UNLOCKING THE FERTILITY POTENTIAL OF HIGH-PRODUCING DAIRY COWS: A NOVEL APPROACH TO ASSESS CONCEPTUS ATTACHMENT AND INSIGHTS INTO EARLY PREGNANCY DETECTION AND LOSSES

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ABSTRACT

Artificial insemination (AI) following a detected estrus results in decreased fertility when compared to hormone-based fertility programs. It is unclear at what stage of gestation pregnancy failure occurs and why it occurs in a greater proportion following AI to a detected estrus. The objective of the present dissertation was to investigate the current fertility potential of high-producing lactating dairy cows. A novel methodology of serial measurements of pregnancy-specific protein B (PSPB) allowed for pregnancy evaluation as soon as day 19 of gestation. Reduced antral age of the pre-ovulatory was associated with greater steroidogenic capacity and more intense estruses. Cows detected in estrus ovulated follicles with greater diameter in comparison to cows synchronized with Double-Ovsynch. Follicle diameter was positively associated with 17β-estradiol to progesterone (E_2 to P_4) ratio, but the ratio was not a predictor of conceptus attachment in estrus cows. Cows that received AI to a detected estrus had decreased fertility at conceptus attachment and pregnancy diagnosis performed between days 31 and 49 post-AI in comparison to Double-Ovsynch. Pregnancy losses occurring prior to this period did not differ between treatments. Hormonal intervention enhanced intrinsic fertility of cows that had at least 2 estrus events detected prior to 1st AI. Induction of accessory corpora lutea post-AI did not improve fertility of cows treated with timed AI programs. The models utilized herein enabled the endocrine, behavioral, and fertility characterization of high-producing dairy cows detected in estrus.

With profound appreciation, I dedicate this endeavor to the mentors whose support and kindness shaped my learning journey.

“For apart from inquiry, […] individuals cannot be truly human.” — Paulo Freire

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AAM	automated activity monitors
aCL	accessory corpus luteum or corpora lutea
AI	artificial insemination
AR	autoregressive
ATP	adenosine triphosphate
BNC	trophoblast binucleate cell
CA	conceptus attachment
CIDR	controlled internal drug-releasing
CL	corpus luteum or corpora lutea
CLO	cloprostenol sodium
CV	coefficient of variation
d	day or days
D	diameter
DIM	days in milk
DO	Double-Ovsynch treatment
DPR	daughter pregnancy rate
E_2	17β-estradiol, estrogen, or estradiol
ESR1	estrogen receptor 1
EGA	embryonic genomic activation
ELISA	enzyme-linked immunosorbent assay
ES	estrus detection treatment
G or GnRH	gonadotropin-releasing hormone

h	hour or hours
hCG	human chorionic gonadotropin
ISG	interferon-stimulated genes
kDa	kilodalton
kg	kilogram
LBF	luteal blood flow
LH	luteinizing hormone
µm	micrometers
mL	milliliters
mm	millimeters
MNC	trophoblast mononucleate cell
MUC1	mucin-1
Mx1	*Myxovirus-resistant gene-1*
Mx2	*Myxovirus-resistant gene-2*
ng	nanograms
OAS1	*2'-5' oligoadenylate synthetase-1*
OPN	osteopontin
Ov.	ovulation
P/AI	pregnancies per artificial insemination
P_4	progesterone
PAG	pregnancy-associated glycoprotein
$PGF_{2\alpha}$ or PG	prostaglandin-$F_{2\alpha}$
PGR	progesterone receptor

PODXL	podocalyxin
PSPB	pregnancy-specific protein B
R	radius
r	correlation coefficient
RAA	reduced antral age treatment
RIA	radioimmunoassay
ROS	reactive oxygen species
SD	standard deviation
SELL	L-selectin
SELP	P-selectin
SELPLG	P-selectin ligand
SEM	standard error of the mean
TAI	timed artificial insemination
TGC	trophoblast giant cell
TMR	total mixed ration
Trt	treatment
VCAM1	vascular cell adhesion molecule 1
vs.	versus

CHAPTER 1

SCIENTIFIC HERITAGE AND THE POWER OF MENTORSHIP

The complexity and fascination of mechanisms leading to pregnancy have propelled the careers of numerous brilliant researchers. As early as 1918, peer-reviewed articles were being published to describe processes leading to pregnancy failure. In 1939, artificial insemination (**AI**) was formally reported as a feasible strategy to achieve pregnancy in cows. In 1948, the first article that methodically described a technique to diagnose gestation in cows manually was published. An increased influx of scientific and clinical articles dealing with pregnancy and pregnancy markers in cows can be traced back to the mid-1950s. Amongst this scientific big bang, research on reproductive physiology advanced at large. In addition to producing excellent science, many of those researchers were recognized as incredible mentors (Figure 1.1).

In 1985, the biannual award “L. E. Casida Award for Excellence in Graduate Education” was installed by the American Society of Animal Science to honor his contributions to science and mentorship of graduate students. In brief, his philosophy for graduate training entailed a sound understanding of the numerous aspects of Animal Science, mastery of techniques to gain new information without ignoring the possibilities of learning new techniques, and an integrated philosophy of research, experimentation and origin of new knowledge. This philosophy well describes the values passed to me during my graduate training. Between 1985 and 2022, 6 awardees of the L. E. Casida Award for excellent graduate training are present in my scientific tree (1985 L.C. Ulberg, 1987 H. A. Tucker, 1995 F. W. Bazer, 1997 W. W. Thatcher, 2015 M. F. Smith, 2022 P. J. Hansen).

Figure 1.1. Illustration of my scientific heritage and the heritage of my Ph.D. mentors. Connections depicted in light orange represent an advising relationship. Connections depicted in yellow denote a graduate training relationship. Connections illustrated in light blue represent postdoctoral training relationships. Connections in white denote an undergraduate training relationship. Finally, connections illustrated in dark blue represent a peers-in-training type of relationship.

The branches of their work reached other researchers, and their efforts gave rise to a highly prolific group of reproductive physiologists who continue to lead the advancements in this field. The award for “Animal Physiology and Endocrinology” was installed by the American Society of Animal Science in 1962 and awarded to 12

individuals represented in my tree (1964 A. V. Nalbandov, 1965 L. E. Casida, 1969 L. C. Ulberg, 1975 J. N. Wiltbank, 1980 F. W. Bazer, 1981 G. D. Niswender, 1983 H. A. Tucker, 1985 W. W. Thatcher, 1992 J. H. Britt, 2002 P. J. Hansen, 2009 M. F. Smith, and 2012 J. S. Stevenson). My scientific heritage nicely depicts the carryover effect and the fierce power of great mentorship.

It was an immense honor to have Dr. Asgerally Fazleabas, Dr. Barry Bradford, Dr. James Ireland, Dr. Ky Pohler, and Dr. Richard Pursley as mentors during my graduate training. Education was an aspiration that was seeded in me from a young age. I was told that through learning, reality could be changed. Having such amazing scientists as my mentors has made this process incredibly educational, fulfilling, and inspiring. I wrote the present dissertation considering the conversations we had, the questions you asked me, and the thoughts you were kind enough to share with me.

CHAPTER 2

REVIEW: THE MOST COMPLEX BINOMIAL VARIABLE TO MEASURE: "PREGNANCY"

INTRODUCTION

The development and maturation of gamete cells is a continuous process in cattle. With its origin in utero, gametogenesis will persist for the entirety of cattle's reproductive or productive lifespan (Garner and Hafez, 2000; Hafez and Hafez, 2000). Dysfunction at any stage during gamete origin, development, and maturation can result in reproductive failure (Budhwar et al., 2017). The study of fertility of cattle focuses on two possible outcomes following the encounter of male and female gametes. The primary outcome is the establishment of pregnancy, while the alternative is the inability to achieve pregnancy. In between these two outcomes lies a nuanced grey zone concerning when and how one of the two becomes factual (Figure 2.1).

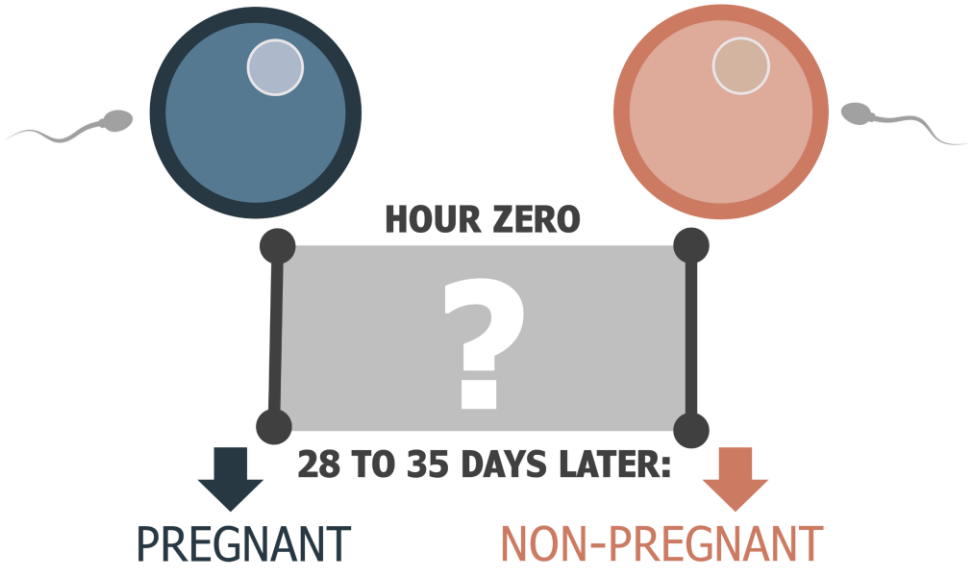

Figure 2.1. Illustration of the paradigm surrounding pregnancy outcomes in cattle. The "grey zone" represents our incomplete knowledge of what factors influence the outcome of the encounter between oocyte and spermatozoa. Our knowledge is limited due to our ability to diagnose pregnancy.

Factors that impact the occurrence or absence of pregnancy are numerous and may occur before, during, or post-conception (Wilmut et al., 1986; Gröhn and Rajala-

Schultz, 2000; Niswender, 2004; Diskin et al., 2006; Aungier et al., 2014; Wiltbank et al., 2016). The main goal of this dissertation was to characterize pregnancy survival in artificially inseminated cows earlier than the current reference diagnoses. The model utilized comprised cows that were allowed to express estrus and received AI. The reference treatment consisted of the estrous synchronization program Double-Ovsynch and timed AI (**TAI**). Another goal was to diminish the knowledge gap, or "gray zone," concerning the factors that lead to failure to achieve pregnancy following AI.

Pregnancy outcome is dependent upon the diagnostic tool employed and the timing of the diagnosis

The accuracy of diagnostic tools directly impacts metrics utilized to report outcomes at a population level. In epidemiology, it is customary to evaluate new techniques against a well-established "golden standard" to determine the sensitivity and specificity of the new technique (Parikh et al., 2008). This golden standard, once novel, underwent comparison against its predecessor. When estimating the outcomes of pregnancy in cattle, the timing of diagnosis affected the accuracy of different techniques; earlier diagnoses in gestation proved less accurate (Silva et al., 2007). While enlightening, terminal studies designed to elucidate biological processes are unsuitable for determining pregnancy survival. Before the advent of physical, image, or laboratory techniques, the most accurate pregnancy diagnosis was a combination of no return to estrus/mating and the parturition of a calf approximately 9 months post-breeding. From a scientific standpoint, data points spanning approximately 21 to 285 days are not elucidative of cow fertility. Nevertheless, this tool was considered the standard for

reporting cattle fertility outcomes in peer-reviewed articles published before 1955 (Trimberger and Davis, 1943; Salisbury et al., 1952).

Pregnancy diagnosis via physical examination of the reproductive tract

The first official report of rectal palpation employed to diagnose pregnancy can be traced back to 1948 (Wisnicky and Casida, 1948). In a review published the same year, A. Cowie mentions non-official records of avant-garde veterinarians who utilized this technique as early as 1815 (Cowie, 1948). Also, according to Cowie's review, Fleming described rectal examination as a safe and useful tool for diagnosing pregnancy in cows in 1896. This technique was refined over the decades, and different methodologies were taught to veterinarians. This technique was taught by Dr. Morrow, professor of Theriogenology, to students at the College of Veterinary Medicine at Michigan State University as early as 1970 (personal communication with alum Dr. Bob Vlietstra). However, the integration of "herd health" as a routine practice in Michigan herds gained prevalence in the late 1970s and early 1980s (personal communication with alum Dr. Bob Vlietstra). An early diagnosis could be performed as early as day 30 post-breeding (Wisnicky and Casida, 1948). A cow may be diagnosed as pregnant if at least 1 out of the 4 positive signs of pregnancy is present (Youngquist, 2006). Namely: 1) Palpation of the amniotic vesicle, 2) fetal membrane-slip test, 3) presence of a fetus, and 4) presence of placentomes.

Pregnancy diagnosis utilizing B-mode ultrasonography

Pierson and Ginther (1984b) daily examined the ovaries of n = 7 heifers during one interestrus period. Reeves, Rantanen and Hauser (1984) outlined a comprehensive methodology for ultrasonic exam of the entire reproductive tract in cattle. Pregnancy was

accurately diagnosed 28 days after conception. In the same year, Pierson and Ginther (1984a) published a second manuscript defining initial guidelines to diagnose pregnancy with ultrasound and to assess the conceptus proper up to 50 days post-AI. The latter publication was the first to define a viable pregnancy based on three criteria: 1) the presence of a non-echogenic area in the horn ipsilateral do the CL; 2) the presence of an embryo within the horn, visualized as an echogenic area; and finally, 3) rhythmic pulsation (heartbeat) within the area defined as the embryo. Forty years later, these guidelines are still utilized in both clinical and academic settings for confirming pregnancy in cattle. Detailed studies of the bovine conceptus characteristics assessed with ultrasound were later published (Curran et al., 1986; Kastelic et al., 1988). These authors reported that the detection of the embryo proper was possible on average 21.4 days of gestation, ranging between days 19 and 27 post-AI.

Ultrasonography has become the golden standard for pregnancy diagnosis since its popularization in the late 1980s (Filteau and DesCoteaux, 1998). This technique provided a consistent and reliable reference diagnosis for key studies that investigated cattle fertility (Pursley et al., 1997a; Moreira et al., 2001; Bello et al., 2006; Souza et al., 2008; Pohler et al., 2016; Borchardt et al., 2018). Accuracy in detecting the presence of an embryo increased with days in gestation (Szenci et al., 1998; Nation et al., 2003). Fertility outcomes have been assessed as early as day 28 (Geary et al., 2016; Reese et al., 2019), but customarily, pregnancy is diagnosed between 30 and 35 days post-AI (Pursley et al., 1998; Martins et al., 2011; Fricke et al., 2016; Filho et al., 2020; Middleton et al., 2022). Pregnancy losses in dairy herds and research have been estimated following this initial diagnosis.

Increased expression of interferon-stimulated genes around maternal recognition of pregnancy

Interferon-tau, a type 1 interferon, is a molecule derived from the trophoblast cells, and it was detected at day 7 of embryonic development (Imakawa et al., 1987; Rashid et al., 2018). Before interferon-tau identification, there was evidence that uterine infusion with day 17 and 18 conceptus homogenates was able to prolong the luteal lifespan in sheep (Rowson and Moor, 1967). Following the isolation of this protein, continuous systemic infusion with interferon-tau directly impeded luteolysis in sheep challenged with prostaglandin-$F_{2\alpha}$ (**$PGF_{2\alpha}$**; Antoniazzi et al., 2013). Interferon-tau is considered the primary embryonic molecule to signal pregnancy to the cow before any cell-to-cell interaction (Farin et al., 1990; Bazer et al., 1997). This signaling mechanism culminated in non-return to estrus and rescue of the corpus luteum (**CL**) by the conceptus (Helmer et al., 1989). The anti-luteolytic effect of interferon-tau involves the inhibition of endometrial oxytocin receptors, disrupting the luteolytic cascade in pregnant cows (Robinson et al., 1999). Concentrations of interferon-tau in the uterine fluid were positively correlated with conceptus area (mm^2) at days 15 (Ribeiro et al., 2016) and 16 of development (Mann et al., 2006).

Interferon-tau induces up-regulation of interferon-stimulated genes (**ISGs**; e.g., ISG15, *Myxovirus-resistant gene-1* and *-2* or **Mx1** and **Mx2**, and *2'-5' oligoadenylate synthetase-1* or **OAS-1**) in the endometrium (Forde et al., 2011), CL (Yang et al., 2010), and mononuclear and/or polymorphonuclear cells (Han et al., 2006; Gifford et al., 2007; Green et al., 2010; Matsuyama et al., 2012; Shirasuna et al., 2012). Interferon-tau also has its signal amplified via other interferons secreted from polymorphonuclear cells

present in the culture media (Fiorenza et al., 2021b). The mRNA expression of ISG15 in peripheral blood mononuclear leucocytes and polymorphonuclear cells was greater in pregnant cows in comparison to non-pregnant counterparts (Han et al., 2006; Gifford et al., 2007; Green et al., 2010, 2011). These differences were present as early as day 5 of pregnancy in polymorphonuclear cells and day 8 in peripheral blood mononuclear leucocytes (Shirasuna et al., 2012). Yet, peak ISG15 mRNA expression in leucocytes was reported to occur on day 20 of gestation (Han et al., 2006). Cows that experienced early embryonic loss had greater ISG15 mRNA expression than non-pregnant cows on day 21 of gestation, with no differences in comparison to pregnant cows (Shirasuna et al., 2012). Culture treatment of 1 and 10 ng/mL of interferon-tau induced a greater % of ISG15 and OAS-1 mRNA in peripheral blood mononuclear leucocytes and polymorphonuclear cells isolated from blood samples collected at different stages of the estrous cycle (Shirasuna et al., 2012).

Recently, a new methodology for estimating ISG15 expression has been proposed. Domingues et al. (2023a) extracted ISG15 mRNA from samples collected at day 19 of gestation. Samples were collected from the cervical os, the vaginal fornix, and leucocytes isolated from whole blood. It became evident that the ISG15 expression differences between pregnant and non-pregnant cows were greater in cervical (96.7-fold) and vaginal samples (31.0-fold) versus leucocytes (5.6-fold). Non-pregnant cows' ISG15 relative abundance was comparable in the three sample sources. This novel technique could allow for the detection of differences in ISG15 expression sooner than day 19 of gestation. Our laboratory has recently collected cervical os samples from n = 24 cows

and n = 11 heifers on days 14 and 19 post-AI. The accuracy of these samples in determining the presence of a conceptus will be retrospectively assessed.

Assessment of luteal function as a secondary marker of pregnancy

The luteal vascular system is composed of an ample bed of capillaries surrounding the periphery of the CL (O'Shea et al., 1977; O'Shea et al., 1979). Non-steroidogenic cells (mainly endothelial cells) totaled 50 – 70% of all cells in the mature CL (Farin et al., 1986). Color Doppler allows for quantitative and/or qualitative classification of the luteal blood flow (**LBF**; Yáñez et al., 2023). Luteal blood flow area was highly correlated with progesterone (**P_4**) concentrations and also predictive of luteolysis (Acosta et al., 2002; Miyamoto et al., 2005; Ginther et al., 2007). Sustained secretion of P_4 was concomitant with maintenance of the CL blood perfusion (Minela and Pursley, 2021). Rescue of the CL by the conceptus between days 18 to 21 of gestation and, as a result, continued P_4 secretion are mandatory steps for pregnancy maintenance in cows (Roberts et al., 1992, 1996a). Thus, the absence of LBF, or the functional regression of the CL, becomes incompatible with pregnancy. The subjective evaluation of luteal function with color Doppler has been employed around days 20 to 24 of gestation as a diagnostic tool (Guimarães et al., 2015; Pohler et al., 2016, 2020; Andrade et al., 2019; Siqueira et al., 2019). Cows with low or absent LBF were deemed non-pregnant with 100% negative predictive value or no false negatives (Guimarães et al., 2015; Dalmaso de Melo et al., 2020; Holton et al., 2022). The possibility to diagnose non-pregnant cows accurately as early as day 20 allowed for fast re-insemination (23 days between services; Palhão *et al.*, 2020) and improved reproductive efficiency (Pugliesi et al., 2017; Bó et al., 2018; Palhão et al., 2020). This tool becomes valuable mostly in herds with seasonal reproductive

management where achieving pregnancy is a time-sensitive milestone (Pugliesi et al., 2017).

Pregnancy-associated glycoproteins: a pregnancy-exclusive embryonic product used to diagnose pregnancy

Pregnancy-associated glycoproteins (**PAGs**) are transcribed by a multigene family (Zoli *et al.*, 1992; Xie *et al.*, 1995; Beckers *et al.*, 1999; Green *et al.*, 2000). Upon search in GENBANK for “pregnancy-associated glycoprotein” 55 results are displayed for the *Bos taurus* species when filtered for mRNA datasets. These results included PAG-encoding genes, PAG-like encoding genes, and likely some pseudogenes (GENBANK, 2024). In GENBANK, there are 21 PAGs identified in *Bos taurus* (boPAG-1, -2, -3, -4, -5, -6, -7, -8, -9, -10, -11, -12, -14, -15, -16, -17, -18, -19, -20, -21, -22). Structurally, these proteins were classified as pertaining to the aspartic proteinase family (Xie et al., 1991). Variants of this glycoprotein have different molecular weights (Xie et al., 1991) and may be more or less glycosylated, hence varying in molecular weight (Klisch et al., 2005). Phylogenetic investigations classified PAGs into an ancient (~87 million years) and a modern group (~52 million years; Green *et al.*, 2000; Hughes *et al.*, 2000; Touzard *et al.*, 2013). Pregnancy-associated glycoproteins reach maternal circulation through a process of migration and fusion with endometrial cells, reviewed in detail in Chapter 3. The first report of detection and partial characterization of this trophoblast-derived protein in cow serum dates to 1982. The protein was referred to as pregnancy-specific protein B (**PSPB**; Butler *et al.*, 1982). Different groups isolated proteins with similar molecular weights and developed immunoassays that were likely based on identical antigens (Xie *et al.*, 1991; Zoli *et al.*, 1992; Mialon *et al.*, 1993). The PSPB variant and the one named PAG-1 were

cloned (Xie et al., 1991; Lynch et al., 1992). The clone's nucleotide sequence was the same. A radioimmunoassay (**RIA**) was developed to detect PSPB as a marker of pregnancy utilizing serum samples from cows (Sasser et al., 1986; Humblot et al., 1988). An enzyme-linked immunosorbent assay (**ELISA**) was developed to measure PAG in serum samples from cows and heifers (Green et al., 2005).

Qualitative and quantitative ELISA were developed and are routinely utilized in dairy herds to detect pregnancy in milk or serum samples (Sasser et al., 2009; Green et al., 2011; Karen et al., 2015; Pohler et al., 2016). In practical trials, the measurement of PSPB concentrations between days 26 and 58 after AI (bioPRYN®) resulted in 98% sensitivity and 97% specificity in comparison to ultrasonography concomitantly performed at sampling (Piechotta et al., 2011). Another study reported that measurement of PSPB at days 37 or 38 post-AI resulted in 100% sensitivity and 84% specificity when cows were re-examined for pregnancy via palpation later in gestation (75 to 90 days). Additionally, bioPRYN had 100% sensitivity and 60.6% specificity on day 34 post-AI when the golden standard utilized was calving (Sasser et al., 2009). In the same study, ultrasound had 98.3% sensitivity (one false negative) and 64.3% specificity (ten false positives). A lower positive predictive value of PAGs as pregnancy markers has been attributed to pregnancy losses occurring between diagnoses (Silva et al., 2007; Holton et al., 2022). Moreover, decreased PAG concentrations at day 24 after AI were associated with the occurrence of pregnancy loss (Reese et al., 2018; Middleton and Pursley, 2019; Filho et al., 2020; Minela et al., 2021).

Day 28 of gestation is the earliest prescribed threshold for pregnancy diagnosis in parous cows with the use of either bioPRYN or IDEXX, two commercial assays. A single

sample collected earlier than the recommended threshold was less accurate in determining pregnancy due to high variability between pregnant cows (Reese et al., 2018; Filho et al., 2020; Pohler et al., 2020). A recent study attempted to utilize a single sample collected at day 24 post-AI to determine pregnancy status in beef heifers and cows (Filho et al., 2020). A PAG concentration cutoff was determined for heifers and cows where specificity and sensitivity to diagnose pregnancy were the highest. Pregnant heifers and cows had greater PAG concentrations in comparison with their non-pregnant counterparts. Yet, utilizing a single sample at day 24 post-AI resulted in 26.0% (cows) and 13.4% (heifers) false negatives in comparison to the reference diagnosis performed at day 30 post-AI. From a management standpoint, identifying non-pregnant cows may be more relevant than identifying pregnant cows. Cows that failed to conceive should be re-inseminated soon after diagnosis (Fricke, 2002). Aggressive re-insemination strategies will generally include the use of $PGF_{2\alpha}$ analog products (Kelley et al., 2016; Carvalho et al., 2018). Thus, falsely diagnosing pregnant cows and heifers as non-pregnant would become a major issue if inducing pregnancy loss.

Multiple sample PAG measurements: providing a within-cow baseline to increase the accuracy of pregnancy diagnosis

Serial sampling regimens provide the opportunity to estimate both hormonal concentrations and percentage/fold increase in concentrations over time. The adoption of a within cow/heifer baseline was proposed to describe dynamic increases in PSPB concentrations during pregnancy (Martins et al., 2018; Middleton and Pursley, 2019). The reference, or baseline sample, was collected before the expected time of PSPB detection in maternal circulation (average of days 16 & 20 and day 17 post-AI, for Martins et al.,

2018 and Middleton and Pursley, 2019, respectively). A second sample was collected following the expected appearance of PSPB in the maternal circulation (days 23 and 24 post-AI). Martins and collaborators estimated the accuracy of two approaches in comparison to a day 28 post-AI PSPB diagnosis: 1) the percentage increase in PSPB concentrations between baseline and day 23; and 2) concentrations of PSPB on day 23 per se. Utilizing a cutoff increase greater than 28% from the baseline had greater accuracy in comparison to utilizing day 23 PSPB concentrations alone (Martins et al., 2018). The second study was designed as a non-pregnancy diagnosis, which aimed to allow for shorter re-insemination periods (Middleton, 2019; Middleton and Pursley, 2019). A cutoff of 10% or greater from the baseline to day 24 post-AI resulted in 100% sensitivity and 94% specificity (non-pregnancy being the outcome measured). Conversely, all cows detected non-pregnant with this methodology, were truly non-pregnant based on ultrasound outcomes at day 34 post-AI. The percent change in PSPB between baseline and day 24 post-AI averaged 2% in non-pregnant cows in comparison with 122% in pregnant cows. It was hypothesized that 6% of pregnant cows experienced pregnancy loss between days 24 and 34 post-AI (Middleton and Pursley, 2019). Two trials have been completed utilizing two within-cow samples and allowed for a 35-day interval between services (Middleton, 2019; Minela et al., 2021).

Our laboratory has reported profiles of serum PSPB concentrations in dairy heifers and cows. Samples were collected from 15 to 35 days post-AI (Middleton et al., 2022). Heifers and cows were diagnosed as pregnant or non-pregnant according to an ultrasound examination performed on day 36 post-AI. Average PSPB concentrations from days 15 to 17 were utilized as the within-cow or within-heifer baseline. The criteria to

determine pregnancy consisted of 1) a day with an increase of 12.5% or greater from the baseline and 2) two more days with 12.5% or greater increases from the day before. All cows that were confirmed pregnant with ultrasound on day 36 met this criteria. Serum PSPB concentrations in pregnant heifers diverged/increased from their non-pregnant counterparts 2 days earlier in comparison to pregnant cows. Pregnant heifers also maintained greater PSPB concentrations from days 23 to 29 post-AI in comparison to pregnant cows. This initial study provided evidence of time differences in PSPB detection in maternal circulation. These temporal differences were associated with parity. Multiparous cows had the longest average time to initial increase in PSPB concentrations in the maternal circulation. Pregnancy-specific protein B concentration profiles during early pregnancy and the daily measurement of this pregnancy marker can impact our understanding of dairy cow fertility. This topic will be further discussed in Chapter 3 and is the fundamental methodology utilized in studies reported in Chapters 5, 6 and 7.

FAILURE TO SUSTAIN PREGNANCY IS A BOTTLENECK FOR COW FERTILITY

Unquestionably, scientific and technological advancements have established effective tools to diagnose pregnancy at different stages, as reviewed in the present chapter. There is a negative linear association between the time to diagnosis and the likelihood of pregnancy (Figure 2.2). Pregnancy losses occurring within one month from AI have been remarkably underdiagnosed until 15 to 20 years ago. The initial step toward furthering the knowledge concerning pregnancy loss occurrence in dairy cows is to employ accurate methods that will define and quantify this phenomenon. Interventions to

improve pregnancy survival following AI will stem from the ability to diagnose this problem.

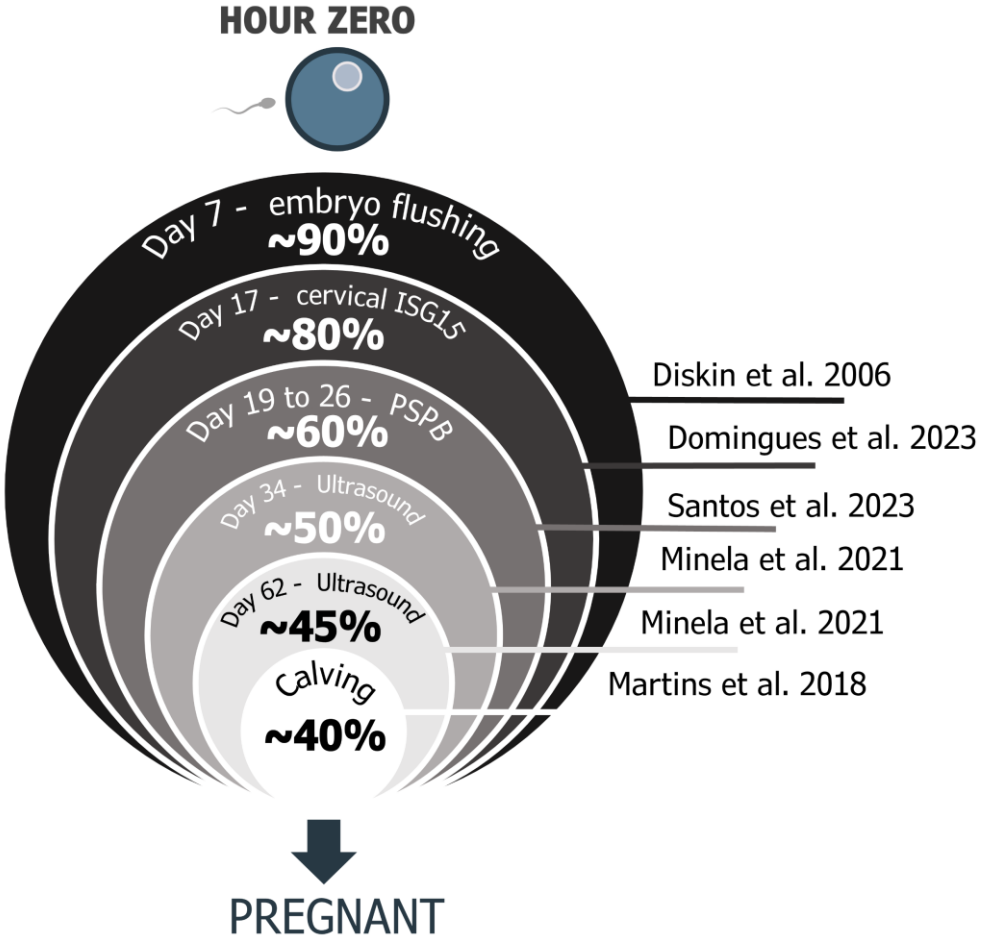

Figure 2.2. Diagram demonstrating the impact of timing to diagnosis and diagnostic tools on the observed pregnancy outcomes (pregnancies per AI; %) of lactating dairy cows. The diagram also depicts how the occurrence of pregnancy losses from the time of fertilization ("HOUR ZERO") to parturition can impact our understanding of dairy cow fertility (Diskin et al., 2006; Martins et al., 2018; Minela et al., 2021; Domingues et al., 2023a; Santos et al., 2023). The black-to-white color scale represents the improvement in the accuracy of diagnostic tools as gestation progresses. Abbreviations: ISG15 (*interferon-stimulated gene 15),* PSPB (pregnancy-specific protein B).

Outcomes from controlled randomized trials that evaluated/reported reproductive efficiency following AI could be deemed "confounded" due to undiagnosed pregnancy losses occurring prior to the primary diagnosis (conventionally performed around 35 days of gestation). This lack of data concerning pregnancy outcomes before the typical day 35

diagnosis will be addressed in this dissertation. Daily PAG measurements provide early evidence of the presence of a conceptus, but also its viability. Biological processes leading to physical and molecular interactions between conceptus and dam, as well as possible dysfunction of these processes are the focus of the literature review in Chapter 3.

CHAPTER 3

REVIEW: THE PARADOXICAL INTERACTIONS BETWEEN THE BOVINE CONCEPTUS AND COW WHICH DEFINE PREGNANCY

SLOW AND STEADY:

THE FIRST WEEK OF LIFE

From the time of fertilization, paternal and maternal genetic and epigenetic characteristics may impact the fate of a newly conceived embryo. Gametogenesis is a lengthy process, and environmental conditions can lead to errors that compromise gamete quality (Rizos et al., 2002; Roth and Hansen, 2005; Pause et al., 2022; Ruebel et al., 2022). Decreased gamete quality has been mainly linked with thermal stress and excessive exposure to reactive oxygen species (**ROS**; Ouellet et al., 2021). Excessive exposure to ROS, low tolerance, or a combination of the two leads to DNA damage presented as single- or double-stranded DNA breaks (Takahashi et al., 2000; Sirini et al., 2017; Musson et al., 2022). Cells have the machinery necessary for DNA repair. Due to cell size differences, spermatozoa and oocytes have different capacities to manage DNA repair (Marchetti et al., 2015). Spermatozoa undergo extensive cytoplasmatic condensation during spermatogenesis and lose most of the cell machinery necessary to repair DNA if needed (Ribas-Maynou et al., 2022). The oocyte, however, has the means to manage DNA repair more efficiently (Carroll and Marangos, 2013). This is important because the cell machinery and mRNAs accumulated during oocyte maturation will be transferred to the embryo and utilized for its development until it becomes self-sufficient (Hyttel et al., 1986; Fair et al., 1997). The zygote can utilize maternal machinery to repair paternal DNA damage that occurred pre- and post-meiosis (Ménézo et al., 2010; Ribas-Maynou et al., 2022). However, repairing DNA takes precedence over mitosis, culminating in delayed embryonic development (Shaltiel et al., 2015). Embryos derived from sperm exposed to increasing levels of oxidative stress had compromised

development between the first cleavage to the blastocyst stage (De Castro et al., 2016). Additionally, induction of DNA damage during oocyte maturation was also deleterious for early embryonic development (Sirini et al., 2017). The embryo itself is highly susceptible to environmental stressors such as heat stress during the first 7 days of life. A heat stress protocol applied throughout the embryo production process (in vitro maturation, in vitro fertilization, and in vitro culture) resulted in decreased cleavage rate, lower interferon-tau gene and protein relative expressions, and greater ROS production in comparison to thermoneutral counterparts (Amaral et al., 2020).

If provided with sufficient mRNAs, the embryo can develop for four days without initiating its transcriptome. The bovine embryo becomes independent of maternal and paternal mRNA at the 8-16 cell stage (Barnes and First, 1991; Tejomurtula et al., 2009; Graf et al., 2014). This event is referred to as embryonic genomic activation (**EGA**). Onwards, the embryo is left “orphan” to produce all the transcripts necessary for further cell proliferation and, more importantly, cell differentiation (Vallée et al., 2009; Sirard, 2012). The blastocyst is the first embryonic structure that contains different cell types with distinct fates. This stage is achieved between days 6 and 7 of development (Winters et al., 1942; Hamilton and Laing, 1946; Lonergan et al., 2016). Activation of different genes will result in different cell types. In bovine embryos, cells in the inner cell mass, or the embryoblasts, have higher relative expression of genes such as *NANOG* and *sex-determining region Y HMG-box 2* or *SOX2* (Ozawa et al., 2012) and will originate the embryo proper. Cells in the trophectoderm, or trophoblasts, were identified as having a higher relative expression of the *CDX2* gene (Ozawa et al., 2012) and will compose the external membranes that support continuous development.

During the first week of life, the embryo must develop steadily to reach the blastocyst stage. Early blastulation was associated with greater post-hatching viability of bovine embryos (Huayhua et al., 2023). However, a balance between fast and adequate development is imperative. Bovine blastocysts that had increased uptake of essential amino acids in the culture media also had more significant DNA damage (Sturmey et al., 2009). This was likely due to oxidative phosphorylation for adenosine triphosphate (**ATP**) production in the pre-implantation embryo (Thompson et al., 1996). Oxidative phosphorylation is highly efficient in producing ATP, but it also results in the production of ROS as a byproduct (Thompson et al., 2000; Harvey et al., 2002). As previously referenced, ROS can cause DNA damage and possibly induce developmental arrest or delay. From these observations arose a theory that besides developing fast, the embryo should do so in an efficient manner, or “quietly,” to avoid DNA damage. Hence the name “The Quiet Embryo Hypothesis” (Leese, 2002; Sturmey et al., 2009; Leese et al., 2022). A recently published study corroborates this hypothesis (Lockhart et al., 2023). Embryos derived from known low-fertility sires had increased autophagy markers compared to high-fertility sires. Autophagy is a cellular mechanism used to eliminate damaged cells or cell organelles. Several genes involved with DNA damage and repair of DNA damage were upregulated in embryos derived from low fertility sires. These embryos also had a greater proportion of development arrest at the 2-4 cell stage and delayed development. Nonetheless, there were no differences in the number of embryoblast and trophoblast cells at the blastocyst stage. This could suggest that despite suboptimal conditions, the embryo could still sustain development. Development past the hatching of the blastocyst

is non-measurable in in vitro conditions. Blastocyst morphology may be unaltered despite the presence of DNA damage and oxidative stress.

1,000-FOLD GROWTH:

FROM BLASTOCYST TO FILAMENTOUS CONCEPTUS

Until this stage of development, the embryo went through several events of cell division and differentiation without increasing in size. A blastocyst has a diameter of approximately 200 µm; this is virtually the size of an unfertilized haploid oocyte (Spencer et al., 2017). Nevertheless, subtle differences in cell number at this stage impacted the embryo's prospect for growth (O'Hara et al., 2014). Elongation of the conceptus commences following the rupture of the zona pelucida (days 8 to 10; Massip and Mulnard, 1980). The trophectoderm is no longer constrained within the zona and can undergo intense proliferation. It grows from 200 µm on day 7 to at least 20 cm on day 19 of development. The 1,000-fold increase in length and weight within 11 to 12 days is attributed to the growth of the trophectoderm rather than the embryo proper (Figure 3.1).

This is the onset of unrestricted co-habitation between the embryo, now termed conceptus, and the uterine environment. Physical connection is not possible yet because of endometrial characteristics that will be reviewed later in this chapter. Nonetheless, the conceptus benefits from growth factors and nutrients in the uterus and rapidly elongates (Spencer et al., 2017). Products of the uterine glands secreted into the lumen are referred to as the histotroph (Roberts and Bazer, 1988; Bazer et al., 2012). Qualities of the histotroph were investigated during the period of conceptus elongation, and results revealed the presence of more than 5,000 biochemicals (Simintiras et al., 2019b). A total of 233 metabolites were significantly enriched in histotroph during conceptus elongation

(Simintiras et al., 2019b). These biochemicals clustered into amino acids (33.9%), lipids (32.2%), carbohydrates (8.6%), nucleotides (8.2%), xenobiotics (6.4%), vitamins and cofactors (5.2%), energy substrates (4.7%), and peptides (0.9%). The quality of the histotroph was modified upon P_4 supplementation (Simintiras et al., 2019c; d; a). Early P_4 supplementation advanced conceptus elongation (Clemente et al., 2009; Rizos et al., 2012). The clusters of biochemicals that were primarily increased with P_4 supplementation were amino acids, carbohydrates, and lipids (Simintiras et al., 2019b; a). Many of the biological pathways activated in day 19 elongated conceptuses were related to metabolism (Mamo et al., 2011).

