USING IN-LINE MILK PROGESTERONE DATA TO DETERMINE OVARIAN ACTIVITY PARAMETERS ASSOCIATED WITH REDUCED FERTILITY IN CANADIAN HOLSTEIN COWS

by

Tony Carreira Bruinje

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Department of Agricultural, Food and Nutritional Science
University of Alberta

ABSTRACT

Improvements in genetic selection and dairy management practices have resulted in dramatic increases in individual milk production over past decades. However, this positive trend has been accompanied by decreased reproductive performance and fertility in dairy cows. Infertility in modern dairy cows is often linked to altered ovarian function; therefore, gaining a better understanding of ovarian activity and factors associated with fertility impairment is of great importance. In this regard, a fully automated technology that measures frequent milk progesterone (P4) concentrations has been recently commercialized. It allows monitoring of real-time ovarian activity and exploration of various components of luteal activity associated with fertility that could not be studied previously.

Three studies were conducted that aimed to evaluate components of ovarian activity parameters associated with reduced fertility, by retrospectively assessing postpartum in-line milk P4 data in Holstein cows. Records of milk P4 were obtained through an automated in-line milk analysis system (Herd Navigator™, DeLaval, Tumba, Sweden) in two (in first and second study) and four (in third study) commercial dairy herds. On average, milk P4 (ng/mL) was measured every 2 d starting at approximately 20 d postpartum until the determination of artificial insemination (AI) outcomes. Variations in adjusted milk P4 values below and above the designated threshold (5 ng/mL) were used to determine the commencement of luteal activity (CLA), length of luteal phases, and pregnancy.

The objectives of the first study (Chapter 3) were to investigate relationships of (1) interval from calving to CLA and (2) luteal phase length and frequency preceding first AI, with parity and AI outcomes in two dairy herds. Primiparous cows had delayed CLA and less abnormal (i.e. short or long) luteal phases than multiparous cows. An early CLA improved fertility in multiparous cows, while having an abnormal luteal phase reduced fertility in primiparous cows. Regardless, the presence of at least two luteal phases preceding first AI greatly improved fertility. In conclusion, a high frequency of luteal phases (including at least one normal luteal phase) preceding first AI is a major factor benefiting AI outcomes.

The second study (Chapter 4) aimed to determine the dynamics of pre- and post-AI milk P4 profiles and their associations with parity and AI outcomes in two herds. Differences in milk P4 profiles between parities and among different AI outcome groups were observed. Primiparous

cows had higher and a more rapid increase in milk P4 levels post-AI than multiparous cows. Also, cows that became pregnant after AI had lower milk P4 levels near the time of AI and higher milk P4 levels beyond d 10 post-AI, compared to cows that were not pregnant after AI.

The third study (Chapter 5) aimed to characterize cut-off values for various luteal activity parameters in the early postpartum period, and before and after AI, that were associated with reduced probability of pregnancy, in a larger population. By identifying cut-off values through receiver operating characteristic curve analysis, several parameters associated with reduced fertility were characterized, such as: (1) low number of cycles preceding first AI, (2) prolonged luteal phase preceding AI, (3) delayed AI following decline in milk P4 levels, and (4) sub-optimal P4 concentrations at different time points before and after AI. In conclusion, modern dairy cows have a high prevalence of abnormal luteal activity parameters associated with reduced probability of pregnancy, which might be the underlying reasons for poor fertility in dairy herds.

The results from this Master's thesis research indicate that monitoring in-line milk P4 profiles can be a valuable approach to identify components of infertility and to benchmark the prevalence of these components in modern dairy herds. Thereby, targeted management strategies to overcome abnormal luteal activity conditions can be implemented to improve fertility, such as (1) inducing luteolysis in cows with prolonged luteal phases preceding AI or with elevated P4 near the time of AI, and (2) supplementing P4 in cows with sub-optimal spontaneous P4 levels before or after AI.

PREFACE

This thesis is an original work by Tony Carreira Bruinje. The principal investigator of the studies addressed in Chapters 3, 4, and 5 was Dr. Divakar J. Ambrose from the Department of Agricultural, Food and Nutritional Sciences, University of Alberta and from the Livestock Research & Extension Branch, Alberta Agriculture and Forestry, Edmonton, AB, Canada.

Chapter 3 of this thesis has been published as *T.C. Bruinje, M.G. Colazo, M. Gobikrushanth and D.J. Ambrose, "Relationships among early postpartum luteal activity, parity, and insemination outcomes based on in-line milk progesterone profiles in Canadian Holstein Cows", Theriogenology, 2017, vol. 100, 32–41.* The candidate was responsible for designing objectives and performing data collection, data management, statistical analysis, results interpretation and manuscript composition. M.G. Colazo reviewed and provided important contributions with concept formation and manuscript edits. M. Gobikrushanth assisted with data analyses and manuscript edits. D.J. Ambrose was the supervisor and corresponding author involved with all steps of concepts and objectives formation, definition, manuscript composition and revisions, and industry liaison.

Chapter 4 of this thesis has been published as *T.C. Bruinje, M. Gobikrushanth, M.G. Colazo and D.J. Ambrose, "Dynamics of pre- and post-insemination progesterone profiles and insemination outcomes determined by an in-line milk analysis system in primiparous and multiparous Holstein cows", Theriogenology, 2017, vol. 102C, 147–153.* The candidate was responsible for designing objectives and performing data collection, data management, statistical analysis, results interpretation and manuscript composition. M. Gobikrushanth assisted with data analyses and manuscript edits. M.G. Colazo contributed by providing thorough reviews and manuscript edits. D.J. Ambrose was the supervisor and corresponding author; he was involved with all steps of concepts and objectives formation, definition, and manuscript composition, edits and revisions, and industry liaison.

Chapter 5 of this thesis, in a modified form, will be submitted as a complete research article for publication in the Journal of Dairy Science with the title "*Using in-line milk progesterone data to characterize parameters of luteal activity associated with reduced fertility in Holstein cows*". The candidate was responsible for designing the research questions,

objectives, and methodology, in addition to performing data processing, statistical analysis, results interpretation, and manuscript composition. The co-authors of the study will be M. Gobikrushanth, for assisting with statistical methods, and M.G. Colazo, for assisting with methodology and manuscript edits. D.J. Ambrose will be the corresponding author and was involved with all steps of concepts formation, manuscript edits and revisions, and industry liaison.

