cows. Based on the P4 profiles observed, which were characterized by greater P4 levels in the O+NCL group, a potential explanation for our findings may be the interplay between the circulating concentrations of P4 at the time of treatment (Figure 4) and the slower rate of circulating P4 decline in cows that received the IVG-PGF treatment. We speculate that some cows in the OCL and O+NCL group with circulating concentrations of P4 in the upper range for this experiment may have not had sufficient time for circulating P4 to decline below the CL regression cutoff. Even though this could explain our observations for cows in the IVG-PGF treatment, the relatively high CL regression rate for the NCL group in the IVG-PGF treatment in comparison with other CL groups and the i.m.-treated group suggests that other mechanisms may affect the success of the IVG treatment. Our findings for the differences in CL regression risk within and across treatments should be interpreted with caution because the relatively limited number of cows in each CL group may have affected the results of the statistical comparisons. Additional research should be conducted with the proper number of IVG-treated cows to test hypotheses related to luteal responses in cows with corpora lutea of different levels of maturity.

CONCLUSION

We concluded that IVG instillation of two 25 mg doses of the natural form of $PGF_{2\alpha}$ Dinoprost was as effective as a single i.m. 25 mg treatment with $PGF_{2\alpha}$ for induction of complete CL regression when used in the presynchronization of the estrous cycle portion of a fertility

protocol such as DO for lactating dairy cows. The similar risk of CL regression was observed in spite of an apparent temporary delay in P4 decline after the IVG treatment. The implications of the delay in rate of P4 decline after the first $PGF_{2\alpha}$ of the DO protocol were likely minor because there were no differences in the proportion of cows detected in estrus and cows with ovulation after GnRH treatment. Although further research is required to evaluate the success of synchronization of ovulation protocols including IVG treatment with $PGF_{2\alpha}$, the results of this experiment indicated that two 25 mg IVG treatments with Dinoprost 12 h apart can be as effective as the regular treatment and route of administration (i.e., 25 mg i.m.) of Dinoprost for induction of complete luteal regression in lactating dairy cows.

ACKNOWLEDGMENTS

This research was supported by Hatch funds under project NYC-127434 from the USDA National Institute of Food and Agriculture and a grant from the Research Innovation Fund from the Cornell Initiative for Digital Agriculture. We would like to thank personnel from the Dairy Unit of the Cornell University Ruminant Center for their help conducting this experiment.

REFERENCES

Abramson, J. H. 2011. WINPEPI updated: computer programs for epidemiologists, and their teaching potential. Epidemiologic Perspectives & Innovations 8:1.

Bartolome, J., J. Van Leeuwen, M. Thieme, O. Sa'Filho, P. Melendez, L. Archbald, and W. Thatcher. 2009. Synchronization and resynchronization of inseminations in lactating dairy cows with the CIDR insert and the Ovsynch protocol. Theriogenology 72:869-878.

Bisinotto, R., E. Ribeiro, L. Martins, R. Marsola, L. Greco, M. Favoreto, C. Risco, W. Thatcher, and J. Santos. 2010. Effect of interval between induction of ovulation and artificial insemination (AI) and supplemental progesterone for resynchronization on fertility of dairy cows subjected to a 5-d timed AI program. J. Dairy Sci. 93:5798-5808.

Bridges, G., L. Helser, D. Grum, M. Mussard, C. Gasser, and M. Day. 2008. Decreasing the interval between GnRH and PGF2α from 7 to 5 days and lengthening proestrus increases timed-AI pregnancy rates in beef cows. Theriogenology 69:843-851.

Brusveen, D., A. Souza, and M. Wiltbank. 2009. Effects of additional prostaglandin F2α and estradiol-17β during Ovsynch in lactating dairy cows. J. Dairy Sci. 92:1412-1422.

Cartmill, J., S. El-Zarkouny, B. Hensley, G. Lamb, and J. Stevenson. 2001. Stage of cycle, incidence, and timing of ovulation, and pregnancy rates in dairy cattle after three timed breeding protocols. J. Dairy Sci. 84:1051-1059.

Chebel, R. C., J. E. Santos, R. L. Cerri, H. M. Rutigliano, and R. Bruno. 2006. Reproduction in dairy cows following progesterone insert presynchronization and resynchronization protocols. J. Dairy Sci. 89:4205-4219.

Edmonson, A., I. Lean, L. Weaver, T. Farver, and G. Webster. 1989. A body condition scoring chart for Holstein dairy cows. J. Dairy Sci. 72:68-78.

Galvão, K., P. Federico, A. De Vries, and G. M. Schuenemann. 2013. Economic comparison of reproductive programs for dairy herds using estrus detection, timed artificial insemination, or a combination. J. Dairy Sci. 96:2681-2693.

Garbarino, E. J., J. Hernandez, J. Shearer, C. Risco, and W. Thatcher. 2004. Effect of lameness on ovarian activity in postpartum Holstein cows. J. Dairy Sci. 87:4123-4131.

Giordano, J., M. Wiltbank, J. Guenther, R. Pawlisch, S. Bas, A. Cunha, and P. Fricke. 2012. Increased fertility in lactating dairy cows resynchronized with Double-Ovsynch compared with Ovsynch initiated 32 d after timed artificial insemination. J. Dairy Sci. 95:639-653.

Giordano, J. O., M. C. Wiltbank, P. M. Fricke, S. Bas, R. Pawlisch, J. N. Guenther, and A. B. Nascimento. 2013. Effect of increasing GnRH and PGF2α dose during Double-Ovsynch on ovulatory response, luteal regression, and fertility of lactating dairy cows. Theriogenology 80:773-783.

Heinonen, K., T. Shiferaw, and M. Heinonen. 1996. Oestrous synchronisation in Ethiopian highland zebu cattle by means of intravaginal cloprostenole administration. Trop. Anim. Health Prod. 28:121-125.

Higgs, R., L. Chase, D. Ross, and M. Van Amburgh. 2015. Updating the Cornell Net Carbohydrate and Protein System feed library and analyzing model sensitivity to feed inputs. J. Dairy Sci. 98:6340-6360.

Kasimanickam, R., M. Asay, P. Firth, W. Whittier, and J. Hall. 2012. Artificial insemination at 56 h after intravaginal progesterone device removal improved AI pregnancy rate in beef heifers synchronized with five-day CO-Synch+ controlled internal drug release (CIDR) protocol. Theriogenology 77:1624-1631.

Lamb, G., C. Dahlen, J. Larson, G. Marquezini, and J. Stevenson. 2010. Control of the estrous cycle to improve fertility for fixed-time artificial insemination in beef cattle: a review. J. Anim. Sci. 88:E181-E192.

Martins, J., R. Policelli, L. Neuder, W. Raphael, and J. Pursley. 2011. Effects of cloprostenol sodium at final prostaglandin F2α of Ovsynch on complete luteolysis and pregnancy per artificial insemination in lactating dairy cows. J. Dairy Sci. 94:2815-2824.

McDougall, S., R. Macaulay, and C. Compton. 2007. Association between endometritis diagnosis using a novel intravaginal device and reproductive performance in dairy cattle. Anim. Reprod. Sci. 99:9-23.

Momont, H. and B. Seguin. 1984. Influence of day of estrous cycle on response to PGF2 alpha products: implications for AI programs for dairy cattle. Pages 336-338 in Proc. Int. Congr. Anim. Reprod. AI, Vol. 3, Champaign, IL. University of Illinois at Urbana Champaign, IL.

Pursley, J., M. Mee, and M. Wiltbank. 1995. Synchronization of ovulation in dairy cows using PGF2α and GnRH. Theriogenology 44:915-923.

Pursley, J., M. Wiltbank, J. Stevenson, J. Ottobre, H. Garverick, and L. Anderson. 1997. Pregnancy rates per artificial insemination for cows and heifers inseminated at a synchronized ovulation or synchronized estrus1. J. Dairy Sci. 80:295-300.

Skenandore, C., A. Pineda, J. Bahr, A. Newell-Fugate, and F. Cardoso. 2017. Evaluation of a commercially available radioimmunoassay and enzyme immunoassay for the analysis of progesterone and estradiol and the comparison of two extraction efficiency methods. Domest. Anim. Endocrinol. 60:61-66.