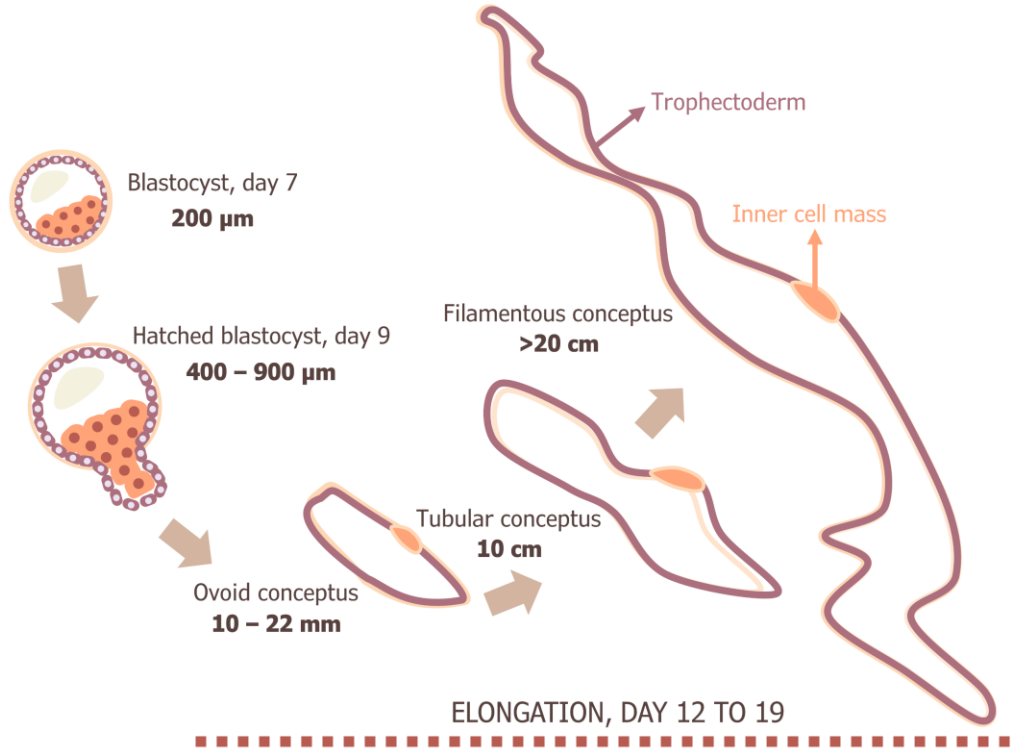

Figure 3.1. Depiction of the exponential growth between the blastocyst stage and elongation of the bovine conceptus. Adapted from Spencer et al. (2017).

Contrarily, an unhealthy uterine environment and improper histotroph secretion may hinder conceptus elongation. Cows with induced endometrial inflammation had

shorter conceptuses 16 days post-AI (Husnain et al., 2023). Cows had received uterine infusions of *Escherichia coli* and *Trueperella pyogenes* 26 days before AI to induce endometrial inflammation. The metabolome of the histotroph was also altered. Conceptus that developed in an unhealthy uterine environment had lower expression of genes involved in nutrient uptake and cell growth, amongst others (Husnain et al., 2023).

Additionally, there is physiological variation in the development rates in seemingly healthy cows. Of 90 recovered conceptuses on day 15 after AI, approximately half were tubular, a quarter were ovoid, and the remainder were filamentous (Ribeiro et al., 2016). Once again, advanced elongation (filamentous vs. ovoid conceptuses) was associated with the upregulation of genes involved with lipid metabolism.

On day 20 of gestation, the conceptus occupied the entire gravid uterus (Chang, 1952). Evolutionarily, this rapid growth could serve two purposes: 1) increasing the output of pregnancy recognition factors that are conceptus-derived and necessary to continue gestation, and 2) increasing the surface area of the external membranes to facilitate physical attachment to the endometrial lining. The approach adopted in the present text is that the former is imperative for the latter.

A MOLECULAR-LEVEL DIALOGUE:

THE INITIAL NETWORK BETWEEN CONCEPTUS AND DAM

Modifying the physicochemical attributes of the endometrium is mandatory for conceptus apposition, attachment, and adherence to the uterus (Guillomot, 1995; Spencer et al., 2004). These events preceded the formation of a more definitive feto-maternal network or the placenta (Imakawa et al., 2017, 2018). The expression of interferon-tau proportionally accompanies the expansion of the trophectoderm (Hansen

et al., 1988; Robinson et al., 2006). As reviewed in Chapter 2, interferon-tau is the main pregnancy recognition factor in cattle. The roles of interferon-tau involved maintaining luteal function (Helmer et al., 1989; Antoniazzi et al., 2013) and modulating immune cells' response to the allogeneic conceptus (Bai et al., 2012; Fiorenza et al., 2021b; a). In bovine conceptuses, interferon-tau mRNA expression peaked at days 17 to 19 of pregnancy and decreased exponentially as conceptus attachment occurred (Farin et al., 1990). Chemically mediated changes in the endometrium occurred while the conceptus was free-floating in the uterus but ultimately favored the physical establishment of feto-maternal communication (Bowen and Burghardt, 2000). Such changes are necessary because apical cell-to-cell interaction is, in essence, a biological paradox (Denker, 1993). The apical endometrium comprises polarized epithelial cells that are highly glycosylated and have non-adherent characteristics (Aplin, 1997; Jones and Aplin, 2009). Changes in these properties constitute what could be termed “uterine receptivity” (Denker, 1993). This concept was proposed in human reproduction 50 years ago and described as the “window of implantation”, implying that timing and synchrony between conceptus and dam are essential for implantation and successful pregnancy establishment (Psychoyos, 1974).

The loss of non-adherence properties leads to uterine receptivity

Mucin-1 (**MUC1**) is a highly glycosylated glycoprotein with an extended extracellular domain anchored throughout the uterine luminal and glandular epithelium (Gendler and Spicer, 1995). The molecular weight of MUC1 ranges from 120 to 225 kDa, but due to the potential for O-glycosylation, it may weigh 250 to 500 kDa (Gendler and Spicer, 1995). The extracellular domain extends from 200 to 500 nm from the plasma membrane (Figure 3.2). Most transmembrane receptors consist of protrusions of about

50 nm (Brayman et al., 2004). Due to these structural properties, MUC1 acts as an anti-adhesion molecule via physical blockage of membrane receptors that could interact with the conceptus (Hilkens et al., 1992). Embryonic implantation in mice seemed to occur concomitantly with decreases in MUC1 mRNA (Surveyor et al., 1995). In sheep, it was demonstrated that in vitro interferon-tau treatment decreased the expression of MUC1 (Wang et al., 2018). Also, in sheep, MUC1 was non-detectable in endometrial epithelia collected near conceptus attachment (Johnson et al., 2001). In a mouse model, estrogen (**E_2**) increased MUC1 expression while P_4 inhibited the E_2-positive feedback (Surveyor et al., 1995). A recent study evaluated the effects of steroidal hormones and interferon-tau in cultured endometrial epithelial cells isolated from cycling cows (Kubota et al., 2021). Progesterone decreased, and E_2 increased the expression of MUC1 in cultured endometrial cells. The co-culture with bovine blastocysts, utilized to incorporate interferon-tau into the media, did not alter MUC1 expression (Kubota et al., 2021). Nevertheless, the interferon-tau produced at the blastocyst stage may not be representative of the interferon-tau secretion attained after elongation. The relative interferon-tau mRNA abundance was diminished at the blastocyst stage compared to a day 16 conceptus (Bertolini et al., 2002). Nonetheless, the interferon-tau function in prolonging luteal lifespan and maintaining P_4 concentrations may synergize with P_4's regulatory mechanisms of MUC1 expression in cows. Moreover, subfertile cows had greater MUC1 and lower interferon-tau relative mRNA expression during diestrus than fertile cows (Kasimanickam et al., 2014; Kasimanickam and Kasimanickam, 2020). This evidence reiterates that altering physicochemical characteristics of the endometrial epithelium is essential for conceptus-maternal interaction and pregnancy survival.

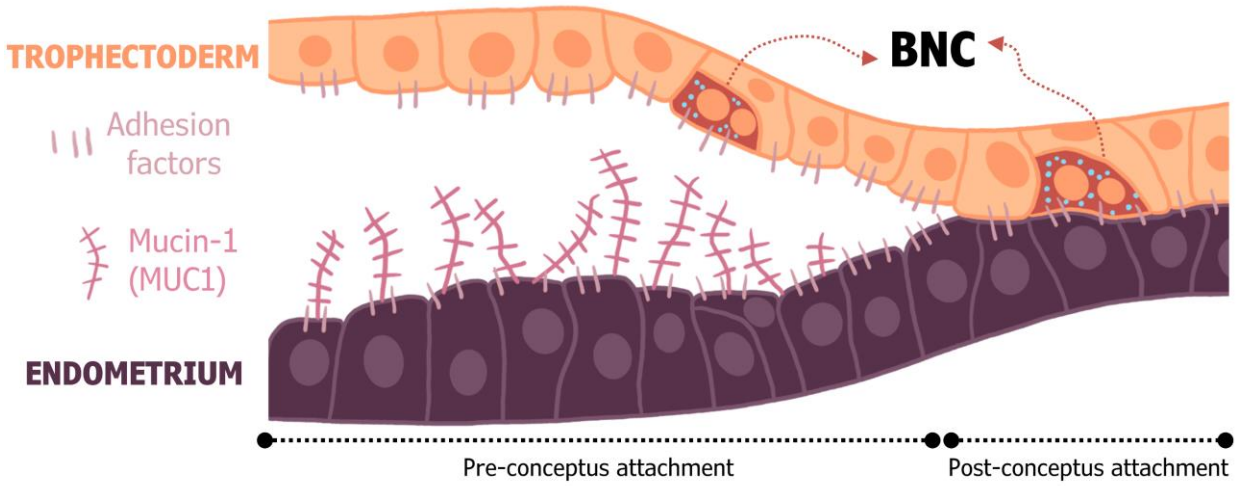

Figure 3.2. Illustration of mucin-1 (MUC1) expression in the endometrium during the pre- and post-conceptus attachment period. Trophectoderm and endometrium express adhesion factors that are unable to interact due to physical blockage of MUC1. Downregulation of MUC1 is part of the processes that lead to uterine receptivity and conceptus attachment. Note the presence of binucleate trophoblast cells (BNC) that are PAG-positive.

Unconstrained molecular connection

The downregulation of MUC1 culminates in the exposure of the endometrial epithelium, and at days 19 to 20 of gestation, the trophectoderm and endometrium are in close apposition (Wathes and Wooding, 1980). Measurement of the intermembrane spaces provided insights into molecules involved in the adhesion process on epitheliochorial placentas (Klisch and Schraner, 2021). In sheep, deer, pigs, and cattle, the intermembrane distances were comparable and greater in size in fetal-fetal compared to maternal-maternal interfaces. This suggested the presence of different-sized molecules at the fetal and maternal interfaces involved in attachment that are likely conserved between species with epitheliochorial placentas (Klisch and Schraner, 2021). Most studies that elucidated the roles of adhesion factors were performed in rodents, humans, pigs, or sheep, with some studies in cattle. The objective of this section is to review the overall function of these factors, regardless of species (Figure 3.3).

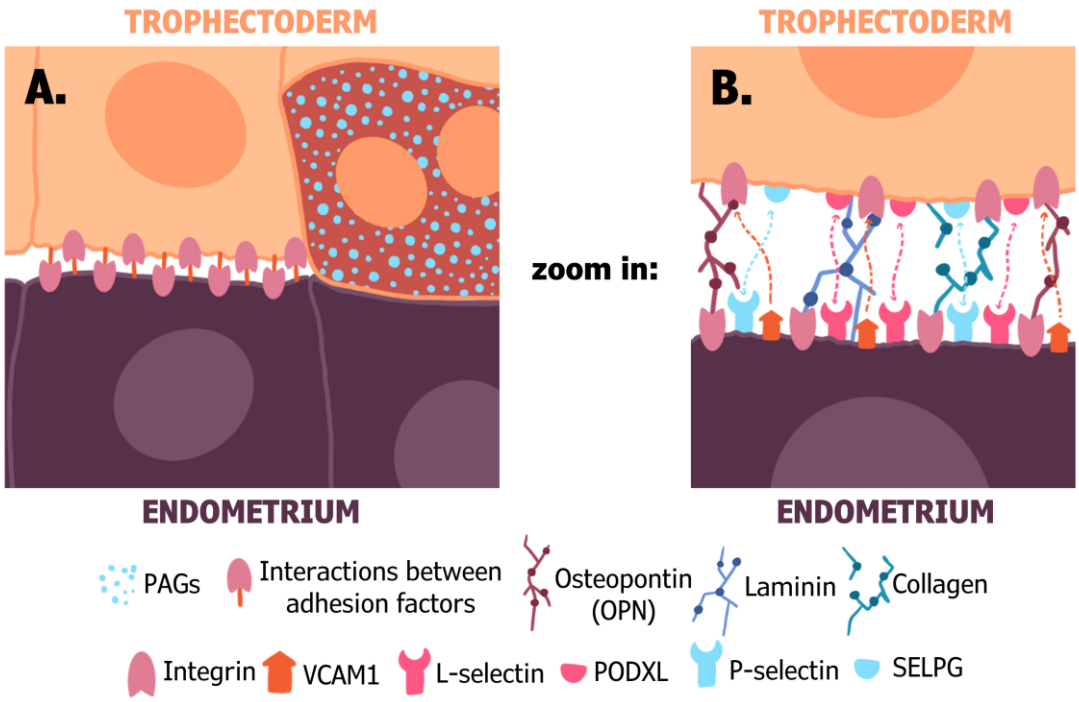

Figure 3.3. Summary illustration of the molecular interactions occurring in the conceptus-maternal interface after elongation of the conceptus. The physical proximity of the opposing epithelia allows for the interactions between adhesion factors and extracellular matrix proteins expressed in the trophectoderm and endometrium (A). Integrins are non-specific and may bind to osteopontin (OPN), laminin, and collagen. It also may interact with vascular cell adhesion molecule 1 (VCAM1) expressed exclusively in the endometrium. Uterine selectins (L- and P-selectin) are expressed in the endometrium, and their ligands podocalyxin (PODXL) and P-selectin ligand (SELPG) are concomitantly expressed in the trophectoderm. The arrows are color-coded according to each molecule and indicate their binding sites (B). Note the presence of binucleate trophoblast cells that are PAG-positive.

Initial apposition and attachment are localized around the embryo proper and ipsilateral to the ovulation/CL (Wathes and Wooding, 1980). Small villi develop in the trophoblasts located in the intercaruncular regions to facilitate the conceptus immobilization (Guillomot and Guay, 1982) and interaction with the endometrium and extracellular matrix. This interactivity was mediated via adhesion molecules (Johnson et al., 2001; Bai et al., 2014, 2015; Kusama et al., 2016). Currently, adhesion molecules identified to be involved in conceptus-maternal apposition and adherence in cattle include

but are not limited to integrins, endometrial selectins (L- and P-selectin), and vascular cell adhesion molecule 1 (**VCAM1**). These adhesion molecules may also interact with extracellular matrix proteins such as osteopontin (**OPN**), laminin and collagen (Figure 3.3). Noteworthy is that these molecules are critical factors in several physiological and pathological adhesion cascades in which the apical surface of a cell adheres to polarized epithelia (Chavakis et al., 2009; Bendas and Borsig, 2012). Conceptus-maternal membrane apposition, attachment, and adhesion involve similar mechanisms of cell-to-cell interaction (D'Occhio et al., 2020).

Integrins are expressed in the trophectoderm (Sutherland et al., 1993) and the endometrium (Bowen et al., 1997). This transmembrane receptor has 26 subunits (18 α- and 8 β-subunits). These subunits can be combined, resulting in the formation of 24 distinct heterodimers. Integrins are non-selective in the sense that they can bind to numerous extracellular matrix proteins (Ruoslahti and Reed, 1994) and are involved in adhesion cascades during pregnancy (Johnson et al., 2023). The extracellular matrix protein OPN was proposed as an intermediary molecule that would bind to integrins from the opposing membranes of the fetomaternal interface working as an "anchor." A series of experiments at Texas A&M University investigated the relationship between OPN and integrins (subunits αv, α4, α5, β1, β3, and β5) expression in sheep. Osteopontin was identified in the apical surface of the uterine luminal and glandular epithelium of pregnant ewes and in the conceptus trophectoderm (Johnson et al., 1999b; a). Similarly, integrin subunits αv, α4, α5, β1, β3, and β5 were localized in those same tissues (Johnson et al., 2001). From these subunits, previously identified receptors for OPN can be assembled (αvβ1, αvβ3, αvβ5, and α5β1; Miyauchi et al., 1991). In ovariectomized ewes, P_4

regulated OPN mRNA expression in glandular epithelium, while a P_4 antagonist ablated OPN expression (Johnson et al., 2000). Co-incubation of OPN-coated beads with uterine epithelium and conceptus trophoblast cells resulted in functional activation of integrin, cytoskeletal remodeling, and focal adhesions (Johnson et al., 2001). Altogether, it is plausible that OPN is one of the extracellular matrix proteins that interact with integrins to promote conceptus apposition and attachment to the endometrium (Bowen and Burghardt, 2000).

Other extracellular matrix components and their affinity for integrins were investigated during the peri-implantation period in cattle (MacIntyre et al., 2002). Laminin and collagen IV were present in pre-attachment trophoblasts but were not detected or less detected as attachment occurred (days 21, 24, and 30 of pregnancy). Analogously, the integrin α1 subunit followed the same expression pattern, with greater expression in pre-attachment trophoblasts. This evidence suggested that in cattle, laminin, collagen IV, and the α1 integrin subunit are involved in initial conceptus attachment (MacIntyre et al., 2002). The α1 subunit can heterodimerize with the β1 subunit. The α1β1 integrin heterodimer was required for human trophoblast interaction with laminin (Hall et al., 1990). The α6 subunit was expressed later in gestation, with linear, greater post-attachment expression (MacIntyre et al., 2002). Given its expression pattern, the authors suggested that the α6 subunit may be involved in trophoblast binucleate cell (**BNC**) migration across the conceptus-maternal interface. The role of BNCs in the process of attachment, adhesion, and placentation is later discussed in this chapter.

Two other molecules were investigated at the University of Tokyo as possible adhesion factors involved in conceptus attachment: endometrial selectins (L-selectin:

SELL and P-selectin: **SELP**) and VCAM1. Two studies demonstrated the roles of SELL (Bai et al., 2015) and VCAM1 (Bai et al., 2014) during conceptus attachment in cattle. The relative fold change in SELL and SELP uterine expression was greater in pregnant cows than in cyclic cows. Uterine SELL was upregulated between days 17, 20, and 22, whereas SELP was upregulated on days 20 and 22 of pregnancy. Possible ligands for these adhesion factors were upregulated in conceptuses. The ligand podocalyxin (**PODXL**) had a greater expression on day 22, whereas the P-selectin ligand (**SELPLG**) was upregulated on days 20 and 22 conceptuses. The concurrent expression of SELL and SELP in endometrial cells with their ligands in trophoblast cells indicates a timely change in the transcription of molecules that lead to attachment and adhesion. In a co-culture system (endometrial epithelial cells + trophoblasts), the expression of SELL and SELP was purposefully decreased via small interfering RNA. The absence of SELL and SELP coincided with greater interferon-tau release from trophoblast cells (Bai et al., 2015). In vitro studies indicated that upon attachment of trophoblast and endometrial cells, interferon-tau is down-regulated (Sakurai et al., 2012). Thus, SELL and SELP appeared indispensable for in vitro cellular attachment. Similarly, VCAM1 had greater expression in endometrial cells of pregnant cows at days 20 and 22 of gestation compared to cyclic cows. Treatment with uterine flushing from pregnant cows increased VCAM1 expression in cultured endometrial cells. Co-culture with trophoblast cells enhanced VCAM1 expression in endometrial cells (Bai et al., 2014). The integrin heterodimer α4β1 functions as a ligand for VCAM1 and was upregulated in day 22 conceptuses but not in endometrial cells.

The complexity of the interactions between transmembrane receptors, adhesion factors, extracellular matrix proteins, and two opposing epithelia that should not interact is far from being fully elucidated in cattle. However, it becomes evident that integrins actively participate in adhesion, and molecules such as OPN, uterine selectins, and VCAM1 may work as intermediary factors.

CONCEPTUS ATTACHMENT:

MOLECULAR TO PHYSICAL INTERACTION

This initial cell-to-cell interaction establishes the membranes' apposition and adhesion as pregnancy advances (Guillomot, 1995). In addition to molecular-level modifications, electron microscopy revealed timely changes in the morphology of the conceptus-maternal interface. A firm attachment between the trophectoderm surrounding the embryo and the endometrium of the pregnant horn was observed as early as day 20 (Wathes and Wooding, 1980). Between days 21 and 22 conceptus attachment was verified at the tip, center, and base of the pregnant horn. Complete interdigitation of the microvillar border of the trophectoderm and endometrium was evidenced on day 28 of gestation. Thus, conceptus attachment and the commencing framework for placentation are established between days 20 and 28 of pregnancy.

A distinction of the ruminant placenta is the presence of BNCs (Wooding and Wathes, 1980), also referred to as trophoblast giant cells (**TGC**; Klisch et al., 1999a). These cells were reported in the chorion as early as day 18 of gestation and represented 6% of the cell population at that stage of pregnancy (Wathes and Wooding, 1980). Throughout gestation, 15-20% of the trophoblast cell population can be classified as BNCs (Wathes and Wooding, 1980; Wooding and Wathes, 1980). Until recently, it was

broadly accepted that any trophoblast mononuclear cell (**MNC**) could become a BNC (Klisch et al., 1999a; Wooding, 2022). Evidence from single-cell RNA-sequence analyses revealed that specific lineages of MNCs give origin to two types of BNCs with divergent gene trajectories (Davenport et al., 2023).

The specialized BNCs form following two mitoses without cell division and likely one bipolar mitosis (Wooding, 1992; Klisch et al., 1999a; b). This results in a binucleated cell with 4 sets of homologous chromosomes in each nucleus and 8C per nucleus (C = 3,000 megabases of DNA, or 24 billion DNA bases per nucleus). This genome amplification in BNCs was suggested as a mechanism for increasing transcriptomic capacity, resulting in the cytoplasmatic accumulation of the many products derived from these cells (Klisch et al., 1999a). Cytoplasmatic granules occupy 50% of the total volume of a mature BNC (Wooding, 1992). These granules contained placental lactogen (Wooding and Beckers, 1987) and PAGs (Zoli et al., 1992a). Such products will reach maternal circulation or be released in the conceptus-maternal interface (Wooding et al., 2005; Wooding, 2022).

The delivery routes of PAGs have been extensively described utilizing light and electron microscope immunocytochemical methodologies (Wathes and Wooding, 1980; Wooding and Wathes, 1980; Wooding, 1983, 1992; Wooding et al., 1986). These studies identified two unique characteristics of BNCs that allowed for systemic delivery of PAGs: 1) a migratory behavior consisting of pseudopodium projected towards the endometrium, also termed “migration front”; and 2) the ability to fuse with single endometrial cells upon apical tight junction, and flattening of the endometrial microvilli, resulting in hybrid multinucleate cells, also termed syncytial cells (Figure 3.4).

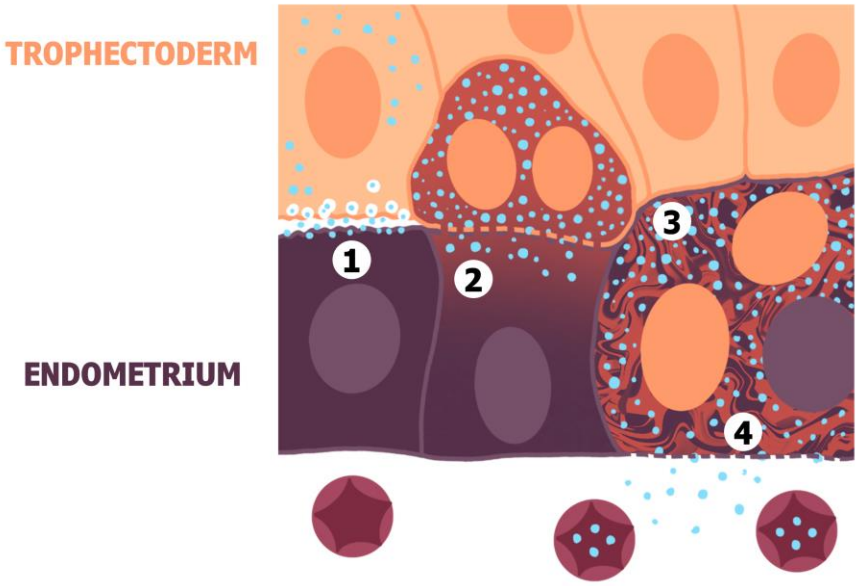

Figure 3.4. Summary illustration of the delivery routes of pregnancy-associated glycoproteins (PAGs; cytoplasmatic granules in light blue) to the conceptus-maternal interface or the maternal circulation. 1) Local release of PAGs from mononucleate trophoblast cells through the formation of cytoplasmatic vesicles and exocytosis. 2) Fusion process of binucleate trophoblast cells and endometrium cells with the transfer of cytoplasmatic contents to the maternal cell. 3) Trinucleate syncytial hybrid cell resulting from the fusion process described in (2). Syncytial cells are multinucleated and PAG-positive. 4) Release of cytoplasmatic granules through the basal membrane. After exocytosis, PAGs transit through the stroma and reach capillary beds below the endometrium. This process leads to the appearance of PAGs in maternal circulation.

Through induction of cell differentiation, MNCs acquired characteristics compatible with the BNC migratory behavior (Polei et al., 2020). These differences were related to extracellular matrix composition and cytoplasmatic membrane receptors that could grant or impede navigation through the conceptus-maternal interface. Integrin genes were differentially expressed in BNCs compared to MNCs (Polei et al., 2020). Another alteration concomitant with the transition from MNC to BNC was the changes in glycocalyx composition of these cells (Polei et al., 2020). BNCs had increased production of sialylated short-chain O-glycans, which may aid in evading the cow's immune system upon migration (Lübbers et al., 2018).

The main delivery route of PAGs: fusion of conceptus and maternal cells

The fusion of BNCs and endometrial cells culminates in the transfer of cytoplasmatic contents from the BNC into the newly formed hybrid cells (Wooding and Beckers, 1987). Pregnancy-associated glycoproteins are released via exocytosis and may reach the maternal capillaries present in the stroma (Figure 3.4; Wooding et al., 2005). The hybrid cells are short-lived and are rapidly demised following fusion and granules release into the maternal system (Wooding, 1982). The MNCs then phagocytize the remainder of the cell debris (Wathes and Wooding, 1980). This process persists throughout pregnancy, with an abrupt decrease in the BNC population around parturition (Wooding, 1983), followed by a peak in PAG concentrations in the maternal circulation (Sasser et al., 1986).

The fusion process and its mediators have been only partially identified. Endogenous retrovirus genes encoded envelope proteins involved in cell fusion and placentation in humans (Blond et al., 2000). In cattle, the relative expression of two candidate endogenous retrovirus genes (*syncytin-Rum1* and *BERV-K1*) was estimated in intercaruncular, caruncular, and fetal tissues at different periods (days 16, 22, 28, 34, 40 and 50 of gestation; McLean et al., 2017). The relative expression of *syncytin-Rum1* and *BERV-K1* was greater in caruncular tissue at day 50 of gestation compared to non-pregnant cows and early pregnancy. Additionally, *BERV-K1* had increased relative expression in intercaruncular regions on day 28 of gestation. Fetal expression of *syncytin-Rum1* showed a linear increase between days 22 and 50 of gestation. *BERV-K1* had a greater relative expression on days 28 and 34 than on day 22 of pregnancy. On days 34 and 50, relative expressions were the highest. The genes *syncytin* and *BERV-K1* were

previously shown to possess fusogenic motifs or fusion peptides in the endogenous retrovirus's domains (Dupressoir et al., 2009; Baba et al., 2011; Cornelis et al., 2012). Their localized and timely expression during pregnancy in cattle indicated a possible functional role in cell-to-cell fusion and progression of placentation. Furthermore, *BERV-K1* was shown to be more fusogenic than *syncytin-Rum1* in an in vitro assay designed to assess the fusion activity of bovine endometrial cells (Nakaya et al., 2013). Additionally, the *BERV-K1* expression was restricted to BNCs of the trophectoderm and differentially expressed in caruncular and intercaruncular tissues dependent on the day of collection (days 78, 156, and 224 of gestation; Nakaya et al., 2013). Following cell differentiation, BNCs had greater expression of *syncytin-Rum1* than MNCs (Polei et al., 2020).

The secondary delivery route of PAGs: localized release in the conceptus-maternal interface

A secondary delivery route was identified as the local release of PAGs in the microvillar tight junctions through the apical diffusion of small cytoplasmatic vesicles (Wooding et al., 2005). The release of PAGs in the maternal interface is functionally more relevant for MNCs, which do not present migratory and fusion behaviors (Figure 3.4). Incidental local release of PAGs may also occur following apoptosis and plasmatic membrane rupture of trophoblast cells. Intense phagocytic activity and cell degeneration were reported in the conceptus-maternal interface starting on day 24 of gestation (Wathes and Wooding, 1980). This also suggests a high degree of cellular reorganization and recycling during early placentation. Considering the non-invasive classification of the bovine placenta (syn-epithelio-chorial), extensive cellular and tissue remodeling processes are unexpected.

Considering the substantial number of encoding PAG genes, the extensive production of these glycoproteins throughout pregnancy, coupled with their local and systemic release, a myriad of possible functions was propounded for these molecules during pregnancy. Nevertheless, no specific PAG functions are established.

ARE PAGs FUNCTIONAL MOLECULES?

Pregnancy-associated glycoproteins are encoded by a multigene family belonging to the aspartic proteinase family (Xie et al., 1991, 1997; Green et al., 1998). The numerous PAGs are differentially expressed throughout gestation (Green et al., 2000). The localization of PAGs in different trophoblast cell populations is associated with their ancient or modern classification. The ancient PAGs are differentially expressed in either MNCs or BNCs. For example, PAG-2, the most transcribed PAG, and PAG-8 were limited to trophoblast MNCs expression, whereas PAG-11 was expressed exclusively in BNCs (Telugu et al., 2009; Touzard et al., 2013; Davenport et al., 2023). Modern PAGs, such as PAG-1, are expressed solely in BNCs (Green et al., 2000). Both PAG-1 and -2 were mainly expressed in cotyledonary regions (Touzard et al., 2013).

These glycoproteins were deemed inactive proteinases due to mutations in the catalytic subsites (Xie et al., 1991; Guruprasad et al., 1996). A bilobed structure was retained, resembling the structure of pepsin (Green et al., 1998). However, there was an indication of proteolytic activity for the ancient PAGs -2 and -12 under acidic pH (Telugu et al., 2010). Regardless of enzymatic function, PAGs seemed to preserve the ability to bind small peptides (7 to 8 amino acids long) like pepstatin A, a protease inhibitor (Guruprasad et al., 1996; Wooding et al., 2005). An adhesive function for locally released PAGs (ancient PAGS) was proposed, given their global expression in the trophectoderm

and the possibility of binding to plasmatic membrane' molecules and extracellular matrix proteins (Wooding et al., 2005; Wooding, 2022).

An endocrine role was proposed upon PAG-induced release of an alpha chemokine (granulocyte chemotactic protein-2) from endometrial explants (Austin et al., 1999). This was a shared effect with the maternal recognition signal interferon-tau (Teixeira et al., 1997). Proposed roles for the granulocyte chemotactic protein-2 included chemotaxis and cell invasion (Proost et al., 1993). No further studies were performed to investigate the physiological significance of this finding during pregnancy. Additionally, PAGs were shown to compete for human chorionic gonadotropin (**hCG**) and luteinizing hormone (**LH**) receptors on CL, myometrium and endometrium (Szafranska et al., 2007).

Immunomodulatory roles were also considered. Hampering the cow's immune response to the histo-incompatible conceptus is imperative for pregnancy. Roberts et al. (1996) hypothesized that PAGs could interfere with antigen presentation to immune cells via the major histocompatibility complex. In vitro treatment with 2,400 and 3,000 ng/mL of purified PAG reduced the cloning efficiency of granulocytes and myeloid cell populations (Hoeben et al., 1999).

A series of in vitro experiments pointed to luteoprotective and luteotropic mechanisms induced with PSPB treatment and mediated via prostaglandin-E_2 (**PGE_2**; Del Vecchio et al., 1990, 1996). Prostaglandin-E_2 is involved in inducing luteolytic resilience and improving the steroidogenic capacity of the CL (Magness et al., 1981; Gimenez and Henricks, 1983; Weems et al., 1985). In addition, PGE_2 may have a role in angiogenic cascades (Gately, 2000), which is critical for proper placentation. Cultured luteal cells secreted greater concentrations of P_4 and PGE_2 in the media following treatment with

PSPB. Similarly, endometrial cells cultured with PSPB released greater concentrations of PGE_2.

Aiding tissue remodeling to accommodate the highly proliferative placental tissue was also one of the roles proposed for PAGs. Recent evidence obtained utilizing immunohistochemical techniques provided novel insight into the global appearance of the uteroplacental interface during the first 2 months of gestation (Seo et al., 2023). This evidence suggested that BNC migration and fusion to form syncytial hybrid cells is more disruptive to the luminal epithelium than previously believed. On day 21 of pregnancy, sporadic PAG-positive cells were observed and incorporated into the luminal epithelium. The stroma was also occasionally stained for PAGs. Migration was disruptive to the cell-to-cell configuration in the endometrium. Epithelial cells appeared disconnected from one another and lost connection with the basement membrane. This decidual-like response was more severe on day 31. Multinucleate cells that were PAG-positive replaced the luminal epithelium, which was absent in large areas of the uteroplacental interface. Cells that were stained for PAGs were also identified in the stroma. At day 31, the placenta could be classified as syndesmochorial rather than epitheliochorial. On day 40, a multi-layered population of PAG-positive cells invaded the caruncular regions and established contact with the stroma. Between days 40 and 67, the caruncular regions seemed to have intense re-organization and remodeling. On day 67, mononuclear cells stained for serine hydroxymethyltransferase 2 were the major cells composing the epithelium separating caruncular and cotyledonary stroma. The authors speculated that these cells may be luminal epithelium cells that survived the “erosion” resulting from trophoblast migration and repopulated the luminal epithelium. Collectively, it becomes clear that cellular and

tissue remodeling are part of the physiological progression of pregnancy and likely necessary for proper placentation.

Treatment with PAGs induced transcriptomic changes in uterine explants from pregnant and non-pregnant cows (Figure 3.5; Wallace et al., 2019). These changes involved the upregulation of metalloproteinases-1 and -3 (**MMP**), which degrade specific extracellular matrix components, including certain collagens and laminin (Mittal et al., 2016). The upregulation of these enzymes was more pronounced in non-pregnant than pregnant endometrial explants. Pregnant endometrial explants had concomitant upregulation of metallopeptidase inhibitor 2 (**TIMP**; Wallace et al., 2019), and this protein was also expressed in BNCs (Figure 3.5; Davenport et al., 2023). Metalloproteinase inhibitor 2 participates in regulatory mechanisms of MMP actions (Murphy et al., 1994). Thus, the cow appears to have mechanisms to induce and to regulate cellular and tissue remodeling. These mechanisms may hinder extensive conceptus invasion in species with epitheliochorial placentas. Pregnancy-associated glycoproteins could be crucial regulators of this process.

Molecular markers of epithelial to mesenchymal transition (EMT), such as cadherin-2 and vimentin, were reported in the trophectoderm of day 22 bovine conceptuses (Yamakoshi et al., 2012). On the same day of gestation, particularly at the attachment areas, epithelial cells also presented cadherin-2 expression. It was suggested that the trophectoderm and epithelial cells underwent a partial/transient epithelial to mesenchymal transition as a mandatory step in advancing conceptus attachment to complete interface adhesion (Figure 3.5).

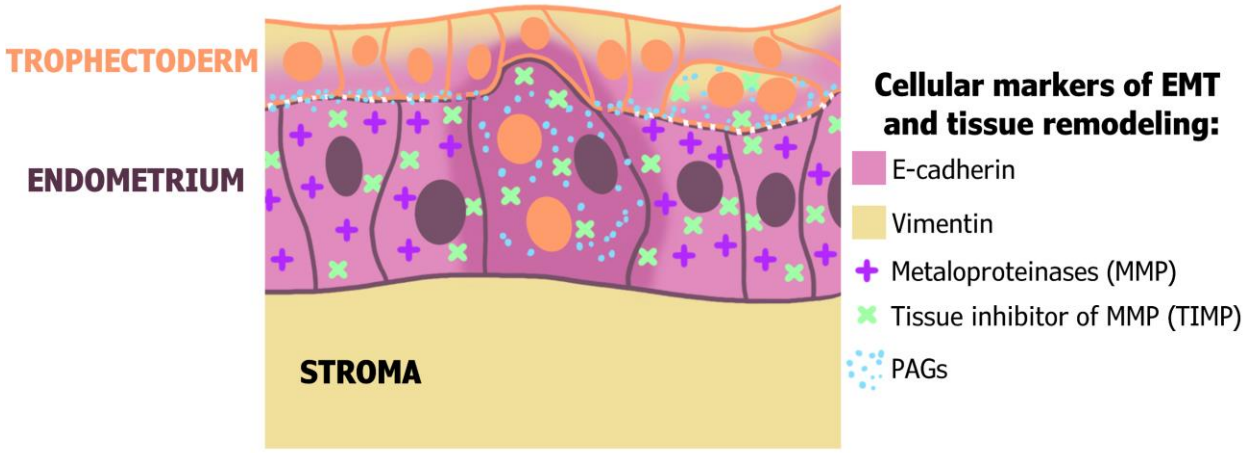

Figure 3.5. Illustration of cellular markers of epithelial to mesenchymal transition (EMT) and regulators of tissue remodeling in the conceptus-maternal interface after conceptus attachment. E-cadherin was expressed in the trophectoderm and endometrium, with stronger staining in adhesion areas. The trophectoderm and maternal stroma expressed vimentin. E-cadherin and vimentin are cellular markers of EMT (Yamakoshi et al., 2012). Pregnancy-associated glycoproteins (PAGs) induced expression of metalloproteinases (MMP) and tissue inhibitor of MMP (TIMP) in pregnant endometrium (Wallace et al., 2019). Binucleate trophoblast cells also express TIMP (Davenport et al., 2023).

Epithelial cells that undergo EMT are granted migratory traits, downregulation of the apicobasal polarity, expression of adhesion glycoproteins, and expression of MMP (Denker, 1993; Hohn and Denker, 2002; Yamakoshi et al., 2012). These changes are compatible with the somewhat disruptive concept of attachment and subsequent placentation described by Seo and collaborators (2023). Epithelial to mesenchymal transition was considered a vital step leading up to placentation, but its regulation may also be crucial to maintain the homeostasis between conceptus and dam (Oghbaei et al., 2022). Up and downregulation of EMT markers are likely involved in orchestrating the timing of cellular changes in both epithelial and trophectoderm cells that lead to healthy placentation (Kusama et al., 2016; Oghbaei et al., 2022). Possible interactions between BNCs migration, PAGs, and EMT markers in cattle have not been investigated.

In summary, conceptus-maternal interactions are mediated via complex and interconnected systems. Important gaps in the knowledge about the main regulators of these processes in cattle are limiting a global interpretation of factors involved in pregnancy success. Timely changes in cellular characteristics of the trophectoderm and endometrium that allow for conceptus attachment may be the first, most critical step to continue pregnancy following maternal recognition of pregnancy. Establishing cell-to-cell dialogue culminates in the delivery of trophoblast products to the maternal circulation or locally at the placental interface. This dialogue can be evaluated via measurements of PAG concentrations in maternal circulation. The timing and level of this ongoing exchange between conceptus and cow were utilized as early pregnancy markers in studies described in Chapters 5, 6, and 7. Furthermore, serial measurements of PAGs were utilized to estimate conceptus viability and competency in establishing and maintaining pregnancy. Despite not having a clear function during pregnancy, the production and transfer of PAGs to the maternal circulation is a clear indicator of a successful establishment of conceptus attachment and initial placentation.

PREGNANCY LOSS AFTER ELONGATION: FAILURE AT WHAT STEP?