To my wife, my parents, and my brother for their endless support

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS

AI	Artificial Insemination
AUC	Area Under the Curve
BHBA	β-hydroxybutyrate
CI	Confidence Intervals
CL	Corpus Luteum
CLA	Commencement of Luteal Activity
DIM	Days in Milk
DPP	Days Postpartum
E2	Estradiol
FSH	Follicle Stimulating Hormone
GH	Growth Hormone
GnRH	Gonadotrophin-Releasing Hormone
IGF-1	Insulin Growing Factor 1
IMAS	In-line Milk Analysis System
LA	Luteal Activity
LH	Luteinizing Hormone
LP	Luteal Phase
mP4	Milk Progesterone
mP4-decline	Milk Progesterone Decline
NEB	Negative Energy Balance

NEFA	Non-Esterified Fatty Acids
NRC	National Research Council
OPEN	Presumed-Open or Non-Pregnant
OR	Odds Ratio
P-LOSS	Presumed-Pregnancy Loss
P/1stAI	Pregnancy per First Artificial Insemination
P/AI	Pregnancy per Artificial Insemination
P4	Progesterone
PGF	Prostaglandin $F_{2\alpha}$
PostPeak	Milk Progesterone Peak Post-AI
PREG	Presumed-Pregnant
PrePeak	Milk Progesterone Peak Preceding AI
ROC	Receiver Operating Characteristics

CHAPTER 1
GENERAL INTRODUCTION

While the world population is expected to grow from 7 to almost 10 billion in 30 years (UNDESA, 2015), dairy production systems are undergoing increasing pressure to reduce greenhouse gas emissions (Tilman and Clark, 2014), restrict the use of hormones to manipulate animal performance (Lane et al., 2008), and yet produce more and better-quality food (Tilman and Clark, 2014). However, as any business enterprise, the aspect that drives a dairy operation is profitability. The profit represents what is left of the revenue after paying all expenses related to its outcomes, and can easily vary from negative to positive margins among similar farms. An economical survey in Alberta showed a range in return to investment from -1.7 to +14.9% among dairy farms (Heikkila and Van Biert, 2014). In the USA, 88% of the incomes of a dairy are from sales of milk, and the main cost is to feed the producing cows, which represents 50% of the total expenses (Santos et al., 2010). These numbers demonstrate how delicate and necessary it is to maximize the efficiency in all aspects of the farm that affect profitability, which is driven by the milk production.

The main aspects related to improved milk yield efficiency are management, feed, herd health, and importantly, reproduction – which is responsible for the initiation of lactation. An important aspect to consider is that a low reproductive performance will affect the proportion of cows in the peak-production period of the lactation, reducing overall herd yearly milk yield (Norman et al., 2009). In addition, low reproductive performance will decrease the number of new births for herd replacement or sales, reduce genetic advancement of the herd (Ribeiro et al., 2012), and increase risk of culling in cows that are not pregnant late in lactation, which also impacts profitability (McDougall and Compton, 2006).

Reproductive efficiency is one of the main factors impacting the profitability of dairy farms. However, the challenge to get cows conceive in a timely manner has dramatically increased over the past decades, concomitantly with the increase in milk production (Lucy, 2001). While the annual milk production per cow has been increasing from approximately 2,000 kg in 1950`s to more than 10,000 kg nowadays (USDA, 2015), the reproductive performance has decreased worldwide (Macmillan et al., 1996; Butler, 1998; Roche et al., 2000; Royal et al.,

2000; Norman et al., 2009). An inverse genetic correlation between milk production and fertility exists (Windig et al., 2006); however, the link between increased milk production and reduced fertility is likely a combination of various physiological and management factors associated with high milk production. Thus, a main challenge of the research community is to better understand these individual factors contributing to reduced fertility and offer solutions to increase the proportion of cows becoming pregnant as early as possible after the postpartum elective waiting period.

The transition between the late gestation and the early postpartum period is characterized by intense endocrine and metabolic changes. These changes involve profound regulations of energy status and liver function to adapt to the abrupt increase in the nutritional demand for the new lactation (Drackley, 1999). As voluntary feed intake is not sufficient to meet the abrupt increase in nutritional requirements, postpartum dairy cows suffer an extensive mobilization of body reserves, leading to a negative energy balance (Bell, 1995). In addition, approximately 50% of the dairy cows develop at least one postpartum health disorder in the postpartum period (Santos et al., 2010). These metabolic challenges prevalent in the postpartum dairy cow are known factors associated with altered ovarian function, such delayed resumption of postpartum cyclicity (Ribeiro et al., 2016a), and occurrence of abnormal (i.e. short or prolonged) estrous cycles (Wiltbank et al., 2006; Chen et al., 2015). In addition to the postpartum disorders, another factor related to the milk production that alters reproductive physiology is the metabolic clearance rate of steroid hormones **(**Wiltbank et al., 2006). Increased dry matter intake in high producing cows causes an increase in the liver blood flow, accelerating its metabolic clearance rate of both estradiol (E2) and progesterone (P4) and reducing their peripheral concentrations (Sangsritavong et al., 2002). Reduced peripheral concentrations of E2 might affect estrus expression (Lopez et al., 2004), and ovulatory processes, while reduced P4 might affect follicular maturation and embryo development (Wiltbank et al., 2006). These altered ovarian processes prevalent in dairy cows are known factors associated with reduced fertility (Lamming and Darwash, 1998; Lucy, 2001; Ribeiro et al., 2016a; Santos et al., 2016a).

To overcome the challenge of achieving high reproductive efficiency, many techniques and strategies have been developed to increase the proportion of cows submitted to AI in a timely manner after the elective waiting period (Stevenson et al., 2001; Chebel and Ribeiro, 2016). Such strategies include programmed breeding methods to control estrous cycle events (i.e.

synchronization of estrus and ovulation) through administration of exogenous hormones (Stevenson et al., 2001; Wiltbank and Pursley, 2014), and electronic aids to detect estrus behaviour (Roelofs et al., 2010; Valenza et al., 2012). Methods for synchronization of ovulation can provide greater reproductive performance and profit per cow compared with AI on detected estrus alone (Galvão et al., 2013; LeBlanc, 2007), and has gained popularity among dairy producers in the past decades (Chebel and Ribeiro, 2016). Although synchronization protocols also benefit cows with an ovarian dysfunction (e.g. anovular cows or cows not displaying estrus) compared to the use of aids that just detect estrus (Fricke et al., 2014; Chebel and Ribeiro, 2016), the proper implementation of synchronization protocols requires considerable labor force training, and the pressure to reduce the use of hormones in food production system is increasing by governmental organizations (Lane et al., 2008).

More recently, a biochemical sensor technology to measure milk P4 in-line during milking to determine spontaneous estrus and pregnancy (Herd Navigator™, DeLaval International, Tumba, Sweden) has become commercially available in Europe (~ 250 units) and in Canada (~ 50 units). As P4 is a key hormone that regulates reproductive cycles, frequent monitoring of in-line P4 values provide real-time physiological information of reproductive function (Saint-Dizier and Chastant-Maillard, 2012; Yu and Maeda, 2017), such as anestrous status, ovulation and luteolytic events, and P4 levels at different time points in the estrous cycle. Thus, the assessment of in-line P4 data offers a novel opportunity to evaluate postpartum ovarian activity in individual cows at whole population level and to better understand the relationship between ovarian function and fertility in modern dairy herds.

Therefore, the present thesis aims to initially review the importance of reproduction in the dairy industry, the physiological events related to ovarian function, and the altered reproductive physiology in modern dairy cows (Chapter 2). Then, three original research studies that aimed to better understand associations between luteal activity and fertility using in-line milk P4 data are presented. The general hypotheses were that different components of altered (i.e. abnormal) luteal activity, determined through in-line milk P4 data, are associated with reduced fertility at AI. The first study investigated relationships of (1) commencement of luteal activity and (2) luteal phase length and frequency preceding first AI, with parity and AI outcomes (i.e. open, pregnancy, pregnancy-loss) (Chapter 3). The objectives of the second study were to determine the dynamics of pre- and post-AI milk P4 profiles and their associations with parity and AI

outcomes (Chapter 4). The third study aimed to characterize cut-off values (i.e. thresholds) of parameters of luteal activity in the early postpartum period and before and after AI associated with reduced fertility in Holstein herds (Chapter 5). Lastly, Chapter 6 presents general discussion, recommendations for future research, limitations, and conclusions.