Souza, A., H. Ayres, R. Ferreira, and M. Wiltbank. 2008. A new presynchronization system (Double-Ovsynch) increases fertility at first postpartum timed AI in lactating dairy cows. Theriogenology 70:208-215.

Stangaferro, M., R. Wijma, M. Masello, M. J. Thomas, and J. Giordano. 2018. Extending the duration of the voluntary waiting period from 60 to 88 days in cows that received timed artificial insemination after the Double-Ovsynch protocol affected the reproductive performance, herd exit dynamics, and lactation performance of dairy cows. J. Dairy Sci. 101:717-735.

Stevenson, J. 2016. Physiological predictors of ovulation and pregnancy risk in a fixed-time artificial insemination program. J. Dairy Sci. 99:10077-10092.

Stevenson, J. and A. Phatak. 2005. Inseminations at estrus induced by presynchronization before application of synchronized estrus and ovulation. J. Dairy Sci. 88:399-405.

Stevenson, J., J. Sauls, L. Mendonça, and B. Voelz. 2018. Dose frequency of prostaglandin F2α administration to dairy cows exposed to presynchronization and either 5-or 7-day Ovsynch program durations: Ovulatory and luteolytic risks. J. Dairy Sci. 101:9575-9590.

Vasconcelos, J., R. Silcox, G. Rosa, J. Pursley, and M. Wiltbank. 1999. Synchronization rate, size of the ovulatory follicle, and pregnancy rate after synchronization of ovulation beginning on different days of the estrous cycle in lactating dairy cows. Theriogenology 52:1067-1078.

Wijma, R., M. Pérez, M. Masello, M. Stangaferro, and J. Giordano. 2018. A resynchronization of ovulation program based on ovarian structures present at nonpregnancy diagnosis reduced time to pregnancy in lactating dairy cows. J. Dairy Sci. 101:1697-1707.

Wijma, R., M. L. Stangaferro, and J. O. Giordano. 2016. Circulating progesterone dynamics after intravaginal instillation of prostaglandin-F2α to lactating dairy cows. Theriogenology 85:1660-1668.

Wiltbank, M. C. and J. R. Pursley. 2014. The cow as an induced ovulator: Timed AI after synchronization of ovulation. Theriogenology 81:170-185.

Zdunczyk, S., P. Andresen, T. Janowski, and A. Chmielewski. 1994. The effectiveness of intravaginal administration of estrumate for cows in silent heat in relation to the progesterone concentration in the blood. DTW. Deutsche tierarztliche Wochenschrift 101:440-441.

CHAPTER VI

AN AUTOMATED CONTROLLED-RELEASE DEVICE FOR INTRAVAGINAL HORMONE DELIVERY

M. Masello, Y. Ren, D. Erickson, and J. O. Giordano

ABSTRACT

Our objective was to develop and validate an electronically-controlled hormone delivery device for reproductive control of cattle. After development and in-vitro testing of a prototype device for intravaginal (**IVG**) hormone release, we aimed to demonstrate the feasibility of inducing luteal regression by automated treatment with PGF2α (**PGF**). The IVG device comprises an outer 3D-printed plastic housing, fluid reservoirs connected to delivery pumps and tubing, a programmable circuit board, and a retention mechanism. For in-vitro testing, 4 pumps were programmed to release different target volumes (0.1, 0.2, 0.5, 1.0, and 2.0 g) in 4 replicates (n = 80). A Bland-Altman plot was constructed to assess the magnitude of disagreement between expected and delivered volumes. Observations fell within acceptable limits of agreement (1.96 SD) >95% of the time, indicating overall good agreement (mean difference=-0.005 g). To assess in-vivo performance of the IVG device, lactating Holstein cows with at least 1 corpus luteum ≥15 mm in diameter were randomly allocated to 1 of 3 treatments: IM-PGF (n=6); two 25 mg intramuscular doses of PGF (Dinoprost) 24 h apart, DEV-PGF (n=6); four 25 mg doses of PGF released automatically by the IVG device every 12 h, and DEV-CTL (n=4); insertion of an empty IVG device (placebo control). Blood samples were collected at 0, 12, 24, 36, 48, and 72 h after treatment. Data was analyzed by ANOVA with repeated measures in SAS. All devices

(10/10) remained in situ until removed at 48-h. Progesterone (**P4**) concentrations from 0 to 72 h were affected by treatment, time, and their interaction. Concentrations of P4 did not differ at time 0 but differed from 24 to 72 h as cows in IM-PGF and DEV-PGF had lesser P4 than cows in DEV-CTL. Conversely, P4 did not differ for IM-PGF and DEV-PGF during the experiment. We conclude that the current IVG hormone releasing device prototype can be programmed to automatically release PGF for successful induction of luteal regression in lactating dairy cows.

Keywords: automation, hormone delivery, dairy cow

INTRODUCTION

Timely and successful insemination of cows after they become eligible for pregnancy or a failed insemination is imperative to achieve optimal herd reproductive performance (Lamb et al., 2010; Wiltbank and Pursley, 2014). Therefore, a method used by many commercial farms to submit cows for insemination consists of synchronizing ovulation followed by timed AI (**TAI**; Pursley et al., 1995; Pursley et al., 1997). Benefits of TAI include insemination by appointment regardless of expression and detection of estrus and the possibility of achieving similar or greater fertility than by AI at detected estrus (Pursley et al., 1997; Santos et al., 2017). A major caveat of implementing TAI programs is the need to administer multiple hormonal treatments as intramuscular (**IM**) or subcutaneous injections. This problem is exacerbated as novel and more complex protocols are developed to maximize fertility, more cows need to be synchronized at the same time in larger herds, or for farms that lack critical resources to facilitate protocol implementation (e.g., dairy herd management software, proper facilities). Implementation of TAI protocols also requires significant human intervention and cow manipulation, which not only

represent a cost burden for farms, but may also affect cow natural behaviors and time budgets (Bolinger et al., 1997).

Thus, a potential strategy to reduce the burden of implementing synchronization of ovulation protocols is to develop an all-encompassing delivery system for releasing all hormones of interest in the sequence, pattern, and dose required to synchronize ovulation. A requirement for successful synchronization of ovulation with an automated device is releasing hormones of interest at pre-defined time intervals at a rate and amount that elicit the desired physiological response. This is critical for reproductive hormones such as PGF2α (**PGF**) and GnRH which exert their biological effects (i.e., LH surge for GnRH and luteal regression for PGF) by reaching target tissues in the form of sudden short-lived surges or pulses (i.e., minutes or a few hours) rather than in a sustained manner with elevated levels for prolonged periods of time (i.e., many hours or days). Another important consideration for the development of automated hormone delivery devices is placement within or on the cow body. Among the different body parts or cavities available for device placement, the vagina offers unique benefits. These include ease of insertion and removal, protection from damage or removal by contact with facilities or by other animals, constant temperature, suitability for extended retention, and efficacy of reproductive hormones after IVG delivery (Wijma et al., 2016; Wijma et al., 2017; Masello et al., 2020). Although previous efforts were made to develop some electronically controlled IVG hormone delivery devices (Cross et al., 2004; Künnemeyer et al., 2004), there is limited information about their performance and suitability for synchronization ovulation in cattle.

Therefore, our objective was to develop a fully automated hormone delivery device for reproductive control of cattle and conduct proof of concept in-vitro and in-vivo validation studies. For the in-vivo validation, we aimed to demonstrate complete luteal regression by

automated delivery of PGF because we have recently demonstrated similar luteal regression risk after IVG or IM administration of PGF (Masello et al., 2020). We hypothesized that automated delivery of PGF by an electronically controlled IVG device would induce luteal regression and the changes in circulating P4 would be similar to those observed after IM injection of PGF.