Pregnancy losses are a major contributor to reproductive inefficiency in dairy cattle herds, leading to increased producer costs (De Vries, 2006; Wiltbank et al., 2016). As discussed in Chapter 2, the impacts of pregnancy losses on cow fertility are heavily dependent upon the time of pregnancy diagnosis and the accuracy of utilized diagnostic tools. A systematic review estimated that pregnancy losses in dairy cows ranged from 19 to 41% when assessed between days 8 and 27 of gestation (Wiltbank et al., 2016). Given the extensive changes induced by the conceptus at a cow level between the first week

and the first month of pregnancy, it is presumptive to assume that pregnancy losses would occur in a high proportion at this stage of gestation. Likely, failure at any of the processes described herein could lead to pregnancy loss. Namely: 1) rescue of the CL via interferon-tau action; 2) induction of uterine receptivity, i.e., changes in physicochemical properties of the endometrium; 3) exposure of transmembrane receptors and adhesion factors; 4) conceptus attachment, with the onset of migration and fusion of BNCs with endometrial cells to form syncytial cells; 5) transfer of conceptus-derived products to the maternal circulation and the conceptus-maternal interface; 6) partial epithelial to mesenchymal transition of the trophectoderm and endometrium that facilitate conceptus and maternal membranes adhesion and remodeling; and 7) tissue and extracellular matrix remodeling to accommodate the developing placenta. There is a lack of published studies that were designed to evaluate pregnancy failure during the period in which these processes are initiated.

The model of daily measurements of PAG, or PSPB in maternal circulation described in Chapter 2, may pose an alternative to diagnose pregnancy near the period of conceptus attachment. This method comprises the identification of individual circulatory PSPB profiles. We speculate that the first day of a significant PSPB increase in maternal circulation is closely related to the onset of conceptus attachment. This assumption is based on previous evidence that conceptus attachment occurred between days 20 and 22 of gestation (Wathes and Wooding, 1980; Yamakoshi et al., 2012). This time window coincides with days to PSPB increase in circulation in most cows. An average of 76.2% of cows had a significant increase in PSPB between days 19 and 21 post-AI. The remaining cows presented a delayed increase phenotype, in which PSPB increased

between days 22 and 26 post-AI (unpublished combined data from 3 studies that utilized this methodology). Thus, this model also allowed for capturing temporal differences for the significant appearance of PSPB in serum samples, which ranged between day 19 and 26 post-AI.

One controlled randomized trial that utilized this methodology has been published (Santos et al., 2023). The first day of PSPB increase was utilized as the initial reference for pregnancy. Pregnancy losses occurring between conceptus attachment and the pregnancy diagnosis at day 34 post-AI were associated with delayed time to PSPB increase and lower concentrations of PSPB between days 23 and 28 of gestation compared to cows that maintained pregnancy. Temporal differences in PSPB appearance in maternal circulation and circulating PSPB concentrations may reflect the quality of the interaction between conceptus and dam. It is unclear if the decreased pregnancy survival is due to this dysfunction in PSPB production and transfer or just an artifact of an underdeveloped conceptus. There are also no reported strategies that could mitigate the occurrence of such phenotypes and possibly improve pregnancy success in lactating dairy cows. The studies described in the present dissertation aimed to improve the understanding of the fertility of lactating dairy cows measured near the period of conceptus attachment. The model of interest comprised cows that received AI to a detected estrus. A secondary aim of these studies was to investigate pregnancy maintenance limiting factors that were cow-, conceptus- or management-related. One study also aimed to evaluate a post-conception intervention that could mitigate pregnancy failure.

Studies described herein focused on different periods concerning the day of AI (before, near, or after) and were ordered accordingly in the subsequent chapters. Chapter 4's study aimed to reduce the ovulatory follicle antral age to evaluate the effects on lactating dairy cows' follicular, endocrine, and estrus characteristics. This was a key study to understand how follicular age can impact estrus behavior in lactating dairy cows and the possible implications for follicle and oocyte quality associated with prolonged follicular development.

The study presented in Chapter 5 aimed to determine differences in time to conceptus attachment between lactating cows that received AI after being detected in estrus with automated activity monitors (**AAM**) and cows treated with the fertility program Double-Ovsynch. This study also investigated the association between pre- and post-conception factors with fertility parameters of cows detected in estrus or synchronized with hormonal intervention.

The main objective of the study reported in Chapter 6 was to determine the reproductive efficiency of lactating dairy cows receiving their first post-partum AI following a detected in estrus or the Double-Ovsynch program. Fertility was first assessed near the period of conceptus attachment with daily PSPB monitoring. Pregnancy losses occurring before the first month of gestation were also estimated with this methodology.

The final study described in Chapter 7 aimed to induce accessory CL (**aCL**) via gonadotropin-releasing hormone (**GnRH**) administration near the period of maternal recognition of pregnancy and conceptus attachment in lactating dairy cows that received TAI. This study helped to further the knowledge about pregnancy survival in cows with sustained P_4 concentrations throughout conceptus attachment.

CHAPTER 4

REDUCED PERIOD FROM FOLLICULAR WAVE EMERGENCE TO LUTEOLYSIS GENERATED GREATER STEROIDOGENIC FOLLICLES AND ESTRUS INTENSITY IN DAIRY COWS

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ABSTRACT

The onset of productive life in dairy cattle, concomitant to parturition, is accompanied by a substantial decrease in fertility in comparison with non-lactating, nulliparous heifers. Follicular growth patterns differ between parous and nulliparous dairy cattle. Nulliparous heifers ovulate follicles with reduced antral age (**RAA**). This study aimed to exogenously reduce ovulatory follicle age in lactating dairy cows from 7 to 5 days old. Cows (n = 80) had their estrous cycles synchronized with the Double-Ovsynch program. At the final portion of this program, luteolysis was induced at either 5 (RAA) or 7 (Control) days following follicular wave emergence. RAA outcomes were estimated in comparison with Controls. RAA resulted in smaller follicles 2 days post-treatment. Despite lower serum concentrations of E_2 before treatment compared with Controls, the rate of increase in this hormone was greater for the RAA treatment. There was no difference in luteolysis rates between treatments. Proestrus (luteolysis onset to estrus onset) was prolonged in RAA cows. Cows with RAA had more intense estruses. Collectively, these results indicate that decreasing the age of the ovulatory follicle may improve the steroidogenic capacity of the dominant follicle and estrus expression intensity in lactating dairy cows.

INTRODUCTION

The transition from nulliparous heifer to lactating cow comes with high costs to fertility (Pursley et al., 1997b). The chance of pregnancy decreases 50% in addition to significant reductions in the behavioral ability of cows to exhibit estrus. Significant changes in ovarian function occur during this transition. Nulliparous heifers have predominantly three follicular waves of follicle development during an estrous cycle

(Wolfenson et al., 2004). After their first calving, these newly lactating cows have primarily two waves of follicular development culminating with an ovulatory follicle that has undergone several more days of development compared to heifers with three follicular waves. The time from emergence of the second follicular wave in cows can be variable between 9 and 14 days of the cycle (Kirby et al., 1997). The third follicular wave of development in heifers begins around day 16 of the cycle (Sirois and Fortune, 1988). Considering heifers have a shorter estrous cycle compared with lactating cows (Wolfenson et al., 2004), the differences in antral age of the ovulatory follicle may be a reason for the differences in fertility between these two groups.

Follicular steroidogenic potential may be altered with longer or shorter periods of antral follicle development. Intrafollicular fluid concentrations of E_2 increased exponentially with follicle diameter in both cows (De los Reyes et al., 2006) and heifers (Nishimoto et al., 2009). Nonetheless, in at least two studies (Sartori et al., 2004; Wolfenson et al., 2004) heifers had greater concentrations of E_2 prior to ovulation compared to cows, despite having ovulatory follicles of smaller diameter (Sartori et al., 2004). The period between follicular emergence and estrus was also shorter in heifers compared to cows (Wolfenson et al., 2004). This could suggest that shortening duration of follicular development in lactating cows to replicate what naturally occurs in heifers may impact endocrine function of the pre-ovulatory follicle and estrus expression.

In an unpublished study from our laboratory, different lengths of time from follicular wave emergence to luteolysis were studied to determine differences in follicular dynamics. These data indicated that follicles with a dominance period of 5 days grew at faster rate from follicular wave emergence to the LH surge (1.9 ± 0.04 mm/day) and had

a greater peak of E_2 following induced luteolysis (2.6 ± 0.3 pg/mL) compared to periods of 6 (1.7 ± 0.05 mm/day, and 2.0 pg/mL) or 7 days (1.6 ± 0.05 mm/day, and 2.1 pg/mL) from wave emergence.

Fertility programs were developed to increase pregnancies per AI (**P/AI**) in lactating dairy cows utilizing TAI. These programs limit the antral age of the ovulatory follicle compared to natural estrus via manipulation of ovarian function to be more like a heifer with three waves of follicular growth. Gonadotropin-releasing hormone and $PGF_{2\alpha}$ pharmaceutical analogs are commonly utilized for synchronization of ovulation in lactating dairy cows. Combining the precise timing of GnRH and $PGF_{2\alpha}$ to induce follicular wave emergence, growth of a new dominant follicle, luteolysis, and ovulation within an 8-h period allowed for AI at the most ideal stage of lactation. This method was first described in 1995, by Pursley and collaborators (Pursley et al., 1995), and named Ovsynch. Ovsynch, and its variations (Moreira et al., 2001; Bello et al., 2006; Souza et al., 2008) also improve fertility via manipulation of luteal function and antral age of the ovulatory follicle. Double-Ovsynch (Souza et al., 2008) is widely utilized for first post-partum AI to improve fertility of dairy cows compared to AI following a detected estrus and is the gold standard for control of follicle and CL function in dairy cows (Santos et al., 2017). Minela et al. (2021) reported that 92% of lactating dairy cows had ovulation and induction of a new follicular wave following the first GnRH of the second Ovsynch of Double-Ovsynch. This allowed 7 days of development prior to the $PGF_{2\alpha}$ induced luteolysis. Luteolysis can be induced on either day 5 or 7 of follicular development in both beef (White et al., 2016) and dairy (Lima et al., 2013) cattle, hence shortening or prolonging the antral age of the pre-ovulatory follicle prior to AI. Pregnancies per AI (Santos et al., 2010) and embryo

quality (Cerri et al., 2009) were both improved in lactating dairy cows when follicular dominance was shortened. Final follicular and oocyte maturation events take place following luteolysis (proestrus phase of the estrous cycle; Fair et al., 1997; Mihm et al., 2006). Longer proestrus length was associated with greater estrus expression prior to TAI and greater P/AI in beef heifers (Núñez-Olivera et al., 2022).

Estradiol secreted by the preovulatory follicle triggers external expression of sexual receptivity (Chenault et al., 1975; Lemon et al., 1975). The primary sign of estrus in cattle is the act of standing to be mounted by another cow. This behavior can be observed in only ~50% of dairy cows, following twice a day visual observation (Pursley et al., 1997b). Electronic estrus detection systems are utilized in beef and dairy operations to improve estrus detection rates (~20% increase compared to visual detection; Fricke et al., 2014). These systems utilize secondary estrus characteristics, such as increase in activity (deviation from a 7-day baseline) and the level/intensity of estrus. The duration of follicular development, follicular characteristics, and endocrine output may impact these characteristics as detected by AAM.

Estrus expression around TAI was associated with increased probability of pregnancy (Tippenhauer et al., 2023). Supplementation of E_2 analogs at the final GnRH of Ovsynch increased the estrus expression (Souza et al., 2007). Madureira et al. (2019) demonstrated that not just estrus expression around TAI but also the level of estrus intensity increased the chances of pregnancy of lactating dairy cows. Occurrence of estrus around AI modified gene expression in both the endometrium and conceptus 19 days post-AI compared to cows with no estrus but with ovulation and normal luteal development (Madureira et al., 2019). These changes were associated with genes

involved in the pre-attachment phase of conceptus development (Davoodi et al., 2016). Moreover, antral follicular age was associated with pregnancy status following AI to a natural estrus. Cows with established pregnancy 35 days after AI had almost one less day of follicular development (emergence to estrus; 7.8 ± 0.2 days) compared to non-pregnant cows (8.6 ± 0.2 days), regardless of number of follicular waves (Bleach et al., 2004). Thus, antral age of the pre-ovulatory follicle at time of the LH surge may play a key role in the fertility potential of the oocyte within that follicle (Cerri et al., 2009).

The objective of this study was to reduce the period from follicular wave emergence to induced luteolysis (i.e., reduce the ovulatory follicle antral age) to test the effects on follicular, endocrine and estrus characteristics in lactating dairy cows. Our novel design allowed for lactating dairy cows to have unadulterated maturation of the ovulatory follicle to gain a greater understanding of the interaction of antral age of the ovulatory follicle at luteolysis and steroidogenic capacity. We hypothesized that reducing time from follicular wave emergence to the induction of luteolysis would result in (1) smaller pre-ovulatory follicles, (2) greater E_2 concentrations following induced luteolysis, and (3) greater intensity and longer periods of estrus. If true, the outcomes could lead to alterations in synchronization programs that may improve fertility of lactating dairy cows. To test this hypothesis cows were randomly assigned to treatments described in Figure 4.1.

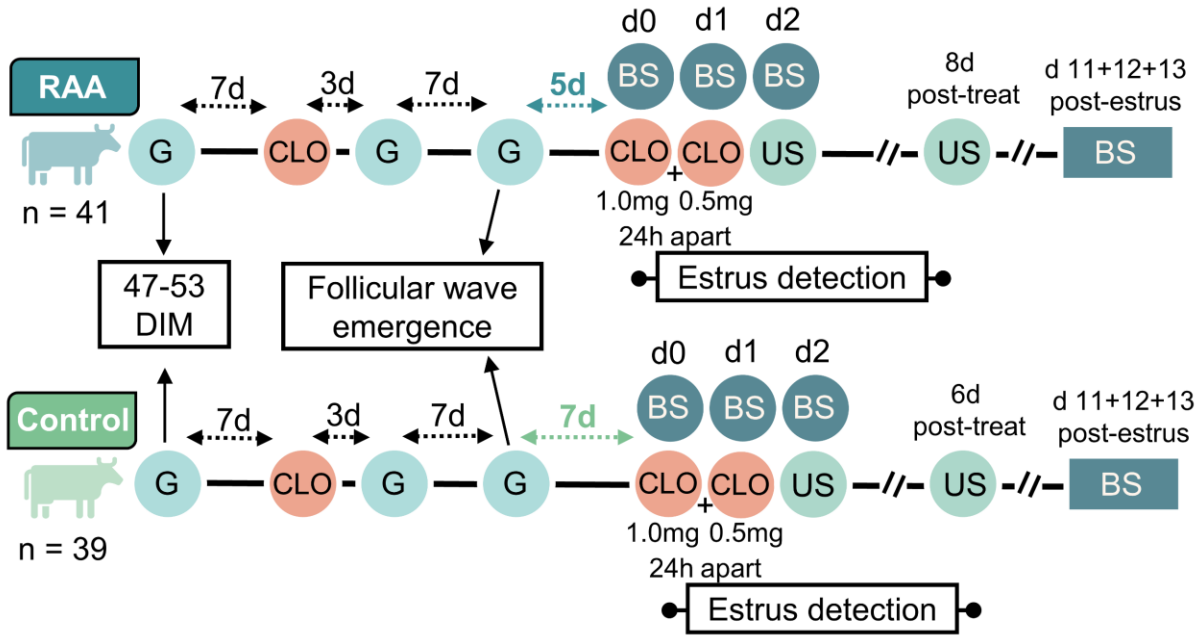

Figure 4.1. Experimental design to determine the effects of shorter (RAA) vs. longer (Controls) development periods of ovulatory follicles utilizing the fertility program Double-Ovsynch in lactating Holstein cows that were detected, or not, in estrus. Double-Ovsynch was initiated between 47 – 53 days in milk (DIM) for both treatments. Gonadotropin-releasing hormone (G) was utilized to synchronize follicular emergence in both treatments. Estrus detection was performed with automated activity monitors following cloprostenol sodium (CLO) administered on day (d) 0. Blood samples (BS) were collected on day 0, 1, 2, from CLO, and at day 11, 12 and 13 after a detected in estrus. Ultrasound (US) was utilized to measure follicular diameter at day 2 post-induction of luteolysis and to confirm ovulation on days 6 or 8 post-induction of luteolysis.

RESULTS

Shortening the duration of follicular development resulted in smaller more steroidogenic follicles and equally steroidogenic CL post-ovulation

Reducing the days between follicular emergence to onset of luteolysis (RAA) effectively resulted in smaller single dominant follicles measured 2 days post-luteolysis in comparison with Controls (13.5 ± 0.4 vs 15.1 ± 0.4 mm diameter; $P = 0.01$). There was an effect of treatment on concentrations of E_2 between day 0 and 2 post-induction of luteolysis ($P < 0.01$; Figure 4.2, panel A). Controls had greater E_2 concentrations at days

0 and 1 in comparison to RAA ($P \leq 0.01$). However, there were no detectable differences in E_2 concentrations at day 2 post-induction of luteolysis ($P = 0.15$). The rate of increase of E_2 concentrations, as a marker of the dominant follicle secretory potential, was greater for RAA compared with Controls between days 0 and 2 post-induction of luteolysis, despite smaller follicular diameter ($P = 0.01$; Figure 4.2, panel B). Previously unpublished data from our laboratory indicated a greater rate of increase in E_2 concentrations (measured every 12 hour) in day 5 follicles versus day 7.

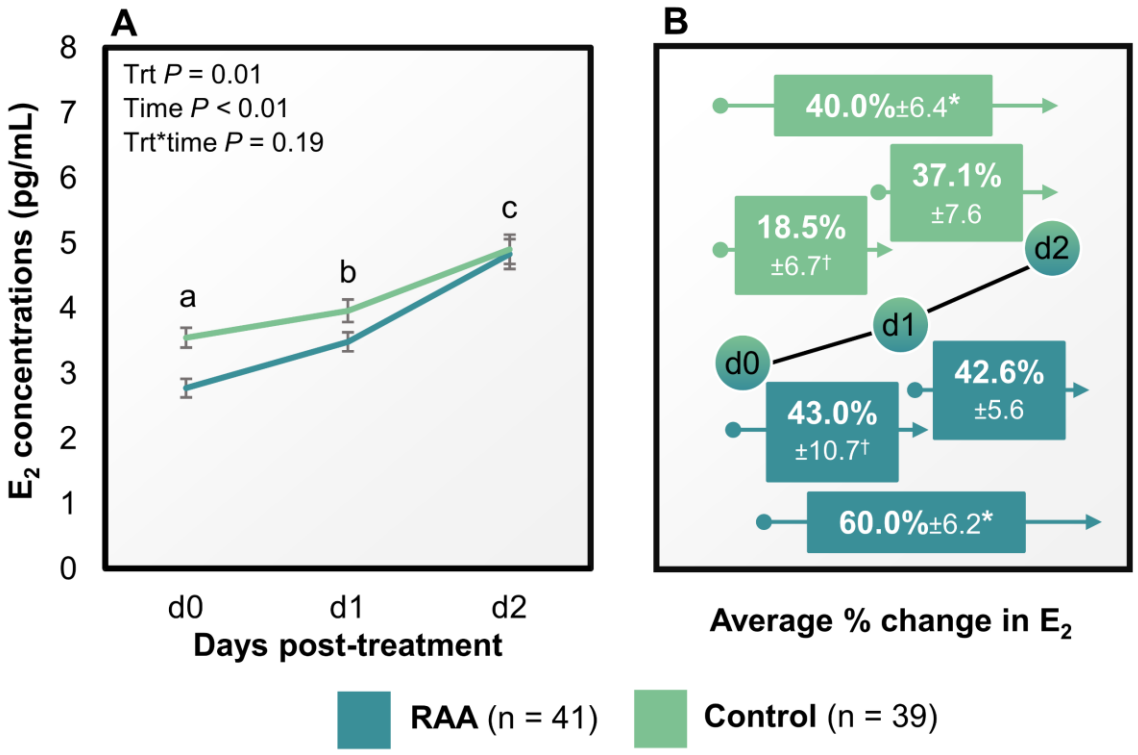

Figure 4.2. Effect of treatment on 17β-estradiol (E_2) concentrations (pg/mL) in lactating Holstein cows following cloprostenol sodium at day 5 (reduced antral age; RAA) or 7 (Control) of follicular development (A). Illustration of the percentage change in E_2 concentrations between days 0 and 1, 1 and 2, and days 0 and 2 in lactating Holstein cows following cloprostenol sodium at day 5 or 7 of follicular development (B). The letter superscript describes the comparison between treatments (Trt) within day post-induction of luteolysis. The letters "a" and "b" denote a $P \leq 0.01$, and "c" denotes a $P = 0.15$. * Indicates a greater % increase in E_2 concentrations between day 0 and 2 in RAA compared to Controls ($P = 0.03$). † Indicates a tendency for greater % increase in E_2 concentrations between day 0 and 1 in RAA in comparison to Controls ($P = 0.08$). The % increase in E_2 concentrations between day 1 and 2 was not different between treatments

Figure 4.2 (cont'd)
(P = 0.53). Data are shown as means ± SEM.

Follicle diameter in cows with a single ovulation was linearly associated with E_2 concentrations in RAA but not Controls, 2 days post-induction of luteolysis (Figure 4.3, panels A and B, respectively). Correlation coefficients were also estimated between E_2 concentrations (days 0, 1, and 2) and follicle diameter 2 days after induction of luteolysis. In this case, there was a tendency for a positive correlation between E_2 concentrations at day 0 and follicle diameter (r = 0.35; P = 0.06) in the RAA treatment. There was a moderately positive correlation between follicle diameter and E_2 concentrations at day 1 (r = 0.76; P < 0.01) and 2 post-induction of luteolysis (r = 0.65; P < 0.01) in the RAA treatment. There was a negative correlation between follicle diameter and E_2 concentrations at day 0 (r = -0.43; P = 0.01), in Control cows. On days 1 and 2, there were no significant correlations between E_2 concentrations and follicle diameter in Control cows (r = 0.27; P = 0.13; and r = 0.26; P = 0.15, respectively).

Mid-cycle P_4 concentrations (average concentrations on days 11, 12 and 13 post-estrus) did not differ between treatments in cows detected in estrus with confirmed ovulations (RAA 5.7 ± 0.6 vs. Controls 5.3 ± 0.6 ng/mL; P = 0.68). Cows with double ovulation tended to have greater mid-cycle P_4 concentrations in comparison with cows with single ovulations (6.7 ± 1.1 vs. 5.0 ± 0.4 ng/mL, respectively; P = 0.08), but no interaction with treatment was detected (P = 0.24). Overall, follicular diameter at day 2 post-induction of luteolysis was not correlated with early mid-cycle P_4 concentrations in cows with single ovulations (r = -0.03, P = 0.83).

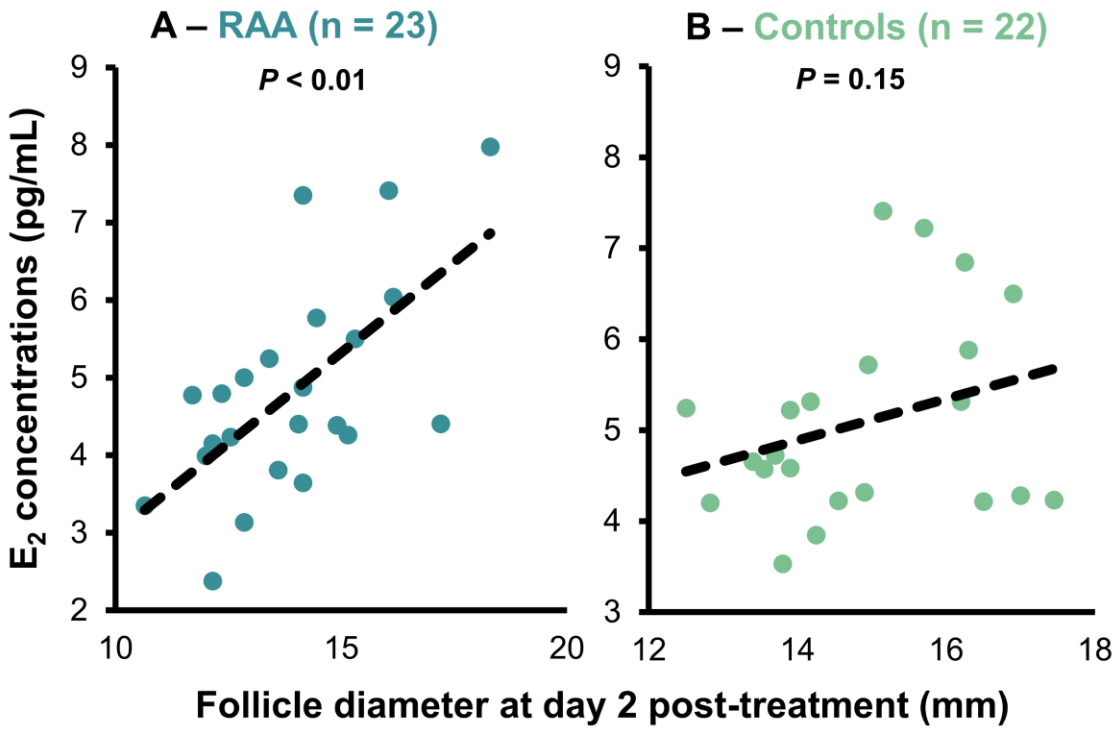

Figure 4.3. Linear relationship within treatment between 17β-estradiol (E_2) concentrations (pg/mL) and follicle diameter (mm) in lactating Holstein cows 2 days post-induction of luteolysis with cloprostenol sodium at (A) day 5 (reduced antral age; RAA) or (B) 7 (Control) of follicular development. Linear regression was estimated only in cows with single ovulations to isolate the relationship between the dominant follicle diameter and its E_2 output.

The experimental design allowed for complete luteolysis in both treatments

Initial induction of luteolysis with 1 mg cloprostenol sodium at time of induction of luteolysis in addition to 0.5 mg cloprostenol sodium one day later induced complete luteal regression in 100% of the cows in both treatment groups. There was no effect of shortening the period from follicular emergence to onset of luteolysis P_4 concentrations at any time (P = 0.68; Figure 4.4, panel A). Rate of decrease in P_4 between days 0 and 1, 1 and 2, and days 0 and 2 were not different amongst treatments ($P \geq 0.25$; Figure 4.4, panel B).

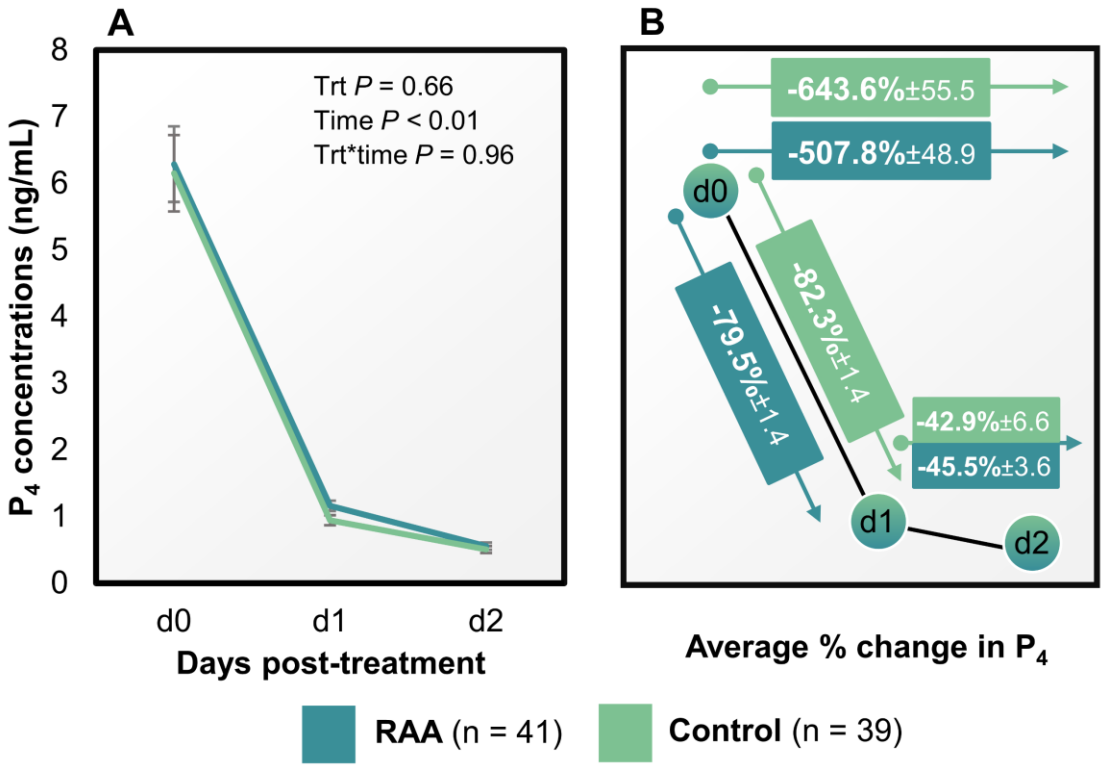

Figure 4.4. Effect of reducing the time from follicular wave onset to cloprostenol sodium (CLO) in lactating Holstein cows on progesterone (P_4) concentrations (ng/mL) 0, 1 and 2 days following induction of luteolysis with CLO (A). Illustration of the effect of treatment (Trt) on percentage change in P_4 concentrations between days 0 and 1, 1 and 2, and days 0 and 2 in lactating Holstein cows following CLO at day 5 (reduced antral age; RAA) or 7 (Control) of follicular development (B). No differences were observed in the % decrease in P_4 concentrations between day 0 and 1, day 1 and 2, and day 0 and ($P \geq 0.25$). Data are shown as means ± SEM.

Concentrations of E_2 were differentially impacted by treatment and double ovulations

Double ovulation occurred in 11/41 and 7/39 cows in RAA and Controls, respectively ($P = 0.40$). The effects of single or double ovulations within treatments on E_2 concentrations and on the rate of increase in E_2 are described in Table 4.1. Concentrations of E_2 were greatest in Controls with double ovulations at days 0 and 1 ($P \leq 0.01$), but no differences were detected at day 2 post-induction of luteolysis ($P \geq 0.35$). The rate of increase in E_2

between days 0 and 2 tended to be lower in Controls in comparison with RAA, in cows with double ovulations (P = 0.097).

Treatment and parity impacted the proportion of cows with behavioral estrus

Overall, 73% (58/80) of cows were detected in estrus during the 8-day period after induction of luteolysis. There was a tendency for a greater percentage of cows in estrus with RAA compared with Controls (80.5 vs 64.1%, P = 0.09). Second parity cows had reduced chances of estrus behavior (55.6%) compared with first (80%; P = 0.04) and third-plus parity cows (81.2%; P = 0.05).

	RAA single ov. n = 23	RAA double ov. n = 11	Control single ov. n = 22	Control double ov. n = 7	Two-way interaction P-value[1]
E_2 (pg/mL)					
Day 0	2.8 ± 0.2^{a*}	2.6 ± 0.2^{a}	3.3 ± 0.1^{a*}	4.5 ± 0.5^{b}	0.01
Day 1	3.5 ± 0.2^{a}	3.7 ± 0.2^{a}	3.8 ± 0.2^{a}	5.0 ± 0.4^{b}	0.047
Day 2	4.8 ± 0.3	5.1 ± 0.5	5.1 ± 0.2	5.9 ± 0.8	NS
% Change in E_2					
Day 0 to 1	42.5 ± 15.7	49.3 ± 13.0	18.8 ± 9.4	19.9 ± 17.4	NS
Day 1 to 2	42.3 ± 8.3	39.4 ± 8.8	45.9 ± 12.4	17.7 ± 8.3	NS
Day 0 to 2	57.0 ± 8.1^{a}	67.2 ± 10.9^{a†}	50.7 ± 7.9^{a}	23.6 ± 15.3^{a†}	0.09

[1] Two-way interaction between number of ovulation and treatment.
NS = non-significant and denotes a $P \geq 0.14$.

Table 4.1. The effect of number of ovulation (ov.) within reduced antral age (RAA) and Control treatments on serum concentrations of 17β-estradiol (E_2) concentrations (pg/mL) and on the % change in E_2 concentrations. Analyses were performed in cows with confirmed single or double ovulations within day, or periods post-induction of luteolysis. Multiple comparisons were only performed in the presence of significant two-way interactions. Letter superscript describes the comparison within day post-induction of luteolysis and between treatment and number of ovulation. Different letter superscripts denote a $P \leq 0.04$. * Denotes a tendency of P = 0.06 for the comparison of RAA single ovulation vs. Control single ovulation on day 0 post-induction of luteolysis. † Denotes a tendency of P = 0.097 for the comparison of RAA double ovulation vs. Control double ovulation on the period between day 0 to 2 post-induction of luteolysis. Data are shown as means ± SEM.

Manipulating the period of follicular development influenced key estrus characteristics

Table 4.2 summarizes the main effects of estrus characteristics assessed with AAM. Cows in RAA had a reduced period between follicular wave emergence and onset of estrus compared to Controls. Additionally, RAA extended the proestrus period compared to Controls. Length of time in estrus, and time to peak activity did not differ between treatments ($P \geq 0.57$). However, estrus intensity, measured as activity peak, was greater for RAA compared to Controls ($P = 0.02$). There was no significant correlation between E_2 concentrations at day 0 and estrus intensity ($r = 0.25$; $P = 0.17$) in RAA cows. Concentrations of E_2 at day 1 ($r = 0.34$; $P = 0.05$) and 2 ($r = 0.32$; $P = 0.07$) post-induction of luteolysis tended to be correlated with estrus intensity in RAA. There was no correlation between estrus intensity and E_2 concentrations in Control cows ($r = -0.02$, $r = 0.08$, and $r = -0.03$, for days 0, 1, and 2, respectively; $P \geq 0.71$).

	RAA (n = 33)	Control (n = 25)	*P*-value
Period from GnRH to estrus (days)[1]	8.5 ± 0.1	10.2 ± 0.1	< 0.01
Proestrus length (hours)[2]	89.4 ± 2.8	81.2 ± 3.5	0.01
Estrus length (hours)[3]	14.7 ± 0.6	14.0 ± 1.0	NS
Time to reach peak activity (hours)[4]	5.5 ± 0.6	5.4 ± 0.8	NS
Estrus intensity[5]	94.6 ± 1.2	86.8 ± 3.1	0.03

NS = non-significant and denotes a $P \geq 0.37$.
[1] Period between GnRH induction of a new follicular wave and estrus onset.
[2] Period between induction of luteolysis (first treatment with CLO) and estrus onset.
[3] Period between estrus onset to re-establishment of normal activity.
[4] Period between estrus onset and the highest/peak value of activity change observed during the estrus event.
[5] Highest/peak value of percentage activity change observed during the estrus event.

Table 4.2. Effect of different intervals to induced luteolysis on estrus characteristics of lactating Holstein cows detected in estrus with automated activity monitors. Reduced antral age (RAA) treatment consisted of a 5-day period between follicular wave emergence and induced luteolysis with cloprostenol sodium (CLO). Controls had a 7-day

Table 4.2 (cont'd)
period between follicular wave emergence and induced luteolysis with CLO. Onset of estrus was defined as the time of increased activity (≥ 35% change) in comparison with a cow 7-day mean activity. All cows that exhibited estrus were included in the analyses, regardless of ovulation status. Data are shown as means ± SEM.

Double ovulations had an impact on estrus characteristics

The effects of double ovulation and the interaction between treatment and double ovulation are described in Table 4.3. Cows in the RAA treatment had no differences in time to exhibit estrus from the day of follicular wave induction, regardless of double or single ovulations (*P* = 0.35).

	Double ov. n = 16	Single ov. n = 40	RAA single ov. n = 21	RAA double ov. n = 10	Control single ov. n = 19	Control double ov. n = 6
Period from GnRH to estrus (days)[1]	8.7 ± 0.2	9.5 ± 0.2	8.7 ± 0.1[ac]	8.2 ± 0.1[a]	10.5 ± 0.1[b]	9.3 ± 0.2[c]
P-values	< 0.01		Two-way interaction: 0.04			
Proestrus length (hours)[2]	70.8 ± 3.7	91.1 ± 2.3	92.9 ± 3.3	77.5 ± 4.0	87.7 ± 3.2	60.7 ± 4.5
P-values	< 0.01		Two-way interaction: 0.17			
Estrus length (hours)[3]	13.5 ± 0.8	14.8 ± 0.7	14.2 ± 0.7	14.4 ± 1.0	14.6 ± 1.3	12.0 ± 1.1
P-values	0.11		Two-way interaction 0.20			
Time to reach peak activity (hours)[4]	5.3 ± 0.6	5.5 ± 0.5	4.9 ± 0.6	6.0 ± 0.7	5.8 ± 0.9	4.0 ± 0.7
P-values	0.31		Two-way interaction 0.17			
Estrus intensity[5]	95.4 ± 2.1	89.7 ± 2.0	93.2 ± 1.7	96.8 ± 2.0	84.8 ± 3.7	~~93.~~ ± 4.6
P-values	0.23		Two-way interaction > 0.20			

[1] Period between GnRH induction of a new follicular wave and estrus onset.
[2] Period between induction of luteolysis (first treatment with CLO) and estrus onset.
[3] Period between estrus onset to re-establishment of normal activity.
[4] Period between estrus onset and the highest/peak value of activity change observed during the estrus event.
[5] Highest/peak value of percentage activity change observed during the estrus event.

Table 4.3. Effect of double ovulation (ov.) and the interactions between treatment and double ovulation on estrus characteristics of lactating Holstein cows detected in estrus with automated activity monitors. Reduced antral age (RAA) treatment consisted of a 5-

Table 4.3 (cont'd)
day period between follicular wave emergence and induced luteolysis with cloprostenol sodium (CLO). Controls had 7 days between follicular wave emergence and induced luteolysis with CLO. Onset of estrus was defined as the time of increased activity (≥35% change) in comparison with a cow 7-day mean activity. Multiple comparisons were only performed in the presence of significant two-way interactions. Different letter superscripts denote a P ≤ 0.01. Only cows that exhibited estrus with confirmed single or double ovulations were included in the analyses. Data are shown as means ± SEM.

However, Control cows with double ovulation had shortened periods to estrus onset in relation to induction of follicular wave in comparison to Control cows that had a single ovulation ($P < 0.01$). Cows with double ovulation had diminished proestrus length in comparison to cows with a single ovulation ($P < 0.01$), but no interaction with treatment ($P = 0.17$). Estrus length, time to reach peak activity, and estrus intensity were no different between cows with double in comparison with single ovulations ($P \geq 0.11$), with no treatment interaction ($P \geq 0.20$).

The rate of increase in E_2 between day 0 and 2 post-induction of luteolysis was predictive of estrus detection

Circulating concentrations of E_2 2 days post-induction of luteolysis tended to predict the probability of estrus detection (Figure 4.5, top panel). Cows with a greater increase in E_2 between days 0 and 2 post-induction of luteolysis were more likely to be detected in estrus (Figure 4.5, bottom panel).

DISCUSSION

Reducing antral age of the pre-ovulatory follicle resulted in smaller, more steroidogenic follicles, as hypothesized. These smaller and younger antral-aged follicles that were 5 days from induction of the follicular wave had a greater increase in E_2 concentrations between days 0 and 2 post-induction of luteolysis compared with the older follicles that were 7 days from induction of the follicular wave (Figure 4.2).

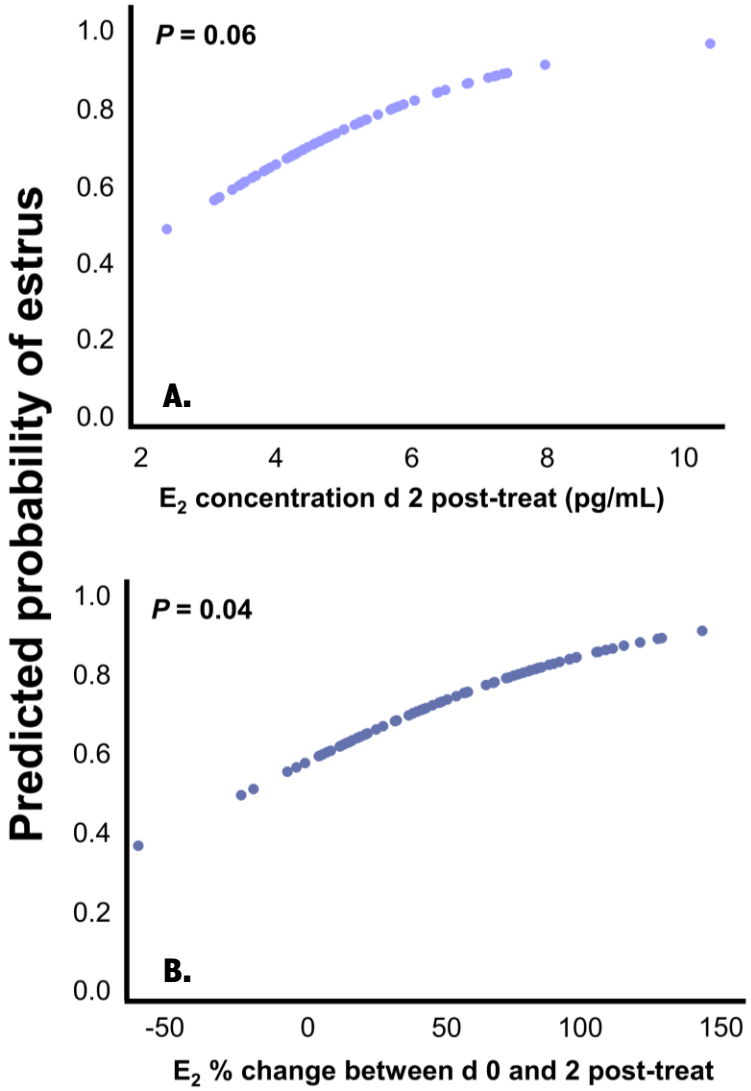

Figure 4.5. Predicted probability of estrus in relation to 17β-estradiol (E_2) concentrations (pg/mL) 2 days post-induction of luteolysis (post-treat; A) and E_2 concentrations % change between days 0 and 2 post-induction of luteolysis (B). Data shown includes all cows from RAA and Control treatments, regardless of ovulation number (n = 80).