CHAPTER 2
LITERATURE REVIEW

2.1. REPRODUCTION IN THE DAIRY INDUSTRY

Artificial insemination (AI) is one of the first technologies that dramatically changed the dairy industry. The establishment of AI as a practical procedure in domestic farm animals was initiated in Russia in early 1900s, becoming widespread worldwide with the publication of the book "The Technique of Artificial Insemination" by Walton, in 1933 (Foote, 2002). The first AI organization for dairy cattle was established in Denmark in 1936, and the impact of AI in the industry was observed in their first year of operation through improved reproductive performance compared to that of natural service (Foote, 2002). This improvement stimulated the advancement of the AI technique in United States, where the use of AI achieved a phenomenal growth in the 1940s after strong collaborations between cooperatives and researchers. The use of AI became a worldwide established technique (Salisbury and VanDemark, 1962; Foote, 2002).

Advancements of AI technique associated with the improvement in testing and selecting bulls for specific traits enabled the industry to boost individual milk production of the dairy cow through rapid genetic progress. Over the last century in the United States, the individual cow milk production has dramatically increased from approximately 1,400 kg per year in 1910 (Pearson, 1917) to more than 10,000 kg nowadays (USDA, 2015), representing an yearly increase rate of more than 7%. In more recent times, there was an increase in the dairy cow milk yield in the United Kingdom of approximately 1.8% per year from 1975 to 1996 (Royal et al., 2000), similar to that in western Canadian reports from 1975 to 1996 (CanWest DHI, 2010). Expectedly during this dramatic change in the milk yield, all aspects related to dairying such as housing system, nutrition, feeding practice, milk harvesting method, animal health, and reproductive management have also dramatically changed (McGuffey and Shirley, 2015). Thus, the steady increase in milk production per cow has occurred due to a combination of improved management, better nutrition, and enhanced reproductive techniques in association with intense genetic selection (Lucy, 2001).

2.1.1. Decreased fertility in the modern dairy cow

Concomitantly with the increase in genetic merit for milk production over the past decades, several reports have demonstrated a gradual decrease in the dairy cow reproductive efficiency worldwide (Macmillan et al., 1996; Royal et al., 2000; Lucy, 2001). In the United States, conception rates were typically reported to be around 55% in the 1950s (Casida, 1961). From 1951 to 1996, Butler (1998) reported a decline in first-service conception rates from approximately 65 to 40%. Reports comparing data between 1970s and 2000s showed that the average 21-d pregnancy rate declined from 22 to 12% (De Vries and Risco, 2005), while conception rates decreased from 55 to 35% (Washburn et al., 2002), and number of services requires per conception increased from 1.6 to 2.9 (Lucy, 2001). During the same period, while the average milk production per lactation increased from 6,500 kg to 9,000 kg, the number of necessary AI per conception almost doubled, and the calving interval increased from by 1.5 months (Lucy, 2001). In another report (DeVries and Risco 2005), the interval from calving to first breeding increased by 20 d from 1980s to 2000s, while milk production per lactation increased 1.5% per year during the same period. From 1996 to 2007, the average intervals from calving to first breeding and from calving to conception increased by 7 and 12 d, respectively, and the overall conception rate decreased by 3% (Norman et al., 2009). A decline in conception rates of approximately 5% in both heifers and cows in Quebec herds was reported between 1990 and 2000 (Bousquet et al., 2004). Comparable trends in decreased reproductive efficiency have also been reported in Europe (Roche et al., 2000; Royal et al., 2000) and Australia (Macmillan et al., 1996).

A negative genetic correlation between milk production and fertility has been reported (Hansen et al., 1983; Roxström et al., 2001). However, the direct cause of poor reproductive performance in modern high producing dairy cows is likely a combination of several factors that influence both milk production and reproduction (Lucy, 2001). Fertility in dairy cows involves complex biological processes that can be influenced by a magnitude of biological, environmental, genetic, and managerial factors (Santos et al., 2010). These factors, such as feed practice, environment, and management, are often related to the intensification of farming systems and increased herd sizes (Lucy, 2001; Barkema et al., 2015). Increased milk production has been associated with increased incidences of diseases (Koeck et al., 2014). However, it is

possible that part of the reason for increased incidence of health disorders (Kaneene and Miller, 1994) was the abrupt increase in herd sizes (Barkeman et al., 2015), which possibly contributed to worsened animal welfare (von Keyserlingk et al., 2009). In this regard, the occurrence of periparturient diseases is a limitation to high fertility in dairy cows (Santos et al., 2010; Ribeiro et al., 2016a). In combination with health disorders and management aspects, the dairy cow fertility is also affected by physiological failures such as prolonged interval from calving to resumption of reproductive cyclicity (i.e. anestrus) and poor estrous behaviour (Santos et al., 2010).

In the 1960s, the interval from calving to resumption of reproductive cyclicity was estimated to be approximately 14–21 d, and the incidence of anestrous was only 5% (Marion and Gier, 1967). More recently, Stevenson (2001) reported that on average, modern dairy cows had anestrus periods of 10 d longer. By evaluating data between 1964 and 2000 in Minnesota, Lucy (2001) reported an increase in the interval from calving to resumption of cyclicity from 29 to 43 d, and an increase in the incidence of anestrous from 0 to 38%. However, it is likely that the increase in the average interval from calving to resumption of cyclicity observed in populations of modern dairy cows is highly influenced by a subpopulation of cows with abnormal physiology (i.e. prolonged anestrus) (Lucy, 2001).

The increase in milk production has also led to an increased challenge in inseminating cows due to poor estrus expression. From 1985 to 1999 alone, estrus detection rates declined from 51 to 42% in Holstein herds in the USA (Washburn et al., 2002). The average duration of behavioural expression of estrus decreased from 15 h in 1970s (Esslemont and Bryant, 1976) to 7 h nowadays (Diskin, 2008; Sveberg et al., 2015). Although the average duration of estrus in modern dairy cows is estimated to be approximately 7 h (Dransfield et al., 1998; Diskin, 2008; Sveberg et al., 2015), 25% of the cows express estruses with low intensity and shorter duration. When comparing to Norwegian Red cows, Holstein cows showed estrus with shorter duration of standing (7 vs. 12 h) and mounting (7 vs. 18 h) periods and with less mounting events per h (18 vs. 46; Sveberg et al., 2015). Thus, the conventional strategy to observe cows in estrus for 30 min twice per d might not be sufficient to detect all cows in estrus (Lucy, 2001), at least in Holstein herds. The reduction in estrus expression is likely to be directly related to the milk production level, as higher producing (46 kg/d) cows show less estrus behaviour than lower producing (36 kg/d) cows (Lopez et al., 2004). In this regard, Diskin (2008) reported that cows

producing 55 kg/d expressed less than 3 h of estrus, while cows producing 25 kg/d expressed almost 15 h of estrus. Likely due to the influence of milk production, some other reproductive dysfunctions have higher incidence in lactating cows compared to heifers, such as multiple ovulation (Sartori et al., 2004), twin pregnancies (Ryan et al., 1991; Sartori et al., 2004), and pregnancy losses (Santos et al., 2010).