MATERIALS AND METHODS

The IVG device (Figure 1) comprises an outer 3D-printed plastic housing (12 x 4.0 x 3.0 cm), two silicone hormone reservoirs (~5 mL) connected to delivery pumps (n = 2; Takasgo Fluidic Systems, Westborough, MA), a printed circuit board (**PCB**) powered by a rechargeable battery, and a retention mechanism. The circuit board is programmed in C language to deliver target doses at a scheduled time. Two GPIO pins are routed and programmed to control the on/off switch of the n-channel MOSFETs (Diodes, Inc., Plano, TX), which controls the power supply for each peristaltic pump. In the "on" setting, liquid solution is pumped out of the hormone reservoirs through tubing that opens up to the exterior at the middle section of the device. Plastic elbows attached to each of the two orifices ensure proper liquid flow to the exterior of the device. Conformal urethane coating (M. G. Chemicals, Surrey, CAN) of the PCB was performed to prevent moisture from reaching the electrical components. To ensure ease of insertion and minimize irritation of the vaginal mucosa, the device is coated with skin-safe silicone rubber (Dragon Skin FX-pro, Smooth-on Inc., Macungie, PA).

Once the final prototype was assembled, the delivery rate for each pump was determined by loading the hormone reservoirs with distilled water and measuring the amount delivered over a 300 s period using a precision scale (Radwag USA LLC., Miami, FL). Delivery curves for four different pumps are presented in Figure 2A. Once the average release rate was defined, each

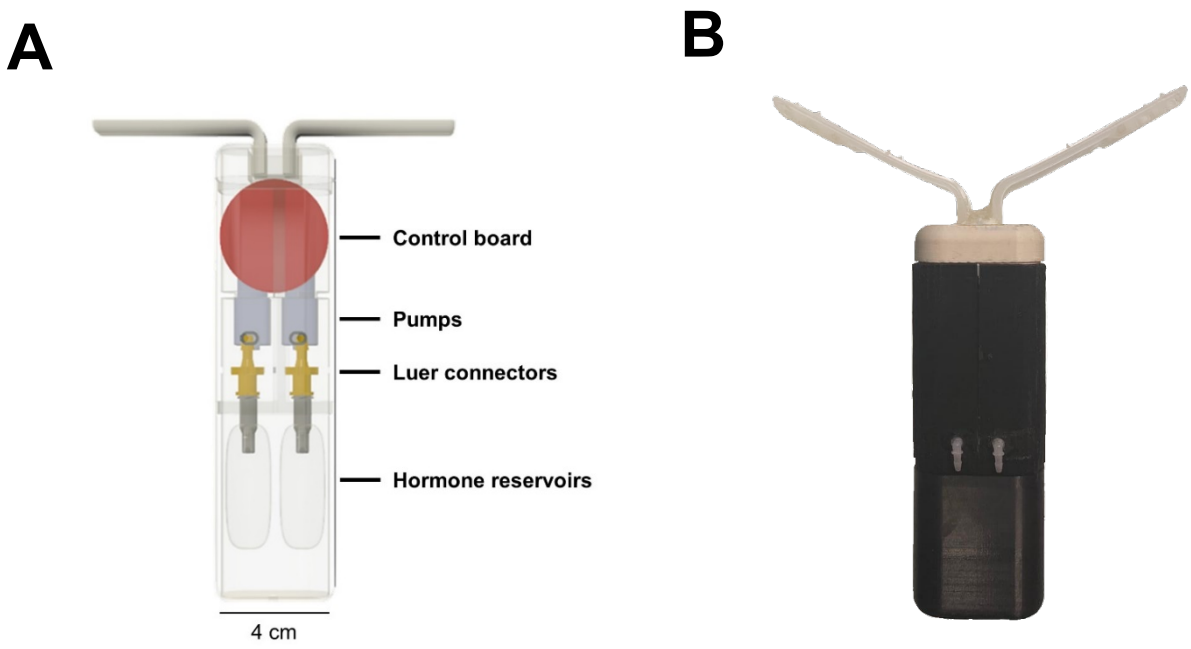


Figure 1. (A) Intravaginal device overview and internal structures. (B) Sample device used in the current experiment. PCB = printed circuit board.

pump (n = 4) was programmed to release a total of 0.1, 0.2, 0.5, 1.0, and 2.0 g of water in four replicates (n = 80 doses). In addition, long-term (i.e., 36 h) functionality was assessed in three replicates by programming IVG devices (n = 2) to deliver four 2.0 g doses following a scheme of one dose every 12 h. The timing and duration of delivery (controlled by the on/off switch) was adjusted for each target dose based on the observed pump release rate (e.g., ~250 s to deliver 2.0 g). To assess the magnitude of disagreement between the expected and delivered amounts and facilitate the detection of trends, a Bland-Altman plot (MedCalc, MedCalc Sofware bvba, Ostend, Belgium) was used to determine differences between target and delivered dose (y-axis) against target dose (x-axis).

To assess IVG device performance in vivo, lactating nonpregnant primiparous and multiparous Holstein cows from the dairy unit of the Cornell University Ruminant Center (Harford, NY) were enrolled in an experiment conducted from October 2019 to November 2019. Cows were housed in freestall barns with concrete flooring, self-locking head gates, and fans and sprinklers in the feedline. Cows were milked thrice daily at approximately 8-h intervals and were fed a TMR once a day with ad libitum access to feed and water. All procedures performed with cows were approved by the Animal Care and Use Committee of Cornell University (Ithaca, NY).

All cows enrolled received a GnRH treatment (200 µg of gonadorelin acetate given i.m., Gonabreed, Parnell Pharmaceuticals, Overland Park, KS, USA) at 40 ± 3 DIM. Seven days later, transrectal ultrasonography (**TUS**) of the ovaries was performed using a 7.5-MHz linear probe (Ibex Pro; E. I. Medical Imagining, Loveland, CO). Cows with ≥ 1 corpus luteum (**CL**) ≥ 15 mm in diameter (n = 16) were randomly allocated to 1 of 3 treatments: IM-PGF (n = 6), DEV-PGF (n = 6), and DEV-CTRL (n = 4). Cows in IM-PGF received 2 treatments of 25 mg of PGF (12.5 mg/mL of dinoprost tromethamine; Lutalyse HighCon, Zoetis, Parsippany, NJ) 24 h apart as a 2-

mL injection in the semimembranosus or semitendinosus muscle. Cows in DEV-PGF received 1 IVG device programmed to automatically release 4 doses of 25 mg of PGF at ~12 h intervals (first dose released at time 0). Cows in the DEV-CTRL treatment received an IVG device without PGF to serve as a placebo control for the presence of the device in the vagina. All devices were removed at 48 h after insertion.

Before device insertion, the vulva and perineal area were washed and disinfected with chlorhexidine solution and dried off with paper towels. Thereafter, vulvar labia were manually opened by one technician while another technician inserted the device using a custom-built applicator. Before device insertion and after removal, a vaginal integrity score (0 = no visible lesions, 1 = superficial lesions, and 2 = erosions of the vaginal mucosa; Walsh et al., 2008) and a mucus score (0 = clear or no mucus, 1 = mucus with flecks of pus, 2 = exudates containing < 50% of pus, and 3 = exudates containing ≥ 50% of pus; Sheldon, 2004) were determined for each cow using a vaginal speculum.

Blood samples (~8 to 9 mL) were collected at time 0 and at 12, 24, 36, 48, and 72 h after treatment by puncture of caudal blood vessels using heparinized evacuated tubes (Vacutainer; BD, Franklin Lakes, NJ). Samples were centrifuged at 2,000 × g for 20 min at 4 ºC. Plasma aliquots were harvested and stored at -20 ºC until assayed for progesterone (P4) in duplicate in 3 RIA assays performed as described in Beam and Butler (1997). The average intra-assay coefficient of variation (**CV**) was 13.2% whereas the interassay CV was 17.0%. For this experiment, the presence of a functional CL was defined as circulating P4 ≥ 1 ng/mL (Ginther et al., 2010), whereas complete CL regression was defined as P4 < 1 ng/mL 72 h after treatment.