Concentrations of E_2 were not greater 2 days following induced luteolysis in the younger vs. older antral aged follicles as our hypothesis anticipated. Earlier induction of final maturation of the pre-ovulatory follicle allowed for a positive correlation between E_2 and follicle diameter 2 days post-induction of luteolysis compared to delaying luteolysis. Luteinizing hormone pulsatility was not measured in this study, so it remains unclear if differences in E_2 secretion from these phenotypically different follicles were due to

differences in LH pulsatility. We speculate that the differences in increases in E_2 would most likely be due to antral age-related dysfunction of granulosa cells. Granulosa cell function is imperative for sustained E_2 secretion during later stages of follicular development (Bergman et al., 1966; Fishman and Goto, 1981; Hsueh et al., 1984). Larger follicles have greater numbers of granulosa cells (Monniaux et al., 1984). It would seem plausible that the larger follicle with more granulosa cells would produce more E_2. Yet, Controls, with larger and more mature follicles, had similar E_2 concentrations compared with cows with younger antral age 2 days after the start of final maturation. Controls had greater time from wave induction to luteolysis. Follicles from these cows had approximately 2 more days following deviation exposed to P_4-suppressed LH pulses. The level of P_4 at the beginning of this period would be produced from at least two CL (mid- and early-cycle) that were initiated during the implementation of the fertility program Double-Ovsynch. This period of high P_4 which decreases LH pulsatility has a detrimental effect on granulosa cell function over time (Mihm et al., 2006). Cows in the RAA group were theoretically only subjected to this high P_4/low LH pulsatility period for approximately one day if deviation took place on day 4 following wave emergence. Cows in this group had follicles that appeared to be more responsive to LH pulses during the peri/post-luteolytic period and had greater increases in serum E_2 concentrations compared to Controls.

These outcomes may help to gain a greater understanding of why estrus detection following a complete estrous cycle is a key problem in dairy cattle reproduction today. The antral age of most cows' ovulatory follicles is approximately 11 days, measured from the start of a new mid-cycle wave to ovulation. This is a significant amount of time under

the high P_4/low LH pulse period. Potential granulosa cell dysfunction due to this in addition to exacerbated steroid metabolism due to high milk production (Wiltbank et al., 2006) could reduce E_2 enough to cause true anestrus, and reduce the percentage of cows detected in estrus.

Co-dominance and double ovulation occurred in a similar proportion in RAA and Controls. Lopez et al. (2005) observed a premature rise in the production of E_2 in cows with co-dominance. The authors suggested that cows with co-dominance had earlier acquisition of cellular characteristics that are concurrent with the dominant follicle phenotype. It appears that in our study Control cows had an earlier increase in E_2 concentrations due to cows with double ovulations compared with RAA (Figure 4.2 and Table 4.1). These differences could be due to longer periods of post-deviation follicular development in Control cows.

There was no linear relationship between E_2 concentrations and follicle diameter assessed 2 days post-induction of luteolysis in the Control cows with confirmed single ovulations (Figure 4.3). In addition, there was a negative correlation between E_2 concentrations before induction of luteolysis and dominant follicle diameter 2 days later. This may suggest that some dominant follicles reached maximum E_2 secretory potential at day 7 of follicular development both functionally (plateau or decrease in E_2 concentration) and morphologically (decrease in diameter). This again could be associated with progressive loss of function that accompanies follicle aging (Fortune, 1994; Mihm et al., 2006). In the present study, 8/41 (RAA) and 11/39 (Control) cows were not detected in estrus with the AAM system. Of these, 5/8 (RAA) and 7/11 (Control) did not ovulate even though all cows had synchronized ovarian function (data not shown).

Current TAI programs, including Double-Ovsynch, induced ovulation 9.5 days after induction of a new follicular wave with GnRH. Approximately 97% of these follicles will ovulate (Minela et al., 2021). This leaves the door open to question the fertility level of some of the oocytes that are “forced-ovulated” with Double-Ovsynch or any other fertility program. Decreased E_2 secretion due to decreased granulosa cell function could impact oocyte maturation that takes place during proestrus. Estrogen, and mainly E_2 receptor 1 in the granulosa cells, are part of the upstream mechanism that assures oocyte meiotic arrest (Liu et al., 2017). Premature meiotic resumption, asynchronous with cytoplastic maturation, as well as early cellular degradation in the follicles would compromise the fertility potential of the oocyte. Reducing antral age resulted in a linear increase of secretory capacity and follicle diameter in cows with single ovulations.

There was no effect of treatment on the steroidogenic potential of the resulting CL after ovulation, measured as P_4 concentrations during early diestrus (day 11, 12 and 13 post-estrus). This was unexpected due to differences in diameters of the ovulatory follicles. Data would suggest that smaller ovulatory follicles tend to develop CL with lower P_4 at day 18 post-ovulation (de Lima et al., 2020). Nevertheless, stage of follicle development could be a source of variation when evaluating the impact of ovulatory follicle diameter on subsequent circulating concentrations of P_4.

It was also hypothesized that cows with the smaller, more steroidogenic, follicles would have greater estrus intensity and longer periods of estrus. Intensity of estrus was greater for cows with younger antral aged follicles at time of induced-luteolysis compared to Controls, as hypothesized (Table 4.2). But the younger, more steroidogenic, follicles did not translate into cows having longer periods of estrus. Greater estrus intensity around

AI, measured as the % change in activity, and the increase in E_2 in the RAA group could impact oocyte cytoplasm and nuclear maturation (Hyttel et al., 1986; de Loos et al., 1992; Mihm et al., 2006; Ferreira et al., 2009) and result in greater fertility (Tippenhauer et al., 2023). This could be a key consideration in changing dynamics of follicle development within fertility programs so that a younger and more steroidogenic pre-ovulatory follicle be considered to enhance P/AI.

The effects of double ovulation on estrus characteristics (Table 4.3) could be due to greater E_2 concentrations in cases of co-dominance (Lopez et al., 2005), a hormonal profile reported herein in Control cows (Table 4.1). Cows with double ovulation had shorter periods to estrus onset, and proestrus length. A greater E_2 output could have induced an earlier E_2 peak and onset of sexual receptivity (Lemon et al., 1975) in these cows. Other key estrus characteristics, such as estrus length and estrus intensity, were not impacted by the occurrence of double ovulations. Based on analyses in the present study, estrus intensity was only weakly correlated with E_2 concentrations, exclusively in RAA treatment. Madureira et al. (2015) also reported no correlation between E_2 concentrations and estrus intensity ($r = 0.02$) for estrus events occurring after a non-manipulated cycle. However, cows with high % change in activity (≥ 90) had greater E_2 concentrations.

Most fertility programs developed for lactating dairy cows utilize a 7-day period between GnRH and induction of luteolysis with $PGF_{2\alpha}$ (Pursley et al., 1995; Moreira et al., 2001; Bello et al., 2006; Souza et al., 2008). A 5-day period between induction of follicular wave and induction of luteolysis is also efficient for estrous cycle synchronization with satisfactory P/AI (Santos et al., 2010). A second dose of cloprostenol sodium can be

administered simultaneously, or dinoprost tromethamine can be administered 24 hours after the induction of luteolysis, to ensure more cows have regressed CL (Brusveen et al., 2009). The physiological justification for both strategies would be to induce complete luteolysis in a greater proportion of cows (Brusveen et al., 2009). Incomplete luteolysis prior TAI substantially decreases pregnancy likelihood of lactating dairy cows (Santos et al., 2010; Martins et al., 2011). Early developing CL (i.e., day 5 of development) have refractory characteristics to induced luteolysis (Wiltbank et al., 1995), and did not regress following a single injection of $PGF_{2\alpha}$ (dinoprost tromethamine; Nascimento et al., 2014). Recent evidence also suggested that cows with multiple CL (regardless of maturity) have decreased luteolytic response and prolonged time to exhibit estrus after administration of a prescribed dose of dinoprost tromethamine (López-Gatius, 2021). In the present study we utilized the $PGF_{2\alpha}$ analog pharmaceutical cloprostenol sodium, of longer half-live than dinoprost tromethamine (Reeves, 1978). Two single doses of cloprostenol sodium, given one day apart, effectively resulted in complete luteolysis of refractory 4-day old CL (Minela and Pursley, 2021). An initial double dose, combined with a single dose 24 hours later, of cloprostenol sodium resulted in 100% luteolysis rates in this study (Figure 4.4). Thus, the treatment regimen utilized for the present study to induce luteolysis was effective in regressing multiple and/or refractory CL. Santos et al. (2010a) reported lower P_4 concentrations at AI in dairy cows treated with two doses of cloprostenol sodium on days 5 and 6 compared to a single dose of cloprostenol sodium administered on day 7. This evidence could indicate that with appropriate amounts of cloprostenol sodium, a period of 5 days from GnRH to $PGF_{2\alpha}$ could be utilized in fertility programs of lactating dairy cows. Decreasing the period of follicular development by two days would result in

ovulation of younger follicles, avoiding potential function compromise occurring within a 7-day interval, as described herein. Adjustments in the period from luteolysis to induced ovulation and subsequent AI would likely have to be assessed alongside this strategy.

The average period between induction of luteolysis to estrus expression (proestrus length) was greater for cows in the RAA group compared to Controls (Table 4.2). The RAA treatment only had an ~8 hour delay to onset of estrus despite a 2-day difference between wave emergence and induced luteolysis. The difference in proestrus length between treatments was likely associated with increased cumulative E_2 exposure in Controls, mostly in cows with co-dominance. Sexual receptivity is a process that requires timely exposure to increasing concentrations of E_2 (Chenault et al., 1975; Lemon et al., 1975). One limitation of the present study was the inability to capture the maximum E_2 concentrations in relation to estrus onset. A study performed utilizing Holstein-Friesian Irish dairy cows reported that E_2 concentrations peaked on average 7 hours before the increase in activity, or estrus onset (Aungier et al., 2015). Finally, greater E_2 concentrations at day 2 post-induction of luteolysis tended to be correlated with greater estrus intensity in RAA, but not Controls. A greater % increase in E_2 concentrations between day 0 and 2 was associated with greater probability of estrus expression. Increasing estrus expression and intensity during TAI programs could be achieved by shortening the period between follicular wave emergence and induced luteolysis.

The proportion of lactating dairy cows detected in estrus tended to be greater in cows with shorter period of follicular development. Contrary to our observation, more dairy cows expressed estrus at TAI following a 7-day in comparison to a 5-day Cosynch (Santos et al., 2010). Additionally, beef heifers synchronized with a 7-day CIDR

(controlled internal drug-releasing P_4 device) Cosynch also had greater estrus expression than a 5-day CIDR Cosynch (Whittier et al., 2013). The difference in outcomes compared to the present study could be related to the amount of time cows were allowed to exhibit estrus. In both studies (Santos et al., 2010; Whittier et al., 2013), ovulation was induced exogenously about 70 hours after luteolysis.

Collectively, these findings would suggest that decreasing the period from follicular emergence to induced luteolysis enhanced the steroidogenic capacity of the pre-ovulatory follicle, despite a smaller diameter. This had a positive impact on estrus characteristics of lactating dairy cows that could be associated with increased fertility.

METHODS

Experimental units

This project was conducted from July 2021 to September 2021 on a commercial dairy farm, (Nobis Dairy Farm, St. Johns, Michigan, USA). The cows' owner has provided informed consent to utilize the animals for data collection. Power analyses concluded that n = 34 lactating dairy cows were needed to detect a 2 mm difference in follicular diameter 2 days following induced luteolysis (14 vs. 16 mm, $\sigma = 2$, $\alpha = 0.05$, $\beta = 0.2$; 5 vs. 7 days of follicular development, respectively). Cows were fed a total mixed ration (**TMR**) consisting of corn, wheat, and alfalfa silages, and corn soybean meal-based concentrate formulated to meet nutrient recommendations for high producing lactating dairy cows (NRC, 2001). The ration was fed once a day in confined free-stall barns with free access to feed and water. The Institutional Animal Care and Use Committee at Michigan State University approved all animal handling and procedures (ID: PROTO202000061). Methods were diligently carried out by authors in accordance with relevant guidelines and

regulations. All animal procedures and methods are reported in accordance with ARRIVE guidelines.

Treatments and model conceptualization

Treatments are described in Figure 4.1. Five weekly cohorts totaling n = 91 Holstein cows ranging from 1st to 9th lactation were available and met enrollment criteria. After enrollment n = 8 cows were culled due to farm management reasons. Cows were distributed across parities as follows: 1st (n = 30), 2nd (n = 28), and 3rd+ (n = 25). All cows received Ovsynch beginning 47 to 53 DIM to pre-synchronize cows to day 0 of the estrous cycle. GnRH was administered to all cows on day 7 of the estrous cycle to initiate a new follicular wave. This GnRH induced a new follicular wave 92% of the time when cows were treated with Double-Ovsynch (Minela et al., 2021). Cows were then blocked by parity and randomly assigned to one of two treatments. Treated cows were administered 1 mg of cloprostenol sodium (Boehringer Ingelheim) 5 days following the GnRH-induced new follicular wave to initiate final follicular maturation of a pre-ovulatory follicle with reduced antral age (RAA; n = 41). Control cows received 1 mg of cloprostenol sodium 7 days after GnRH to induce final maturation of the pre-ovulatory follicle (Control; n = 42). All cows received an additional 0.5 mg of cloprostenol sodium to ensure complete luteolysis 24 hours following the initial administration. Following luteolysis cows were allowed to express natural estrus activity up to 8 days post-induction of luteolysis and received AI from 73 to 83 DIM (Figure 4.1).

All treatments were administered intramuscularly with 1 ½” 20 gauge single-use needles in either the semitendinosus or semimembranosus muscles. Cows that did not have functional CL (P_4 < 1.0 ng/mL) at time of induction of luteolysis with CLO were

removed for analyses. Final analyses included n = 80 cows (RAA, n = 41; Control, n = 39).

Activity monitoring – estrus characteristics

All cows were equipped with a neck collar fitted with an activity monitoring system (Heatime® Pro+, Allflex Livestock Intelligence). Activity monitoring systems continuously measure acceleration forces and report increases in physical activity to detect behavioral signs of estrus in cattle (Fricke et al., 2014). Individual cow activity change data were recorded with DataFlow II software (Allflex Livestock Intelligence) in 2-hour time increments. Software reports were received via email twice daily. Cows with a 35% activity increase compared to their past 7-day activity average would be considered in estrus. Onset of estrus was recorded at the time cows went above this threshold. The periods between estrus onset to estrus peak (maximum activity % change for that estrus event), and time to peak, were recorded in hours. Time from estrus onset to normalization of activity (activity below the high activity threshold; estrus length) was also recorded in hours. Maximum percentage increase in activity was considered a measurement of estrus intensity. Cows that were detected in estrus received AI according to am/pm guidelines (Trimberger and Davis, 1943). Pregnancy outcomes were not considered in the power analyses and were not evaluated in this study.

Blood samples and hormonal analyses – E_2 and P_4 determination

Blood samples were collected to evaluate E_2 and P_4 prior to, and 1 and 2 days, following induction of luteolysis, and only P_4 11, 12 and 13 days post-estrus. All samples were harvested from the coccygeal vein or artery with serum separation tubes (Venous Blood Collection Tubes: SST, BD Vacutainer). All samples were stored at 4°C until

processing 24 hours later. Samples were centrifuged at 2,000 × *g* for 20 minutes to allow serum separation. Serum aliquots were frozen at -18°C until hormonal analyses. Measurement of E_2 and P_4 were performed with RIA (Dr. George Perry, Texas A & M University). Progesterone RIAs were previously described by Engel et al. (2008). All samples were run in duplicate. Intra- and inter-assay CVs were 8.9% and 6.8%, respectively. The assay sensitivity was 0.08 ng/mL. Estradiol RIA were previously described by Perry and Perry (2008). All samples were run in duplicate, and intra- and inter-assay CVs were 1.8% and 4.0%, respectively. Assay sensitivity was 0.5 pg/mL.

Dominant follicle secretory capacity and complete luteolysis determination

The change over time (rate of increase/decrease) in concentrations of E_2 and P_4 were utilized to estimate the dominant follicle secretory capacity, and complete luteolysis, respectively. Concentrations of E_2 and P_4 immediately before CLO-induction of luteolysis were considered as baseline concentrations for each cow. A percentage change (*% change* = (*observed – baseline/baseline*)**100*) was calculated between day 0 and 1, day 0 and 2, and day 1 and 2, for both E_2 and P_4. Additionally, complete luteolysis was defined as either P_4 < 1 ng/mL 2 days after induction of luteolysis, or a reduction of > 500% between induction of luteolysis and 2 days later.

Ultrasonography – follicle diameter and confirmation of ovulation

Linear array ultrasonography was used to map ovaries and describe ovarian structures (MyLab Gamma, Esaote). Luteal and follicular structures > 8 mm in diameter were measured and mapped 2 days following induction of luteolysis. All measurements were performed using built-in calipers. The first diameter measurement was measured horizontally at the greatest width and the second diameter was measured perpendicular

to the first diameter, at the greatest height. The final follicular diameter was reported in mm as the average of both measurements. Cows were reassessed with ultrasound at either 8 (RAA) or 6 (Control) days after induction of luteolysis to determine the appearance of newly formed CL and ovulation (Figure 4.1). Two cows in RAA failed to ovulate following estrus detection. Cows with double ovulation were removed from all analyses that included follicle diameter measurements (RAA, n = 11; Control, n = 7).

Statistical analyses

All statistical analyses were performed utilizing SAS 9.4. Binary variables were analyzed with a generalized linear mixed model fitted with PROC GLIMMIX. Differences in proportions were tested using the chi-square test of independence. Treatment and parity were included in the model as fixed effects.

Continuous variables were analyzed utilizing PROC MIXED. Concentrations of E_2 and P_4 at days 0, 1 and 2 were estimated with PROC MIXED and with the REPEATED statement to account for measurements performed over time. These analyses included two-way and three-way interactions between time, treatment, and double ovulation. An auto-regressive model was also utilized (**AR**1). All models included treatment and parity as fixed effects. Double ovulation was specified as a fixed effect in analyses that included all cows. Interaction terms of treatment and double ovulation were maintained in the models when $P < 0.20$. Degrees of freedom were estimated utilizing Kenward-Roger approximation. Differences within fixed effects were sliced using the LSMEANS statement and adjusted with Tukey-Kramer in the event of multiple comparisons. Linear regression between follicle diameter and E_2 concentrations at 2 days after induction of luteolysis was estimated with PROC REG. Pearson correlation coefficients between two continuous

variables were estimated with PROC CORR. Predicted probability of estrus as a function of E_2 concentrations and E_2 concentrations % change was estimated with PROC LOGISTIC. Predicted probability of double ovulation as a function of P_4 concentrations on day 0 was estimated with the same methodology. The significance level was set at $P \leq 0.05$ for all analyses. *P*-values were rounded to nearest hundredth.

CHAPTER 5

E_2 TO P_4 RATIO IS ASSOCIATED WITH CONCEPTUS ATTACHMENT IN DAIRY COWS RECEIVING AI AFTER DOUBLE-OVSYNCH BUT NOT ESTRUS DETECTION

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ABSTRACT

Determining conceptus attachment's timeframe via daily PSPB monitoring is innovative in predicting lactating dairy cow pregnancy survival. Factors contributing to reduced fertility in dairy cows receiving AI following estrus detection remain unclear. This study aimed to determine differences in time to conceptus attachment in lactating cows treated with the fertility program Double-Ovsynch compared to cows that were detected in estrus. Additionally, we investigated various pre- and post-conception factors potentially influencing fertility outcomes. We hypothesized AI following estrus would lead to an extended time to conceptus attachment and lower PSPB concentrations post-attachment compared to Double-Ovsynch. There were no differences in the average time to conceptus attachments between treatments. However, cows inseminated post-estrus detection that experienced pregnancy loss exhibited diminished PSPB concentrations on days 2 and 3 following conceptus attachment. Further analyses revealed that cows with pregnancy loss between conceptus attachment and 60 - 66 days post-AI displayed lower cumulative PSPB concentrations during the initial 3 days post-attachment compared to cows maintaining pregnancy. Steroid hormone interactions were assessed with ratios of E_2 to P_4 concentrations on the day of the LH surge. Notably, the E_2 to P_4 ratio proved predictive of conceptus attachment in cows subjected to Double-Ovsynch but not in those inseminated post-estrus detection surge. In conclusion, the E_2 to P_4 ratio, measured around the time of the pre-ovulatory LH surge, emerges as a potentially effective tool for estimating the fertility potential of lactating dairy cows undergoing TAI, particularly in the context of the Double-Ovsynch program.

INTRODUCTION

A significant gap remains in the understanding of the poor fertility of dairy cows. Manipulating ovarian function to limit the ovulatory follicle's antral age improves dairy cows' fertility compared to cows receiving AI following estrus (Santos et al., 2017; Sitko et al., 2023). Yet, it is unclear why these differences exist and if the time to conceptus attachment may be a limiting factor.

A systematic review of fertility data published before 2016 estimated that pregnancy losses occurring from day 8 to 27 ranged between 19 to 41% (Wiltbank et al., 2016). This period encompasses the critical establishment of the commencing physical communication between conceptus and dam, or conceptus attachment (Wathes and Wooding, 1980). Time to conceptus attachment was highly associated with pregnancy loss (Santos et al., 2023). Pregnancy-specific protein B is produced from BNCs (Butler et al., 1982; Wooding et al., 2005) and has been successfully utilized as a pregnancy marker in maternal circulation beginning near the time of conceptus attachment (Middleton et al., 2022; Pursley et al., 2023; Santos et al., 2023). Middleton et al. (2022) reported that most cows experienced a significant PSPB increase on day 20 or 21 of gestation in comparison to a within-cow baseline. Considering the timing of PSPB increase, it appears this event occurs at or soon after conceptus attachment. This observation corroborates previously published morphological evidence (Wathes and Wooding, 1980; Guillomot and Guay, 1982). Cows with a delay (≥ 22 days post-ovulation) in conceptus attachment had greater chances of pregnancy loss compared to most cows that had conceptus attachment on days 19 to 21 (Santos et al., 2023). The prospective discovery of utilizing serial measurements of a conceptus-exclusive pregnancy marker as early as 19 days post-AI

reveals a substantial gap in our comprehension of pregnancy losses in lactating dairy cows. This knowledge gap is significant as it also encompasses pre- and post-conception factors that may influence pregnancy outcomes when utilizing an earlier baseline for pregnancy assessment.

There are key measurable pre- and post-conception factors that have been positively and negatively associated with fertility. They are ovulatory follicle antral age or follicle diameter (Savio et al., 1993; Revah and Butler, 1996; Bello et al., 2006), E_2 and P_4 dynamics prior to ovulation (high E_2, concomitant with low P_4) (Brusveen et al., 2009; Shimizu et al., 2010; Martins et al., 2011; Colazo et al., 2017; Borchardt et al., 2018; Ciernia et al., 2018), reproductive tract size (Baez et al., 2016; Young et al., 2017; Madureira et al., 2020), maintenance of the CL during maternal recognition of pregnancy (Meyer et al., 1995; Siqueira et al., 2013; Dalmaso de Melo et al., 2020), as well as conceptus-driven PAG secretion (Pohler et al., 2016; Filho et al., 2020; Minela et al., 2021). It was not clear, however, if time from AI to an initial increase in PAGs in maternal circulation could explain why cows treated with fertility programs have a greater chance for pregnancy compared with AI following natural estrus.

The main objective of this study was to determine differences in time to conceptus attachment in lactating dairy cows treated with the fertility program Double-Ovsynch compared with cows that were detected in estrus with AAM. Our secondary objectives were to determine the effect of treatment on pre- and post-conception factors (E_2 and P_4 near ovulation, uterine size, CL blood flow, and serum PSPB) and how these relate to fertility outcomes. We hypothesized that AI following a natural estrus would result in

prolonged time to conceptus attachment and lower PSPB concentrations following attachment compared with Double-Ovsynch.

MATERIALS AND METHODS

Experimental units

Data were collected at Nobis Dairy Farm in St. Johns, Michigan, USA. Power analyses revealed that at least n = 22 pregnant lactating dairy cows per treatment were necessary to detect an average 1.5-day difference between treatments in time to conceptus attachment ($\alpha = 0.05$, $\beta = 0.20$, $\sigma = 1.97$). During the study period, n = 162 lactating dairy cows were due for first service and were available for inclusion in the study. Cows were considered ineligible for treatment assignment based on culling/selling decisions (n = 16) or health-related conditions (n = 1). Thus, n = 145 cows were assigned to treatments during six weeks, ranging from 1st to 7th parity. A total of n = 5 cows were excluded due to ovulation failure following AI. Cows were fed a TMR once daily with free access to feed and water and were confined in a free-stall barn. The TMR consisted of corn, wheat, and alfalfa silages and corn-soybean meal-based concentrates formulated to meet or exceed nutrient recommendations for high-producing lactating dairy cows (NRC, 2001). Nobis Dairy has a rolling herd average of 16,009 kg milk and 627 kg fat. The Institutional Animal Care and Use Committee at Michigan State University approved all animal handling and procedures.

Treatments

Cows were blocked by lactation and randomly assigned to treatments in a skewed ratio (60% Estrus, 40% Double-Ovsynch), estimating that 70% of estrus cows would be detected in estrus and inseminated. Cows in the Double-Ovsynch group (**DO**; n = 54) were

synchronized with GnRH (100 µg of gonadorelin; Cystorelin, Boehringer Ingelheim Animal Health) and $PGF_{2\alpha}$ (0.5 or 1.0 mg of cloprostenol sodium; Synchsure, Boehringer Ingelheim Animal Health) and received TAI as described in Figure 5.1. Cows in the estrus detection group (**ES**; n = 86) received GnRH between 47 and 53 DIM and $PGF_{2\alpha}$ 7 days later, between 54 and 60 DIM (Figure 5.1) to initiate cyclicity.

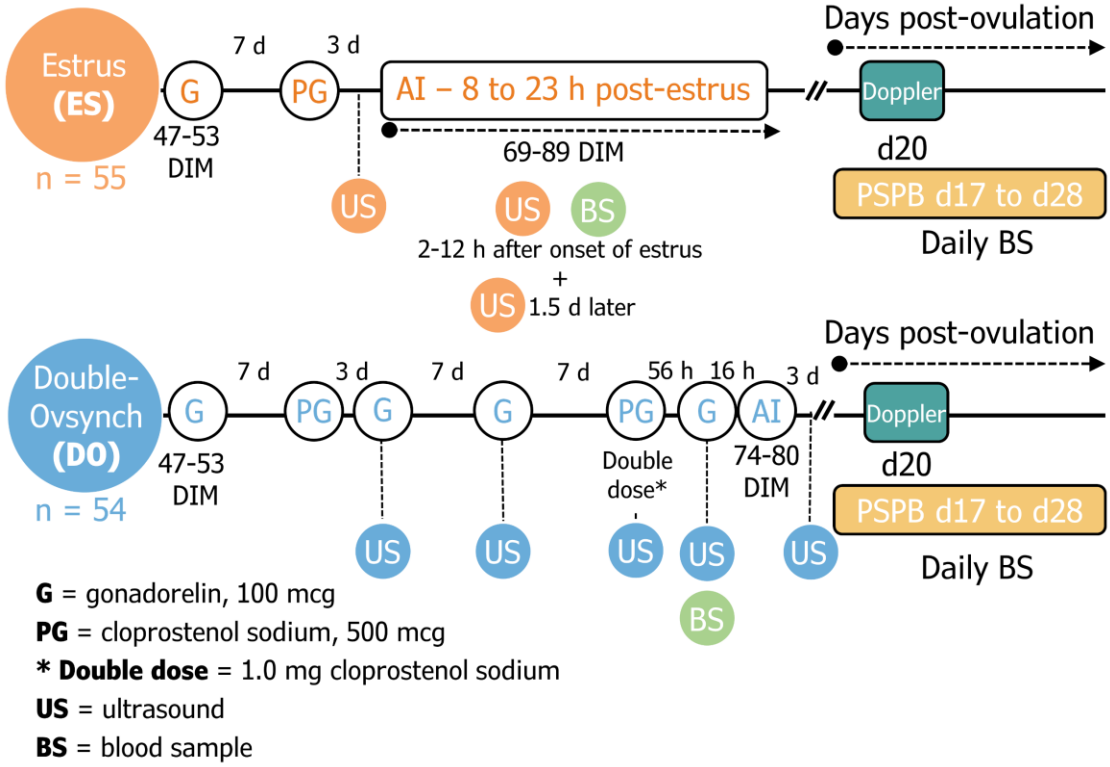

Figure 5.1. Description of treatment protocols utilized to artificially inseminate (AI) lactating Holstein cows (n = 109) for first service. Gonadorelin (G) and cloprostenol sodium (PG) were utilized in both treatments. In the ES group, an initial treatment of G – 7 days (d) – PG was given to resolve cyclicity. AI was performed between 69 – 89 days in milk (DIM), 8 to 23 hours (h) after estrus onset was detected utilizing automated activity monitors. The DO cows received G and PG administrations as described. Timed AI was performed 16 h after the final G, between 74 – 80 DIM. Ultrasound (US) exams were performed on the day of final G (DO) or 2 – 12 h after estrus onset (ES), and a blood sample (BS) was collected concomitantly. Ovulation was confirmed in a second US exam, either 3 days after AI in DO cows or 1.5 days after estrus onset in ES cows. Daily BSs were collected to measure pregnancy-specific protein B (PSPB) between days 17 to 28 post-ovulation. On day 20, luteal function was assessed with color Doppler.

Cows in ES were fitted with a collar and mounted with an activity and rumination monitor (Heatime Pro+ system, powered by Allflex). Percentage activity change data was updated in 2-hour intervals. Estrus onset was defined as the first time point that activity increased 35% from the 7-day activity average of each cow. Overall, 70.9% (61/86) of cows were detected in estrus and received first service. Only cows detected in estrus between 70 and 89 DIM and receiving AI between 8 and 23 hours from onset of estrus were included in the analyses (n = 55). Two AI technicians performed AI. Commercial semen from multiple sires (n = 4) purchased by the farm was utilized and evenly distributed across treatments.

Ovarian dynamics and measurement of ovarian structures and uterine horns

Linear array ultrasonography (7.5 MHz, MyLabVet Delta, Esaote) was used to map ovaries and assess ovarian dynamics. All measurements were determined using built-in calipers. The first diameter measurement for each structure was horizontal, at the greatest length, and the second was perpendicular to the 1st measurement, at the greatest height.

Assessment of CL volume, follicle, and uterine horn diameters before AI was performed on the day of final GnRH (DO) or 2-12 hours after estrus onset (ES). Luteal volume was reported in mm³ and calculated with the formula Volume = (4/3) π R^3, where R was the radius, or ½ the diameter in mm. The volume of cavities, if present, was calculated with the same formula and subtracted from the final luteal volume. The ovulatory follicle diameter (mm) and the uterine horn diameter (mm) were calculated as the average between its horizontal and perpendicular diameters. Average ovulatory follicle diameter was calculated only in cows with single ovulations (n = 42 for ES and n = 46 for DO). Uterine horns were measured cross-sectionally immediately cranially to the uterine

bifurcation. If observed, the lumen diameter was measured and further subtracted from the final average diameter. For analyses, only the size of the horn ipsilateral to the ovulation was utilized. In cows with bilateral ovulations, the average diameter of the right and left horns was calculated. Ovulation was confirmed in all cows included in the dataset, either 4 days after the final GnRH administration for the DO treatment or 36 to 48 hours after estrus onset for ES treatment (Figure 5.1). An additional exam was performed 20 days post-AI to assess LBF (luteal blood flow) as a morphological indicator of luteal function. Luteal blood flow was measured in individual CL utilizing color Doppler. Images were saved and processed to select the colored pixel area, as previously described (Minela and Pursley, 2021). Luteal blood flow was reported as the average colored pixel area from two pictures for each CL. If more than one CL was present, CL's blood flow was added.

Blood samples for determination of E_2, P_4, and PSPB concentrations

Samples of blood were collected in 8.5 mL tubes from the coccygeal artery or vein into tubes coated with clot-activator and separator gel (BD Vacutainer). Before AI, samples were collected on the day of final GnRH (DO) and 2-12 hours following estrus onset (ES). The post-AI sampling regimen included daily samples between days 17 to 28 post-ovulation. Samples were allowed to clot at 4°C for 24 hours before being centrifuged at 2,000 × *g* for 20 minutes for serum separation. A 1.5 mL aliquot of serum was stored at -18°C until shipment to external laboratories for analyses (Dr. George Perry's laboratory at Texas A&M and bioTRACKING). Samples collected before AI were assayed for P_4 and 17β-estradiol (E_2) with previously validated RIA (Engel et al., 2008; Perry and Perry, 2008). Intra- and inter-assay CVs for the P_4 assays were 3.5 % and 9.2 %, respectively. The

assay sensitivity was 0.08 ng/mL. The E_2 assays had intra- and inter-assay CVs of 4.4 % and 8.4 %, respectively. Assay sensitivity was 0.5 pg/mL. The minimum E_2 concentration in the DO treatment was 1.14 pg/mL (known time of LH surge). This value was utilized as the minimum expected threshold in ES cows (estimated time of LH surge). A total of n = 10 ES cows were removed from these analyses.

A commercial enzyme-linked immunosorbent assay kit (bioPRYN, bioTRACKING), developed by Sasser et al. (1986), was utilized to measure serum concentrations of PSPB in samples collected daily between days 17 and 28 post-ovulation. The lowest threshold of the assay was 0.2 ng/mL. The intra- and inter-assay CV were 4.9% and 8.3%, respectively.

Criteria to determine the first day of continuous PSPB increase

A baseline concentration value was calculated for each cow as the average of samples collected on days 17 and 18 post-ovulation for each cow. The lowest detectable amount (0.2 ng/mL) was utilized as a baseline value when both day 17 and 18 samples were below this sensitivity cutoff. The day of a significant increase in PSPB concentrations was defined as the first day in which PSPB increased ≥ 12.5% from the baseline (Santos et al., 2023). The PSPB concentration on that day also had to be above the lowest detectable amount. Day of the initial PSPB increase is hereafter referred to as conceptus attachment, or day of conceptus attachment. Two additional days of ≥ 12.5% increase from the previous day were utilized to confirm that PSPB was continuously increasing within the cow. The period from the initial day of PSPB increase, or conceptus attachment, in addition to the following 2 days in which PSPB continuously increased, will be referred to as the conceptus attachment “confirmatory period.”

Validation of differential steroid hormone dynamics near the LH surge

A ratio between E_2 and P_4 was calculated (Ratio = E_2 concentrations in pg/mL / P_4 concentrations in ng/mL) to determine the interactions between these hormones. The dataset was segmented into tertiles within treatments (ES: bottom n = 15, mid n = 15, and top n = 14; DO: bottom, mid and top n = 18/tertile). This data stratification was utilized due to the clear variability between tertiles within each treatment. Each E_2 to P_4 tertile differed significantly from one another (ES $P \leq 0.02$; DO $P < 0.01$).

Calculation of additional variables to describe embryo viability

Different daily PSPB concentration metrics were proposed to address the non-normal distribution of this variable as it relates to pregnancy status and embryonic competency. The first approach was to investigate PSPB concentrations during the confirmatory period (first, second, and third day from conceptus attachment). The second approach was to sum up the observed concentrations for the extent of the confirmatory period (3-day period), which may be referred to as cumulative PSPB concentrations.

Cows were also grouped across treatments into three categories of pregnancy status: cows with no conceptus attachment (No-CA), cows with conceptus attachment that maintained pregnancy until the second pregnancy diagnosis (Maintained), and cows with conceptus attachment that lost pregnancy at any time point up to the second pregnancy diagnosis (Lost). Pre- and post-conception factors with possible detrimental effects on pregnancy establishment and survival were investigated between treatment and pregnancy status.

Pregnancy diagnosis

Cows that returned to estrus after AI were considered non-pregnant and re-inseminated and thereby not checked for pregnancy. The remaining cows were checked by the herd veterinarian between days 31 to 37 post-ovulation. This is referred to as the first pregnancy diagnosis. Cows were re-confirmed between days 60 to 66 post-ovulation. This is referred to as the second pregnancy diagnosis. Each of these pregnancy diagnoses was performed via transrectal ultrasonography.

Statistical analyses

All statistical analyses were performed using SAS (version 9.4, SAS Institute Inc.). The level of significance was set at $P \leq 0.05$. A tendency was described as a *P*-value ranging from > 0.05 to ≤ 0.10. Multiple comparisons were adjusted with the Tukey-Kramer test for multiplicity. Descriptive statistics utilized features of PROC FREQ and PROC MEANS. All continuous variables were reported as means ± SEM.

Analysis of residuals from continuous variables was utilized to assess normality with PROC UNIVARIATE and SGPLOT. Shapiro-Wilk was utilized as an objective normality test. A Box-Cox transformation analysis was employed utilizing PROC TRANSREG on variables that presented a non-normal distribution to establish the value of the λ-exponent. The following variables were transformed with the recommended λ exponents in parenthesis: follicle diameter (-0.85), total CL volume prior to AI (0.3), horn diameter (log), E_2 concentrations (0.2), P_4 concentrations (0.3), ratio E_2 to P_4 (0.25), total pixels at day 20 post-AI (0.6), PSPB concentration (-0.15), and sum PSPB concentrations during confirmatory period (log). Further analyses were performed with the transformed

variable. Figures were built in Excel (Microsoft Office) with untransformed variables to aid data interpretation.

All continuous outcomes are reported as mean ± SEM. Analyses were performed utilizing PROC MIXED. All statistical models included the fixed effects of treatment and parity (primiparous n = 36, multiparous n = 73). When applicable, pregnancy status and double ovulation were also included as fixed effects. Lactation (1^{st} to 7^{th}) was included as the block variable in the random statement. The classification of conceptus attachment day was included as a random effect (d 19, 20, 21, and ≥ 22) when analyzing the sum of PSPB concentration during the confirmatory period.

Repeated measurements were analyzed with PROC MIXED and included time in the REPEATED statement. Models included time, treatment, and parity as fixed effects, in addition to pregnancy status when applicable. The random statement included lactation, day of conceptus attachment classification, and ID nested within treatment. ID nested within treatment was also specified in the subject option. A first-order AR1 covariance structure was utilized.

Predicted probabilities were estimated with PROC LOGISTIC. Wald chi-square was used to evaluate the association between pre- and post-conception continuous variables and outcome of interest (conceptus attachment or pregnancy loss). The linear relationship between two continuous variables was estimated with PROC REG utilizing a univariate model with a two-tailed t-test. PROC CORR was utilized to estimate Pearson correlation coefficients between two continuous variables.

RESULTS

Effect of treatment on days to conceptus attachment

There was no difference in the distribution of days to conceptus attachment between treatments (Figure 5.2; *P* = 0.23). The average days to conceptus attachment did not differ between ES vs. DO treatments (21.1 ± 0.2 vs. 20.9 ± 0.3; *P* = 0.19) or between primiparous and multiparous cows (21.2 ± 0.2 vs. 20.8 ± 0.2; *P* = 0.13). Conceptus attachment occurred in 31/55 ES and 30/54 DO cows.