Another physiological dysfunction prevalent in modern lactating dairy cows is the reduced peripheral concentrations of steroid hormones associated with high milk production (Wiltbank et al., 2006). Lactating cows have lower estradiol (E2) and progesterone (P4) concentrations than non-lactating cows (Sangsritavong et al., 2002). Lower circulating E2 and P4 might affect the regulation of the hypothalamus-ovarian-axis responsible for ovarian processes, and cause abnormal ovarian activities (Wiltbank et al., 2006). In this regard, several studies have reported an increased incidence of abnormal luteal phases (i.e. ovarian activity) in modern dairy cows (Opsomer et al., 1998; Blavy et al., 2016). Opsomer et al. (1998) reported high-yielding Holstein cows having increased incidences of anestrus (21 vs. 7%) and of prolonged luteal phases (20 vs. 3%) compared to moderate-yielding Friesian cows. Moreover, the prevalence of cows having at least one abnormal cycle prior to first service increased from approximately 30% in 1980s (Lamming and Darwash, 1998) to more than 50% nowadays (Shrestha et al., 2004; Ranasinghe et al., 2011). The occurrence of abnormal ovarian activity (i.e. anestrus and prolonged luteal phases) prior to first service is well known to be associated with reduced fertility (Lamming and Darwash, 1998; Opsomer et al., 2000; Shrestha et al., 2004; Ranasinghe et al., 2011; Ribeiro et al., 2016a).

It is commonly accepted that the estrous cycle length in domestic cattle (*Bos taurus*) is 21 d, with an expected range between 18 and 24 d (Savio et al., 1990; Forde et al., 2011). However, recent studies have reported increased estrous cycles lengths in modern dairy cows. Royal et al. (2000) showed that from 1970s to 1990s, the average interovulatory interval increased from 20 to 22 d. Although this was still within the expected range of 18–24 d, the increased average in the cycle length may represent an increased variability in the estrous cycles among cows, which might be one extra factor associated with low fertility (Royal et al., 2000). More recently, Remnant et al. (2015) evaluated 114,572 inter-service intervals from 42,252 cows in 159 herds in the United Kingdom and reported that the average interval between services was 22 d rather than the expected 21 d, with 90% of the observations ranging between 18 and 28 d. In addition, Blavy

et al. (2016) evaluated luteal phases from different dairy breeds and observed a high variability in the luteal phase length [80% of the luteal phases ranged between 13 and 31 d, while the expected luteal phase length in *Bos taurus* is between 14 and 18 d (Forde et al., 2011)]. It is becoming evident that the commonly suggested estrous cycle length of 18 to 24 d might not reflect the real estrous cycle length in modern lactating dairy cows (Remnant et al., 2016).

2.2. REPRODUCTIVE PHYSIOLOGY

The reproductive function of a female bovine is characterized by estrous cycles that start after puberty (approximately 6 to 12 months of age) and continue through most of her life (Forde et al., 2011**)**. Cattle are polyestrous animals and have continuous cycles approximately every 21 d. The endocrine mechanism that controls the cyclicity is mainly regulated by the hypothalamus [Gonadotrophin-Releasing Hormone GnRH)], pituitary [(Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH)], ovaries (P4, E2, and inhibin), and the uterus [Prostaglandin $F_{2\alpha}$ (PGF)], through positive and negative feedback controls (Roche, 1996). The mechanism can be altered by several factors such as pregnancy, nursing, and season of the year (in some species), and can be affected by environmental, nutritional, metabolic, or pathological conditions. The estrous cycle is distinguished into four stages: proestrus, estrus, metestrus, and diestrus, or may be divided into follicular (proestrus, estrus) and luteal (metestrus, diestrus) phases of the cycle. The proestrus stage (2-3 d long) is the transition between the cessation of the previous estrous cycle (characterized by a luteolysis and decline in P4 to its basal levels) and the initiation of the new cycle (Forde et al., 2011).

The first reproductive event of the estrous cycle is the estrus. It is characterized by the presence of a growing dominant follicle that produces increasing amounts of E2. High levels of E2 in combination with low (basal) P4 levels induce profound behavioural changes and physiological alterations in the reproductive tract for sexual receptivity (Roelofs et al., 2010). The duration of behavioural sexual receptivity, characterized by increased standing and mounting activity, varies between 8 and 18 h in cattle and can be affected by a variety of factors at cow- or environmental-level. Cow factors include lactation status (López-Gatius et al., 2005), level of milk production (Lopez et al., 2004), health status (Walker et al., 2008), and stress (Dobson and Smith, 2000; Wolfenson et al., 2000). Environmental factors include nutrition

(Orihuela, 2000), seasonality (Wolfenson et al., 2000; Lopez-Gatius et al., 2005), housing type (Vailes and Britt, 1990), and herd size, as cows from larger herds are less likely to be visually detected in estrus (Hurnik et al., 1975). Once E2 reaches its peak concentration during estrus, it triggers a positive feedback signalling the release of GnRH by the hypothalamus (Allrich, 1994). The GnRH acts on the pituitary gland triggering the LH surge responsible for inducing ovulation of the dominant, pre-ovulatory follicle 20 to 30 h later (Roelofs et al., 2010). The combination of the presence of a pre-ovulatory follicle, basal P4 levels, and LH pulses occurring every 40 to 70 min for 2 to 3 d, are all factors determining the ovulation of the dominant follicle (Roche, 1996). This cascade of events related to the ovulation culminates in the release of the oocyte into the oviduct. During the next 3 to 4 d following ovulation, the post-ovulatory follicular cells transform into luteal tissue, when P4 starts to be gradually produced in the metestrus stage.

The metestrus stage is the transition from E2 dominance to P4 dominance phases, characterized by the onset of ovulation, intense cellular and structural remodeling of the follicular tissue, and the formation of a functional intra-ovarian endocrine gland called CL (Forde et al., 2011). The preovulatory LH surge is responsible for alterations in the follicular cells, stimulating luteinisation of the theca and granulosa cells into luteal cells that produce P4 instead of E2. During the ovulatory process, an invagination of follicular wall and rupture of basal membrane allows the migration of theca cells, fibroblast and endothelial cells to the centre of the newly formed CL. In dairy cows, the CL has one of the greatest blood flow per unit of tissue among the entire organism, consuming oxygen in the cellular-level 2 to 6 times more than that for other important organs such as liver, kidney or heart (Robinson et al., 2008). Although 50% of the CL cells are endothelial, the luteal cells (30%) are the responsible for its main function: production and secretion of P4 (Miyamoto et al., 2010) throughout the luteal phase to maintain pregnancy if fertilization occurs and a conceptus is present (Forde et al., 2011).