The effect of experimental treatments on P4 concentrations was evaluated by ANOVA with repeated measures using the MIXED procedure of SAS (version 9.4; SAS institute Inc.,

Cary, NC) with a model that included treatment, time, and the treatment × time interaction as fixed effects, whereas cow within treatment was included as a random effect. In addition, cow within treatment was the subject of repeated-measures analysis using a spatial power covariance structure to adjust for the varying time intervals at which blood was collected. Results are presented as LSM ± SEM. Significance was declared at $P < 0.05$.

RESULTS

Device Performance In-vitro

A minor bias across the range of target doses was observed (Figure 2B). For doses in the 0.1 to 1.0 g range, all observations fell within the limits of agreement, whereas one observation from pump 1 (difference = -0.06 g, equivalent to 3% of target dose) and one observation from pump 2 (difference = 0.03 g, equivalent to 1.5% of target dose) fell outside the limits of agreement for the 2.0 g dose. The overall difference between target and actual dose averaged -0.005 g, indicating overall good agreement. For the long-term assessment, both devices were able to accurately (< 5% average error) release 2.0 g of distilled water every 12 h (Figure 2C).

Device Performance In-vivo

At the time of device insertion, all cows presented either clear or no vaginal mucus (mucus score = 0), with no visible lesions of the vaginal mucosa (vaginal integrity score = 0). At the time of device removal 4/10 cows presented mucus with flecks of pus (mucus score = 1), and 5/10 cows presented mild irritation of the vaginal mucosa (vaginal integrity score = 1). Irritation seemed to be located in areas where plastic elbows protruding from the device were in contact with the vaginal mucosa. Nevertheless, none of the cows had a score of 2 or erosions of the vaginal mucosa. In addition, none of the cows presented noticeable signs of distress or

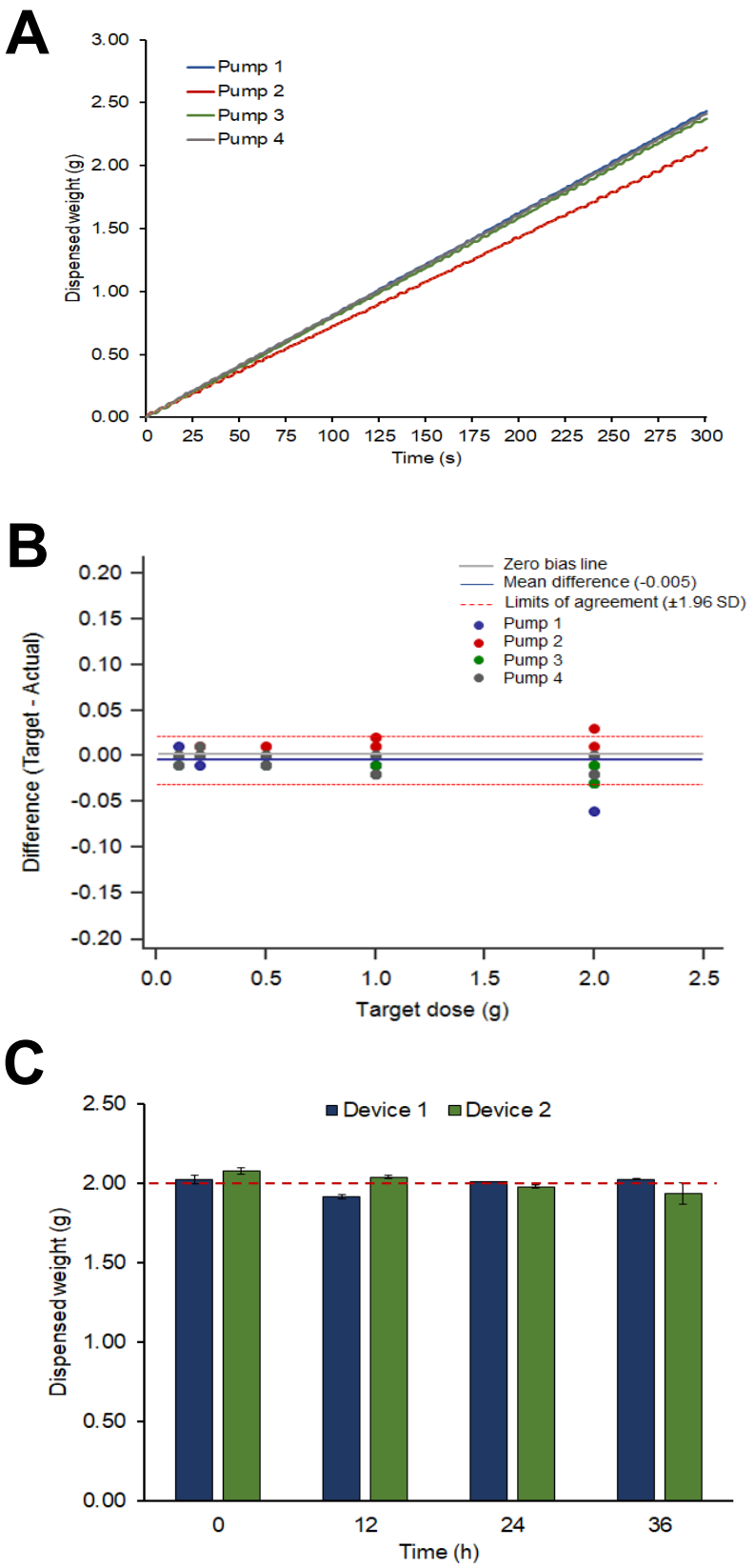


Figure 2. (A) Representative delivery curves of 4 pumps showing amount (g) of distilled water delivered over a 300 s period. (B) Bland-Altman plot, with the differences between target and delivered dose plotted against target dose. Pumps (n = 4) were programmed to release different target volumes (0.1, 0.2, 0.5, 1.0, and 2.0 g) in 4 replicates (n = 80 doses). The mean difference (-0.005g) is represented by the solid blue line and the 95% confidence limits by the red dashed lines, whereas the solid grey line represents the zero bias line (i.e., 0 g). (C) Graph depicting the average amount (g) delivered by two IVG devices programmed to release four 2.0 g doses (target dose; red dashed line) following a scheme of one dose every 12 h.

abnormalities in behavior at any time during the study period. All IVG devices (10/10) remained in situ for the 48-h study period (100% retention rate). Hormone reservoirs for all devices recovered from cows in the DEV-PGF group contained no fluid at the time of removal suggesting complete release while inserted.

The effect of treatment on circulating P4 concentration profiles is presented in Figure 3. There was an effect of treatment ($P = 0.003$), time ($P < 0.001$), and an interaction between treatment and time ($P = 0.003$). From 24 to 72 h, cows in the DEV-PGF and IM-PGF treatments had lesser concentrations of P4 than cows in the DEV-CTRL treatment (negative control). In contrast, concentrations of P4 did not differ for the DEV-PGF and IM-PGF treatments during the entire sampling period. Circulating P4 profiles for individual cows are presented in Supplementary Figure 1. Cows treated with PGF had a 67 to 92% reduction in concentrations of P4 by 36 h after the first treatment. Except for one cow from the DEV-PGF (P4 = 1.20 ng/mL) and one cow from the IM-PGF treatment (P4 = 1.21 ng/mL), all other cows had P4 <1 ng/mL at 36 h after treatment. Thereafter, concentrations of P4 continued to decline up to the end of the sampling period (0.14 to 0.50 ng/mL) when, except for one cow from the IM-PGF treatment (P4 = 0.50 ng/mL), all cows had P4 concentrations below < 0.5 ng/mL. In contrast, cows in the DEV-CTRL treatment did not experience a decline in P4 concentrations at any point after device insertion and average P4 concentration for the group was never below 1 ng/mL.