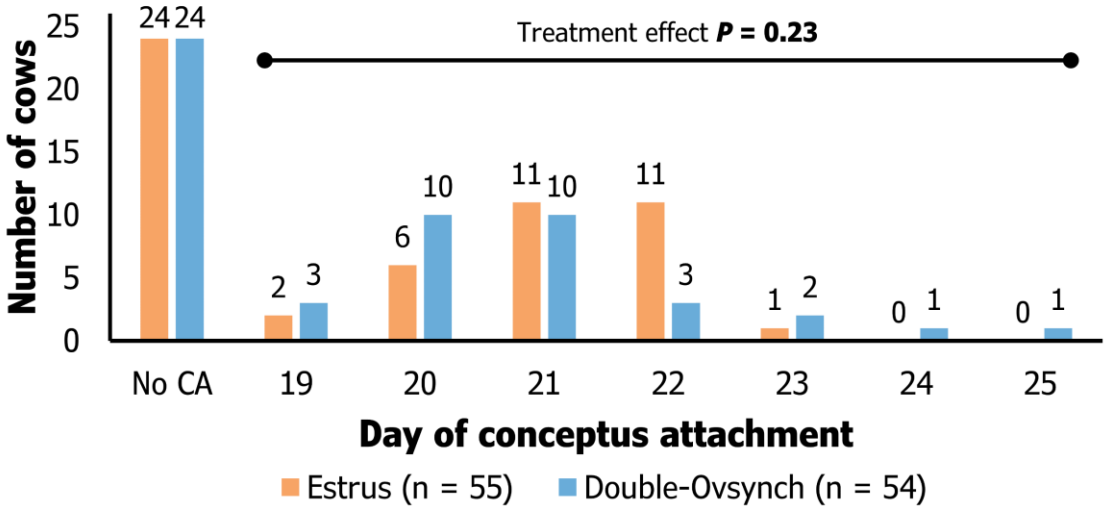

Figure 5.2. The effect of treatment on the distribution of day of conceptus attachment in lactating Holstein cows (n = 109). Treatments consisted of AI following either estrus detection (ES, n = 55) or the fertility program Double-Ovsynch (DO, n = 54).

Average days from AI to conceptus attachment in cows that maintained vs. lost pregnancy from conceptus attachment to pregnancy diagnosis with ultrasound on day 60 to 66 post-AI was 21.1 ± 0.2 vs. 21.1 ± 0.5 for ES and 20.8 ± 0.3 vs. 21.8 ± 1.1 for DO (*P* ≥ 0.35). From the day of conceptus attachment until the second pregnancy diagnosis between day 60 to 66 post-AI, 8/31 ES and 4/30 DO cows lost pregnancy. There was a tendency for greater pregnancy losses following ES compared with DO (7/31 vs. 1/30; *P* = 0.06) between the time of conceptus attachment and the first pregnancy diagnosis.

Effect of treatment on serum concentrations of PSPB during the 1st three days of conceptus attachment

Concentrations of PSPB during the confirmatory period (1st 3 days of conceptus attachment) are described in Figure 5.3. There were no differences (P = 1.0) in concentrations of PSPB during the confirmatory period between treatments in cows that maintained pregnancy. Cows in the ES treatment that lost pregnancy had lower PSPB concentrations on the second and third day of the confirmatory period in comparison with ES and DO cows that maintained pregnancy ($P \leq 0.04$). On the third day, there were greater PSPB concentrations in DO compared to ES (P = 0.04) in cows that lost pregnancy. Parity did not affect PSPB concentrations during the confirmatory period (P = 0.53).

Effect of treatment on pre-conception factors

Follicle and uterine horn diameters, serum E_2 and P_4 concentrations, and the E_2 to P_4 ratios were measured near the time of the LH surge in each treatment (Figure 5.1). Follicle diameter was greater for ES in comparison with DO (18.4 ± 0.5 vs. 14.9 ± 0.3 mm; $P < 0.01$). Horn diameter tended to be greater in ES cows in comparison with DO cows (22.5 ± 0.6 vs. 21.8 ± 0.6 mm; P = 0.08). Concentrations of E_2 (4.0 ± 0.4 vs. 3.9 ± 0.3 pg/mL; P = 0.78) and P_4 (0.49 ± 0.04 vs. 0.60 ± 0.04 ng/mL; P = 0.78) did not differ between ES and DO. However, there was a tendency for an effect of treatment on the E_2 to P_4 ratio (10.1 ± 1.1 vs. 7.7 ± 0.7, for ES vs. DO, respectively; P = 0.054). No effect of parity was observed for any of the analyzed pre-conception variables ($P \geq 0.18$).

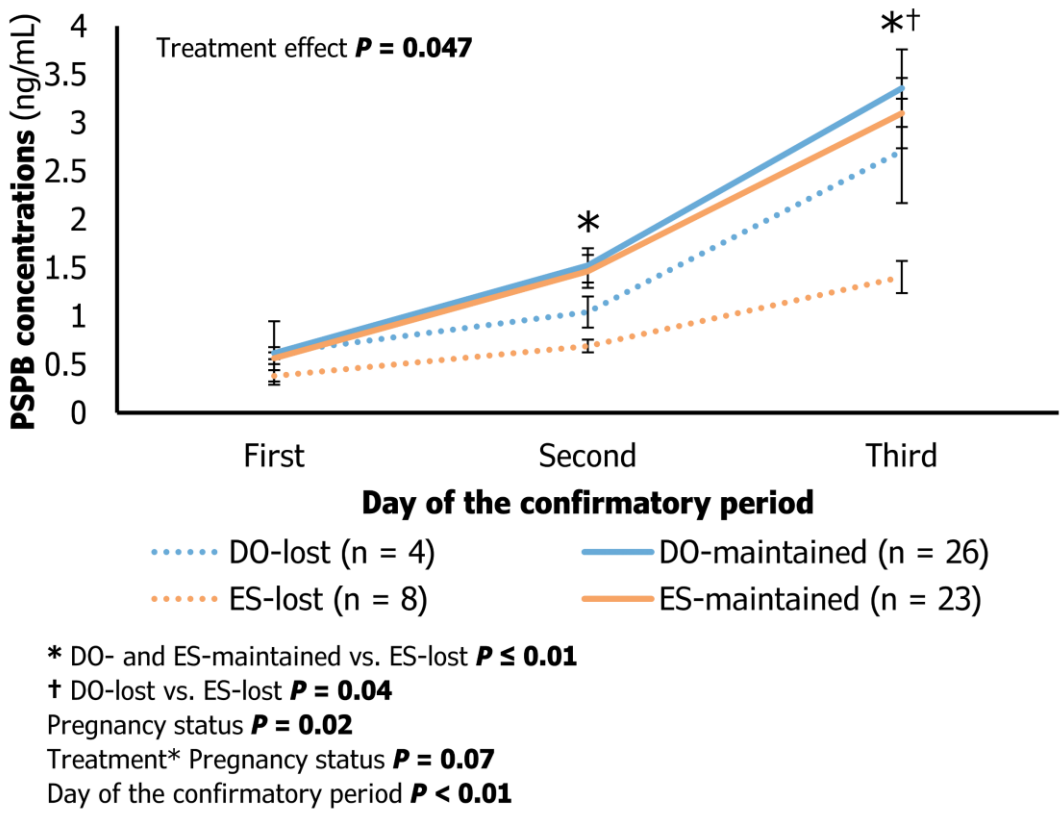

Figure 5.3. The effect of treatment (Trt; Estrus detection – ES, or Double-Ovsynch – DO) on concentrations of pregnancy-specific protein B (PSPB; ng/mL) during the confirmatory period. The confirmatory period consisted of the first day of significant PSPB increase in the maternal circulation ("first" or day of conceptus attachment), in addition to 2 more days in which PSPB was continuously increasing ("second" and "third"). The pregnancy status "maintained" included cows with conceptus attachment that sustained pregnancy to 60 – 66 days after AI.

Validation of the E_2 to P_4 ratio as a descriptor of steroid hormone dynamics

There was a wide range in the E_2 to P_4 ratio near the time of the LH surge within both ES and DO treatments. In the ES treatment, the average E_2 to P_4 ratio ranged from 3.7 (bottom tertile) to 18.6 (top tertile). In the DO treatment average E_2 to P_4 ranged from 3.3 (bottom tertile) to 13.7 (top tertile). Additionally, and as expected, concentrations of E_2 increased, and P_4 decreased from the lowest to highest tertiles of E_2 to P_4 ratios (Figure 5.4). Whether ovulation induction was manipulated through $PGF_{2\alpha}$ and GnRH or the LH

surge occurred endogenously, the distribution of cows within each E_2 to P_4 ratio tertile and the patterns of E_2 and P_4 within those ratios remained consistent.

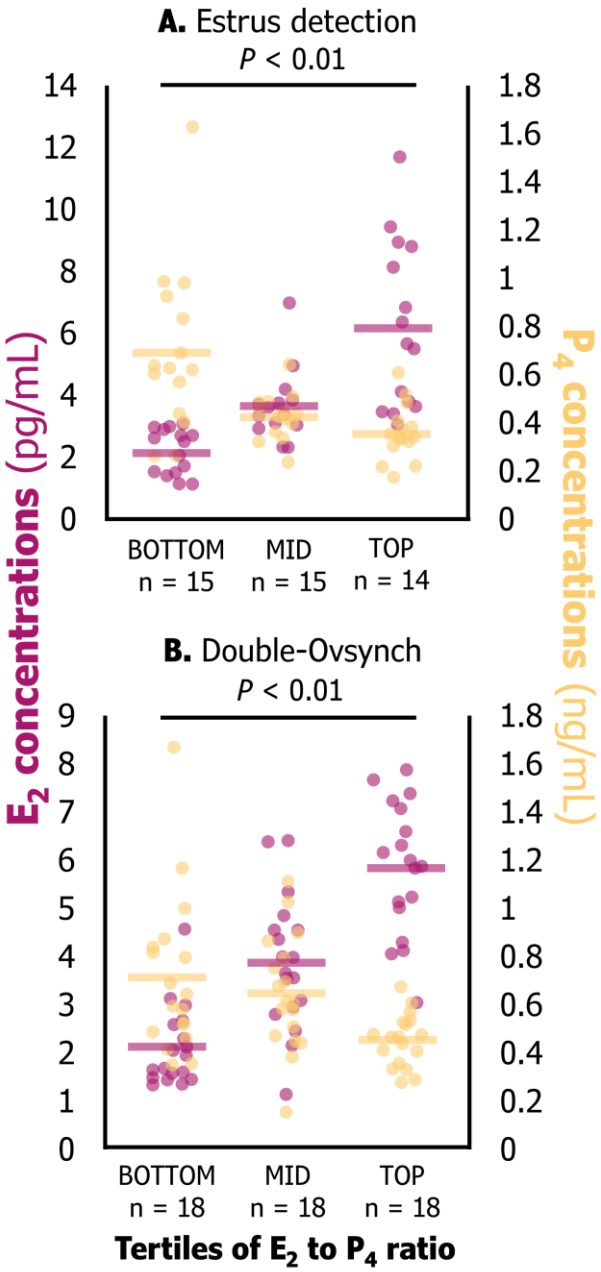

Figure 5.4. Description of the relationship between circulating concentrations of 17β-estradiol (E_2; in pink) and progesterone (P_4; in gold) within assigned E_2 to P_4 ratio tertiles (bottom, mid and top) and treatments (A. Estrus detection or B. Double-Ovsynch). Concentrations of E_2 and P_4 were measured in serum from samples collected on the day of the final gonadorelin (Double-Ovsynch) or 2 – 12 hours following estrus onset (Estrus detection). Each circle represents an observation, and the lines denote the average for

Figure 5.4 (cont'd)
E_2 (in pink) or P_4 (in gold) concentrations within tertile and treatment. Average E_2 and P_4 concentrations differed between tertiles ($P < 0.01$) for comparisons performed within treatments.

There was no linear relationship between luteal volume measured on the day of estrus or the final GnRH and the E_2 to P_4 ratio ($P = 0.66$). There was an overall positive linear relationship between ovulatory follicle diameter and the E_2 to P_4 ratio ($P = 0.01$). Ovulatory follicle diameter did not differ between tertiles within ES and DO treatments (Figure 5.5). However, ES had a greater average ovulatory follicle diameter in comparison with DO in each tertile of E_2 to P_4 ratios ($P \leq 0.04$).

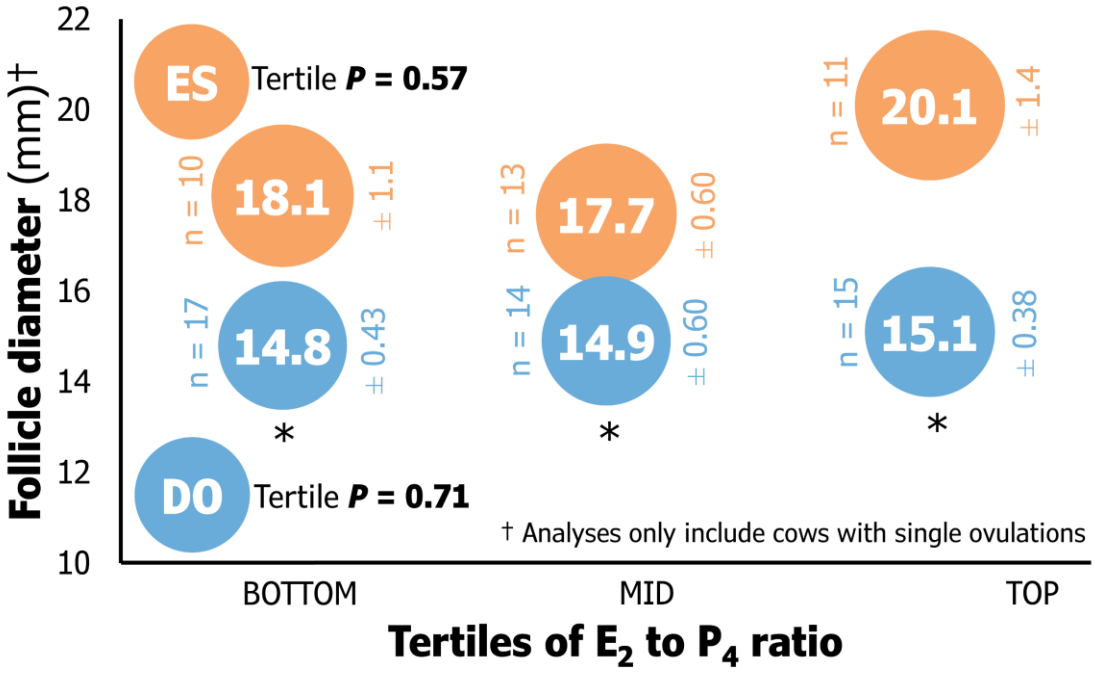

Figure 5.5. The relationship between ovulatory follicle diameter (mm; in cows with confirmed single ovulations) and tertiles of the ratio of 17β-estradiol (E_2) to progesterone (P_4) with each treatment (Estrus detection or ES, and Double-Ovsynch or DO). Follicle diameter was measured with ultrasound on the day of the final gonadorelin (DO) or 2 – 12 hours following estrus onset (ES). Follicle diameter did not differ between tertiles in analyses performed within treatments ($P \geq 0.56$). The symbol * denotes $P \leq 0.04$ for comparisons of DO vs. ES within tertiles.

Effect of treatment on post-conception factors

Treatment and parity effects were determined for the post-conception factors LBF and cumulative PSPB concentrations during the confirmatory period. There were no differences in LBF measured as colored pixels at day 20 post-AI between treatments (9,831.2 ± 921.9 for ES vs. 9,547.2 ± 820.2 for DO; $P = 0.76$). Luteal blood flow was predictive of conception attachment in both treatments ($P < 0.01$). Finally, no treatment effects were observed for the sum of PSPB concentrations during the confirmatory period (4.4 ± 0.5 vs. 5.3 ± 0.6 ng/mL for ES vs. DO, respectively; $P = 0.14$). Cumulative PSPB during the confirmatory period was associated with the predicted probability of pregnancy loss in ES but not DO ($P = 0.02$ vs. $P = 0.66$). No significant parity effects were observed for any of the analyzed post-conception variables ($P \geq 0.16$).

Associations of conceptus attachment outcome and pre- and post-conception factors

Follicle diameter did not emerge as a significant predictor of conceptus attachment for either treatment (Figure 5.6, panels A and B). However, the E_2 to P_4 ratio exhibited a positive association with the probability of conceptus attachment in DO but not in ES (Figure 5.6, panels C and D).

Distinct variations between treatments were evident in the average E_2 to P_4 ratio among cows experiencing conceptus attachment (Maintained), those with conceptus attachment followed by pregnancy loss (Lost), and those without conceptus attachment (No-CA; Figure 5.7, panels A and B). Cows in the DO group with conceptus attachment that maintained pregnancy to 60 – 66 days post-AI displayed a greater average E_2 to P_4

ratio compared to cows with conceptus attachment and subsequent pregnancy loss, as well as those without conceptus attachment.

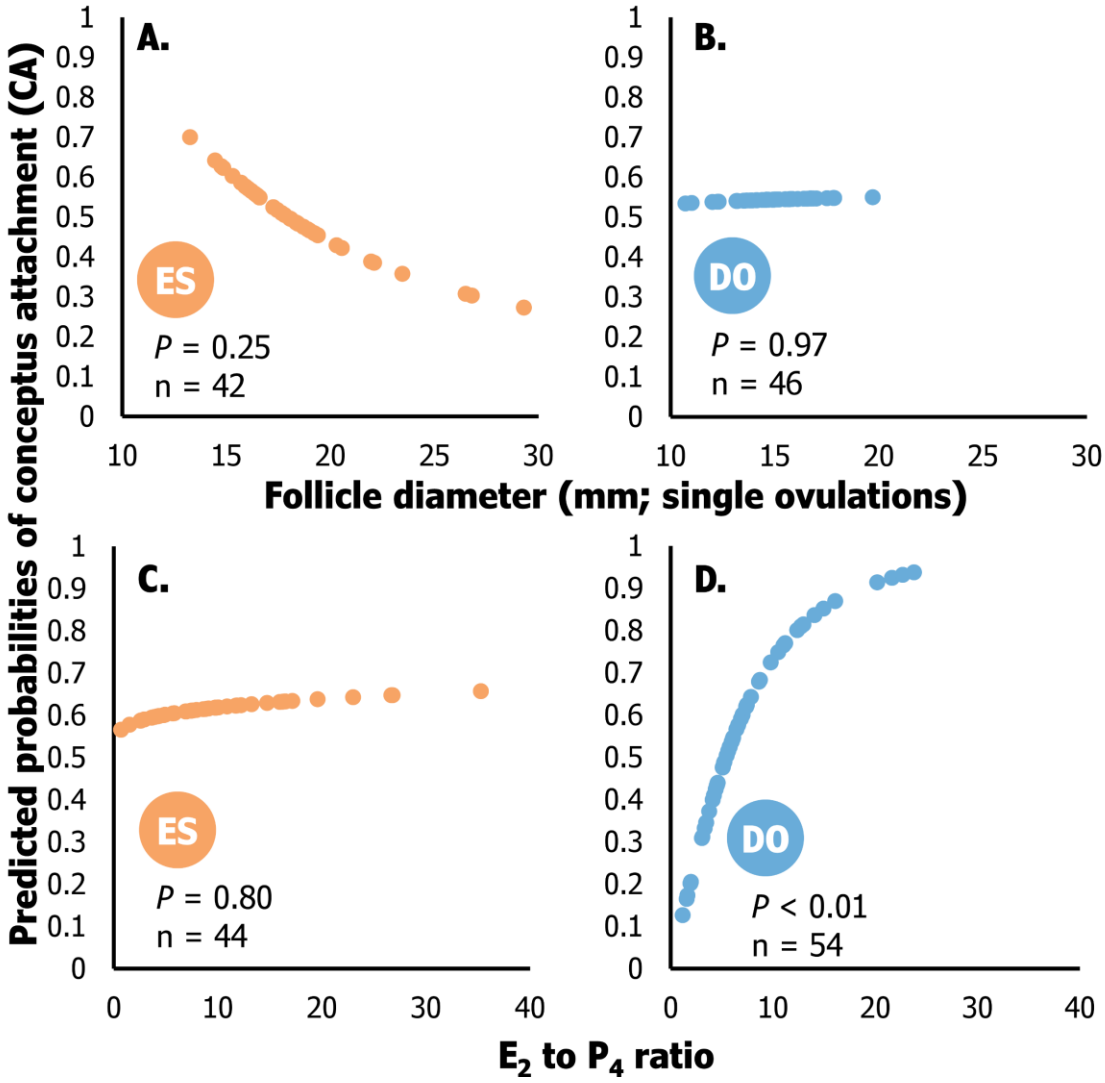

Figure 5.6. The predicted probabilities of conceptus attachment based on ovulatory follicle diameter (panels A and B) and the ratio of 17β-estradiol (E_2) to progesterone (P_4; panels C and D) within treatment (Estrus detection – ES, or Double-Ovsynch – DO) in lactating dairy cows. All variables were measured on the day of the final gonadorelin (DO) or 2 to 12 hours after estrus onset (ES).

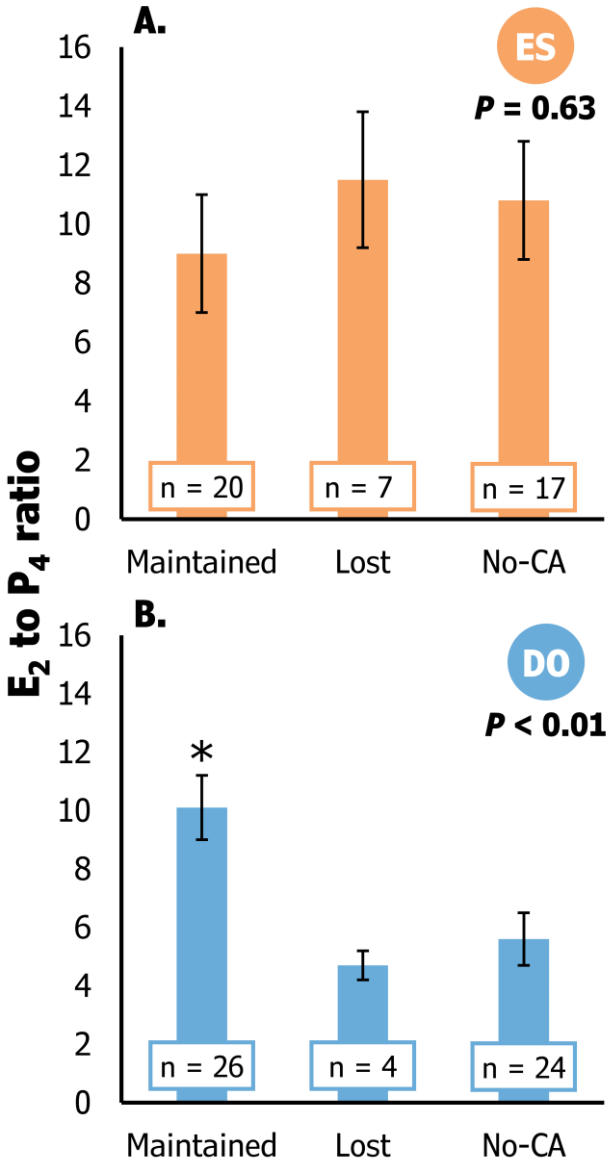

Figure 5.7. A description of the 17β-estradiol (E_2) to progesterone (P_4) ratios in cows with and without conceptus attachment and in cows that had pregnancy loss following conceptus attachment within each treatment (Estrus detection or ES, and Double-Ovsynch or DO). Pregnancy statuses were determined utilizing the day of conceptus attachment (CA) as the initial baseline. The "Maintained" status included cows with conceptus attachment that sustained pregnancies up to the second pregnancy diagnosis (day 60 to 66 post-artificial insemination – AI). The "Lost" status included cows that had conceptus attachment and lost pregnancy at any time point up to the second pregnancy diagnosis. And cows in the "No-CA" status were cows with undetected PSPS increase in maternal circulation. * Denotes a $P \leq 0.053$ for within DO comparisons between Maintained vs. Lost and Maintained vs. No-CA.

Conversely, E_2 to P_4 ratios were not different in cows with pregnancy loss compared to both cows that maintained pregnancy or that had no conceptus attachment ($P \geq 0.35$). Luteal blood flow was not different in cows with pregnancy loss compared to cows that maintained pregnancy ($P = 0.35$). Still, both groups with conceptus attachment had greater LBF compared to cows with no conceptus attachment ($P < 0.01$). The cumulative PSPB concentrations during the confirmatory period were greater for cows that maintained pregnancy compared to cows with pregnancy loss following conceptus attachment ($P = 0.01$).

Relationships between cow- and conceptus-related factors

Ovulatory follicle ($P \geq 0.10$) and uterine ($P \geq 0.79$) diameters were not correlated with days to conceptus attachment or cumulative PSPB during the confirmatory period. However, horn diameter had a significant negative correlation with cumulative concentrations of PSPB in ES but not DO treatments (ES r = -0.36, $P = 0.049$; DO r = -0.03, $P = 0.87$). Concentrations of E_2 and P_4 near the time of LH surge were not correlated with the conceptus-related investigated factors ($P \geq 0.13$ and $P \geq 0.14$, respectively). There was no relationship observed between E_2 to P_4 ratio and days to conceptus attachment ($P \geq 0.26$). Still, a greater E_2 to P_4 ratio tended to have a negative correlation with the cumulative PSPB concentrations in ES but not DO-treated cows (ES r = -0.32, $P = 0.08$; DO r = 0.1, $P = 0.60$). There was no significant correlation between LBF at day 20 post-AI and days to conceptus attachment, or cumulative PSPB concentrations over the confirmatory period ($P \geq 0.15$).

	Maintained n = 49	Lost n = 12	No-CA n = 48	*P*-value
Pre-conception factors				
Follicle diameter (mm)	16.0 ± 0.3[a]	16.5 ± 0.7[a]	17.1 ± 0.6[a]	0.89
Uterine horn diameter (mm)	21.2 ± 0.6[a]	23.3 ± 1.4[a]	22.9 ± 0.6[a]	0.12
E_2 concentrations (pg/mL)	4.0 ± 0.3[a]	3.4 ± 0.5[a]	3.4 ± 0.4[a]	0.55
P_4 concentrations (ng/mL)	0.46 ± 0.02[a]	0.45 ± 0.06[a]	0.64 ± 0.05[a]	0.61
E_2 to P_4 ratio*	9.3 ± 0.8[a]	8.4 ± 1.6[a]	7.4 ± 1.1[a†]	0.09
Post-conception factors				
LBF (×1,000 pixels)	13.24 ± 0.78[a]	10.96 ± 1.55[a]	5.78 ± 0.76[b]	< 0.01
Time to CA (days)	20.9 ± 0.2[a]	21.3 ± 0.5[a]	-	0.17
Cumulative PSPB (ng/mL)**	5.3 ± 0.4[a]	3.1 ± 0.4[b]	-	0.03

* Indicates a tendency for a treatment by pregnancy status interaction in analyses of 17β-estradiol (E_2) to progesterone (P_4) ratio (*P* = 0.052).
** Indicates a tendency for a treatment by pregnancy status interaction in analyses of cumulative PSPB (*P* = 0.08).
† Indicates a tendency for a difference between Maintained vs. No-CA (*P* = 0.07).

Table 5.1. Relationship of pregnancy status with pre- and post-conception factors. Pregnancy statuses were determined utilizing the day of significant pregnancy-specific protein B (PSPB) increase or the day of conceptus attachment (CA) as the initial baseline. The "Maintained" status included cows with conceptus attachment that sustained pregnancies up to the second pregnancy diagnosis (day 60 to 66 post-artificial insemination – AI). The "Lost" status included cows that had conceptus attachment and lost pregnancy at any time point up to the second pregnancy diagnosis. Cows in the "No-CA" status had undetected PSPB increase in maternal circulation.

DISCUSSION

The objective of this study was to gain a greater understanding of the mechanisms underlying improved P/AI of fertility programs, such as Double-Ovsynch (Santos et al., 2017), compared to AI following the detection of estrus. Our laboratory developed a novel model for determining the time of conceptus attachment, approximately around day 20 post-AI (Middleton et al., 2022). This innovation enabled us to investigate the developmental aspects of embryonic growth two weeks earlier than the conventional day

~35 pregnancy diagnosis. A delay in the timing of conceptus attachment clearly indicates an elevated risk of pregnancy loss (Santos et al., 2023). Such losses would go unnoticed at the standard 35 days post-AI pregnancy evaluation.

Contrary to our hypotheses, there were no differences between treatments in time to conceptus attachment or in cumulative serum concentrations of PSPB during the 1st three days of conceptus attachment (Figures 2 and 3). In a previous study, more than 85% of cows with conceptus attachment on days 20 and 21 post-AI (estimated based on initial PSPB increase) sustained their pregnancy (Santos et al., 2023). The ability of a conceptus to secrete products into the maternal circulation in a timely manner is suggestive of proper development and successful activation of molecular pathways involved with conceptus attachment. Adhesion is the initial process that leads to stable cell-to-cell interaction, conceptus attachment, and placentation (Wathes and Wooding, 1980; Guillomot and Guay, 1982; D'Occhio et al., 2020). This process is mediated through several molecules, but integrins and cadherins were largely characterized during the peri-attachment period in ruminants (Johnson et al., 2001; Sakurai et al., 2012; Yamakoshi et al., 2012). The extended period to detect PSPB in the maternal circulation (conceptus attachment ≥ 22 days; 19/61 cows) in the current study could be associated with malfunction or delay in initial steps of conceptus attachment and BNC differentiation (Nakano et al., 2005). The phenotype of delayed increase in PSPB was less prevalent in previously sampled populations (Middleton et al., 2022; Santos et al., 2023). Thus, it becomes highly likely that subtle differences in average days to conceptus attachment are uncaptured in small sample sizes, even though power analyses were performed using these previous data to determine sample size.

Cows in the ES group that lost pregnancy had diminished concentrations of PSPB following conceptus attachment, a phenotype not observed in DO cows that also lost pregnancy in this study. Santos et al. (2023) reported a clear reduction in PSPB secretion during the 1st week of conceptus attachment in cows with early pregnancy loss. The occurrence of compromised PSPB secretion, concurrent with unsuccessful pregnancies, may be associated with the premature pregnancy losses observed in 7/8 cows in the ES treatment (before the first pregnancy diagnosis). Lower concentrations of PSPB at day 24 post-AI were an early predictor of pregnancy loss (Pohler et al., 2016; Minela et al., 2021). The present study is the first report of a trend for greater pregnancy loss as a plausible explanation for reduced P/AI following estrus detection. Studies with greater numbers of cows are needed to evaluate the effect of ES and DO on the proportion of early pregnancy losses occurring past conceptus attachment.

The biological significance of reduced PSPB in serum of cows that lost pregnancies is not clear. Is this due to a slowed development of the conceptus or a flaw in the ability of BNCs of a healthy and normally developing conceptus to produce adequate quantities of PSPB? Thus, are adequate quantities of PSPB needed for conceptus attachment or are the lower concentrations of the PAG a consequence of a retarded conceptus? The role of PAGs in pregnancy establishment and survival has not been fully characterized. *In vitro* evidence demonstrated that uterine transcriptome can be altered with PAG exposure. Genes associated with tissue remodeling were upregulated in both pregnant and non-pregnant uterine explants upon treatment with PAG (Wallace et al., 2019). The same authors proposed a maternal-conceptus interface localized action. Pregnancy-associated glycoproteins could function as regulators of tissue remodeling that accompanies

conceptus attachment and subsequent placentation. The presence of PAG-positive trinucleate trophoblast cells incorporated into the uterine endometrium was reported as early as day 20 (Wooding and Wathes, 1980), alongside firm attachment of the trophectoderm to the endometrial lining between days 20 and 21, ipsilateral to the CL (Guillomot and Guay, 1982). Below average and/or delayed PAG exposure to the maternal interface during this critical time may be a limiting factor to sustaining pregnancy. Pregnancies presenting these types of PSPB secretion profiles were more likely to be terminated before a day 34 pregnancy diagnosis (Santos et al., 2023). There was about 1 ng/mL less PSPB being secreted during the first 3 days following conceptus attachment in the ES in comparison with DO treatment. This decreased exposure to PSPB could be deleterious to proper pregnancy survival. In this study, the cumulative PSPB concentrations during the confirmatory period were predictive of reduced pregnancy survival in ES but not in DO. This is likely due to the trend for more losses following conceptus attachment in the ES group. Overall, conceptuses of failed pregnancies had ~2 ng/mL less PSPB over the confirmatory period in comparison with conceptuses of sustained pregnancies. Thus, this metric is a valid marker of not only presence of a conceptus but also embryonic viability of high-risk pregnancies.

In the current study, we measured various fundamental pre- and post-conception factors that can impact fertility of lactating dairy cows (Brusveen et al., 2009; Shimizu et al., 2010; Pohler et al., 2013; Young et al., 2017; Ciernia et al., 2018; Dalmaso de Melo et al., 2020; Filho et al., 2020; Madureira et al., 2020; Minela et al., 2021). Follicle diameter prior to ovulation, a key determinant of fertility (Perry et al., 2005; Bello et al., 2006), was greater in the ES treatment compared with cows in the DO treatment. The efficiency of

Double-Ovsynch in controlling the onset of follicular development, luteolysis (day 7 of development), and ovulation (9.5 days of development) results in consistent ovulation of follicles around ~16 mm. In this study, ES cows ovulated follicles on average 3.5 mm larger in comparison with DO. Considering a daily growth of 1.2 mm (Sartori et al., 2004), this finding could be interpreted as the ovulation of follicles that were almost 3 days older in the ES group. This difference in ovulatory follicle diameter could be the result of more frequent LH pulses that likely lead to greater aged and prematurely matured oocytes compared to Double-Ovsynch (Fricke and Wiltbank, 2022). Consequently, this suboptimal physiological state leads to decreased embryo quality following estrus detection (Ahmad et al., 1995). Yet, concentrations of E_2, which were shown to impact fertility (Perry et al., 2007; Ketchum et al., 2023), were not different between treatments. Also, there was no association between the ovulatory follicle diameter and PAG concentrations between days 24 to 60 post-AI in suckled beef cows (Pohler et al., 2013). In this study, cows were sampled between 2 and 12 hours in relation to estrus onset. Thus, it is possible that E_2 was past peak concentrations at the time of sampling. Nevertheless, the E_2 secretory capacity of the pre-ovulatory follicle tended to be positively correlated with its diameter ($r = 0.35$, $P < 0.01$, data not shown) and E_2 to P_4 ratios were not different between treatments. We also only utilized cows in the ES group for data analysis with a minimum concentration cutoff of E_2 relative to the lowest measurement of E2 in the DO group.

The work presented herein proposed a combined metric to assess steroid hormone dynamics around the LH surge in both treatments. This metric consisted of a ratio between E_2 and P_4 concentrations (ratio E_2 to P_4) at a critical time following luteolysis and near the time of the LH surge. An optimal scenario at this stage of the estrous cycle would be an

inverse relationship between E_2 and P_4, thus a high E_2 to P_4 ratio. Dynamic secretion and timely exposure to steroid hormones before ovulation are part of mechanistic priming of the endometrium (Shimizu et al., 2010) and were attributed to high fertility following both fertility programs (Fricke and Wiltbank, 2022) and estrus detection (Madureira et al., 2019). Complete luteolysis and reduction of P_4 concentrations occurring synergistically with final follicular development and peaking E_2 secretion (Lemon et al., 1975) are events that set what could be referred to as the “uterine clock” (Shimizu et al., 2010). Four clusters of genes were upregulated in E_2-exposed endometrium in a model that mimicked estrous cycle changes in P_4. These genes were associated with embryonic development, cell division/differentiation/adhesion/migration, gastrulation, organogenesis, angiogenesis, invasive growth, epithelial to mesenchymal transition, and migration, all essential to pregnancy establishment and maintenance (Shimizu et al., 2010). Motta et al. (2020) reported a greater rate of augmentation in the endometrial area during proestrus in cows with the highest E_2 and lowest P_4 concentrations. This classification could be considered equivalent to the top tertiles of E_2 to P_4 ratio in both treatments. An increased endometrial thickness the day before AI was associated with greater fertility in lactating dairy cows (Souza et al., 2011). Thus, suboptimal and/or non-timed exposure to steroid hormones could offset the uterine clock and result in an inappropriate environment for pregnancy in cows. As previously stated, cows in the top E_2 to P_4 ratio tertile had the highest E_2 and lowest P_4 concentrations. The opposite was observed on the bottom tertile of the ratio distribution. This metric described extreme scenarios of optimal and suboptimal steroid hormone interaction that were accordingly associated with fertility in the DO treatment. Cows without conceptus attachment had a lower mean E_2 to P_4 ratio in comparison with

cows that had conceptus attachment and maintained pregnancy, regardless of treatment. This suggests that an imbalanced interaction of E_2 and P_4 around AI may be more limiting to conceptus attachment than pregnancy survival following conceptus attachment.

Color Doppler ultrasonography was utilized to assess luteal function and predict non-pregnant cows as early as day 20 post-AI (Siqueira et al., 2013; Guimarães et al., 2015; Dalmaso de Melo et al., 2020). In the present study, LBF at day 20 post-AI was predictive of pregnancy establishment in both treatments. Luteal rescue during maternal recognition of pregnancy is mediated through interferon-tau secretion in trophectoderm cells (Meyer et al., 1995; Bai et al., 2012; Pate, 2020). On day 21 of gestation, no differences in mRNA expression of ISG15 were reported between cows that either maintained or lost pregnancy (Shirasuna et al., 2012). In the present study, LBF at day 20 post-AI was not predictive of pregnancy loss. It appears that luteal rescue mechanisms were in place regardless of whether conceptuses were maintained or lost after conceptus attachment. Alternate explanations of pregnancy loss not associated with conceptus developmental issues or LBF may be explained by pathways related to post-attachment processes, such as the epithelial-mesenchymal transition (Yamakoshi et al., 2012), tissue remodeling (Guillomot and Guay, 1982; Salamonsen, 1999), and angiogenesis leading up to placentation (Reynolds et al., 2006).

In summary, time to conceptus attachment did not differ between treatments. The small number of cows in this study did not lead to an explanation of why differences in fertility occur in cows receiving AI following estrus vs. the fertility program Double-Ovsynch. However, concentrations of PSPB during the first 3 days following conceptus attachment were a powerful predictor of pregnancy loss. This was evident in ES but not

DO. The ratio of E_2 to P_4 was positively associated with the probability of conceptus attachment in DO treatment but not ES. In the DO treatment, cows that maintained pregnancy had greater E_2 to P_4 ratios in comparison with non-pregnant cows, or cows that lost pregnancy. This implies that maintaining adequate steroid hormone dynamics before AI positively influences the probability of both establishing and sustaining pregnancy in fertility programs. Residual P_4 following luteolysis appears to be a confounder of pregnancy, as does insufficient E_2. Thus, it appears that fertility of lactating dairy cows is at least partially dependent upon ensuring complete luteolysis in addition to a well-controlled antral-aged pre-ovulatory follicle with the greatest steroidogenic capacity to allow for the greatest E_2.

CHAPTER 6

DETERMINING THE TRUE FERTILITY POTENTIAL OF AI FOLLOWING ESTRUS

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INTRODUCTION

Hormonal intervention may be utilized to orchestrate processes that happen physiologically during the estrous cycle of cows. The use of analog pharmaceuticals became a viable strategy with the approval of analog GnRH (1978) and $PGF_{2\alpha}$ (dinoprost tromethamine in 1979 and cloprostenol sodium in 1982). The use of hormone analogs allowed for either the induction of luteolysis and estrus expression (Plunkett et al., 1984) or the combined induction of a new follicular wave, luteolysis, and ovulation (i.e., Ovsynch; Pursley et al., 1995). The latter was considered the first fixed-time AI program. Exogenous ovarian manipulation allowed for AI of all eligible cows without visual detection of estrus (Pursley et al., 1997b). Estrus detection rates were 50% at best when performed by experienced personnel at least twice a day (Trimberger and Davis, 1943; Senger, 1994; Pursley et al., 2012). Decreased service rate may be a major variable negatively impacting 21-day pregnancy rates (Fricke et al., 2014; Borchardt et al., 2021).

Several breakthroughs regarding estrous synchronization and cow fertility emerged from the Ovsynch study. Those included: 1) the differential synchronization ability depending on ovulation to the initial GnRH administration, thus the necessity of pre-synchronization (Vasconcelos et al., 1999); 2) the importance of increasing P_4 concentrations during the development of ovulatory follicles (Lopez et al., 2005; Bisinotto et al., 2010; Wiltbank et al., 2012); 3) the need for complete luteolysis prior to AI and strategies to ensure luteolysis (Brusveen et al., 2009; Borchardt et al., 2018; Minela et al., 2021); and 4) the use of TAI programs as fertility treatments that could be used on a herd level to improve reproductive health and fertility (Carvalho et al., 2018). Fertility programs such as Double-Ovsynch (Souza et al., 2008) and Presynch-10/11 (Moreira et

al., 2001) yielded greater P/AI in comparison to a detected estrus (Strickland et al., 2010; Santos et al., 2017; Rial et al., 2022; Sitko et al., 2023).