2.2.1. Increasing progesterone following ovulation

Progesterone (4-pregnene-3,20-dione) is a steroid hormone secreted from cholesterol (low- and high-density lipoproteins) by the luteal cells (Fitz et al., 1982). In bovines, the quantity of P4 secreted by the CL depends on the steroidogenic capacity and quantity of luteal cells in the luteal tissue. In dairy cows, larger ovulatory follicles develop into larger CL post-ovulation, producing

more P4 levels than smaller CL resulting from smaller ovulatory follicles (Vasconcelos et al., 2001). The CL in high-producing dairy cows secretes a daily amount of P4 that results in approximately 5.6 ng/mL of P4 per d in plasma (Wiltbank et al., 2012a). Considering that high-producing dairy cows have a metabolic clearance rate of P4 at 3,100 L/h, the total P4 produced by the CL was estimated to be approximately 416.2 mg per d (Wiltbank et al., 2012a).

After ovulation, the CL starts to gradually secrete P4, reaching elevated concentrations approximately 2 to 5 d after ovulation, initiating the diestrous stage (Forde et al., 2011) with P4 concentrations > 8 ng/mL in plasma or > 20 ng/mL in milk (Roelofs et al., 2006). The diestrus is the longest stage of the estrous cycle, characterized by the period in which the CL is fully functional and secreting high amounts of P4 during the entire luteal phase of the cycle, which ranges from 14 to 18 d in length; Forde et al., 2011), or for the entire duration of a pregnancy. If fertilization occurred following estrus, increasing P4 concentrations would promote a quiescent uterine environment suitable for early embryo development, attachment of the conceptus to the endometrium, nourishment and survival of the embryo/fetus, and normal parturition (McDonald et al., 1952). In this regard, a fast increase in P4 levels following ovulation was reported to be a major factor benefiting conceptus elongation and development (Carter et al., 2008; Clemente et al., 2009). On the other hand, a slow rise in P4 concentrations retarded embryo development (Forde et al., 2011). This influence of increasing P4 concentrations on embryo development is likely caused by enhanced endometrial environment, as exogenous P4 supplementation after insemination advances expression of endometrial genes responsible for energy source and composition of histotroph (Forde et al., 2009). When a successful pregnancy establishment does not occur, a spontaneous luteolysis will regress the CL resulting in the initiation of a new estrous cycle.

The mechanism responsible to cause CL regression is coordinated by endometrial pulses of prostaglandin $F_{2\alpha}$ (PGF) at the end of the diestrous stage. The PGF is released by the endometrium and its synthesis is regulated by the prolonged uterine exposure to P4 during the diestrous stage (at approximately d 14 of the luteal phase). The PGF reaches the ovary through the utero-ovarian vein and ovarian artery (Hixon and Hansel, 1974). One to two pulses of PGF in 24 h are required to promote regression of the CL (Wiltbank and Ottobre, 2003), and this pulsatile pattern of PGF is coordinated by oxytocin regulators in the uterus and in the CL (Silvia et al., 1991). However, when fertilization occurs and a conceptus is present, the embryonic

trophectoderm cells produce interferon-tau, which is a Type I interferon constituted of a single chain amino-acids. The interferon-tau is released into the uterus and it acts blocking the upregulation of oxytocin and E2 receptors in the endometrium, preventing the pulsatile secretion of endometrial PGF (Forde et al., 2009). In addition, the interferon-tau promotes the release of interferon-stimulated genes in the endometrium, which are associated with conceptus fixation and maintenance. Both the suppression of luteolytic mechanism and promotion of interferon-stimulated genes release by the endometrium are dependent on P4 (Spencer et al., 2007). Thus, a successful maintenance of pregnancy in cattle will be characterized by the presence of a CL and a high P4 environment starting from a few d after ovulation throughout the approximate 280 d of gestation.

2.2.2. Follicular development and progesterone priming

During most of the stages of the estrous cycle, the pituitary gland is releasing FSH in response to GnRH, which induces waves of follicular growth. Dairy cows usually have 2 follicular waves during a regular estrous cycle (Sartori et al., 2004; Forde et al., 2011). During each emergence of a follicular wave, a group of 2 to 4 mm follicles begin to grow and, after 2 to 3 d and when FSH reaches its peak concentration, normally only one follicle is selected to become dominant (with approximately 8.5 mm). This dominant follicle continues to grow (Pierson and Ginther, 1988) until it ovulates with 10–20 mm (Wiltbank et al., 2002). After dominance, there is a decline in FSH levels due to E2 and inhibin being secreted by the dominant follicle and by all other follicles ≥ 5 mm (Gibbons et al., 1997)**.** In modern dairy cows, the emergence of the second follicular wave occurs at approximately d 11 and the spontaneous luteolysis occurs at approximately d 19 after ovulation, and the interval between spontaneous luteolysis and next ovulation is 5 d (Sartori et al., 2004). Sartori et al. (2004) reported an average interval between two consecutives ovulations (i.e. inter-ovulatory interval; indicating the estrous cycle length) of 23 d. Usually during the first follicular wave, the dominant follicle is mature during the diestrous stage, so the high circulating P4 keeps the LH pulses at low level, preventing the dominant follicle from ovulating, which then undergoes atresia (Rage et al., 1980).

Progesterone plays an important role in the follicular development. Circulating P4 levels indirectly regulate LH frequency, which controls the development and maturation of the dominant follicle. The LH pulse frequency is lower during luteal phases (period with high circulating P4) than during follicular phases (period with low circulating P4; Forde et al., 2011; Endo et al., 2012). Also, Goodman et al. (1982) treated ovariectomized ewes with exogenous P4 and reported decreased frequency of LH pulses in the treated group (i.e. high P4 group). Although the direct effect of P4 on follicular development is unclear, P4 receptors have been localized in bovine oocytes and cumulus cells (Aparicio et al., 2011). However, the accepted mechanism of P4 regulation of follicular development is through indirect effects on LH pulse frequency (Endo et al., 2012). Dominant follicles growing under lower circulating P4 levels might have advanced maturation in relation to the resumption of meiosis, reducing oocyte quality and subsequent embryo survival. For instance, Revah and Butler (1996) reported that oocytes from persistent (i.e. over-matured) follicles had morphological signs of germinal vesicle rupture before ovulation, which might have been caused by an over-exposure to high frequency LH pulses.

2.2.3. Late gestation and early postpartum

Since the initiation of the diestrous period following the fertilization, the entire gestation period is characterized by high concentrations of circulating P4. Similarly, circulating E2 begins to increase at approximately 60 d of gestation, and remains elevated throughout the gestation (Crowe et al., 1998). The combination of both high circulating P4 and E2 suppresses the hypothalamic-pituitary axis function associated with reproductive events by inhibiting secretion of GnRH by the hypothalamus and release of LH by the pituitary (Nett, 1987). Towards the end of the gestation period, there is a change in hormonal profile and circulating P4 concentrations start to gradually decrease, while E2 progressively increases until a high peak (50 to 1000-fold higher concentrations) immediately preceding parturition (Patel et al., 1999).