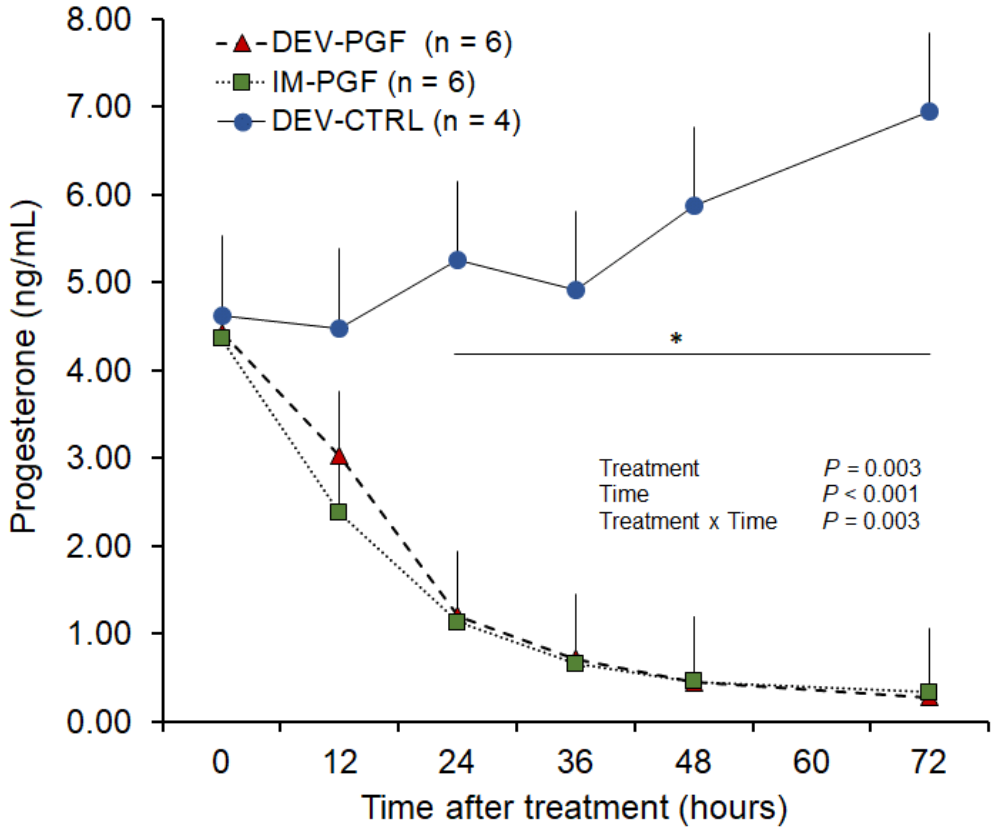


Figure 3. Circulating concentrations of progesterone (P4) from 0 to 72 h after application of treatments. Lactating Holstein cows with at least 1 corpus luteum ≥15 mm in diameter were randomly allocated to 1 of 3 treatments: IM-PGF (n=6); two 25 mg intramuscular doses of PGF 24 h apart, DEV-PGF (n=6); four 25 mg doses of PGF released automatically by the IVG device every 12 h, and DEV-CTL (n=4); insertion of an empty IVG device (placebo control). Values are presented as LSM ± SEM. *Circulating P4 differed from 24 to 72 h as cows in IM-PGF and DEV-PGF had lesser P4 than cows in DEV-CTL.

DISCUSSION

In the present study, we developed and evaluated a programmable, reusable IVG device for controlled hormone delivery in cattle. The current prototype device is capable of automatically delivering up to two different types of hormones at predefined time points. Despite a minor bias observed with increasing amounts of target doses, in-vitro results showed that the current device permits precise control of released hormone amount and timing, and that performance does not seem to decrease overtime (at least for up to 36 h). This is relevant because causing the desired biological response with reproductive hormones such as PGF and GnRH depends on hormone release in the right amount at the right time with periods of several days in between treatments. Although our proof of concept results with PGF were encouraging, additional research is required to test the ability of the developed device to release more than one hormone following typical schedules of synchronization of ovulation protocols for TAI.

In support of our hypothesis, the device was able to automatically deliver PGF and induce complete luteal regression in lactating dairy cows. The differences in P4 profiles with the placebo controls and the lack of significant differences with IM-treated cows suggested that CL regression was caused by the PGF released by the device rather than the potential physical effect of the presence of the device in the vagina. These results were expected because we recently demonstrated similar P4 profiles and luteal regression risk in cows that received PGF through the IVG or IM route of administration (Wijma et al., 2016; Masello et al., 2020) and there are no obvious biological reasons to expect that the presence of the device in the vagina would cause CL regression. Additional research is needed to determine the optimal dose, frequency, and timing of PGF to achieve CL regression rates similar than those observed with IM injection of

PGF as the purpose of this first experiment was to provide proof of concept of automated PGF release.

A concern with the use of an electronic device for vaginal insertion is the potential detrimental effect of moisture and temperature on electronic components (Cross et al., 2004). In the current experiment, our prototype device did not seem to be negatively affected by the vaginal environment as evidenced by the successful release of all the PGF loaded in the device reservoirs. This was likely because of the conformal coating of the PCB and the extra protection provided by the external silicone rubber coating, which prevented moisture from affecting the device function. In addition, high retention rates and the long-lasting battery supply also contributed to the successful delivery of all doses of PGF. One of the limitations of the current experiment, however, was that the exact timing of PGF delivery could not be confirmed because the current version of the device was not designed to communicate with external sources. In-vivo monitoring of the timing of hormone release would have required device removal and re-insertion multiple times. This was avoided due to the potential irritation of the vaginal mucosa and cow discomfort due to repeated insertion and removal. Thus, future versions of the device will include wireless communication to enable real-time in-situ monitoring of hormone release.

We observed mucopurulent vaginal discharge in approximately half the cows that received a device. These findings were expected because it is known that insertion of IVG P4 releasing devices such as the PRID and CIDR-B might result in mucopurulent vaginal discharge in a substantial proportion of cows (Chenault et al., 2003; Walsh et al., 2007; Von Krueger and Heuwieser, 2011). The observed vaginal discharge, however, seemed to be inconsequential for fertility (Walsh et al., 2007; Von Krueger and Heuwieser, 2011). In the current experiment, we also observed mild irritation of the vaginal mucosa in half the cows with an IVG device. We

speculate this was caused by the plastic elbows protruding from the device because irritation was highly localized in small areas of the vaginal mucosa that may have been in contact with these elements of the device. Thus, future device optimization will focus on minimizing vaginal irritation.

Results from this proof of concept experiment, which demonstrated the feasibility of automated delivery of PGF and successful induction of complete CL regression in lactating dairy cows are encouraging. Nevertheless, before automated electronically controlled IVG hormone-releasing devices can be deployed for control of reproduction in cattle, additional research is necessary to characterize device performance and implementation in detail. In particular, demonstrate the ability of the device to release more than one hormone of interest (e.g., GnRH, PGF and P4) for a longer period of time (e.g., ~10 to 20 d) and following more complex drug delivery profiles. Once optimized, these intravaginal hormone delivery devices may be an alternative to the injection methods presently used to administer hormones for synchronization of ovulation. On-farm use of this automated delivery system might simplify herd management and reduce animal disruption.

ACKNOWLEDGMENT

This research was supported by the Research Innovation Fund of the Cornell Initiative for Digital Agriculture project NYC-127439 from the National Institute of Food and Agriculture, U.S. Department of Agriculture. We thank German Granados, Claudio Bartolomeu, and Susanne Pelton from the Department of Animal Science of Cornell University (Ithaca, NY) for their help with blood sampling and hormone assays. We also thank personnel from the Dairy Unit of the Cornell University Ruminant Center (Harford, NY) for helping with cow management.

REFERENCES

Beam, S. W. and W. Butler. 1997. Energy balance and ovarian follicle development prior to the first ovulation postpartum in dairy cows receiving three levels of dietary fat. Biol. Reprod. 56:133-142.

Bolinger, D., J. Albright, J. Morrow-Tesch, S. Kenyon, and M. Cunningham. 1997. The effects of restraint using self-locking stanchions on dairy cows in relation to behavior, feed intake, physiological parameters, health, and milk yield. J. Dairy Sci. 80:2411-2417.

Chenault, J., J. Boucher, K. Dame, J. Meyer, and S. Wood-Follis. 2003. Intravaginal progesterone insert to synchronize return to estrus of previously inseminated dairy cows. J. Dairy Sci. 86:2039-2049.