Milk production in the USA increased from 2,074 kg/cow/year in 1944 to 11,043 kg/cow/year in 2023 (USDA, 2023). This astonishing increase in milk production came at the cost of metabolic adaptations that interfered with the reproductive endocrinology of high-producing lactating dairy cows (Wiltbank et al., 2006). High milk production and elevated hepatic metabolism resulted in decreased circulating levels of steroid hormones (Sangsritavong et al., 2002; Lopez et al., 2004). Depletion of these hormones impacted estrus behavior and fertility following TAI (Lopez et al., 2004; Bello et al., 2006). A biological model for this syndrome and its detrimental effects on fertility was recently proposed (Fricke and Wiltbank, 2022).

Management strategies based on hormonal intervention improved fertility to comparable levels to those reported in low-producing lactating dairy cows which composed the U.S. herd 80 years ago. The success of fertility programs, however, is highly dependent on compliance, and it can become costly in terms of labor (Galvão et al., 2013). An alternative to diminish the labor associated with these programs is the use of automated systems to detect secondary signs of estrus (Fricke et al., 2014). These devices are fitted in cattle as collars or ear tags and track the baseline activity. An estrus event is characterized as a 35% increase or greater from a within-cow 7-day average activity. The onset of estrus, as defined by the increase of activity above the 35% threshold, is closely associated with the surge of LH (Valenza et al., 2012; Aungier et al., 2015). Yet, this tool only allows for a ~70% detection rate without correcting possible

intrinsic subfertility stemming from high metabolic rates of steroid hormones (Valenza et al., 2012).

Pregnancy outcomes are usually measured with ultrasound at ~35 days post-AI. The diminished P/AI following a detected estrus is based on this initial diagnosis. It is unclear at what stage pregnancy failure occurs and why it occurs in greater proportion following estrus detection. The daily measurement of PSPB concentrations provides a reliable indication of pregnancy status as early as day 19 of gestation (Middleton et al., 2022). Time to detection and concentrations of this conceptus-exclusive protein in the maternal circulation were associated with the likelihood of pregnancy loss (Santos et al., 2023). A preliminary study of our laboratory indicated that the proportion of cows with conceptus attachment, based on the serial measurements of PSPB, was not different between estrus detection and the Double-Ovsynch program (Chapter 5). That study was not appropriately powered to assess binary fertility outcomes. Nonetheless, there was a tendency for greater early pregnancy losses in the estrus detection treatment. These losses occurred between conceptus attachment and a pregnancy diagnosis with ultrasound on days 31 to 37 post-AI. This was the only study that estimated fertility outcomes (i.e., P/AI and pregnancy losses) following a detected estrus earlier than an initial diagnosis performed ~30 days post-AI. Based on this initial evidence, pregnancy losses occurring before the first pregnancy diagnosis may be contributing to diminished fertility following a detected estrus.

Given the lack of fertility outcomes comprising the period of conceptus attachment in lactating dairy cows, we designed a study to compare AI following a detected estrus or the fertility program Double-Ovsynch. The main objective was to assess if the differences

in fertility between these two AI strategies were also observed during the period of conceptus attachment. We hypothesized that estrus detection would result in lower P/AI measured around 35 days post-AI in comparison to the fertility program Double-Ovsynch. We also hypothesized that decreased fertility following estrus detection would be a result of greater pregnancy losses occurring before the ultrasound pregnancy diagnosis around day 35 post-AI. An additional hypothesis was that pregnancy losses in the estrus detection treatment would be associated with greater time to conceptus attachment in comparison to Double-Ovsynch.

MATERIALS AND METHODS

Experimental units

This project was conducted from May 2022 to January 2023 on a commercial dairy herd (Green Meadows Dairy Farm, Elsie, Michigan, USA). The herd milked ~4,020 Holstein dairy cows with a rolling herd average of 14,288 kg of energy corrected milk. Cows were fed a TMR thrice a day with free access to feed and water and were confined in a ventilated free-stall barn. The TMR consisted of corn silage, alfalfa haylage, ground corn, canola oil, soy hulls, soybean meal, mineral additives, and whey, formulated to meet nutrient recommendations for high-producing lactating dairy cows (NRC, 2001). The Institutional Animal Care and Use Committee at Michigan State University approved all animal handling and procedures. Power analyses revealed that at least n = 170 lactating dairy cows per treatment were necessary to detect a 15% difference in P/AI (reference 35% P/AI following a detected estrus). Given that treatment differences may be lower and P/AI greater when assessed earlier in gestation, it was estimated that at least n = 309 lactating dairy cows would be necessary to detect an 11% difference in P/AI (reference

55% P/AI following a detected estrus). During the study period, n = 1,124 lactating cows eligible for first AI were available for enrollment. Treatments were randomly assigned between 28 and 34 DIM. A total of n = 151 cows left the study due to reasons that included culling/selling decisions that were either health or production-driven (n = 96), health events (n = 23), and non-compliance to the study protocols (n = 24). Additionally, cows in the Double-Ovsynch treatment that had spontaneous estrus before the final GnRH were removed from the study (n = 8). Thus, n = 973 lactating Holstein cows were eligible to receive AI.

Treatments

Cows were blocked by parity and cohort and randomly assigned to treatments in a skewed ratio (70% Estrus, 30% Double-Ovsynch). The skewed ratio aided in achieving comparable numbers of cows receiving AI in each treatment. It was assumed that 70% of cows in the estrus group would be detected in estrus and inseminated. Cows in the Double-Ovsynch treatment (**DO**; n = 322) were synchronized with GnRH (100 µg of gonadorelin; Cystorelin, Boehringer Ingelheim Animal Health) and $PGF_{2\alpha}$ (0.5 mg or 1.0 mg of cloprostenol sodium at the final $PGF_{2\alpha}$; Synchsure, Boehringer Ingelheim Animal Health) as previously described (Souza et al., 2008). The first GnRH was administered between 47 and 53 DIM and TAI was performed between 74 and 80 DIM, 13 to 21 hours following induced ovulation (Figure 6.1). Cows in the estrus detection treatment (**ES**; n = 651) did not receive hormonal intervention. ES cows were considered eligible for AI between 69 and 94 DIM. During these 25 days, 51.9% (338/651) of cows were detected in estrus and received AI on average 15.5 hours after estrus onset (Figure 6.1). Eight

technicians performed AI. Commercial semen from multiple sires (n = 41) purchased by the farm was utilized. Mating was assigned according to cow genetic merit.

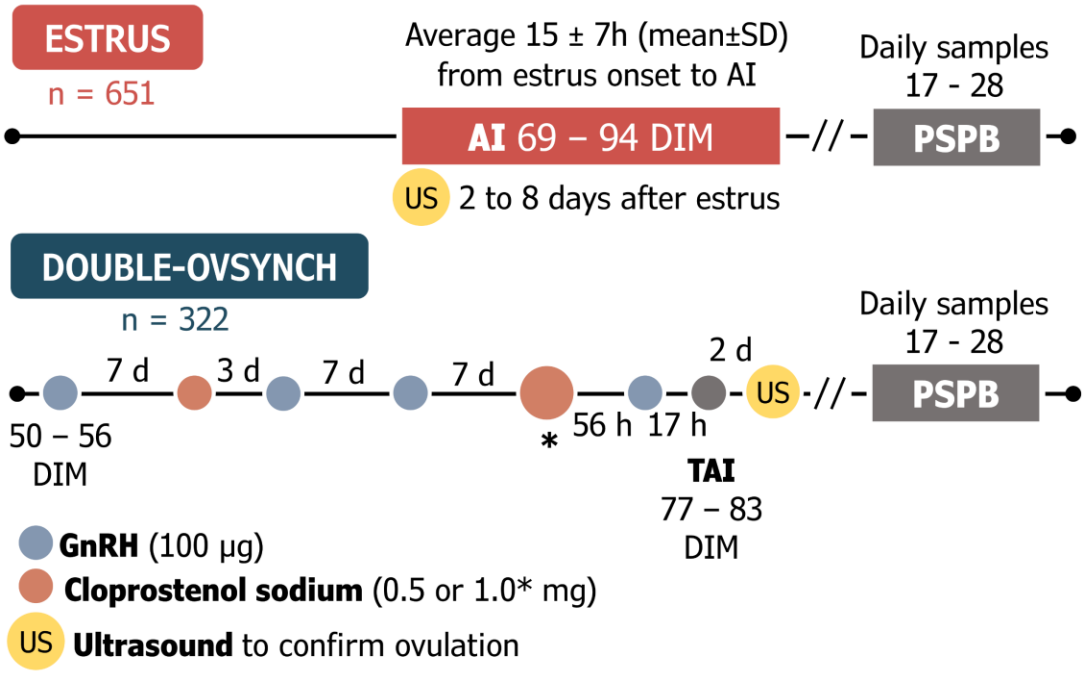

Figure 6.1. Experimental design to determine the effects of natural estrus or estrous cycle synchronization with gonadotropin-releasing hormone (GnRH) and cloprostenol sodium on the reproductive efficiency of lactating Holstein cows receiving first post-partum artificial insemination (AI). Cows were randomly assigned to receive first AI between 69 and 94 DIM upon a detected estrus, or first AI between 77 and 83 days in milk (DIM) following the fertility program Double-Ovsynch. Estrus was detected with an automated activity monitor (AAM). Ovulation was confirmed in all cows that received AI with ultrasonography (US) 2 to 8 days after estrus onset as determined by the AAM or 2 days after the final GnRH of Double-Ovsynch. Cows with confirmed ovulation had daily blood samples collected between days 17 to 28 after the expected day of ovulation. Samples were utilized to determine the circulating concentrations of pregnancy-specific protein B (PSPB, ng/mL).

Estrus detection with AAM

All cows in the study were equipped with an ear tag that detected basal activity and rumination (Heatime® Pro+, Allflex Livestock intelligence). Individual activity data were obtained in 2-hour time increments from DataFlow II software (Allflex Livestock Intelligence). An estrus event was indicated in the software when basal activity increased

at least 35% in comparison to a 7-day baseline. Cows in the ES group were inseminated in relation to estrus onset, or the time that activity increased above the threshold. Cows in the DO were considered also to have estrus if the event occurred after the final GnRH of Double-Ovsynch and before TAI. The percentage change in activity for the AI estrus event was utilized as a marker of estrus intensity (ranging between 36 and 100. Estrus events occurring before AI were recorded for all cows. Cows were classified in categories according to the number of estrus events prior to AI. The categories comprised no estrus (0; cows that did not have any estrus event before AI), one estrus (1; cows with 1 estrus event prior to AI), at least two estruses (≥2; cows that had 2 to 4 estrus events before AI). Cows were classified into two categories according to estrus intensity at AI, as described by Madureira et al. (2015). Cows with “high estrus intensity” (estrus intensity > 88; n = 241) and cows with “low estrus intensity” (estrus intensity ≤ 88; n = 90).

Ultrasonography – confirmation of ovulation

Linear array ultrasonography (MyLab Gamma, and MyLab Delta, Esaote) was utilized to confirm ovulation in all cows. In DO cows, ovulation occurrence was confirmed 3 days after the final GnRH. In ES cows, ovulation was confirmed between 2 and 8 days from estrus onset. The appearance of newly formed CL characterized ovulation. Color Doppler was utilized to ensure the presence of high blood flow, indicating functional CL. In case of an inconclusive exam, cows were re-checked 2 or 3 days following the first exam. The number and side of ovulation were also recorded. Cows without ovulation after estrus (n = 6) or at the end of the Double-Ovsynch program (n = 10) were removed from all post-AI outcome analyses. Additional n = 3 cows were removed from the DO treatment due to apparent incomplete luteolysis despite having ovulation. Thus, n = 309 cows in the

DO treatment and n = 332 cows in the ES treatment were considered for post-AI outcome analyses.

Blood sample collection

Blood was collected from the coccygeal artery or vein into 8.5 mL tubes coated with clot-activator and separator gel (SST^{TM} Venous Blood Collection Serum Tube, BD Vacutainer®). Samples were collected daily from all cows between days 17 to 28 in relation to the day of estimated ovulation (24 hours following estrus onset or final GnRH of Double-Ovsynch). Samples were kept in a refrigerated container until transport to the laboratory. Samples were refrigerated at 4°C for 24 hours before being centrifuged at 2,000 × *g* for 20 minutes for serum separation. Aliquots of 1.5 mL of serum were stored in identified microtubes and froze at -18°C until analyses were performed.

Determination of PSPB concentrations

A commercial ELISA kit (bioPRYN®, bioTRACKING) was utilized to measure serum concentrations of PSPB. Most of the analyses were performed in-house. About 15% of samples were shipped to the bioTRACKING laboratory for analyses with the same methodology. A seven standards concentration curve was utilized and included the expected concentrations of 8, 4, 2, 1, 0.5, 0.25 and 0.125 ng/mL of PSPB. All samples from each cow were assayed in the same plate. Each plate contained one cow per treatment. The minimum detectable concentration of the assay was 0.2 ng/mL.

Estimated day of conceptus attachment

The estimated day of conceptus attachment, or the first day of significant PSPB increase, was performed following the methodology described in Middleton et al. (2022). A baseline value was calculated for each cow as the average of days 17 and 18

concentrations. If both days resulted in undetectable concentrations of PSPB the assay minimum detectable concentration of 0.2 ng/mL was assumed as their baseline value. The criteria to estimate day of conceptus attachment included: 1) an increase of ≥ 12.5% compared to the baseline value and 2) two additional days of ≥ 12.5% compared to the day before. For example, if a cow had at least a 12.5% increase from the baseline on day 20, we expected an increase of 12.5% or greater between days 21 and 20, and an increase of 12.5% or greater between days 22 and 21. This three-day period is referred to as the confirmatory period. Cows were also classified into categories according to the day of conceptus attachment: 19 and 20 (early conceptus attachment), 21 (intermediate time to conceptus attachment), and 22 or greater (delayed time to conceptus attachment).

Pregnancy diagnosis

Cows detected in estrus after AI were considered non-pregnant and re-inseminated according to farm guidelines and thus not checked for pregnancy. The remaining cows were transrectally examined with ultrasound by the herd veterinarian. Pregnancy was diagnosed upon the visualization of a fetal heartbeat. The tentative range for first diagnosis was between 31 and 37 days post-AI. Farm staff failed to separate cows for pregnancy diagnosis (n = 32). Out of those, n = 13 were examined with ultrasonography 3 days later, by the first author. The remaining cows were examined the following week by the herd veterinarian or the first author. This resulted, on average, 35 days to diagnosis, ranging between days 31 and 49 post-AI. This diagnosis will be referred to as “first pregnancy diagnosis” for convenience. All cows diagnosed pregnant at the first pregnancy diagnosis had also met the criteria for conceptus attachment based on PSPB concentrations.

Statistical analyses

All statistical analyses were performed using SAS (version 9.4, SAS Institute Inc., Cary, North Carolina, USA). Significance level was set at $P \leq 0.05$ for all analyses. A *P*-value > 0.05 and ≤ 0.10 was considered a tendency.

Binomial variables (service rate, PR/AI, pregnancy loss, and total pregnant cows after first service) were analyzed with a generalized linear mixed model in PROC GLIMMIX. Proportions were compared using the chi-square test of independence. The effects of treatment, parity, number of estrus events prior to AI, and estrus intensity (only ES treatment) were assessed for these fertility outcomes. Interactions with treatment were included when estimating the effects of parity and number of estrus events prior to AI on fertility outcomes. The LSMEANS statement was utilized to slice fixed effects. Data are reported as the unadjusted proportions obtained with PROC FREQ. The distribution of days to attachment between treatment, and pregnancy loss frequency occurring at different days to attachment categories within treatment were also estimated with PROC FREQ. The *P*-values were obtained with the chi-square test of independence.

Continuous variables (days to conceptus attachment and estrus intensity) are reported as mean ± SEM. Data were analyzed with PROC MIXED. Models estimated the fixed effects of treatment, parity, and pregnancy status. The LSMEANS statement was utilized to slice fixed effects. Multiple comparisons were adjusted with Tukey-Kramer test for multiplicity. PROC CORR was utilized to estimate Pearson correlation coefficients between two continuous variables.

RESULTS

Treatment and parity impacted reproductive performance

The effects of treatment on reproductive efficiency are summarized in Figure 6.2. All cows had confirmed ovulation after the final GnRH or a detected estrus.

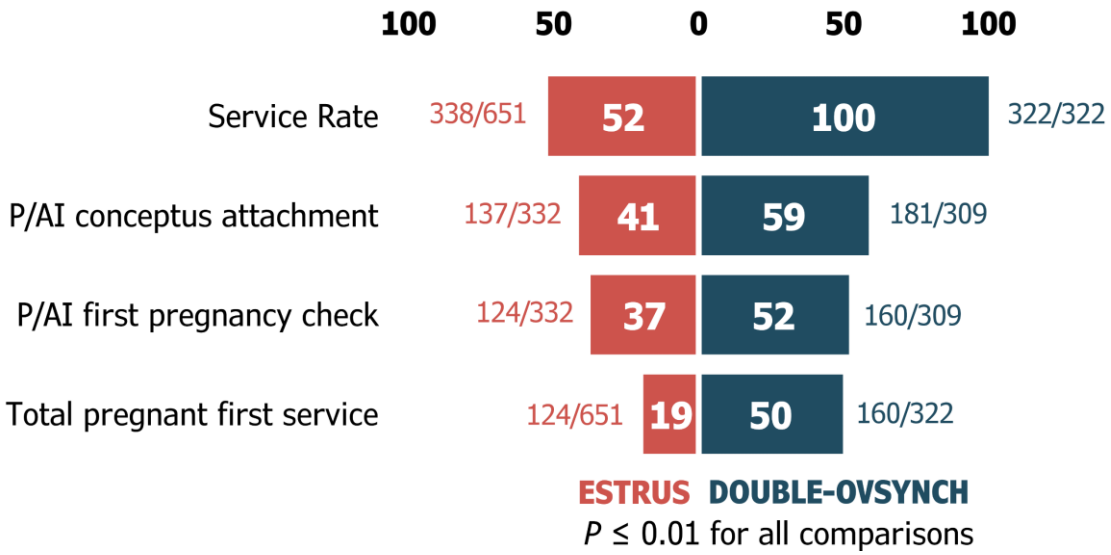

Figure 6.2. The effect of AI following automated activity monitor detected estrus (red) vs. controlling follicular and luteal development using Double-Ovsynch (blue) on proportion of lactating dairy cows (%) that: received first AI between 69 and 94 days post-partum (Service rate); were pregnant near conceptus attachment (pregnancies per AI – P/AI conceptus attachment); were pregnant at the first pregnancy diagnosis performed between day 31 and 49 post-AI (P/AI first pregnancy check); and proportion of cows pregnant following first service considering the entire enrolled population (Total pregnant first service). Outcomes of P/AI were only estimated in cows that had confirmed ovulation and with apparent complete luteolysis diagnosed with ultrasonography.

Cows in the ES treatment had decreased service rate, P/AI at conceptus attachment and P/AI at the first pregnancy diagnosis compared to cows in DO treatment ($P \leq 0.01$). This resulted in a lower proportion of pregnant cows following the first service in ES compared to DO cows ($P < 0.01$). Cows in the ES treatment were eligible for first service between 69 and 94 DIM and averaged 80.6 ± 7 days (mean ± standard deviation, **SD**) to first service. First services performed outside of this range were not considered in

the present study. Time from estrus onset to AI ranged between 3 and 37.5 hours and did not impact fertility outcomes of ES cows (P = 0.15). Parity negatively affected overall fertility of lactating dairy cows, regardless of treatment. Differences between primiparous and multiparous cows treated with ES and DO are described in Table 6.1.

Outcome (%, n/n)	Estrus		Double-Ovsynch		***P*-value** Parity	***P*-value** Trt*Parity
	Primi	Multi	Primi	Multi		
Service rate	68.7[a] 158/230	42.8[b] 180/421	100[c] 137/137	100[c] 185/185	<0.01	0.99
P/AI conceptus attachment	47.4[a] 74/156	35.8[b] 63/176	62.5[c] 85/136	55.5[ac] 96/173	0.04	0.55
P/AI first pregnancy diagnosis	44.9[a] 70/156	30.7[b] 54/176	59.6[c] 81/136	45.7[a] 79/173	<0.01	0.88
Total pregnant first service	30.4[a] 70/230	12.8[b] 54/421	59.6[c] 81/137	42.7[d] 79/185	<0.01	0.91

Table 6.1. The effect of parity (primiparous – Primi, and multiparous – Multi) within treatments (Trt; Estrus or Double-Ovsynch) on fertility parameters of lactating Holstein cows that received first artificial insemination (AI) following a detected estrus or the Double-Ovsynch program. “Service rate” denotes the proportion of cows that received AI between 69 and 94 days post-partum. “Pregnancies per AI (P/AI) conceptus attachment” refers to the proportion of cows that had a significant increase in pregnancy-specific protein B in circulation near the period of conceptus attachment. “P/AI first pregnancy check” refers to the proportion of cows diagnosed pregnant between days 31 and 49 post-AI. “Total pregnant first service” refers to the proportion of cows pregnant at the first pregnancy check considering the entire enrolled population. Different letter superscripts denote a $P \leq 0.04$ for multiple comparisons within fertility outcomes.

Fewer multiparous cows received first service in the ES treatment compared to primiparous ES cows and DO treatment (in which all cows were artificially inseminated). Multiparous cows that received AI following a detected estrus had lower P/AI at conceptus attachment, and the first pregnancy diagnosis compared to primiparous ES cows and DO cows. In contrast, multiparous cows treated with the fertility program Double-Ovsynch had

no differences in P/AI at conceptus attachment, and first pregnancy diagnosis compared to primiparous cows in the ES treatment. At the first pregnancy diagnosis, primiparous DO cows had greater P/AI than multiparous treatment counterparts, and ES cows. These outcomes translate into an overall greater proportion of both primiparous and multiparous cows pregnant at first service in DO compared to ES treatment, but with a greater proportion of primiparous cows pregnant within each treatment.

Time to conceptus attachment was prolonged in cows treated with Double-Ovsynch

Average days to conceptus attachment was 0.5 days longer in the DO treatment (Figure 6.3). Despite a lower proportion of conceptus attachment, a greater percentage of conceptuses in the ES treatment had attachment on days 19 and 20. Cows in the Double-Ovsynch treatment had a greater proportion of conceptus attachment and a distribution of days to attachment that was comparable to previously reported data (Santos et al., 2023). Parity impacted days to conceptus attachment. Overall, multiparous cows had prolonged time to conceptus attachment in comparison with primiparous cows (20.8 ± 0.10 vs. 20.3 ± 0.08; $P < 0.01$). There was no interaction between parity and treatment on days to conceptus attachment ($P = 0.35$).

Pregnancy loss was a multicausal outcome

Overall, treatment did not impact the proportion of pregnancy losses occurring between conceptus attachment and the first pregnancy diagnosis (9.5% ES vs. 11.6% DO; $P = 0.71$). Overall, multiparous cows had a greater proportion of early pregnancy loss (16.3%, 26/159) in comparison with primiparous cows (5.0%, 8/159; $P < 0.01$). Parity and treatment did not interact in analyses of pregnancy loss ($P = 0.76$).

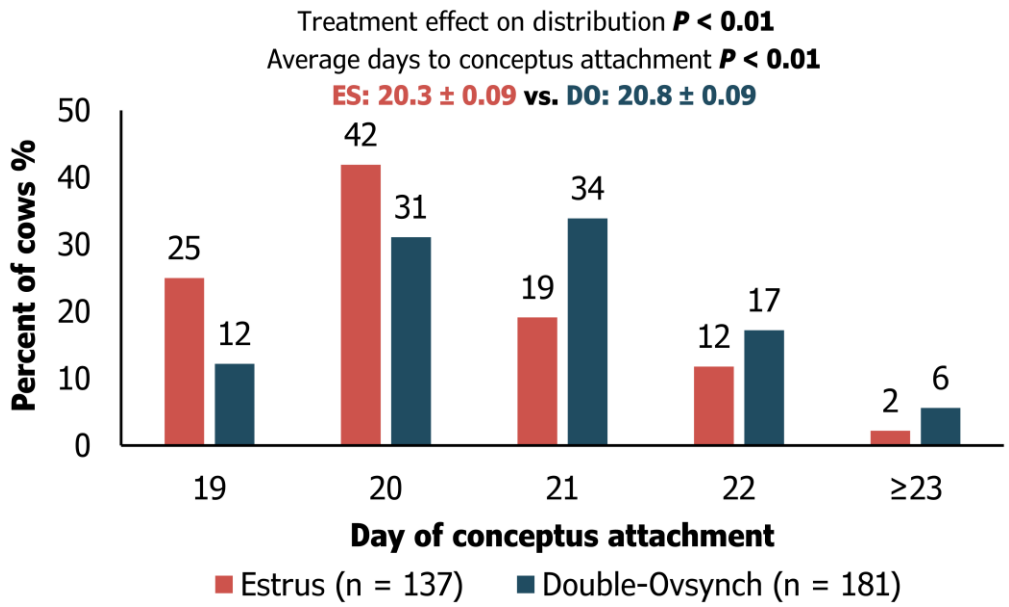

Figure 6.3. The effect of artificial insemination (AI) following estrus vs. Double-Ovsynch on the distribution of days to conceptus attachment and average days to conceptus attachment of lactating Holstein cows following first service. First AI was performed between 69 and 94 days post-partum in the Estrus treatment and between 77 and 83 days post-partum in the Double-Ovsynch treatment. All cows in the analyses had confirmed ovulation.

Day of conceptus attachment was associated with pregnancy loss ($P < 0.01$). Cows with conceptus attachment on day 22 or greater had greater pregnancy losses in comparison with cows that had attachment on days 19 and 20 (23.3 vs. 5.3%; $P < 0.01$). Cows with attachment on day 21 did not differ from cows with early or late conceptus attachment (12.6%; $P \geq 0.09$). Additionally, cows that maintained pregnancy following conceptus attachment had fewer days to conceptus attachment compared to counterparts that lost pregnancy (20.4 ± 0.06 vs. 21.5 ± 0.27; $P < 0.01$).

Treatment did not impact the frequency of early pregnancy losses within day of conceptus attachment

There was no association between treatments and days of conceptus attachment when estimating pregnancy losses (Figure 6.4).

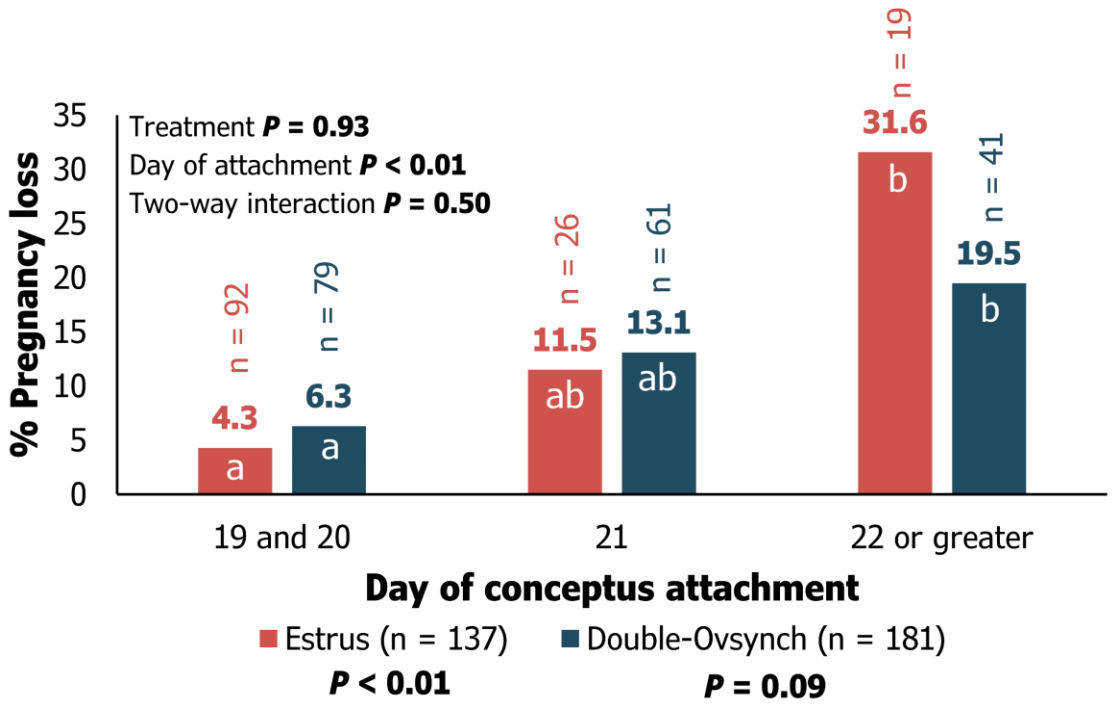

Figure 6.4. The effect of artificial insemination following estrus vs. Double-Ovsynch on the proportion of pregnancy losses occurring between conceptus attachment and the first pregnancy diagnosis within day of conceptus attachment classification (19 and 20, 21 and 22 or greater). The *P*-values under "Estrus" and "Double-Ovsynch" refer to the distribution of losses between day of conceptus attachment within treatment. Different letter superscripts inside the bars denote a $P \leq 0.03$ of comparisons performed between treatments and day of conceptus attachment classification.

The frequency of pregnancy loss following conceptus attachment was impacted by day of conceptus attachment in the ES treatment ($P < 0.01$). Cows that received AI following estrus detection and had late conceptus attachment (≥ day 22) had greater proportion of pregnancy loss than treatment counterparts with early conceptus attachment (day 19 and 20; $P < 0.01$). There was also a tendency for an association between the frequency of pregnancy losses occurring after conceptus attachment and days to conceptus attachment in DO treatment ($P = 0.09$). Similarly, the proportion of pregnancy loss was greater in DO cows with attachment on days 22 and greater in comparison to cows with attachment on days 19 and 20 ($P = 0.01$). Altogether, no

differences were observed in the frequency of pregnancy losses when comparing ES and DO within day of attachment classification ($P \geq 0.20$).

Number of estrus events prior to first service was associated with fertility and estrus intensity at AI

Average number of estruses detected prior to first AI was not different between treatments (ES 1.2 ± 0.06 vs. DO 1.3 ± 0.06; $P = 0.56$). Primiparous cows had greater average number of estruses detected before first AI compared to multiparous cows (1.6 ± 0.06 vs. 0.92 ± 0.05; $P < 0.01$) without a treatment interaction ($P = 0.15$). There was no effect of treatments in the distribution of cows that had zero, one or two or more estrus events prior to first service (Figure 6.5; $P = 0.39$).

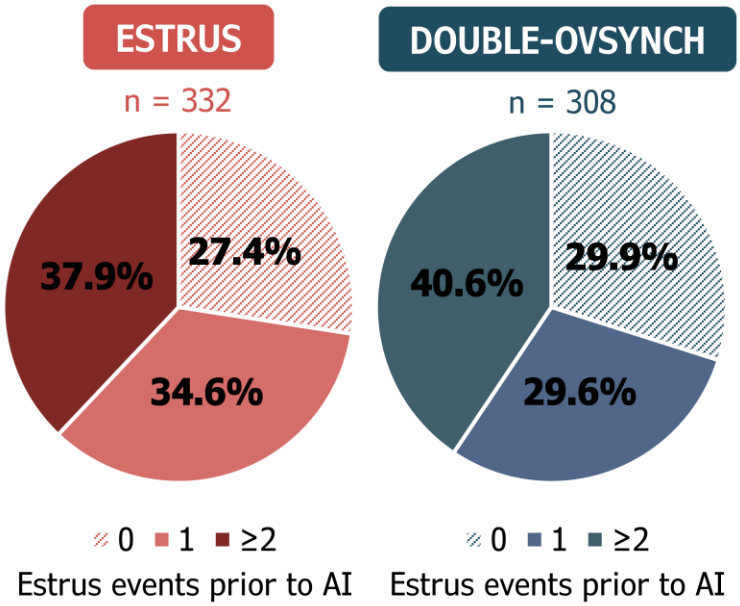

Figure 6.5. The effect of artificial insemination (AI) following estrus vs. Double-Ovsynch on the proportion of cows with zero, one or two or greater (0, 1, and ≥ 2) estrus events recorded prior to first AI. Estrus events were detected with automated activity monitors. An estrus event was defined as a cow with an increase in activity equal to or greater than 35% in comparison to a within-cow 7-day activity average. The distribution of cows assigned to each category (0, 1, and ≥ 2) was not affected by treatments $P = 0.39$.

A greater number of estrus events was positively associated with fertility of lactating dairy cows (Table 6.2). Cows in the ES treatment with zero estrus events recorded prior to first service had the lowest P/AI at conceptus attachment and at first pregnancy diagnosis compared. Occurrence of one or two or more estrus events did not improve fertility of ES cows in comparison to cows with zero estrus events recorded prior to first service. In contrast, DO cows that had two or more estrus events before AI had the greatest P/AI at conceptus attachment and first pregnancy diagnosis in comparison to treatment counterparts and ES treatment.

No. of estrus events prior AI %, n/n	**Estrus**			**Double-Ovsynch**			
	0	1	≥ 2	0	1	≥ 2	*P*-value[1]
P/AI conceptus attachment	36.3[a] 33/91	46.1[ab] 53/115	40.5[ab] 51/126	50.0[ab] 46/92	53.9[b] 49/91	68.0[c] 85/125	0.07
P-value[2]			0.087				
Days to conceptus attachment Mean ± SEM	20.2[ab] ± 0.2	20.4[abc] ± 0.1	20.1[a] ± 0.1	20.9[bc] ± 0.2	21.0[c] ± 0.2	20.5[abc] ± 0.1	0.03
P-value			0.85				
Pregnancy loss	15.1[a] 5/33	14.8[a] 8/54	7.4[a] 4/54	12.5[a] 6/48	16.3[a] 8/49	10.6[a] 9/85	0.29
P-value			0.79				
P/AI first preg. check	30.8[a] 28/91	40.0[ab] 46/115	39.7[ab] 50/126	45.7[b] 42/92	45.1[b] 41/91	60.8[c] 76/125	0.04
P-value			0.23				

[1] Fixed effect of number of estrus events prior to first service.
[2] Two-way interaction between treatment and number of estrus events occurring prior to first service.

Table 6.2. The impact of number (No.) of estrus events detected with automated activity monitors (AAM) prior to the first artificial insemination (AI) on fertility of lactating Holstein cows treated with AI following estrus vs. Double-Ovsynch. Cows were assigned to categories based on the number of estrus events detected prior to first AI: 0 (no estrus prior to AI), 1 (one estrus event before AI) and ≥ 2 (at least 2 estrus events before AI).

Table 6.2 (cont'd)
"Pregnancies per AI (P/AI) conceptus attachment" refers to the proportion of cows that had a significant increase in pregnancy-specific protein B (PSPB) in circulation near the period of conceptus attachment. "Days to conceptus attachment" refers to the average days to have a significant increase of PSPB in maternal circulation. "Pregnancy loss" refers to the proportion of cows that had a significant increase in PSPB, or conceptus attachment, but lost pregnancy by the first pregnancy diagnosis between 31 and 49 days post-AI. "P/AI first pregnancy check" refers to the proportion of cows diagnosed pregnant between days 31 and 49 post-AI. Different letter superscripts denote a $P \leq 0.048$ for multiple comparisons within fertility outcome.

Occurrence of estrus prior to first service was not associated with pregnancy losses after conceptus attachment, regardless of treatment. Additionally, at first pregnancy diagnosis, the fertility treatment Double-Ovsynch improved P/AI in cows with zero, and one estrus events prior to AI in comparison to ES cows that were not detected in estrus prior to first service. ES cows that were not detected in estrus before AI with AAM had less intense estruses at AI in comparison with cows that had 2 or more estrus events (86.1 ± 1.9 vs. 93.8 ± 0.9; $P < 0.01$). Estrus intensity at AI in ES cows that had a single estrus detected before AI (90.3 ± 1.4) did not differ from cows with no estrus or cows with 2 or greater estrus events ($P \geq 0.13$).

Relationship between estrus intensity and fertility outcomes

Only a small proportion of cows (n = 88) were detected in estrus around TAI in the DO treatment. Thus, associations between estrus intensity were only assessed within the ES treatment. Average estrus intensity did not differ between cows with conceptus attachment in comparison with cows that had no conceptus attachment (92.2 ± 0.99 vs. 89.3 ± 1.16; $P = 0.11$). However, cows that experienced pregnancy loss between conceptus attachment and the first pregnancy diagnosis had lower estrus intensity at AI compared to cows that maintained pregnancy (92.7 ± 1.02 vs. 87.5 ± 3.03; $P = 0.049$). This translated in an association between pregnancy diagnosed at the first pregnancy

diagnosis and estrus intensity at AI (92.7 ± 1.02 in pregnant vs. 89.1 ± 1.11 in non-pregnant cows; *P* = 0.03). No differences in P/AI at conceptus attachment (42.3 vs. 37.8%; *P* = 0.46) or the first pregnancy diagnosis (39.4 vs. 31.1%; *P* = 0.17) were observed between cows with high (intensity > 88; n = 241) or low estrus intensity (intensity ≤ 88; n = 90). Days to conceptus attachment did not differ between cows with high or low estrus intensity (20.2 ± 0.10 vs. 20.4 ± 0.18; *P* = 0.40). Estrus intensity and days to conceptus attachment were not correlated (r = -0.07, *P* = 0.42).

DISCUSSION

Our methodology ensured that cows detected in estrus had ovulation following the estrus event detected with AAM. Fertility was only assessed in ES cows that had a true estrus followed with an ovulation. Time to AI from estrus onset did not impact P/AI in ES cows. Thus, lack of proper estrus detection and incorrect timing of AI relative to estrus onset were not the cause of low fertility of cows receiving AI following an estrus.

Cows in the DO treatment that were detected in estrus before receiving the final GnRH were removed from the study. Fertility was only assessed in DO cows with an exogenously induced LH surge and confirmed ovulation following GnRH administration. We believe that this experimental design allowed for the characterization of the true fertility of AI following a detected estrus assessed as early as day 19 post-AI compared to the industry standard fertility program Double-Ovsynch.

In agreement with our hypothesis, cows that received AI following a detected estrus had decreased reproductive efficiency and fertility compared to the Double-Ovsynch program. Cows in the ES treatment had decreased chances of receiving their first post-partum AI between 69 and 94 DIM. This was an expected result, considering

that 100% of the cows enrolled in a TAI program receive AI (Pursley et al., 1997a). Nonetheless, the average service rate in the ES treatment was ~20% points lower compared to published literature (Valenza et al., 2012; Sitko et al., 2023). This was due to a negative effect of parity. The service rate in multiparous cows was 42.8%, while primiparous received first post-partum service at an expected rate when utilizing AAM (68.7%). A deleterious effect of parity on service rates was previously reported (Rial et al., 2022). When estrus detection is utilized as the preferred strategy for the first post-partum service, adapted management (likely hormonal intervention) for multiparous cows may be necessary. Different than our hypothesis, cows in the ES treatment had a diminished proportion of cows with conceptus attachment in comparison to the DO treatment (41% vs. 59%). Data from the preliminary study described in Chapter 5 suggested that fertility following a detected estrus was not impaired in comparison to Double-Ovsynch when assessed near the period of conceptus attachment (56% vs. 56%). The proportion of pregnant cows remained lower in ES treatment at the first pregnancy diagnosis in comparison to DO. This finding corroborates previously published evidence (Santos et al., 2017; Sitko et al., 2023). Contrary to our hypothesis, the decreased fertility observed on the first pregnancy diagnosis was not due to greater early pregnancy losses in ES treatment. In the present study, pregnancy losses occurring between conceptus attachment and the first pregnancy diagnosis were not different between treatments. Evidence reported herein indicates that cows that receive AI following a detected estrus fail to establish or maintain pregnancy at developmental stages that precede conceptus attachment.

In agreement with the latter statement and contrary to our hypothesis, conceptuses in the ES treatment established functional conceptus attachment (transfer of PSPB into maternal circulation) half a day earlier compared to DO cows. A total of 67% of ES cows had conceptus attachment on days 19 and 20 post-AI. Earlier PSPB increase in maternal circulation has been linked to a greater probability of pregnancy survival (Santos et al., 2023). Moreover, it was a phenotype reported in nulliparous heifers (Middleton et al., 2022), which had greater fertility than parous cows (Pursley et al., 1997b). Thus, conceptus viability and competence did not seem impaired in cows that received AI following estrus detection. The fertility caveat was that fewer conceptuses developed to the stage of conceptus attachment and were able to initiate the transfer of PSPB into the maternal circulation after day 19 of gestation. The percentage of viable embryos recovered from AI of thermoneutral cows after a detected estrus was 47.1% (days 5, 6 or 7 of development; Wiltbank et al., 2016). A greater proportion of embryos recovered from lactating dairy cows detected in estrus were classified as non-viable and had a reduced proportion of live blastomeres in comparison to embryos recovered following synchronization programs (Cerri et al., 2009). This implies that the first week of life may be the most critical stage to pregnancy success in cows that received AI following a detected estrus. This decreased embryonic viability could be associated with poor oocyte quality (Krisher, 2004).