Although release of LH is suppressed during pregnancy, changes in FSH levels are less evident during gestation and are usually similar to that of cycling cows. However, FSH surges are less frequent during the last wk preceding parturition (Crowe et al., 1998), resulting in the absence of follicular wave emergence during this period (Ginther et al., 1996). Preceding

calving, P4 levels start to gradually decrease, while E2 concentrations increase, achieving its peak (2-fold higher concentrations) in the last 24 h. Most of this high circulating E2 is caused by the placental E2, and E2 levels sharply decline to basal levels right after calving (Patel et al., 1999). As the CL regresses, the endocrine environment right after calving is characterized by basal concentrations of both P4 and E2, allowing the hypothalamic-pituitary axis to return to its regular function by releasing GnRH and consequently stimulating the release of FSH and LH (Crowe et al., 1998).

As early as 3 d after calving, the FSH surges and leads a first new follicular wave, which promotes the growth of a follicle until it reaches dominance (approximately 8.5 mm; Pierson and Ginther, 1988). Beyond this point, the dominant follicle depends on LH pulses to continue its development to the pre-ovulatory stage. Then, this follicle is expected to produce high concentrations of E2 to induce the LH surge that triggers the ovulatory process (Crowe et al., 1998), restarting the postpartum cyclicity. However, the LH reserves in the pituitary are often still very low right after calving, taking up to 3 wk to restore adequate amounts of LH. While LH pulses are unavailable, the development of dominant follicle is largely affected and, consequently, the dominant follicle is not capable of producing quantities of E2 necessary to trigger the LH surge and the first ovulation (Sakaguchi, 2011). As the lack of pulsatile LH might affect first dominant follicle development, this follicle might regress (in approximately 35% of cases) or become anovulatory (in approximately 20% of cases), developing into a cyst (Savio et al., 1990; Beam and Butler, 1997). The fate of the dominant follicle in the first postpartum follicular wave is a primary factor affecting the reestablishment of postpartum ovarian activity (Peter et al., 2009).

2.3. TRANSITION PERIOD AND EARLY POSTPARTUM OVARIAN ACTIVITY

From late gestation to early postpartum, the dairy cow undergoes several endocrine and metabolic changes that require high amounts of energy, nutrients and minerals to adapt to the new lactation (Santos et al., 2010). This transition period (3 wk before and 3 wk after calving) is crucial to regulate health status and milk production. This regulation of energy status, liver

function and mammary gland demand are key components to adapt to the new lactation (Drackley, 1999). This period is characterized by a rapid fetal growth in the last wk of gestation concomitant with colostrogenesis and onset of lactation, dramatically increasing the cow's nutrient needs (Bell, 1995). However, the dramatic increase in nutrient requirement is not followed by a proportional increase in feed intake. In addition, in order to account for the increasing milk production, the partitioning of glucose usage is proportionally greater to the mammary gland than to the other physiological processes (Bell, 1995; Santos et al., 2010).

2.3.1. Health disorders

The challenge of this transitional adaptation often results in a high incidence of diseases and metabolic disturbances in the postpartum period. These health disorders are known to be associated with fertility problems (Gröhn and Rajala-Schultz, 2000; Santos et al., 2010; Ribeiro et al., 2016a; Santos et al., 2016), such as anestrus (Peter et al., 2009; Santos et al., 2016), delayed ovulations (Wiltbank et al., 2006), and lower embryo quality (Cerri et al., 2009). Evaluating records from almost 6,000 dairy cows in the US, Santos et al. (2010) reported only 56% of healthy cows in early postpartum, with the high incidence of diseases that occurred during first 60 d postpartum negatively affecting fertility. The most common disorders reported were fever (21%), clinical endometritis (21%), metritis (16%), dystocia (15%), mastitis (12%), ketosis (10%) and lameness (7%). Greater impact on fertility was seen in cases of: (1) metritis, declining the likelihood of being cyclic at 65 d postpartum by 20%; (2) clinical ketosis, reducing conception rate at first AI from 51 to 29%; and (3) lameness, increasing odds of pregnancy losses by 17.5% (Santos et al., 2010).

Several other studies also reported that cows suffering from metabolic or uterine diseases had prolonged anestrus ((Peter and Bosu, 1988; Lamming and Darwash, 1998; Opsomer et al., 2000; Vercouteren et al., 2015), lower conception rates, and greater incidence of pregnancy losses than healthy cows (Ribeiro et al., 2016a). The association between occurrence of diseases and reproductive dysfunction is likely caused by altered function of the hypothalamic-pituitary-ovarian axis. Uterine diseases might suppress the hypothalamic GnRH secretion, possibly impairing pituitary LH release and delaying folliculogenesis on ovarian function (Peter and Bosu, 1988), which is likely caused by inflammatory responses on the reproductive tract

(Sheldon and Dobson, 2004). For instance, cows suffering from subclinical infections tend to experience a prolonged uterine secretion of PGF in the first 3 wk postpartum, which might prevent the onset of ovarian cyclicity and prolong the anestrus period (Peter and Bosu, 1988). In addition, the reproductive function of dairy cows is extremely sensitive to physiological stressors (Dobson and Smith, 2000) such as heat stress (Wolfenson et al., 2000; Hansen, 2009), which has an additive effect when the cow is lactating due to increased metabolic rate, causing reproductive losses such as ovulation failures (Lopez-Gatius et al., 2005), and lower pregnancy rates (Ambrose et al., 1999). These findings indicate that metabolic disorders play an important role to reproductive function of the modern dairy cows. Furthermore, cows undergoing health disorders may experience a decreased appetite, reducing feed consumption and consequently inadequate nutrient intake, leading to a postpartum negative energy balance (Butler, 2003).

2.3.2. Negative energy balance

For the modern cow producing almost 11,000 kg of milk per year (USDA, 2015), the mammary gland demands expressive amounts of nutrients, which results in less partition of nutrients to others biological events in the early postpartum period, such as reproduction (Santos et al., 2010). Furthermore, the decline in feed consumption in late gestation results in less feed intake early postpartum. Thus, postpartum lactating cows are not capable of acquiring the required nutrients to meet the energy needs for milk production and maintenance (Drackley, 1999). The demand for calories exceeds that obtained by the feed intake, and approximately 90% of the calories obtained through feed is consumed by the mammary gland alone (Bell, 1995; Drackley, 1999). For instance, a cow producing 50 kg/d requires 53 Mcal/d, compared to 12.5 Mcal/d for a non-lactating cow (NRC, 2001). These events cause an excessive mobilization (i.e. loss) of body reserves, resulting in a negative energy balance (NEB). The NEB is more intense in cows with excessive body condition (i.e. fat cows) at calving, probably due to a lower appetite compared to cows with lower body condition (i.e. thin cows). The NEB may extend until 10 to 12 wk postpartum (Butler, 2003), usually reaching its nadir in the second wk (Bell, 1995).