Cross, P. S., R. Künnemeyer, C. R. Bunt, D. A. Carnegie, and M. J. Rathbone. 2004. Control, communication and monitoring of intravaginal drug delivery in dairy cows. Int. J. Pharm. 282:35-44.

Ginther, O., H. Shrestha, M. Fuenzalida, A. Shahiduzzaman, M. Hannan, and M. Beg. 2010. Intrapulse temporality between pulses of a metabolite of prostaglandin F2α and circulating concentrations of progesterone before, during, and after spontaneous luteolysis in heifers. Theriogenology 74:1179-1186.

Künnemeyer, R., P. Cross, C. Bunt, D. Carnegie, and M. Rathbone. 2004. Electronically controlled, intravaginal drug delivery. Proceedings of the Institution of Mechanical Engineers, Part B: Journal of Engineering Manufacture 218:1409-1415.

Lamb, G., C. Dahlen, J. Larson, G. Marquezini, and J. Stevenson. 2010. Control of the estrous cycle to improve fertility for fixed-time artificial insemination in beef cattle: a review. J. Anim. Sci. 88:E181-E192.

Masello, M., M. Scarbolo, M. Schneck, M. Perez, E. Schillkowsky, E. Sitko, F. S. Hernandez, E. Cabrera, R. Rossi, and J. Giordano. 2020. Intravaginal instillation of prostaglandin F2α was as effective as intramuscular injection for induction of luteal regression in lactating dairy cows. J. Dairy Sci. 103:2743-2755.

Pursley, J., M. Mee, and M. Wiltbank. 1995. Synchronization of ovulation in dairy cows using PGF2α and GnRH. Theriogenology 44:915-923.

Pursley, J., M. Wiltbank, J. Stevenson, J. Ottobre, H. Garverick, and L. Anderson. 1997. Pregnancy rates per artificial insemination for cows and heifers inseminated at a synchronized ovulation or synchronized estrus1. J. Dairy Sci. 80:295-300.

Santos, V., P. Carvalho, C. Maia, B. Carneiro, A. Valenza, and P. Fricke. 2017. Fertility of lactating Holstein cows submitted to a Double-Ovsynch protocol and timed artificial insemination versus artificial insemination after synchronization of estrus at a similar day in milk range. J. Dairy Sci. 100:8507-8517.

Sheldon, I. M. 2004. The postpartum uterus. Veterinary Clinics: Food Animal Practice 20:569-591.

Von Krueger, X. and W. Heuwieser. 2011. Effect of CIDR® on 4-day-service-rate, pregnancy rate and vaginal irritation in dairy heifers. Tierärztliche Praxis Ausgabe G: Großtiere/Nutztiere 39:277-280.

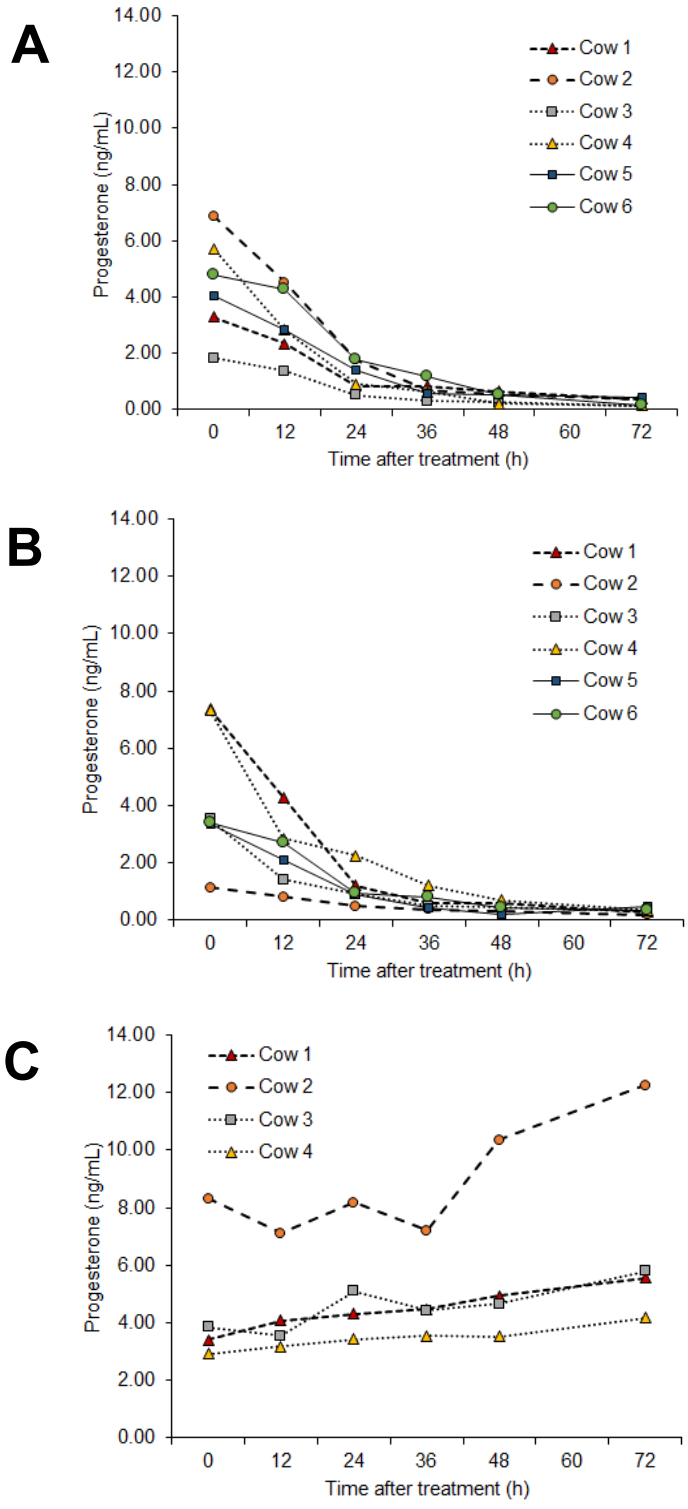
Walsh, R., S. LeBlanc, T. Duffield, D. Kelton, J. Walton, and K. Leslie. 2007. Synchronization of estrus and pregnancy risk in anestrous dairy cows after treatment with a progesterone-releasing intravaginal device. J. Dairy Sci. 90:1139-1148.

Walsh, R. B., S. J. LeBlanc, E. Vernooy, and K. E. Leslie. 2008. Safety of a progesterone-releasing intravaginal device as assessed from vaginal mucosal integrity and indicators of systemic inflammation in postpartum dairy cows. Can. J. Vet. Res. 72:43.

Wijma, R., M. Stangaferro, M. Masello, M. Elmetwally, G. Granados, F. Amovilli, and J. Giordano. 2017. Intravaginal instillation of gonadotropin-releasing hormone analogues with an absorption enhancer induced a surge of luteinizing hormone in lactating dairy cows. J. Dairy Sci. 100:7626-7637.

Wijma, R., M. L. Stangaferro, and J. O. Giordano. 2016. Circulating progesterone dynamics after intravaginal instillation of prostaglandin-F2α to lactating dairy cows. Theriogenology 85:1660-1668.

Wiltbank, M. C. and J. R. Pursley. 2014. The cow as an induced ovulator: Timed AI after synchronization of ovulation. Theriogenology 81:170-185.



Supplemental figure 1. Individual plasma progesterone profiles from 0 to 72 h after treatment for (A) cows in DEV-PGF, (B) cows in IM-PGF, and (C) cows in DEV-CTRL.

SECTION III

GENERAL CONCLUSION

CHAPTER VIII

OVERALL CONCLUSIONS AND FUTURE RESEARCH

1. Strategies to maximize reproductive and economic performance of replacement dairy heifers.

1.1. Overall conclusions

Although previous studies have evaluated the effect of different reproductive management strategies for replacement heifers, it remained unclear whether all-TAI programs for first service improve reproductive and economic performance of heifers relative to all-AIE programs. In addition, programs that combine an intensive period of AIE after PGF-induced estrus followed by submission to TAI (i.e., combined programs) were not previously explored for heifers. Therefore, we conducted a randomized controlled experiment to compare reproductive performance (Chapter II) and herd profitability (Chapter III) for heifers managed with first AI service programs that relied primarily on AIE, TAI, or a combination of both. We hypothesized that systematic use of reproductive management programs that hasten insemination through TAI (i.e., ALL-TAI) or programs that combine AIE and TAI (i.e., PGF+TAI) would reduce time to pregnancy relative to a program that relied primarily on AIE (i.e., PGF+AIE). We also expected that programs incorporating more aggressive use of TAI would be more expensive to implement but would result in improved overall economic performance of the heifer herd.