Cows detected in estrus had greater follicle diameter compared to the Double-Ovsynch program, which indicates prolonged development (Chapter 5). Extending antral age of the pre-ovulatory follicle impaired follicular function (Lucy et al., 1992; Mihm et al., 1999; Minela et al., 2023). Greater days between the emergence of the follicular wave to

estrus were associated with decreased fertility (Bleach et al., 2004) and poor oocyte and embryo quality in cows artificially inseminated in estrus (Ahmad et al., 1995; Cerri et al., 2009). Prolonging the period between follicular wave emergence and ovulation resulted in premature oocyte maturation (Revah and Butler, 1996; Mihm et al., 1999). Estrogen and its receptor are involved in mechanisms responsible for the maintenance of oocyte meiotic arrest (Liu et al., 2017). Estrogen also regulated transcriptome in endometrial cells that were compatible with early conceptus development (Shimizu et al., 2010). Thus, it appears that depletion of steroid hormones in high-producing lactating dairy cows could be involved in prolonging the antral age of pre-ovulatory follicles, which consequently resulted in oocytes of poor quality and low fertility in cows in the ES treatment (Fricke and Wiltbank, 2022).

Double-Ovsynch had an expected distribution of days to conceptus attachment, with most of the cows having a significant PSPB increase on days 20 and 21 post-AI. This observation agrees with the studies described in Chapters 5 and 7 and with published evidence (Santos et al., 2023). An average of 20.8 days to conceptus attachment in DO cows was still compatible with greater fertility in comparison to ES cows that had conceptus attachment earlier (52% vs. 37% P/AI at the first pregnancy diagnosis). Despite the differences in time to conceptus attachment, early pregnancy loss was not different between treatments. Nonetheless, cows that lost pregnancy had delayed conceptus attachment. The proportion of pregnancy losses increased as days to attachment increased in both treatments. The delayed phenotype in PSPB increase stems from unknown mechanisms. Decreased concentrations and prolonged time to appearance of PSPB in the maternal circulation could be expected in underdeveloped

conceptuses that have diminished chorionic surface to establish conceptus attachment. An underdeveloped conceptus may also be unable to initiate physicochemical interactions with the endometrium (Ribeiro et al., 2016). Nonetheless, the almost obligatory early appearance of PSPB in the maternal circulation for a successful pregnancy could indicate a role for PAGs during early placentation. Treatment with PAGs induced transcriptomic changes that were concurrent with tissue remodeling in pregnant endometrium (Wallace et al., 2019). Day 19 conceptus that developed in cows that expressed estrus near TAI had increased expression of genes associated with adhesion and tissue remodeling (Davoodi et al., 2016). In view of the reported changes in cytoskeletal composition of the commencing conceptus-maternal interface (Yamakoshi et al., 2012) and extensive tissue remodeling during early conceptus attachment and placentation (Seo et al., 2023) PAGs become a likely mediator of this process. A model of functional ablation of PAG-7 induced delayed attachment and resulted in embryos with diminished trophectoderm area in vitro (poster IETS 2024; Moreno et al., 2023). This was a pioneer experiment that demonstrated the direct contribution of PAGs in cell-to-cell interaction processes. This function was proposed in a review almost 20 years ago (Wooding et al., 2005), and now it has been demonstrated in vitro.

Failure or delay to resume cyclicity is one of the main hindering factors for post-partum reproductive success of dairy cows (Santos et al., 2009; Borchardt et al., 2021). Estrus detection before the voluntary waiting period was proposed as an efficient tool to select subgroups of cows that may benefit from fertility treatments (Rial et al., 2022; Gonzalez et al., 2023). One limitation of this strategy is that AAM systems were more accurate in detecting anovular cows than cycling cows during the first 30 DIM (Borchardt

et al., 2023). Specificity was considered high but limited to 84%. Cows with at least one estrus event before 49 DIM were more likely to be detected in estrus again and receive AI (Rial et al., 2022). Absence of estrus events detected with AAM during the first 40 DIM was a strong predictor of prolonged days to first service and decreased fertility compared to cows that had at least 1 estrus event during that period (Borchardt et al., 2021). The present study utilized AAM to detect number of estrus events occurring before first service in cows. Multiparous cows had lower number of estrus events detected prior to AI, regardless of treatment. This outcome disagrees with another study, where more multiparous cows were deemed to have resumed cyclicity before 65 DIM via serial P_4 measurements (Santos et al., 2009). This could be partially due to multiparous cows having silent ovulations and without external estrus behavior (Crowe et al., 2014). Hormonal intervention in the DO treatment did not impact the number of estrus events detected before first AI. Treatment also did not impact the proportion of cows assigned to number of estrus categories (0, 1 and ≥ 2). For both treatments, a greater proportion of multiparous cows were assigned to the 0 category, whereas a greater proportion of primiparous cows were assigned to the ≥ 2 category (data not shown). In the present study, there were no differences in P/AI at conceptus attachment or the first pregnancy diagnosis between number of estrus events categories within the ES treatment. This could be due to lack of power when stratifying the data into categories. Nonetheless, the observed proportions of P/AI at the first pregnancy diagnosis for cows with 0 (30.8%) and ≥ 2 (39.7%) estrus events were numerically relatable to outcomes of a properly powered study (29.4% vs. 37.8%, respectively; Borchardt et al., 2021). The cows classified herein in the ≥ 2 estrus events categories were equivalent to cows deemed not to necessitate

hormonal intervention in studies that attempted to utilize targeted reproductive management (Rial et al., 2022; Gonzalez et al., 2023). Yet, our data would suggest that hormonal intervention was beneficial for this subgroup of cows and further enhanced their intrinsic fertility. Cows with ≥ 2 estrus events prior to the Double-Ovsynch TAI had enhanced fertility at conceptus attachment and the first pregnancy diagnosis compared to all other estrus categories within treatment. Hormonal intervention also improved the fertility of cows with zero or a single estrus event before TAI compared to ES cows that had no estrus events before AI. These data suggested that cyclicity prior to AI directly impacts fertility of lactating dairy cows receiving first post-partum service following a detected estrus or the Double-Ovsynch program. Hormonal intervention mitigated the deleterious effects of lack of cyclicity and further enhanced fertility of cows with proper reproductive health compared to estrus detection alone. This improvement is likely due to the timely control of induced luteolysis and reduced age of pre-ovulatory follicles that developed under high P_4 (Minela et al., 2021).

Prolonged antral age resulted in less intense estruses (Minela et al., 2023). More intense estruses (as measured as activity change during an estrus event) were associated with greater fertility in lactating dairy cows diagnosed for pregnancy after the first month of gestation (Tippenhauer et al., 2023). Estrus expression near TAI resulted in longer conceptuses recovered on day 19 of gestation (Davoodi et al., 2016). A more extensive chorionic surface would likely favor conceptus attachment. Cows in the ES treatment with conceptus attachment had no difference in average estrus intensity compared to cows without conceptus attachment. The occurrence of pregnancy loss was associated with less intense estruses. This culminated in average lower estrus intensity

in cows that failed to establish or to maintain pregnancy by the first month of gestation, corroborating previously reported evidence (Madureira et al., 2019; Tippenhauer et al., 2023). However, P/AI at the first pregnancy diagnosis did not differ between cows classified as having high or low estrus intensity. Only n = 90 cows were classified as having low intensity estruses (≤ 88 activity change).

In conclusion, lactating dairy cows that received AI following a detected estrus had decreased fertility compared to the Double-Ovsynch program. These cows were less likely to have conceptus attachment but were not at a greater risk of pregnancy loss compared to DO cows. Most of the ES cows that were successful in establishing conceptus attachment did so on days 19 and 20, which culminated on average earlier conceptus attachment than DO treatment. In agreement with our previous observations, delayed conceptus attachment was associated with pregnancy losses. Multiparous cows had delayed conceptus attachment compared to primiparous cows. Hormonal intervention with the Double-Ovsynch program was able to mitigate the negative effects of lack of cyclicity on the fertility of lactating dairy cows. Treatment DO was able to enhance the intrinsic fertility of lactating dairy cows that had at least 2 estrus events prior to their first postpartum service. Identifying groups of cows that are less likely to express estrus and receive AI will likely become a mandatory practice in herds that utilize estrus detection as a first service strategy.

CHAPTER 7

EFFECT OF GnRH ADMINISTRATION BEFORE AND AFTER CONCEPTUS ATTACHMENT ON CONCEPTUS ATTACHMENT AND PREGNANCY SURVIVAL OF LACTATING DAIRY COWS

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INTRODUCTION

Non-return to estrus is the earliest visible change in cow reproductive physiology associated with pregnancy. Maternal recognition of pregnancy depends on the timely secretion of interferon-tau, a signaling molecule produced by the developing conceptus (Meyer et al., 1995). This mandatory chemical communication precedes any cell-to-cell interaction (Sakurai et al., 2012). It culminates in the CL rescue and maintenance of P_4 concentrations (Helmer et al., 1989; Antoniazzi et al., 2013), essential to support continued conceptus development (Niswender, 2004). The developmental capacity of conceptuses may vary depending on P_4 concentrations (Forde et al., 2009; Ribeiro et al., 2016). Ninety out of 160 dairy cows receiving AI were classified as pregnant on day 15. Of those pregnancies, about half of the recovered conceptuses were tubular, a quarter were ovoid, and the remaining quarter were classified as filamentous. There was a positive association between conceptus length, conceptus area, and interferon-tau concentrations (Rizos et al., 2012; Ribeiro et al., 2016). A minimum interferon-tau concentration threshold or minimum area of trophectoderm necessary to rescue the CL and continue pregnancy has not been determined (Hansen et al., 2017). However, interferon-tau's ability to induce changes in the uterine transcriptome was dose and time-dependent (Talukder et al., 2023).

The luteolytic mechanism involves the positive feedback of E_2 on the expression of oxytocin receptors and, consequently, the action of oxytocin (Nancarrow et al., 1973; Mccracken et al., 1999; Robinson et al., 2001). These combined factors will culminate in the release of $PGF_{2\alpha}$ (Armstrong and Hansel, 1959; McCracken, 1980; Walters and Schallenberger, 1984). Continuous infusion with interferon-tau prolonged CL lifespan in

cyclic ewes treated with $PGF_{2\alpha}$ (Bott et al., 2010; Antoniazzi et al., 2013). Interferon-tau inhibits the expression of the oxytocin receptor in pregnant cows as early as day 16 of gestation. The regulation of E_2 type 1 receptor (**ESR1**) via interferon-tau did not precede oxytocin receptor inhibition (Robinson et al., 1999). Overall, the expression of ESR1 was greater in deep gland epithelium, with no differences in expression between pregnancy status at day 15 of gestation (Robinson et al., 2001). Progesterone and E_2 up-regulate the P_4 receptor (**PGR**) expression, whereas P_4 downregulates E_2 receptor expression (Meyer et al., 1988; Wathes et al., 1996). Indeed, by day 18 of gestation, ESR1 was not detected in pregnant cows, and PGR was continuously increased in expression from day 14 to 20 of gestation (Robinson et al., 1999, 2001). In cyclic endometrium explants, the oxytocin receptor was upregulated in the absence of E_2 (Leung and Wathes, 2000). Yet, prostaglandin-E_2 and -$F_{2\alpha}$ secretion increased upon E_2 treatment in vitro (Li et al., 2020). Treatment with 1 ng of exogenous E_2 at day 15 of the estrous cycle induced earlier luteolysis in non-pregnant Nelore heifers (Rio Feltrin et al., 2024).

The presence of a dominant follicle that secretes E_2 around the time that the conceptus is initiating an anti-luteolytic process may be disadvantageous for pregnancy. Yet, no characterization of the role of E_2 (positive or negative) around the time of maternal recognition of pregnancy and conceptus attachment was defined in cows. Induction of ovulation prior to the expected time of the start of luteolysis, e.g., day ~18 of the cycle, would pose an alternative to remove the E_2 source and avoid any possible contribution of this hormone to the luteolytic cascade. Additionally, an aCL (accessory CL) would develop post-ovulation and sustain high circulating concentrations of P_4. Concomitant treatment with P_4 and oxytocin decreased the release of $PGF_{2\alpha}$ in endometrium explants

compared with oxytocin treatment alone (Bogacki et al., 2002). The oxytocin receptor expression increased proportionally to the decrease in P_4 concentrations and peaked at day 21 of the estrous cycle (Meyer et al., 1988). Thus, sustained concentrations of P_4 could directly disrupt the luteolytic cascade (Lopes et al., 2013).

Administration of GnRH ahead of pregnancy diagnosis has been proposed as a pregnancy-safe strategy to decrease days between services (Lopes et al., 2013). Ovulation to the first GnRH of Ovsynch administered on day 25 post-AI improved the chances of synchronization of a pre-ovulatory follicle in non-pregnant cows on day 32 post-AI (Dewey et al., 2010). An ovulation rate of 66% was reported in non-pregnant cows following GnRH administered on day 18 post-AI. The following GnRH administration on day 25 post-AI resulted in ovulation in 81% of treated cows (Minela et al., 2021). Cows that ovulated to the first GnRH of Ovsynch have greater fertility following TAI (Bello et al., 2006; Chebel et al., 2006). However, a recent study reported a low overall response to GnRH administered as a resynchronization strategy starting on day 25 post-AI (average 28.7%), dependent on the pregnancy status (Leão et al., 2023). Pregnant cows had lower ovulation response in comparison to their non-pregnant counterparts. The authors associated this finding with the hampering effects of high P_4 on the induced LH surge (Giordano et al., 2012; Motta et al., 2020). In addition to this mechanism, in vitro studies demonstrated that PAGs functioned as signal molecules that could interact with gonadal and endometrial gonadotropin receptors in pigs (Szafranska et al., 2007). This has not been demonstrated in cows but could help to explain why ovulation rates to GnRH in pregnant cows are attenuated compared to non-pregnant cows.

A greater increase in P_4 during early diestrus and higher P_4 plateau were associated with viable conceptuses that were interferon-tau positive at day 16 of gestation (Mann and Lamming, 2001). The effects of early P_4 supplementation on histotroph and embryo development were investigated and well-characterized (Simintiras et al., 2019b). Despite the benefits of early P_4 on advancing conceptus elongation (Clemente et al., 2009; O'Hara et al., 2014), exposure to above physiological P_4 concentrations resulted in shorter cycles when supplemented between days 1 to 4 (Garrett et al., 1988) but prolonged cycle length when administered 4 times/day starting on days 8, 12 and, 16 of the cycle (Ginther, 1970). Supplementation with P_4 also resulted in earlier down-regulation of endometrial PGR expression (Milgrom et al., 1973; Garret et al., 1988; Wathes et al., 1996; Okumu et al., 2010). To our knowledge, there is no published evidence of the effects of increasing P_4 via induced ovulations near the time of conceptus attachment on cattle fertility.

This study aimed to induce aCL via GnRH administration on days 18 and 25 or day 25 post-AI in lactating dairy cows that received TAI. We hypothesized that the induction of aCL would enhance pregnancy survival in cows with greater luteal volume during the period of maternal recognition of pregnancy and conceptus attachment and improve synchronization rates for cows diagnosed as not pregnant at day 34 post-AI.

MATERIALS AND METHODS

Experimental units

This study was conducted on a commercial dairy herd from April through July 2021 (Nobis Dairy Farm, St. Johns, Michigan, USA). Power analyses revealed that at least n = 124 lactating dairy cows per treatment were necessary to detect a difference of one day

in time to PSPB increase between treatments ($\alpha = 0.05$, $\beta = 0.20$, $\sigma = 1.97$). There were n = 425 cows available for enrollment in the trial. Following treatment assignment, n = 66 cows were removed from the study. Reasons included diagnosed health problems, culled, no ovulation following final GnRH, spontaneous ovulation prior to final GnRH of Ovsynch, or no pregnancy diagnosis on day 34 post-AI. Outcomes from treatments were based on n = 358 lactating Holstein cows. All cows were fed a TMR once a day with free access to feed and water and were confined in a free-stall barn. The TMR consisted of corn, wheat, alfalfa silages, and corn-soybean meal-based concentrates formulated to meet or exceed nutrient recommendations for high-producing lactating dairy cows (NRC, 2001). The Institutional Animal Care and Use Committee at Michigan State University approved all animal handling and procedures.

Experimental design

Lactating Holstein cows were blocked by parity (1st to 9th) and service number (1st to 7th) and randomly assigned to three treatment groups: GnRH (2 mL, or 100 µg of gonadorelin acetate; Cystorelin, Boehringer Ingelheim) on days 18 and 25 (**G18+25**; n = 136), saline (2 mL of sodium chloride solution 0.9%, VeltiVex) on day 18 and GnRH on day 25 post-AI (**G25**; n = 114), or saline on days 18 and 25 post-AI (**Controls**; n = 108) following either 1st service with Double-Ovsynch or a re-synchronization strategy for 2nd through 7th AI (Figure 7.1). Resynch could be an Ovsynch starting on day 32 post-AI or one of the three treatments in cows diagnosed non-pregnant after treatment (G18+25, n = 26; G25, n = 15; Control, n = 15). All cows were treated with GnRH on day 32 post-AI for the initial GnRH of Ovsynch. The final $PGF_{2\alpha}$ of Double-Ovsynch and Ovsynch consisted of a double dose of cloprostenol sodium (4 mL or 1.0 mg of Synchsure,

Boehringer Ingelheim). A group of non-pregnant cows, without ovulation to any of the GnRH treatments (n = 46), was selected as a “negative control.” Negative control cows were included in analyses of PSPB concentrations and luteal volume.

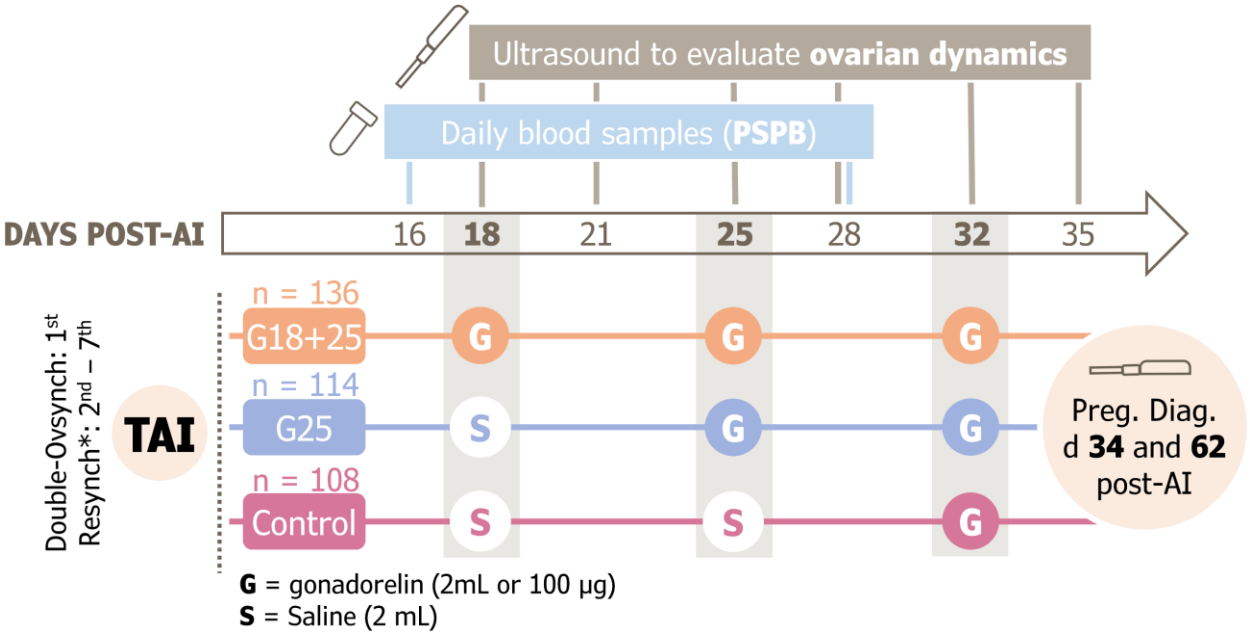

Figure 7.1. Experimental design to determine the effects of gonadotropin-releasing hormone (GnRH or G) post-timed artificial insemination (TAI) treatments on conceptus attachment and pregnancy survival. Lactating Holstein cows were synchronized with the fertility program Double-Ovsynch for 1st service and Ovsynch for 2nd to 7th service. After AI, cows were randomly assigned to receive GnRH on days 18, 25, and 32 (G18+25, n = 136), GnRH on days 25 and 32 (G25, n = 114), or a single GnRH on day 32 post-AI (Controls, n = 108). Saline (S) was administered on day 18 to G25 cows and on days 18 and 25 post-AI to Control cows. Daily blood samples were collected between days 16 to 28 post-AI to measure concentrations of pregnancy-specific protein B (PSPB). Ultrasound was utilized to monitor ovarian dynamics on days 18, 21, 25, 28, 32, and 35 post-AI. Ultrasound was also utilized on days 34 and 62 post-AI to diagnose pregnancy. * Resynch could be an Ovsynch starting on day 32 post-AI or one of the three treatments in cows diagnosed non-pregnant after treatment (G18+25, n = 26; G25, n = 15; Control, n = 15).

Ultrasonography evaluation of ovulation, follicle size, and luteal volume

All cows were evaluated using ultrasonography (7.5 MHz linear array probe, MyLabVet Gamma, Esaote) to assess ovarian function. The diameter of follicles > 8 mm and all functional CL were recorded. Exams were performed on the day of the final GnRH

of Double-Ovsynch or Ovsynch to measure pre-ovulatory follicle diameter and 2 days post-GnRH to confirm ovulation. Ovarian evaluations were also performed on the day of each post-AI GnRH (d 18, 25, 32) and 3 days later to confirm ovulation in treated cows (days 21, 28, and 35; Figure 7.1). Spontaneous or induced ovulation was recorded upon the disappearance of ovulatory-size follicles and the appearance of new CL in the subsequent examination. Additionally, cows without a follicle ≥ 8 mm on the day of GnRH treatment were considered ineligible to ovulate. Thus, not included in the estimations of ovulation response.

Luteal volume was calculated for the original CL and aCL and reported as mm^3. The formula for the estimation of volume consisted of $V = (4/3)\ \pi\ R^3$. Radius (R) was calculated based on the average diameter between the horizontal (D^a) and perpendicular (D^b) diameter measurements of the CL parenchyma ($r = ([D^a + D^b]/2)/2$). The volume of luteal cavities was calculated with the same formula and subtracted from the parenchymal luteal volume. “Total luteal volume” refers to the sum of all functional CL volumes present at the time of examination.

Formation of aCL was classified according to the response to GnRH treatments (induced on day 18, days 18+25, day 25). The presence of aCL was also classified according to the timing of induction of ovulation. Cows with “pre-conceptus attachment aCL” were cows with ovulation to the GnRH administered on day 18 post-AI. Cows with “post-conceptus attachment aCL” were cows that ovulated to either day 25 or 32 post-AI GnRH. Cows with “no aCL” did not ovulate to any of the administered GnRH on days 18, 25 and 32 post-AI.

Collection of blood samples for PSPB measurements

Blood samples were collected from the coccygeal vein or artery utilizing vacuum tubes coated with clot activator and separator gel. Samples were collected daily between days 16 and 28 post-AI. Samples were placed in ice upon collection and refrigerated for 24 hours at 4° C to allow for clotting. Samples were centrifuged for 20 min at 2,016 x *g* at 4° C. Serum aliquots were harvested and transferred to 1.7 mL tubes and stored at -20°C. Frozen samples were shipped to the bioTRACKING laboratory for PSPB quantification.

A commercial ELISA kit (bioPRYN, bioTRACKING), developed by Sasser *et al.*, 1986, was utilized to measure serum concentrations of PSPB in samples collected daily between days 16 and 28 post-AI. The lowest threshold of the assay was 0.2 ng/mL. The intra- and inter-assay CV were 4.9% and 8.3%, respectively.

A baseline concentration value was calculated for each cow as the average of samples collected on days 16, 17 and 18 post-ovulation for each cow. The lowest detectable amount (0.2 ng/mL) was utilized as a baseline value when day 16 to 18 samples were below this sensitivity cutoff. The day of a significant increase in PSPB concentrations was defined as the first day in which PSPB increased ≥ 12.5% from the baseline in addition to two days of ≥ 12.5% increase from the previous day to confirm that PSPB was continuously increasing within the cow. The day of the initial PSPB increase is henceforth referred to as conceptus attachment or day of conceptus attachment.

The period from the day of conceptus attachment, in addition to the following 2 days in which PSPB continuously increased, will be referred to as the conceptus attachment “confirmatory period.” The cumulative concentrations of PSPB during this

period (sum of concentrations from first, second, and third days of the confirmatory period) were utilized as a marker of pregnancy viability.

Pregnancy diagnoses

Outcomes of PSPB were utilized to determine conceptus attachment occurring between 19 and 25 days post-AI. The herd's veterinarian diagnosed pregnancy with ultrasonography at days 34 and 62 post-AI. A positive pregnancy diagnosis was concurrent with the presence of anechoic fluid in the horn ipsilateral to the ovulation and detection of a heartbeat. Pregnancy losses were estimated between conceptus attachment and the first pregnancy diagnosis at day 34 post-AI ("early loss") and between days 34 and 62 post-AI ("late loss").

Statistical analyses

Statistical analyses were performed using SAS (version 9.4, SAS Institute Inc.). The significance level was set at $P \leq 0.05$ for all analyses. A P-value > 0.05 and ≤ 0.10 was considered a tendency. The P-values of multiple comparisons were adjusted with the Tukey-Kramer test for multiplicity. Figures and tables were generated in Excel (Microsoft Office) based on SAS outputs.

Binomial variables (ovulation to GnRH treatments, conceptus attachment, P/AI, and pregnancy loss) were analyzed in PROC GLIMMIX with a generalized linear mixed model. Differences in proportions were estimated with the chi-square test of independence. Treatment, parity (primiparous and multiparous), presence of aCL (pre-conceptus attachment, post-conceptus attachment, no aCL), pregnancy status (conceptus attachment or not), response to previous GnRH treatments (ovulation or not) were investigated as independent variables. Random effects included the blocking

variables of lactation and TAI program. The LSMEANS statement was utilized for slicing fixed effects. Data are reported as the unadjusted proportions obtained with PROC FREQ. The effect of treatment on the distribution of days to conceptus attachment was also estimated with PROC FREQ, and the *P*-values were obtained with the chi-square test of independence.

Continuous variables (days to conceptus attachment and cumulative PSPB concentrations during the confirmatory period) are reported as mean ± SEM. Models estimated the fixed effects of treatment, parity, and presence of aCL in PROC MIXED. The LSMEANS statement was utilized for slicing fixed effects.

Serum PSPB concentrations (ng/mL) and total luteal volume (mm^3) were analyzed using PROC MIXED with the REPEATED statement to account for measurements over time. The models included treatment, time (days post-AI), treatment by time interaction, and parity as fixed effects. The RANDOM statement included cow nested within treatment, lactation and TAI program.

Analyses of PSPB concentrations were performed with an AR1 covariance structure. Analyses of total luteal volume were fitted with a spatial power [sp(power)(time)] covariance structure due to unevenly spaced measurements. Interactions were sliced with PROC PLM using an output from PROC MIXED. Multiple comparisons within the interaction of treatment by time were performed between treatment groups and sliced by time.

% (n/n)	**G18+25 (n = 136)**	**G25 (n = 114)**	**Control (n=108)**	*P*-value TRT
		OVERALL		
Ovulation to G18	52.2 (71/136)	-	-	-
Ovulation to G25	58.0 (76/131)	28.4 (31/109)	-	< 0.01
Ovulation to G32	40.0 (54/135)	46.9 (53/113)	50.0 (54/108)	0.27
Cows with aCL	77.9 (106/136)	57.9 (66/114)	50.0 (54/108)	< 0.01
		Cows with CA		
Ovulation to G18	48.1 (37/77)	-	-	-
Ovulation to G25	43.2 (32/74)*	24.0 (18/75)	-	0.02
Ovulation to G32	21.1 (16/76)*	38.7 (29/75)*	33.9 (21/62)*	0.65
		Cows without CA		
Ovulation to G18	57.6 (34/59)	-	-	-
Ovulation to G25	77.2 (44/57)	38.2 (13/34)	-	< 0.01
Ovulation to G32	64.4 (38/59)	63.2 (24/38)	71.7 (33/46)	0.05
		Cows with ovulation to previous G		
Ovulation to G18	-	-	-	-
Ovulation to G25	71.6 (48/67)*	-	-	-
Ovulation to G32	53.9 (41/76)*	58.1 (18/31)	-	0.83
		Cows without ovulation to previous G		
Ovulation to G18	-	-	-	-
Ovulation to G25	43.7 (28/64)	-	-	-
Ovulation to G32	21.8 (12/55)	42.9 (33/77)	-	0.02

Table 7.1. The effect of treatment on the ovulatory response of lactating Holstein cows that received post-artificial insemination (AI) GnRH (G) treatments on days 18, 25, and 32 (G18+25), days 25 and 32 (G25), and day 32 post-AI (Control). The percentage of cows with accessory corpora lutea (aCL) was estimated regardless of what GnRH treatment cows ovulated to. The ovulatory response was also evaluated within treatment in cows that had conceptus attachment (CA) or not and cows that had ovulated to the previous GnRH treatment or not. The discrepancies in the number of cows within day of treatment are due to the removal of cows without a follicle ≥ 8 mm at the time of treatment. The symbol * denotes a significant difference in comparisons performed within day of GnRH treatment (Ovulation to G18, G25 and G32) between cows with or without CA and

Table 7.1 (cont'd)
cows with or without ovulation to previous G.

RESULTS

Ovulation rates to GnRH treatments depended on the presence of a conceptus and previous ovulation

Overall, the proportion of cows with induced aCL near the conceptus attachment period (day 18 and 25 post-AI) was greater in G18+25 (72.8%) in comparison with G25 (27.2%) and Controls (0.0%; $P < 0.01$). Ovulation to GnRH treatments depended upon conceptus attachment outcome (attachment or no attachment) and ovulation to the previous GnRH administration (Table 7.1). Cows without conceptus attachment had greater ovulation rates at day 25 for the G18+25 treatment and at day 32 for all treatments compared to their treatment counterparts with conceptus attachment. Cows in the G18+25 treatment that ovulated to a previous GnRH administration had greater ovulation rates at days 25 and 32 compared to G18+25 cows without previous ovulation. But at day 32 post-AI, the proportion of cows with ovulation did not differ between treatments.

Treatment with GnRH near the time of conceptus attachment did not impact pregnancy survival

Overall, treatment with GnRH near conceptus attachment did not affect pregnancy survival in comparison with Controls (Table 7.2). Treatment did not impact the proportion of cows pregnant at day 34 or 62 post-AI. The proportion of pregnancy losses occurring early (between conceptus attachment and day 34 post-AI) or late (between day 34 and 62 post-AI) was also not different between treatments.

% (n)	Conceptus attachment	Early loss	Preg. Diag. day 34	Late loss	Preg. Diag. day 62
G18+25	56.6 (136)	15.6 (77)	47.8 (136)	12.3 (65)	41.9 (136)
G25	65.8 (114)	12.0 (75)	57.9 (114)	9.1 (66)	52.6 (114)
Control	57.4 (108)	12.9 (62)	50.9 (106)	13.0 (54)	44.3 (106)
P-values	0.28	0.80	0.27	0.77	0.22

Table 7.2. The effect of treatment on fertility of lactating Holstein cows that received post-AI GnRH treatments on days 18, 25 and 32 (G18+25), days 25 and 32 (G25), and day 32 post-AI (Control). Fertility was estimated near the period of conceptus attachment (between day 19 to 24 days post-AI), at day 34 (Preg. diag. day 34) and day 62 (Preg. diag. day 62) pregnancy diagnoses with ultrasound. Pregnancy losses were classified as occurring early (Early loss; losses occurring between conceptus attachment and the day 34 pregnancy diagnosis) or late (Late loss; losses occurring between day 34 and 62 pregnancy diagnoses). The *P*-values refer to the effect of treatment within outcome.

The average time to conceptus attachment did not differ between treatments (Figure 7.2). Delayed time to conceptus attachment was positively associated with a higher predicted probability of early (Wald Chi-square: $P < 0.01$) and late (Wald Chi-square: $P = 0.02$) pregnancy loss. The proportion of cows with late conceptus attachment (≥ 22 days) did not differ between treatments (G18+25: 31.2% vs. G25: 18.7% vs. Control: 32.3%; $P = 0.12$).

The ovulatory response was not associated with luteal volume

The occurrence or absence of ovulation to GnRH administered on days 18, 25 and 32 post-AI was not associated with total luteal volume (Figure 7.3). On days 18, 25, and 32 post-AI, cows that ovulated had non-different total luteal volume compared to cows that failed to ovulate, independent of whether conceptus attachment occurred or not.

Decreased ovulatory response on days 25 and 32 post-AI was associated with the occurrence of conceptus attachment but not on day 18 post-AI.

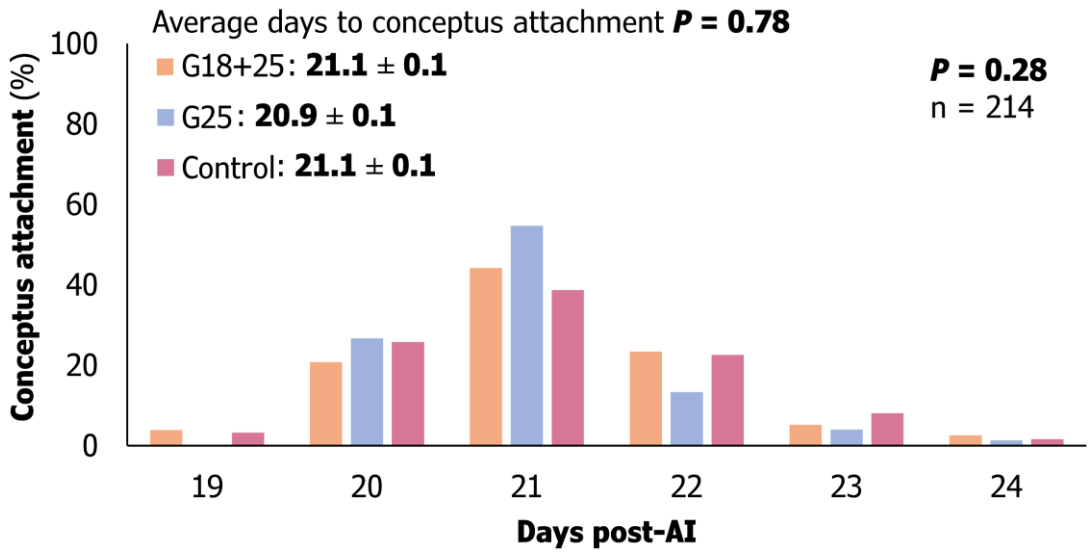

Figure 7.2. Effect of treatment on the percentage of lactating Holstein cows with conceptus attachment on days 19, 20, 21, 22, 23, and 24 post-AI and average days to conceptus attachment. The day of conceptus attachment was determined based on the first day of a significant increase in pregnancy-specific protein B in maternal circulation, and this initial day had to be followed by two additional days of continuous increases.

Treatment with GnRH on days 18 and 25 of gestation did not increase total luteal volume in cows with early pregnancy loss

The treatment G18+25 induced aCL in a greater proportion of cows. Thus, it also increased total luteal volume compared to Controls (Figure 7.4). This treatment effect was observed in cows that maintained pregnancy and cows with late pregnancy loss but not in cows with early pregnancy loss. Thus, G18+25 treatment appeared ineffective in improving/maintaining luteal function in cows that experience early pregnancy loss. Total luteal volume did not differ between G25 and Control cows after treatment (days 28, 32, and 35 after AI), independent of pregnancy outcome. This lack of treatment effect could be attributed to the overall low ovulation response after the administered GnRH on day 25 post-AI in G25 cows.

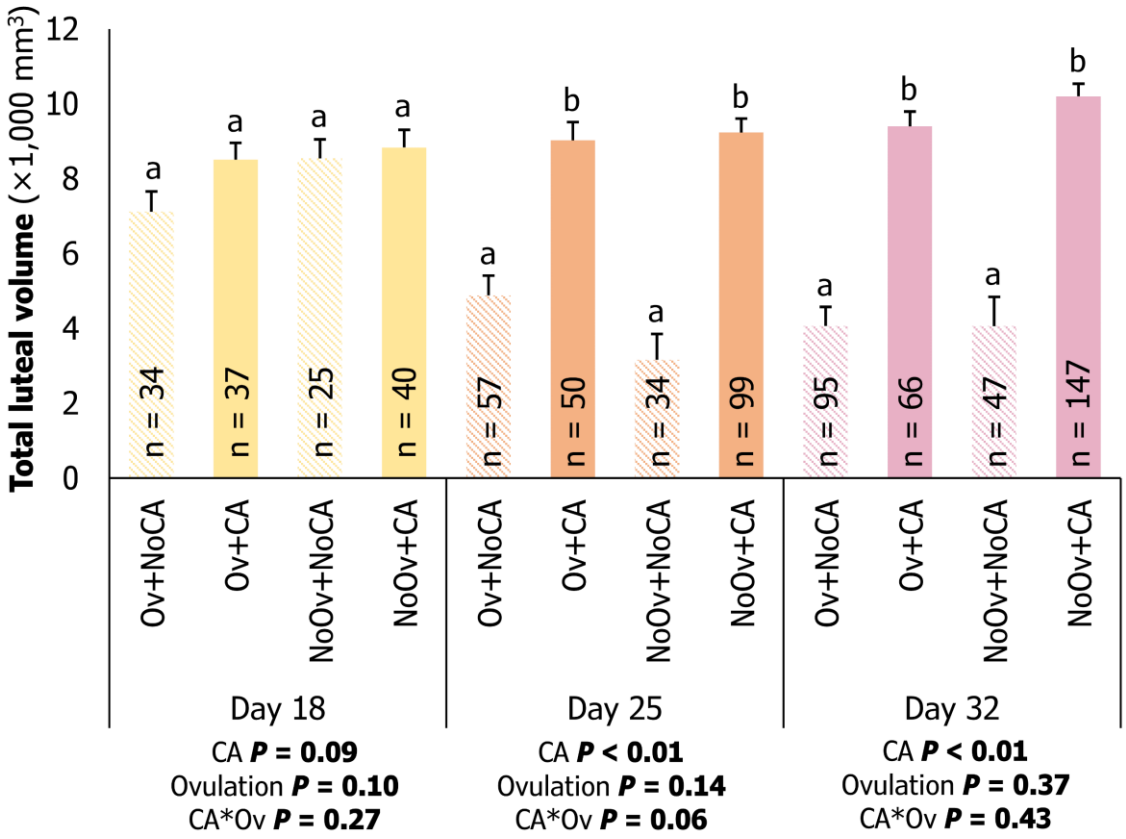

Figure 7.3. The effect of conceptus attachment (CA or NoCA) and ovulatory response (Ov or NoOv) following GnRH treatments administered on day 18, 25 and 32 post-AI on total luteal volume (mm^3) of artificially inseminated lactating Holstein cows. Solid color bars represent cows with conceptus attachment (CA), and hashed bars include cows with no conceptus attachment (NoCA). The total luteal volume included the volume of all functional corpora lutea (CL; main CL and accessory CL). Different letter superscripts within day of GnRH treatment denote a $P < 0.01$. Non-significant multiple comparisons had a $P \geq 0.06$.

Treatment impacted average concentrations of PSPB in cows with pregnancy loss

Mean concentrations of PSPB between day 18 and 28 post-AI varied between treatments, whether cows with conceptus attachment had early or late pregnancy (Figure 7.5). Cows with early loss in G18+25 and G25 treatments had lower PSPB concentrations on days 23 and 24 compared to Controls. Cows in G18+25 and G25 also took one day longer to differ from negative control cows in comparison to Control cows.