Sufficient feed consumption is required to stimulate adequate glucose production and insulin secretion and maintain all the biological processes (Drackley, 1999). Insufficient feed intake and a NEB leads to a shortage of exogenous glucogenic precursors and, consequently,

hypoinsulinemia (Grummer et al., 2004). Hypoinsulinemia is associated with high systemic concentrations of catecholamine and glucocorticoids, which stimulates hormone-sensitive lipases in adipose tissue to mobilize fatty acids (Drackley, 1999). In addition, these changes in the partition of nutrients stimulate the utilization of endogenous precursors, such as increasing hepatic gluconeogenesis, decreasing peripheral glucose utilization through insulin resistance, and increasing muscular amino acid mobilization and lipolysis (Bell, 1995). The lipolysis of tri-acyl glycerides (stored as fat tissue) releases glycerol and non-esterified fatty acids (NEFA) in circulation, an important source of energy for the cow in NEB (Drackley, 1999; Fukao et al., 2004). The NEFA are mainly absorbed by the mammary gland, but they are also used by the liver for its own energetic needs by oxidizing the NEFA to synthesize adenosine triphosphate. However, when there is an exacerbated mobilization of adipose tissues in the cow in NEB, there is an excessive hepatic oxidation of NEFA (Drackley, 1999). When the oxidative capacity exceeds, the liver starts to partially oxidize NEFA into ketone bodies, such as β-hydroxybutyrate (BHBA), which can be used as an alternative energy source by the tissues (Drackley, 1999; Fukao et al., 2004). Another pathway of excessive NEFA in the liver is its re-esterification in tri-acyl glycerides and re-storage as adipose tissue, or its release in the form of very low-density lipoprotein (Drackley, 1999).

A cow during NEB is characterized by low circulating concentrations of glucose and insulin, high circulating concentrations of NEFA and BHBA, and consequently low concentrations of circulating insulin-growth factor (IGF-1) (Butler, 2003). The low IGF-1 during period of NEB might impair follicular steroidogenesis, as cows exposed to hyperinsulinemic clamp during NEB had increased concentrations of plasma IGF-1 (Butler et al., 2004). In this regard, IGF-1 seems to be essential for adequate expression of steroidogenic enzymes and E2 production by the follicle, as increased concentrations of E2 were seen in cows supplemented with insulin (Butler et al., 2004). Greater concentrations of IGF-1 were also associated to improved ovulatory capacity (Butler, 2003) and conception rates (Taylor et al., 2004). Also, other studies demonstrated that inducing high insulin concentrations shortened the postpartum anestrus (Gong et al., 2002; van Knegsel et al., 2007), potentially enhancing follicular quality and ovulatory capacity. In addition to E2, insulin and IGF-1 are also responsible to induce expression of LH receptors and improve responsiveness to LH, which are essential factors for follicle growth and oocyte maturation (Lucy, 2000; Webb et al., 2004). Cows genetically

selected for higher milk production have the somatotropic axis affected, and have reduced expression of growth hormone (GH) receptors in the liver (Lucy et al., 2009). The reestablishment of GH receptors is highly dependent on insulin (Butler 2003), which is also responsible for IGF-1 synthesis.

The NEB can directly affect the hypothalamic secretion of GnRH. As both reproduction and feed intake are controlled by similar neurological centres, hormonal mediators and modulators (Schneider, 2004), a deficit in oxidizable metabolic fuels (i.e. in NEB) would affect the hypothalamus by impairing GnRH secretion. In this regard, pubertal heifers under an induced NEB (approximately 23% of body loss) had prolonged anestrus (Diskin et al., 2003)**.** These mechanisms suggest that, from an evolutionary perspective, animals under low availability of nutrients (i.e. NEB) might favour survival over the reproductive processes; while under nutrient abundance, reproductive processes might be optimized (Schneider, 2004).

Energy status is capable of dramatically changing ovarian processes and delaying the resumption of cyclicity after calving (Beam and Butler, 1999). Especially during NEB, the dominant follicle from the first follicular wave is more likely not to ovulate, either regressing or becoming anovulatory, and the NEB was seen to contribute by extending the first ovulation to 40 to 50 d postpartum (Beam and Butler 1997). The incidence of non-ovulated cows at 50 d postpartum can be up to 55% (Beam and Butler, 1999). Even after undergoing ovulation, cows that had delayed first ovulation have their subsequent reproductive performance affected (Ambrose and Colazo, 2007; Santos and Rutigliano, 2009). The NEB is also associated with poor neutrophil function (Grinberg et al., 2008), which might impair postpartum uterine involution and uterine health, compromising subsequent reproductive performance (Wathes et al., 2009).

As NEB has detrimental effects on regulation of GnRH, LH, insulin, and IGF-1, cows under NEB might have increased risk of having prolonged anestrus (Butler 2003), abnormal cycle lengths (Chen et al., 2015), and lower fertility (Santos et al., 2009). Chen et al. (2015) reported that cows that had abnormal estrous cycles (longer than 24 d) were those experiencing a worse postpartum energy balance and metabolic status (lower glucose, insulin and IGF-1, and higher BHBA), compared to cows that had normal cycles (between 18 and 24 d in length). Similarly, Hommeida et al. (2005) reported a more severe NEB in cows with prolonged luteal

phases. Although the mechanisms associating NEB and abnormal luteal phases are not fully understood, it is likely that prolonged luteal phases are caused by uterine diseases, which compromises PGF secretion (Opsomer et al., 2000, Shrestha et al., 2004, Ranasinghe et al., 2011). In this regard, cows having more uterine diseases were possibly those experiencing a more severe NEB. Another mechanism associating NEB with the occurrence of abnormal cycles is the reduced circulating E2 due to reduced IGF-1 in cows under NEB. This reduced E2 could result in decreased synthesis of P4 receptors, reducing the period of P4 dominance in the uterus. This could lead to early synthesis of oxytocin that initiates positive feedback to PGF release, causing a premature luteolysis (Mann and Lamming, 2000) and, consequently, a short (i.e. abnormal) luteal phase.

Several studies have reported NEB being associated with prolonged anestrus (Shrestha et al., 2004; Wathes et al., 2007) and poor fertility at AI (Butler 2003; Santos and Rutigliano, 2009). Cows with greater body condition during the first 14 d after calving had an earlier resumption of cyclicity postpartum (Chen et al., 2015). Also, greater body condition at initiation of the reproductive program (i.e. after the elective waiting period) was associated with greater P4 concentrations during the estrous cycles, resulting in improved conception rates (Stevenson et al., 1999). Despite the indirect effects of NEB, lipid metabolites involved in the regulation of energy status have been reported to negatively affect ovarian cellular function in-vitro by modulating proliferation and steroidogenesis in granulosa cells (Vanholder et al., 2005). Also, in-vitro cultured oocytes in media with greater concentrations of NEFA and BHBA had delayed maturation, fertilization, and lower cleavage rates (Leroy et al., 2006).