Based on the results obtained from our randomized controlled experiment with ~1,000 heifers enrolled in two commercial dairy herds, the greatest effect of the programs evaluated was a shift in timing of pregnancy. This shift was almost entirely due to the different insemination rate caused by the experimental treatments because overall P/AI to first service did not differ. More specifically, programs that relied more on TAI had reduced days to conception, with the greatest hazard of pregnancy for ALL-TAI, intermediate for PGF+TAI, and lowest for the PGF+AIE program.

From an economic standpoint, we expected that the reduction in time to pregnancy observed for the programs that relied more on TAI would translate into reduced rearing cost and increased first lactation revenue. We also expected that these economic benefits would offset the greater reproductive cost associated with implementation of TAI. Our hypotheses were supported as we observed greater reproductive cost but increased overall cash flow for the programs that used TAI (i.e., favored ALL-TAI and PGF+TAI relative to PGF+AIE). In addition, results from the stochastic analysis indicated that the economic benefits for the programs using TAI are likely to be observed under a wide range of market conditions. Notably, minor monetary differences for some outcomes included in the cash flow estimation and compensation among other items not directly affected by timing of pregnancy (e.g., replacement cost) resulted in lack of statistically significant differences in cash flow for the 15-mo study period. In addition, substantial heifer-to-heifer variation in milk income and replacement costs may have also contributed to the lack of significant differences among treatments. Assuming the numerical differences observed in cash flow (+$52 per slot for PGF+TAI and +$42 per slot ALL-TAI relative to PGF+AIE) are repeatable, it seems reasonable to suggest that the two programs that relied more on TAI may be more convenient than relying solely on AIE for dairy farms with similar management conditions

and reproductive performance than those of our experiment. In addition, considering that the success of AIE-based programs is highly contingent upon effective and proactive estrus detection, for farms with poor estrus detection efficiency the magnitude of the economic differences may favor TAI programs even more.

In summary, results presented in Section I of this dissertation showed that incorporation of TAI through either all-TAI or combined programs has the potential to improve the reproductive and economic performance of replacement dairy heifers. While implementation of such programs may result in greater reproductive costs, these are easily offset by the economic benefits of earlier pregnancy. Therefore, as long as programs that incorporate TAI reduce days to pregnancy, the additional investment to synchronize ovulation for TAI in replacement dairy heifers seems justified.

1.2. Possible future directions for research

Although our experiment provided valuable insights regarding the effect of different reproductive strategies on the reproductive and economic performance of replacement heifers, it did not evaluate programs for second and subsequent AI services. This is relevant because despite reasonable first-service fertility, a substantial proportion (~55%) of heifers needed reinsemination. By design, all heifers in our experiment that were not reinseminated at spontaneous estrus were submitted to the 5-d Cosynch protocol for TAI to ensure timely reinsemination. Many commercial farms, however, prefer to reduce their reliance on TAI by maximizing reinsemination of heifers at detected estrus. For these farms, programs that promote AIE after nonpregnancy diagnosis (**NPD**) rather than blanket use of TAI may be a more viable alternative. Such programs typically include treatment with PGF either to all nonpregnant cows

or only cows with a CL present at NPD to promote estrus expression. In the latter type of program, PGF is only given to cows that are likely to express estrus in response to treatment, whereas those without a CL are submitted directly to TAI. In addition, cows that are not detected in estrus within a certain number of days (usually 7 d) are submitted to TAI to avoid delayed reinsemination. Unfortunately, while these types of programs have been tested for lactating cattle (McArt et al., 2010; Giordano et al., 2015; Masello et al., 2020), they have not been extensively evaluated and are rarely used for replacement dairy heifers.

Thus, an experiment could be conducted to assess the reproductive performance of heifers managed with a resynchronization program aimed at promoting AIE rather than TAI after NPD (**R-AIE**). This program can be compared with a resynchronization strategy similar to that implemented in our experiment (Chapter II), in which all nonpregnant heifers were submitted to the 5-d Cosynch protocol (**R-TAI**). Moreover, it can also be compared to a strategy where all heifers are reinseminated based on spontaneous estrus (i.e., no intervention; **R-CTRL**), which represents a somewhat typical resynchronization strategy used for heifers (NAHMS, 2018). Briefly, after first service heifers would be randomly assigned to one of the three resynchronization strategies (i.e., R-AIE, R-TAI or R-CTRL). For the R-AIE group, nonpregnant heifers with a CL observed by transrectal ultrasonography at NPD would receive PGF to induce estrus. Heifers not AIE within 7 d would be enrolled in the 5-d Cosynch protocol for TAI. Conversely, heifers without a CL present at NPD will be immediately submitted to TAI. For the R-TAI group, all heifers diagnosed not pregnant at NPD will be submitted to TAI. Lastly, heifers in R-CTRL would be reinseminated relying solely on AI after spontaneous estrus. For the latter group, heifers not displaying estrus within ~45 d after a previous AI would be submitted to TAI to avoid excessively delayed reinsemination.

To determine the effect of treatments on pregnancy dynamics during the AIP, heifers would need to remain in the same treatment group for the entire experimental period (e.g., 100 d after first service). If estrus detection efficiency is as high as in the experiment presented in Chapter II, then it is reasonable to expect that the R-AIE program would increase the proportion of heifers reinseminated after detected estrus without negatively affecting time to pregnancy relative to R-TAI. On the other hand, if fertility of AIE services is greater than that of TAI (as observed in our experiment), it may be possible that the R-AIE program improves P/AI and therefore shortens time to pregnancy. In either case, the greatest benefit of this type of resynchronization program would be to reduce reliance on hormonal treatments, which may be relevant for farms with poor facilities or labor constraints. At the same time, this program would also ensure timely insemination of all heifers by submission to TAI of those not detected in estrus within a short (7 d) period of time. This would likely improve insemination rate and thereby pregnancy rate when compared to the R-CTRL program.

2. Development and integration of new technologies in cattle reproductive management

2.1. Overall conclusions

Section II of this dissertation includes our work on the development and validation of novel technologies for cattle reproductive management. The tools developed are aimed at improving farm management and cattle performance by reducing labor needs, improving animal handling and well-being, and facilitating the development of novel management strategies and treatments. Once fully developed and optimized, these technologies may be beneficial to overcome many of the limitations and challenges associated with implementation of current reproductive management practices in both dairy and beef farms. One such practice is the determination of

reproductive status of cows (e.g., pregnancy, nonpregnancy, anovulation, estrus), which can be either fully or partially accomplished by determining the presence of a functional (circulating P4 ≥ 1 ng/mL) CL on the ovaries. Typically, this is accomplished by veterinarian-administered exams or shipment of cow bodily fluids (i.e., milk or blood) for P4 quantification with laboratory assays. While some technologies for on-farm assessment of P4 have been developed, most are not easily accessible or practical for on-farm use due to cost, difficulty of implementation, low accuracy, or a combination thereof.

Thus, the main objective of the study described in Chapter V was to develop a novel platform for on-farm determination of functional CL presence. The system comprised a disposable fluorescence-based LFIA test strip along with a portable imaging device to assess circulating concentrations of P4 in plasma. Once developed, the objective was to validate the platform and its accuracy for determining functional CL presence. Using lactating dairy cow plasma samples (n = 58), the LFIA system showed high sensitivity and specificity for differentiation of samples with ≥ 1 or < 1 ng/mL of P4, with an overall accuracy of 90%. Additional improvements of this system may lead to the development of a rapid, low-cost, cow-side tool to be used as an alternative to existing on-farm methods for determination of functional CL in cattle. Such technology could be used to: (1) confirm ovulation and luteal regression in synchronization of ovulation protocols, (2) determine onset or resumption of ovarian cyclicity, (3) as a method for nonpregnancy diagnosis, (4) confirm estrus events, and (5) tailor synchronization of ovulation treatments to individual cow needs.