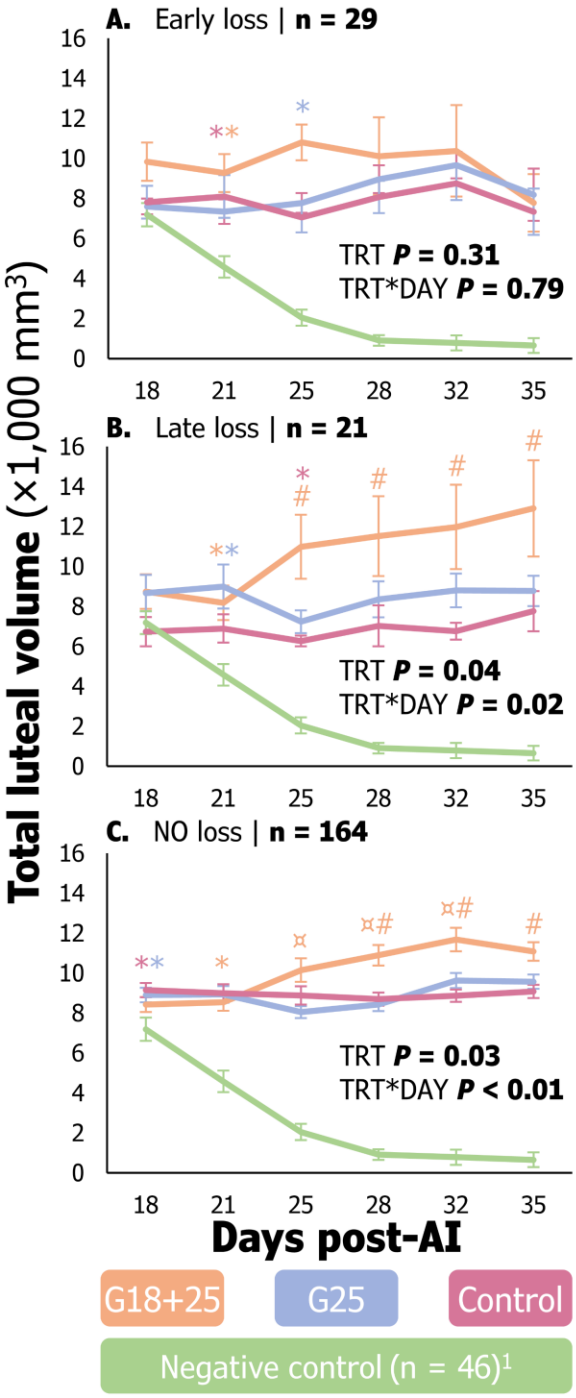

Figure 7.4. The effect of treatment on total luteal volume (mm^3) of lactating Holstein cows with early pregnancy loss (panel A; loss between conceptus attachment to day 34 post-AI), late pregnancy loss (panel B; loss between day 34 to 62 post-AI), and cows that maintained pregnancy (panel C; conceptus attachment and no pregnancy loss). The total luteal volume included the volume of all functional corpora lutea (CL; main CL and accessory CL). Analyses to obtain treatment and treatment by day interaction *P*-values were performed with G18+25, G25, and Control as treatments. [1]Negative control (in green) consists of n = 46 cows that had no conceptus attachment and no formation of

Figure 7.4 (cont'd)
accessory CL. This was included for reference and to determine the 1st day in which treatments differed from Negative Controls. * Denotes a $P \leq 0.03$ for the comparison between treatments and the Negative control. # Denotes a $P \leq 0.02$ for the comparison between G18+25 and Control. ¤ Denotes a $P \leq 0.01$ for the comparison between G18+25 and G25. Symbols are color-coded according to treatment colors.

In contrast, cows with late pregnancy loss in the G18+25 and G25 had greater PSPB concentrations on days 23 and 24 post-AI compared to Controls. On day 23, G18+25 also had greater PSPB concentrations in comparison to G25. Cows with late pregnancy loss in the Control treatment differed from negative controls on day 23, one day later compared to G18+25 and G25. There was no effect of treatments in serum concentrations of PSPB during this period in cows that maintained pregnancy to day 62 post-AI (Figure 7.5).

Ovulatory response before and after conceptus attachment was associated with decreased fertility in lactating dairy cows

Cows were classified according to response to GnRH treatments and the formation of aCL (Figure 7.6). The possible outcomes in terms of response to GnRH administered on days 18, 25, and 32 post-AI were: aCL induced before the period of conceptus attachment (ovulation upon GnRH administration on day 18 post-AI, or pre-conceptus attachment aCL), aCL induced after the period of expected conceptus attachment (ovulation upon either GnRH administration on days 25 and 32 post-AI, or post-conceptus attachment aCL), and no aCL (cows that failed to ovulate to GnRH treatments).

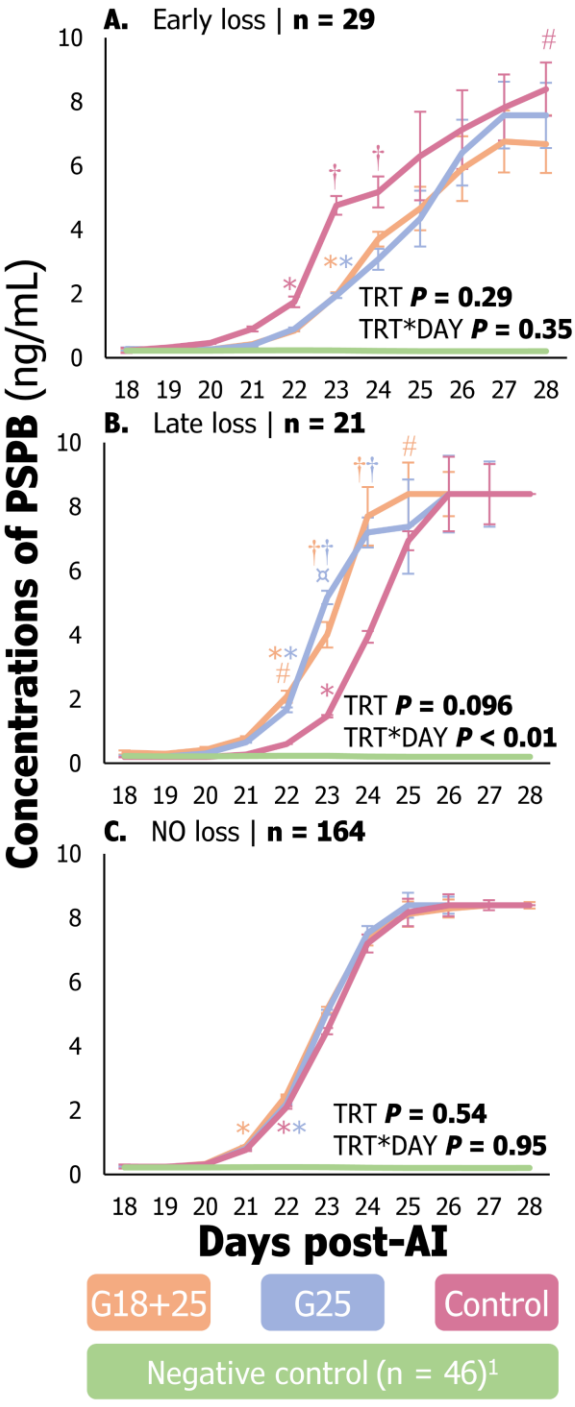

Figure 7.5. The effect of treatment on serum concentrations of pregnancy-specific protein B (PSPB; ng/mL) of lactating Holstein cows with early pregnancy loss (panel A; loss between conceptus attachment to day 34 post-AI), late pregnancy loss (panel B; loss between day 34 to 62 post-AI), and cows that maintained pregnancy (panel C; conceptus attachment and no pregnancy loss). Analyses to obtain treatment and treatment by day interaction *P*-values were performed with G18+25, G25, and Control as the treatments. Negative control (in green) consists of n = 46 cows that had no conceptus attachment and no formation of accessory CL. This was for reference and to determine the first day

Figure 7.5 (cont'd)
in which treatments differed from Negative Controls. * Denotes a $P \leq 0.02$ for the comparison between treatments and the Negative control. # Denotes a $P \leq 0.01$ for the comparison between G18+25 and Control. † Denotes a $P \leq 0.05$ for G18+25 and G25 compared to Control. ¤ Denotes a $P = 0.02$ for the comparison between G18+25 and G25. Symbols are color-coded according to treatment colors.

Cows with induced aCL pre- and post-conceptus attachment had decreased fertility at conceptus attachment and on days 34 and 62 post-AI compared to cows that failed to ovulate to GnRH treatments (Table 7.3). The decreased fertility observed on days 34 and 62 post-AI was not associated with pregnancy losses occurring after conceptus attachment.

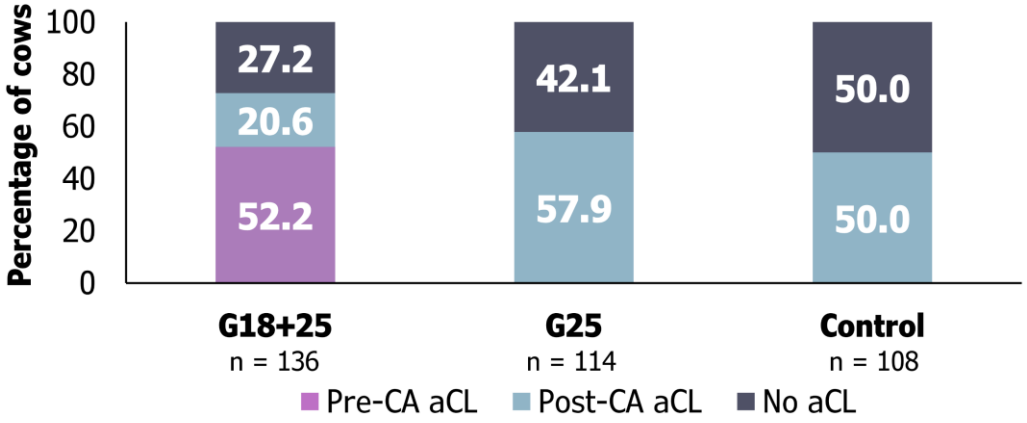

Figure 7.6. The effect of treatment (G18+25, G25 and Control) on the distribution (% of cows) of lactating Holstein cows with accessory CL (aCL) pre-conceptus attachment (pre- CA), post-CA aCL or no aCL ($P < 0.01$). Pre-CA aCL included cows that ovulated to the GnRH administered on day 18 post-AI. Post-CA aCL included cows that ovulated following GnRH treatment on days 25 and 32 post-AI or both. Cows with no aCL were cows that did not ovulate to GnRH treatments administered on days 18, 25, and 32 post-AI.

Serum concentrations of PSPB during the first three days post conceptus attachment were indicative of pregnancy loss

Overall, cows with early pregnancy loss had less cumulative PSPB during the first 3 days (confirmatory period) following conceptus attachment (4.80 ± 0.5 ng/mL) compared to cows that maintained pregnancy (6.81 ± 0.3 ng/mL; $P = 0.01$) during that

period. Cumulative PSPB concentrations during the confirmatory period were not different in cows with late pregnancy loss (6.17 ± 0.7 ng/mL) in comparison with cows that maintained or lost early ($P \geq 0.33$). There was no treatment effect ($P = 0.51$) or an effect of the aCL classification ($P = 0.49$) on the cumulative PSPB concentrations during the confirmatory period. Cows with aCL induced pre-conceptus attachment aCL had about 1 ng/mL more cumulative PSPB concentrations (7.4 ± 0.6 ng/mL) compared to cows with post-conceptus attachment aCL (6.3 ± 0.4 ng/mL) and no aCL (6.3 ± 0.4 ng/mL) but with no significant differences ($P \geq 0.49$).

% (n)	**Conceptus attachment**	**Early loss**	**Preg. d 34**	**Late loss**	**Preg. d 62**
aCL induced:					
Pre-CA[1]	52.1 (71)[a]	18.9 (37)[a]	42.3 (71)[a]	10.0 (30)[a]	38.0 (71)[a]
Post-CA[2]	46.6 (148)[a]	15.7 (70)[a]	40.4 (48)[a]	11.9 (59)[a]	35.6 (146)[a]
No aCL	77.7 (139)[b]	11.1 (108)[a]	69.1 (139)[b]	11.5 (96)[b]	61.1 (139)[b]
P-values	< 0.01	0.48	< 0.01	0.97	< 0.01

[1] Cows that ovulated following GnRH administered on day 18.
[2] Cows that ovulated following either GnRH given on day 25, 32 or both.

Table 7.3. The effect of accessory corpora lutea (aCL) on the fertility of lactating Holstein cows that were artificially inseminated. Cows with aCL were classified as having an aCL induced pre-conceptus attachment (pre-CA; ovulation following day 18 GnRH) or post-conceptus attachment (post-CA; ovulation to day 25 and 32 GnRH, or both). Fertility was estimated near the period of conceptus attachment (between day 19 to 24 days post-AI), at day 34 (Preg. diag. day 34) and day 62 (Preg. diag. day 62) pregnancy diagnoses with ultrasound. Pregnancy losses were classified as occurring early (Early loss; losses occurring between conceptus attachment and the day 34 pregnancy diagnosis) or late (Late loss; losses occurring between day 34 and 62 pregnancy diagnoses). Different letter superscripts denote a $P \leq 0.03$ for comparisons within columns.

DISCUSSION

Previous data from our laboratory (Minela et al., 2021) indicated that re-synchronization with GnRH on days 18 and 25 (GGPG) led to greater than expected P/AI for inseminations previous to and after this re-synch strategy. These data were the impetus to gaining a greater understanding of how well this re-synchronization strategy may: 1) synchronize follicular development for non-pregnant cows to be re-inseminated, 2) induce aCL following GnRH administered on day 18 post-AI that could enhance the probability of conceptus attachment for the previous AI, and 3) enhance the probability of maintenance of pregnancy following conceptus attachment in cows that ovulate to GnRH administered on day 25.

Contrary to our hypotheses, re-synchronization with GnRH on days 18 and 25 post-AI did not enhance the percentage of cows that ovulated to the 1st GnRH of Ovsynch (day 32 post-AI GnRH) in non-pregnant cows (Table 7.1). Cows that received a similar re-synchronization strategy had greater ovulation to 1st GnRH of Ovsynch and greater chances for pregnancy (Dewey et al., 2010). However, ovulatory response following a GnRH administered on day 25 after AI occurred on only 28.7% of treated cows (Leão et al., 2023). In the present study, ovulation to GnRH administered on day 25 improved ovulation response to the first GnRH of Ovsynch in the G18+25 treatment. However, the overall ovulation rate was no different than untreated Controls on day 32 post-AI, or at the first GnRH of Ovsynch. Considering this outcome, the labor and GnRH product costs associated with G18+25 and G25 strategies may not justify their utilization.

The hypothesis that fertility would be improved in cows with aCL emerged from the concept that sustained P_4 concentrations near maternal recognition of pregnancy and

conceptus attachment would favor pregnancy establishment. Progesterone participates in mechanisms associated with modulating uterine receptivity (Bazer et al., 2009), while E_2 participates in the luteolytic cascade, which could terminate pregnancy (Nancarrow et al., 1973; Mccracken et al., 1999). However, the fertility of lactating dairy cows was not enhanced in cows treated with G18+25 and G25 compared to Controls (Table 7.2). Furthermore, cows with conceptus attachment had reduced chances for ovulation. Leão et al. (2023) reported that administration of GnRH on day 25 did not improve the P/AI of the previous AI, and ovulation was reduced in pregnant cows. Thus, it seems plausible that upstream mechanisms that allow for ovulation may be altered, but not completely shut down, during pregnancy.

Progesterone regulates the release of gonadotropins on a hypothalamic level. Ovariectomized cows challenged with E_2 responded with a surge of LH detected in circulation. A combined challenge with P_4 and E_2 inhibited this response (Schoenemann et al., 1985). The LH release from the anterior pituitary and the LH surge amplitude were GnRH dose-dependent (Schams et al., 1974; Giordano et al., 2012). The amplitude of the LH peak was positively associated with ovulatory response to GnRH and negatively associated with P_4 (Oliveira e Silva et al., 2023). Intracarotid infusion with a GnRH antagonist impacted the release of LH from the anterior pituitary in a dose-dependent manner (Wise et al., 1984). Expression of GnRH receptors was downregulated in ovariectomized rats challenged with P_4 and upregulated upon E_2 exposure (Bauer-Dantoin et al., 1995). Removal of P_4's negative feedback via induced luteolysis improved the ovulatory response (Oliveira e Silva et al., 2023). These mechanisms could justify the lower ovulatory response in cows with conceptus attachment, which have intrinsically high

P_4 concentrations. However, the occurrence or absence of ovulation in pregnant cows was not associated with luteal volume on the day of GnRH treatment (Figure 7.3). We speculate that mandatory changes within the follicle that lead to ovulation may also be altered during pregnancy. Hyperprolactinemia has inhibitory effects on LH release via altered signaling on GnRH neurons (Grattan et al., 2007). Conceptuses at day 15 of development have no expression of prolactin. Prolactin linearly increases in conceptuses between days 17, 19, 21, 23 and 25 of gestation (Kessler et al., 1991).

Another possibility is a shift in the pattern of follicular growth during pregnancy. Considering that luteolysis is inhibited during pregnancy, follicular dominance could be extended during the first “cycle” after AI. If so, the second wave dominant follicle could become non-functional as late as day 21 (Vasconcelos et al., 1999). A new follicular wave would emerge within 24 hours from atresia (Ginther et al., 1989). Treatment with GnRH on day 25 would be administered near the time of dominance acquisition (between days 3 or 4), and the ovulation response may be diminished at that time (Bello et al., 2006). In the present study, only cows with follicles ≥ 8 mm (presumably a dominant follicle) were considered eligible to ovulate. However, with the frequency of examinations it was difficult to determine the functionality of the largest follicle present at treatment. Moreover, ovulation on day 18 post-AI enhanced the ovulatory response of the day 25 GnRH, reiterating that the presence of a follicle on day 6 or 7 of development can improve the ovulatory response to GnRH.

Also, contrary to our hypothesis, induction of aCL near the period of maternal recognition of pregnancy and conceptus attachment did not improve chances for or maintenance of pregnancy. Cows with aCL induced before or after the period of

conceptus attachment had decreased fertility compared to cows that did not have aCL (Table 7.3). Santos et al. (2023) demonstrated that inducing aCL early in the cycle with hCG decreased the chances for conceptus attachment. The latter study modified follicular dynamics and induced aCL much sooner than the present study, in a period of limited interaction between dam and conceptus (2 and 5 days post-ovulation). In contrast, cows that ovulated to a GnRH administered 5 days after AI had greater P/AI on days 32 and 67 post-AI and lower pregnancy loss compared to cows that did not ovulate (Baez et al., 2017). Our model intended to achieve uninterrupted P_4 exposure following conceptus elongation and upon the prospect of conceptus attachment. This period marks the onset of extensive molecular and physical interactions between conceptus and dam. Products secreted by the conceptus induced local and systemic changes in maternal physiology (Mansouri-Attia et al., 2009; Bott et al., 2010; Forde et al., 2011; Antoniazzi et al., 2013). Changes in follicular function during early pregnancy have not been investigated. Nonetheless, oocytes collected from pregnant beef heifers and cows maintained their fertility potential (Merton et al., 2012). It is unclear from the data reported herein and from published data if the “intrinsic quality” of the pregnancy could have interfered with the ovulatory response. One possibility is that a viable elongated conceptus, which can initiate mechanisms of luteal rescue, have conceptus attachment, and sustain pregnancy, is also more efficient in shutting off mechanisms that lead to cyclicity rather than pregnancy. Luteolysis and ovulation are two of the main physiological processes that cease with pregnancy. Thus, the ability to ovulate may reflect inadequacies in the pregnancy itself. This was reflected in the present dataset as lower P/AI at conceptus attachment, day 34 and 62 post-AI in cows that managed to ovulate to GnRH treatments.

The relationship between ovulation occurrence and poor fertility could also be associated with a proposed function of PAGs. An in vitro study provided evidence of competitive binding of cotyledonary proteins (PAG-positive) to gonadotropic receptors upon challenge with LH or hCG (Szafranska et al., 2007). Cotyledonary proteins purified from later than 30 days of gestation had a greater ability to compete for gonadotropic receptors. This competitive binding was verified in luteal, myometrium and endometrium tissues collected during the luteal phase of cyclic sows. If true, the presence of an incompetent conceptus, which may have impaired PAG secretion, could favor ovulatory responses in these cows of lower fertility. In fact, a delayed time to PSPB increase in the maternal circulation was associated with increased chances of early and late pregnancy loss. However, the occurrence of pregnancy losses was not associated with the formation of pre- or post-conceptus attachment aCL compared to Controls. Ovulation rates on days 18, 25, and 32 post-AI also did not differ whether conceptus attachment occurred early (≤ 21 days post-AI) or late (≥ 22 days post-AI; data not shown). Overall, cows with conceptus attachment had decreased ovulation rates on days 25 (33.6%) and 32 (31.0%) in comparison with day 18 post-AI (48.1%), suggesting that ovulatory response may decrease as pregnancy progresses.

We hypothesized that cows at risk of pregnancy loss would benefit from sustained P_4 concentrations early during pregnancy via the formation of aCL. However, treatments G18+25 and G25 failed to increase total luteal volume compared to Controls in cows with early pregnancy loss. The treatment G18+25 increased total luteal volume in cows that had late pregnancy loss and cows that maintained pregnancy. The low ovulation rates in the G25 treatment were also reflected in the lack of treatment effects on total luteal

volume. Pregnancy loss may occur before or after the loss of luteal support (Domingues et al., 2023b). During the period of ovarian activity monitoring (days 18 through 35 post-AI), n = 1 cow with conceptus attachment had luteolysis and spontaneous ovulation between days 28 and 32 post-AI. At day 35 post-AI, n = 5 out of n = 18 cows that had early pregnancy loss without aCL appeared to have luteal regression. In the preceding examinations, luteal function was non-different compared to cows that maintained pregnancy. Thus, it appears that other factors could impact the pregnancy fate other than luteal function. In the present study, an earlier increase in PSPB, as well as greater cumulative PSPB concentrations in maternal circulation during the confirmatory period, were associated with successful pregnancy outcomes. The positive relationship between early and greater exposure to PAGs with pregnancy success has been reported in dairy and beef cows (Filho et al., 2020; Minela et al., 2021; Santos et al., 2023). Proper conceptus attachment is the initial step for placentation, which will begin after day 28 of gestation (King et al., 1980; Wooding and Burton, 2008). Pregnancy-associated glycoproteins detected in maternal circulation are an indicator of this ongoing process.

Cows with greater concentrations of P_4 between days 8 and 61 of gestation did not have greater concentrations of PAG measured between days 18 and 33 of gestation or earlier time to PAG increase in maternal circulation (Domingues et al., 2023b). Progesterone concentrations were enhanced via aCL induction on days 7 and 13 post-AI. A positive effect of P_4 on PAG concentrations was observed later in gestation, on days 47 and 61 post-AI. In the present study, treatment did not affect PSPB concentrations in cows that maintained pregnancy (Figure 7.5). Cows in the G18+25 treatment had greater concentrations of PSPB compared to negative control cows one day earlier than Controls.

Cows with early and late pregnancy loss had treatment-dependent profiles of PSPB concentrations. Cows with early pregnancy loss in the treated groups had decreased PSPB on days 23 and 24 post-AI and later conceptus attachment compared to Controls. It was unclear why the treatment G25 (an untreated control group before 25 days post-AI) had the same PSPB profile as the G18+25 treatment. In cows with late loss, a similar response was observed. However, the G18+25 induced greater PSPB concentrations on day 23 compared to G25 and on days 22 to 25 post-AI compared to Controls. This could suggest a possible benefit in treating cows with GnRH on day 18 post-AI in cows that are at risk of losing pregnancy. However, G18+25 treatment did not prevent pregnancy loss from occurring.

In summary, treatment with GnRH after AI to induce aCL did not improve pregnancy survival of lactating dairy cows. Cows that ovulate to a previous GnRH treatment were more likely to ovulate to following treatment, but ovulation rate to the 1st GnRH of Ovsynch did not differ between treatments. Cows with conceptus attachment were less likely to respond to GnRH treatments. However, total luteal volume did not differ in cows with conceptus attachment that ovulated or not. We speculate that pregnancy interfered with mechanisms involved in follicular function and ovulation. Treatment with GnRH on days 18 and 25 post-AI did not increase luteal volume in cows that had early pregnancy loss. Cows that had aCL induced pre-conceptus attachment and post-conceptus attachment had decreased fertility in comparison to cows without aCL. The interesting combinations of reduced ovulation rates to GnRH in pregnant cows, the lack of increase in luteal mass in the pregnant cows that did ovulate, and the surprising reduction of cows with conceptus attachment that had a GnRH-induced aCL at day 18

post-AI may indicate an evolutionary protective mechanism in place. These data are sufficient to forewarn others when developing aggressive re-synchronization programs with the intent to induce new aCL.

CHAPTER 8

FINAL REMARKS

ESTRUS DETECTION IN DAIRY HERDS:

PROGRESS OR RETROGRESS?

Chapter 6 of this dissertation aimed to investigate the true fertility potential of high-producing lactating dairy cows that received AI following a detected estrus. We deemed it relevant to inquire about the reproductive efficiency and physiology of estrus detection in view of the increasing AAM utilization in dairy herds. Since the early 2000s, there has been an increasing use of hormonal protocols to synchronize estrous cycles and AI cows in a fixed time. The reproductive efficiency of dairy herds has undoubtedly improved because of the use of this management tool (Fricke and Wiltbank, 2022; Chebel et al., 2024). This improvement was a result of 100% service rates, combined with enhanced fertility after the development of pre-synch Ovsynch-type programs (i.e., Presynch-11/10, G6G, and Double-Ovsynch, 2001 to 2008). Before the development of Ovsynch in 1995, estrus detection was the sole option for AI of cattle. Thus, reproductive benchmarks (e.g., service rate, days non-pregnant, 21-day pregnancy rate) achieved in 1990 dairy herds may be a great reference to infer the current efficiency of this strategy when utilized apart from hormonal intervention. Nonetheless, the lactating dairy cow of 1995 is vastly different from the 2024 dairy cow. Differences between these cows can be attributed to genetic merit, increased milk production due to advancements in nutrition, and metabolic changes that accompany that phenotype.

Cows with greater milk production have decreased circulating concentrations of steroid hormones (Lopez et al., 2004). Progesterone and E_2 have regulatory endocrine roles on a hypothalamic, pituitary and uterine level. Steroid hormones control or assist in mechanisms that lead to follicular development, oocyte maturation, luteolysis, estrus

expression, and ovulation (Nancarrow et al., 1973; Lemon et al., 1975; Mccracken et al., 1999). During fertility programs, these events are exogenously controlled with the final objective of making a better oocyte and preparing the uterine environment. Dysfunction in the release of these hormones may be involved with decreased fertility of lactating dairy cows that receive AI following estrus detection (Figure 8.1).

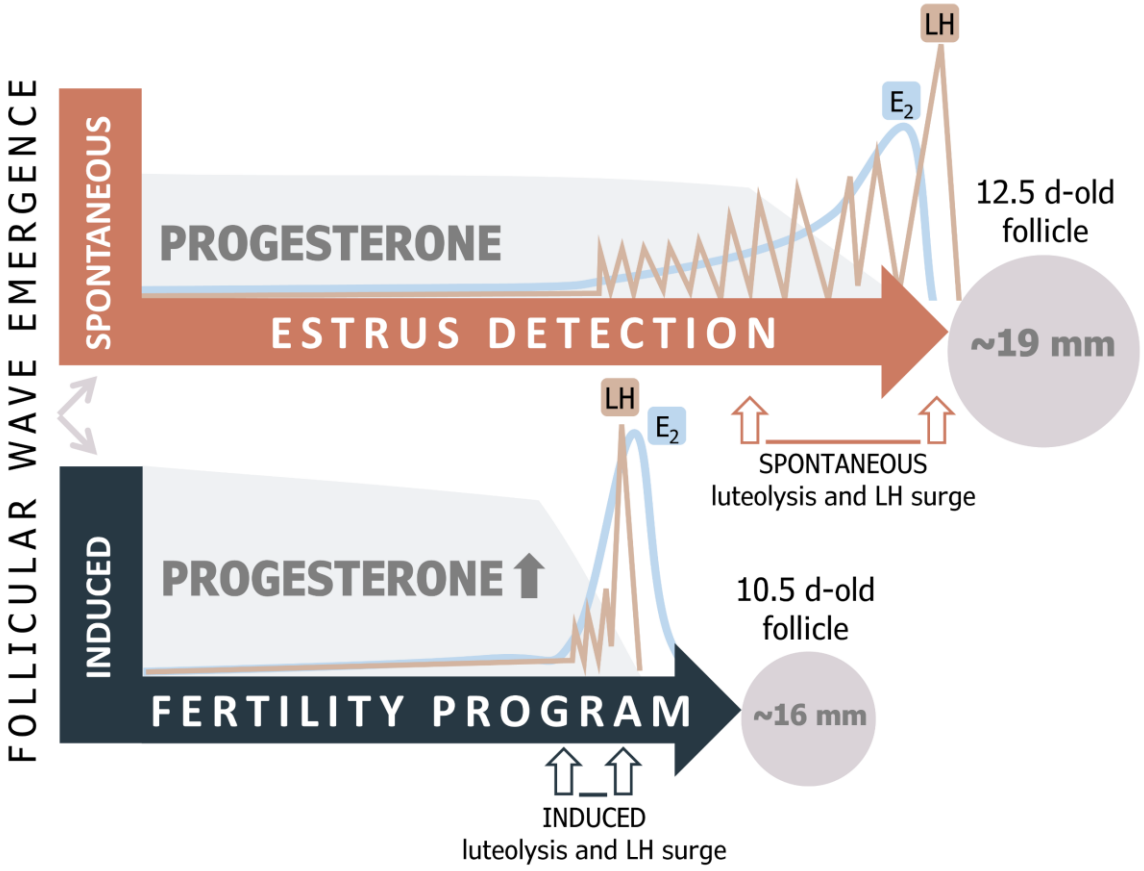

Figure 8.1. Schematic representation of the detrimental effects of increased steroid hormone metabolism during natural estrous cycles that lead to prolonged periods of follicular development. Fertility programs correct such dysfunction via increased progesterone during the development of the pre-ovulatory follicle, inhibiting luteinizing hormone (LH) pulsatility. Luteolysis is induced in a timely manner when follicles are near peak functionality and can have quick increases in estrogen (E_2) output. The LH surge and, consequently, ovulation are exogenously induced, regardless of E_2 peak and estrus expression.

Are hormonal treatments necessary to manage reproduction in the 2020's dairy herds?

High milk production altered estrus behavior in lactating dairy cows (Lopez et al., 2004). The study described in Chapter 4 investigated how antral age may impact steroid hormone dynamics and estrus characteristics as detected with AAM. This study indicated that only 70% of cows were detected in estrus, regardless of previous synchronization of the follicular waves and exogenous induction of luteolysis. Cows that had pre-ovulatory follicles with reduced antral age had follicles with greater steroidogenic capacity, tended to be more likely detected in estrus, and had greater estrus intensity. Cows that had greater increases in E_2 between induction of luteolysis to 2 days later were more likely to be detected in estrus. These findings could imply that during natural estrous cycles, the period between the onset of follicular development, luteolysis, E_2 peak, LH surge, and ovulation will directly impact follicular endocrinology and estrus characteristics as detected with AAM. In this scenario, the high-producing lactating dairy cow is the sole regulator of when and if these key physiological processes occur.

Evidence from Chapter 5 clearly indicated differences in follicular characteristics between cows that were allowed to have natural estruses and cows synchronized with Double-Ovsynch. Cows in the estrus detection treatment ovulated follicles that were, on average, 3.5 mm larger in diameter compared to the Double-Ovsynch treatment. Considering a daily growth rate of 1.5 mm per day, follicles of a cow in estrus had developed for at least 2 days longer. It has been hypothesized that to induce the E_2 peak in circulation and external sexual receptivity, high-producing dairy cows would require a greater period of follicular development (Fricke and Wiltbank, 2022). In corroboration to

that hypothesis, follicular diameter was positively associated with greater E_2 to P_4 ratios in the estrus detection treatment. In contrast, cows in the Double-Ovsynch treatment consistently ovulated follicles between 14 and 16 mm in diameter, which has been correlated with enhanced fertility (Bello et al., 2006). Follicular diameter was not associated with E_2 to P_4 ratios in the Double-Ovsynch treatment. The ratio of E_2 to P_4 was associated with pregnancy survival in cows in the Double-Ovsynch treatment but not in the estrus detection treatment. This finding reinforced the importance of the occurrence of complete luteolysis during manipulated cycles. Timely luteal regression is mandatory for final follicular development, and increasing concentrations of E_2 are indispensable to preparing the uterine environment for the early embryo (Shimizu et al., 2010).

Before the completion of the projects described in Chapters 5 and 6, it was our understanding that the intrinsic fertility of the enrolled experimental units (high-producing dairy cows) would be diminished following AI to a detected estrus. The initial hypothesis was that decreased fertility would be associated with the phenotype of prolonged days to conceptus attachment in estrus detection cows. These studies were pioneers in describing fertility outcomes earlier than the typical initial pregnancy diagnosis (between 30 to 40 days post-AI) in cows that received AI after estrus.

The study in Chapter 5 was designed to investigate differences in days to conceptus attachment. Average days to conceptus attachment did not differ between treatments. Additionally, the proportion of cows that had conceptus attachment was precisely the same in estrus detection and Double-Ovsynch treatments. This initial evidence suggested that the fertility potential of lactating dairy cows was non-different near the period of conceptus attachment, whether they received AI following a detected

estrus or a synchronized ovulation. We questioned ourselves: Is decreased fertility following estrus detection a result of greater pregnancy losses occurring after conceptus attachment? The sample size utilized in Chapter 5 was not sufficient to answer that question. However, a statistical trend for greater losses in the estrus detection treatment was the impetus to repeat this study with an appropriate sample size.

Chapter 6 comprised a straightforward experimental design where cows received AI after estrus detection without hormonal treatments or the Double-Ovsynch program. Cows received AI within a similar DIM range between treatments, and all cows had confirmed ovulation (thus were truly in estrus or had induced ovulation), which we believe is a first when comparing estrus to a fertility program. Until now, most scientists would agree that the reason for low fertility following a detected estrus dealt with the imperfection of detecting estrus and/or the mistiming of AI relative to ovulation. In this study, not only did all cows receiving AI ovulate following estrus, but there were no differences in P/AI as it related to the timing of AI. It was a design that essentially described the true reproductive potential of utilizing estrus detection to AI the 2020 high-producing lactating dairy cow. The novelty of this study was the detection of the first day of significant PSPB increase in maternal circulation or the day of conceptus attachment. This methodology allowed for the estimation of pregnancy losses occurring before the first month of gestation. The findings from Chapter 5 were non-repeatable in Chapter 6. Cows that received AI following a detected estrus had decreased fertility at conceptus attachment compared to Double-Ovsynch. The proportion of pregnancy losses occurring before the first month of gestation did not differ between treatments. Most conceptuses that reached the stage of conceptus attachment appeared competent in initiating the transfer of PSPB to maternal

circulation between days 19 and 20 post-AI. This resulted in average earlier conceptus attachment following estrus detection compared to Double-Ovsynch. Collectively, it was concluded that pregnancy failure in cows that received AI to a detected estrus occurred mainly before the period of conceptus attachment. Pregnancy failure occurring before the elongation of the conceptus was linked to poor oocyte quality (Wiltbank et al., 2016). Oocyte health was compromised in cows with extended follicular antral age and greater follicular diameter (Revah and Butler, 1996; Mihm et al., 1999; Bleach et al., 2004). Although oocyte quality was not assessed in the present dissertation, the evidence presented in Chapters 4 and 5 would suggest that cows detected in estrus had follicular phenotypes compatible with compromised oocyte health.

Considering the large gap in the proportion of cows that became pregnant to the first service reported in Chapter 6 (estrus detection 19% vs. Double-Ovsynch 50%), it seems unlikely that reproductive management without hormonal intervention would ever become feasible again. That is unless the dairy industry adapts current benchmarks for excellence in reproduction. The Dairy Cattle Reproduction Council awards herds that presented excellent reproductive outcomes. All previous winners of this award had above 35% 21-day pregnancy rates. To achieve such results the equation would necessarily include high service rates and high P/AI. A recent report (Tippenhauer et al., 2023) provided current reproductive outcomes from n = 9 herds that opted to breed most cows following estrus detection with AAM and keep hormonal treatments at a minimum. These herds were averaging 51.2% service rates and 37.2% P/AI. The average 21-day pregnancy rate was 19.0%. These outcomes are almost identical to data reported for the

estrus treatment in Chapter 6. These parameters are also almost identical to reproductive outcomes achieved in top dairy herds in 1995.

Nonetheless, herds may be able to improve their efficiency upon early identification of subgroups that could benefit from fertility treatments. Studies have demonstrated that when associated with early targeted hormonal intervention estrus detection may have comparable efficiency to an all timed-AI program (Rial et al., 2022; Gonzalez et al., 2023). In Chapter 6, the occurrence of estrus events before the first service was associated with the subsequent fertility of lactating dairy cows. Our interpretation was that hormonal intervention mitigated the negative effects of lack of cyclicity before AI. Additionally, it further enhanced the intrinsic fertility of lactating dairy cows that could be deemed not to necessitate hormonal intervention (at least 2 estrus events before 1st AI). Upon synchronization with Double-Ovsynch, those cows had 68% P/AI at conceptus attachment and 60% P/AI at the first pregnancy diagnosis. These are astonishing numbers considering the overall fertility potential of U.S. Holstein cows.

Based on the outcomes reported herein and data collected in our laboratory for the past 6 years, it becomes clear that Double-Ovsynch is highly efficient in achieving pregnancy. Chapter 7 aimed to test a strategy that could support pregnancy survival and perhaps enhance the already high fertility achieved through hormonal intervention. Induction of aCL following AI did not improve the P/AI of lactating dairy cows. Treatments did not increase luteal volume in cows that experienced early pregnancy loss. Ovulatory response was hindered in pregnant cows in comparison to cows with no conceptus attachment, but not associated with luteal volume. We speculated that pregnancy may

either alter follicular dynamics or shut down mechanisms associated with the ovulatory cascade since those processes are non-compatible with pregnancy physiology.

Data presented in Chapter 7 indicated that pregnancy loss was associated with phenotypes of delayed conceptus attachment and decreased concentration of PSPB in maternal circulation. Until recently, these pregnancy losses occurring between conceptus attachment and an exam performed ~35 days post-AI were undiagnosed. Data presented in this dissertation compromised some of the first reports of high-producing dairy cow fertility assessed near the period of conceptus attachment. We hope that these data will support future research that aims at improving fertility through the reduction of pregnancy losses. Developing strategies that could mitigate the occurrence of delayed conceptus attachment and decreased PSPB concentrations phenotypes and, with that, decrease the likelihood of pregnancy losses would have a tremendous positive impact on cow fertility. It is our understanding that such strategies would involve hormonal intervention. Once again, the utilization of exogenous hormonal intervention to orchestrate processes that occur naturally could help advance dairy cow fertility even further.

Reproduction drives milk production!

Greater reproductive efficiency may accelerate the genetic gain of herds. Genetic improvement is likely the main contributor to the increase in milk production observed on a national level. Average U.S. milk production increased from 4,572 lb/cow/year in 1944, 16,433 lb/cow/year in 1995, to 24,345 lb/cow/year in 2023 (USDA, 2023). It is estimated that AI allowed between 1.0 and 1.5% genetic gain/year. About 50% of the increase in milk production can be attributed to the use of AI. The remaining 50% are attributed to enhanced feeding strategies and adequate animal husbandry (Moore and Thatcher,

2006; Bertolini and Bertolini, 2009). Targeted selection for increased milk production was inversely proportional to fertility (Royal et al., 2000; VandeHaar and St-Pierre, 2006; Bach, 2019). The inclusion of the reproductive trait “Daughter pregnancy rate (DPR)” in the year 2000 just recently hindered the downward trend in dairy cow fertility (Lucy, 2007; Fricke and Wiltbank, 2022).

The state of Michigan was the top state in production/cow with 27,430 lb/cow/year in 2022. Michigan dairy producers have been adopting technologies that support high reproductive efficiency since the early 2000s with a very progressive approach. The lactating dairy cows utilized in studies described in Chapters 4, 5, and 7 are part of the 3rd most productive Holstein herd in the state of Michigan (37,571 lb/cow/year of energy corrected milk). Over the years, this herd maintained excellent reproduction and achieved pregnancies in a timely manner with the use of fertility programs. Our research herd, Nobis Dairy, is a notable example of a herd that has most of its cows within the “High Fertility Cycle.” Nobis’ cows are proof that high milk production does not necessarily come at the cost of fertility. Our outstanding reproductive outcomes at Nobis Dairy are partially responsible for this herd being ranked #3 in the state of Michigan for rolling herd averages. The very highest-producing Holstein cows can be extremely fertile when fertility programs and great compliance are present.

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