Another common reproductive management practice that poses numerous challenges for dairy and beef cattle operations is synchronization of ovulation for TAI. Implementation of synchronization protocols not only requires a significant amount of labor and cow handling, but

it can also disrupt cattle due to the multiple intramuscular injections required. Therefore, Chapters VI and VII describe experiments conducted with the long-term goal of developing and validating an electronically controlled IVG delivery device for automated reproductive control in cattle. Because this IVG device can be successful only if hormones used for synchronization of ovulation elicit the desired physiological response when given intravaginally, we conducted the experiment presented in Chapter VI. Our hypothesis was that luteal regression risk and P4 profiles would be similar for cows that received IVG or IM treatment with PGF during the presynchronization portion of a fertility protocol for TAI. At the time this experiment was conducted, we did not have yet developed a reliable IVG prototype device; therefore, IVG-PGF was delivered through a catheter in the cranial portion of the vagina.

Collectively, results from this experiment confirmed previous findings from our laboratory suggesting that two IVG doses of PGF 12 h apart successfully induced complete luteal regression in lactating dairy cows. Notably, we did not observe differences in luteal regression risk between IVG and IM administration of PGF. We only observed a slight delay for the initial decline in P4 after induction of luteolysis for IVG-treated cows. In spite of this delay, P4 levels did not differ at 48 and 72 h after treatments when P4 concentrations should be the lowest after PGF treatment. We speculated that the potential implications of the observed delay in P4 decline were likely minor because we did not observe differences in relevant reproductive outcomes (i.e., estrus expression, ovulation in response to GnRH, and P/AI). Thus, we concluded that IVG delivery of two PGF doses 12 h apart was as effective as an IM treatment with PGF to induce complete luteal regression in cattle and thereby could be used as an alternative route of PGF administration.

After we demonstrated the feasibility of inducing luteal regression after IVG treatment with PGF, we developed and validated a programmable, reusable IVG device for controlled hormone delivery in cattle (Chapter VII). The prototype device comprised an outer 3D-printed plastic housing, two fluid reservoirs connected to delivery pumps and tubing, a programmable circuit board, and a retention mechanism. Results from the proof of concept in-vitro and in-vivo validation studies suggested that the prototype device developed could be programmed to deliver PGF at predefined time points and successfully induce complete luteal regression in lactating dairy cows. We expect that once optimized, our IVG device may be an alternative tool to the needle-injection methods presently used to synchronize ovulation in cows. This automated system could potentially benefit farms and cattle in many ways including but not limited to: (1) minimizing labor required to synchronize ovulation by reducing the number of handlings required, (2) improving cow performance and well-being by minimizing disruption of natural behaviors, (3) maximizing synchronization of ovulation protocol compliance by controlling timing and accuracy of hormone delivery, and (4) optimize synchronization protocols by mimicking cow physiology and natural hormone release patterns. The latter could not only result in improved reproductive management and performance but might also help reduce hormone use and protocol implementation costs.

In summary, Section II of this dissertation describes the development and validation of two novel technologies for use in cattle; (1) a LFIA-based portable platform to monitor functional CL presence, and (2) an IVG hormone delivery device to automate synchronization of ovulation. Even though each of these technologies is focused on tackling different challenges associated with current management practices, the underlying long-term goal is the same; to

increase productivity and sustainability of dairy and beef cattle operations through optimization and simplification of reproductive management of females.

2.2 Possible future directions for research

One of the limitations of our current LFIA system is the reduced accuracy for determining functional CL presence when P4 levels are in the range of 0.2 to 0.8 ng/mL. Although the reason for this reduced performance is currently unclear, one possible explanation may be cross-reactivity of the P4 antibody with P4 metabolites present in bovine serum. In addition, it could also be possible that the analytical sensitivity of the assay is not sufficient to accurately detect functional CL presence when P4 levels are similar to that of the threshold used to classify samples (i.e., 1 ng/mL). In this regard, a possible way to improve analytical sensitivity is by using reporter labels, such as Europium, that provide brighter, more stable colorimetric signals while minimizing background noise. Hence, future research should focus on further optimizing the current assay format by testing other P4 antibodies and reporter labels to minimize cross-reactivity and increase analytical sensitivity, respectively.

Another limitation of our current LFIA system is the need to process whole blood samples to obtain plasma. This format not only extends total assay time but also precludes cow-side testing. Although unprocessed whole blood has been previously used as a biomatrix for LFIA testing, test strips need to incorporate a specialized sample pad capable of filtrating red blood cells. Thus, future work should focus on identifying a sample pad that enables determination of P4 concentrations in unprocessed whole blood samples.

Finally, an additional modification needed to enable immediate and cow-side readouts is to automate image processing and interpretation of results. Future research should be directed towards developing and validating algorithms that accurately determine the intensity of test and control lines and sequentially compute T/C ratios. Once developed, such algorithms can be integrated into a smartphone app to interpret and display the results to the end-user. Collectively, these modifications would enable the development of an all-encompassing, easy-to-use system that requires no trained personnel or sampling processing to determine functional CL presence in cattle. Thereafter, large-scale on-farm studies can be conducted to evaluate the overall system performance under field conditions.

Future research for the development of the automated hormone delivery device could explore the value of adjusting PGF dosage based on cow-specific factors such as CL maturity at the time of treatment (i.e., new, old, or a combination of both) and parity (primiparous vs. multiparous). Indeed, an interesting finding from the study presented in Chapter VI was that luteal regression was compromised for IVG-treated cows that presented both a new and an old CL at the time of treatment. Similarly, we observed reduced luteal regression risk (statistical tendency) for multiparous than primiparous cows (regardless of route of hormone delivery). Thus, future research could explore the value of tailoring the amount, frequency, and timing of PGF given to cows. This could be easily accomplished with the electronically controlled IVG device described in Chapter VII. Ultimately, we could establish novel protocols to maximize the proportion of cows with complete luteal regression during synchronization of ovulation and thus improve overall fertility to TAI services.

Although our current IVG device prototype was capable of accurately delivering PGF in-vivo, only an initial proof of concept experiment was conducted and is presented in Chapter VII.

Thus, additional research with more cows is required to characterize device performance and implementation in detail. In particular, future work should also focus on evaluating device performance using other hormones of interest (e.g., GnRH and P4) and the execution of longer and more complex drug delivery schemes. Equally important, future work should also explore device design optimization including but not limited to: (1) communication with external sources to enable in-situ monitoring of hormone delivery, (2) housing design and external coating to minimize vaginal irritation, and (3) retention mechanism to ensure high retention rates for prolonged periods of time (~10 to 20 d).

3. References

Giordano, J., M. Stangaferro, R. Wijma, W. Chandler, and R. Watters. 2015. Reproductive performance of dairy cows managed with a program aimed at increasing insemination of cows in estrus based on increased physical activity and fertility of timed artificial inseminations. J. Dairy Sci. 98:2488-2501.

Masello, M., B. Ceglowski, M. Thomas, and J. Giordano. 2020. A reproductive management program aimed at increasing reinsemination of nonpregnant dairy cows at detected estrus resulted in similar reproductive performance compared with a program that favored timed artificial insemination. J. Dairy Sci.

McArt, J., L. Caixeta, V. Machado, C. Guard, K. Galvao, O. Sá Filho, and R. Bicalho. 2010. Ovsynch versus Ultrasynch: reproductive efficacy of a dairy cattle synchronization protocol incorporating corpus luteum function. J. Dairy Sci. 93:2525-2532.

NAHMS. 2018. Dairy 2014, Part III: Reference of Dairy Cattle Health and Management Practices in the United States, 2014. #696.0218. Centers for Epidemiology and Animal Health, USDA: Animal and Plant Health Inspection Service: Veterinary Services, Fort Collins, CO.