2.3.3. Anestrus and pregnancy maintenance

Several studies demonstrated that a delayed resumption of postpartum ovarian activity has detrimental effects on subsequent conception rates (addressed in Chapter 3) and embryonic and fetal survival (Wiltbank et al., 2014; Ribeiro et al., 2016a). The mechanism associated with anestrus and lower conception rates is likely related to the lower P4 concentrations during the growth of the ovulatory follicle (Wiltbank et al., 2014). Furthermore, the effects of anestrus on pregnancy losses might be related to shifts in gene expression observed in conceptus cells from cows that underwent prolonged anestrus (Ribeiro et al., 2016a). Elongated conceptuses on d 15

of development from cows that were in anestrus had a downregulation of transcripts involved with IGF-1 regulation, such as phosphatidylinositol/AKT (Ribeiro et al., 2016b)**.** This might affect energy metabolism, cell survival, proliferation, and migration during establishment of pregnancy (Kim et al., 2010; Santos et al., 2016b). Transcripts associated with DNA repair were also downregulated in embryos from anovular cows, such as TOP1 and NBN, which are two transcripts essential for embryo survival in mouse models (Morham et al., 1996). Furthermore, embryos from cows that underwent anestrus had upregulation of transcripts that were associated with apoptosis, autophagy, and consequently embryo mortality and pregnancy losses (Toder et al., 2002; Santos et al., 2016b)

The differences in gene expression in embryos from cows that had been anestrus suggest that some of the negative effects of anestrus on embryo survival might be expressed later in gestation. This would explain the higher prevalence of pregnancy losses in cows that had prolonged anestrus (Santos et al., 2016; Chapter 3). Another pathway linking anestrus and poor embryo survival is the occurrence of postpartum diseases (Santos et al., 2010). Embryos from cows that suffered inflammatory diseases had upregulation of major histocompatibility complex-I heavy chain BOLA gene (Ribeiro et al., 2016b), suggesting that these embryos are more vulnerable to the maternal immune system and less likely to be maintained to term (Ribeiro et al., 2016b).

2.3.4. Primiparous vs. multiparous cows

Although lactating cows under NEB have an altered regulation of metabolites that cause reproductive failures (such as anestrus and prolonged estrous cycle), effects of NEB seem to be inconsistent among cows of different parities. Usually, the ideal dairy heifer achieves the first calving at about 24 months of age. Because at 24 months of age heifers are not yet physically mature (Coffey et al., 2006), their metabolic state requires nutrients for their body growth in addition to that for fetal growth, colostrum and milk production. Thus, primiparous cows are expected to have different control of tissue mobilization due to nutrient partitioning for growth and for the first lactation (Wathes et al., 2007). For instance, primiparous cows have greater plasma concentrations of IGF-1 (Wathes et al., 2007), glucose (Santos et al., 2001), and NEFA (Vandehaar et al., 1999), but lower BHBA concentrations (Wathes et al., 2007) in the

periparturient period than multiparous cows. The changes in the circulating concentrations of IGF-1, insulin, NEFA, and BHBA over the calving period are similar and coordinated for both parity groups; however, the control of tissue mobilization is different between primiparous and multiparous cows, as primiparous cows may limit partitioning of nutrients into milk production (Wathes et al., 2007). These differences suggest that the regulation of the hypothalamic-ovarian axis associated with postpartum reproductive function might also differ among different parities.

Several studies reported that primiparous cows were associated with delayed resumption of ovarian activity postpartum compared to multiparous cows (Bulman and Lamming, 1978; Vercouteren et al., 2015; Santos and Rutigliano, 2009; addressed in Chapter 3). For instance, Santos and Rutigliano (2009) reported that fewer primiparous cows had initiated ovarian activity by 65 DIM compared to multiparous cows (70 vs. 81%), although primiparous cows still had greater conception rates (38 vs. 30%) and lower pregnancy losses (9 vs. 17%) than multiparous cows. Similarly, Vercouteren et al. (2015) reported that multiparous cows had 1.5 greater odds of being cyclic at 21 DIM than primiparous cows. Although differences in partition of nutrients related to NEB between primiparous and multiparous cows suggest that primiparous cows suffer less from severe NEB, primiparous cows are more prone to develop calving problems and uterine diseases than multiparous cows (Vercouteren et al., 2015), which are risk factors for prolonged anestrus (Peter at al., 2009; Santos et al., 2016).

2.4. METABOLIC CLEARANCE RATE OF STEROID HORMONES

The NEB does not seem to explain all cases of anovulation, although the main mechanism associated with anestrus and failures in ovulatory processes the early postpartum period is the pulsatile LH secretion likely affected by NEB (Wiltbank et al., 2006). For instance, delayed first ovulation is not clearly associated with level of milk production (Lopez et al., 2005). Also, milk production does not seem to directly and consistently affect fertility parameters such as conception rate (Lucy, 2001; Washburn et al., 2002). However, lactating cows have much lower conception rates than heifers (Lucy, 2001; Royal et al., 2000; Washburn et al., 2002), and can produce less viable embryos than non-lactating cows (Sartori et al., 2002). Thus, it seems that the lactating status (i.e. lactating vs. non-lactating) influences conception rate more than the level of

milk production, as in a same herd with similar management, higher producing cows can have higher conception rates (LeBlanc, 2010) than lower producing cows.

Although effects of milk production on conception rates are inconsistent, it is clear that milk production level is associated with the duration of estrus expression (Lopez et al., 2004; Wiltbank et al., 2006). Lopez et al. (2004) reported a negative correlation between milk production and duration of estrus activity ($r = –0.51$) and between milk production and E2 concentrations ($r = –0.57$). In that study, higher producing cows (approximately 46 kg/d) had shorter estrus duration (6 vs. 11 h), less standing events (6 vs. 9 events), and less standing time (22 vs. 28 s) of estrus activity compared to lower producing cows (approximately 33 kg/d). In addition, higher producing cows had lower circulating E2 and larger pre-ovulatory follicles than lower producing cows (Lopez et al., 2004). High milk production is also directly linked to double ovulation rate, which is an underlying mechanism causing increased twinning rates in lactating dairy cows (Silva del Río et al., 2006; Wiltbank et al., 2006). It has been reported that double ovulation rates increase with increasing milk yield. Lopez et al. (2005) reported that a 50% double ovulation rate were seen in cows producing above 50 kg/d. Fricke and Wiltbank (1999) reported that cows producing greater than 41 kg/d had 20% incidence of double ovulation, and cows producing less than 41 kg/d had only 7% incidence of double ovulation, regardless of lactation number. Unfortunately, the mechanisms responsible for the increase in double ovulation rates above a certain critical amount of milk production are still unclear (Wiltbank et al., 2006).

Higher producing cows have larger ovulatory follicles (Lopez et al., 2004) and larger CL (Lopez et al., 2005), but lower circulating E2 (Lopez et al., 2004) and P4 (Lopez et al., 2005) than lower producing cows. The most accepted explanation for high producing cows having larger structures but lower circulating E2 and P4 is the increased liver metabolism of steroid hormones (Wiltbank et al., 2006). The liver is the major site of both E2 (Sangsritavong et al., 2002) and P4 (Parr et al., 1993) catabolism. Experiments evaluating different levels of feed consumption observed that an increased plane of feed intake increased the liver blood flow (Sangsritavong et al., 2002) and the excretion of P4 metabolites (Lopez et al., 2005). Sangsritavong et al. (2002) observed a correlation ($r = 0.92$) of the metabolic clearance rate between bromosulphthalein (an indicator of liver metabolic function; West, 1995) and P4 or E2 after different feeding quantities. The liver blood flow was greater in lactating (1561 L/h) than in

non-lactating (747 L/h) cows (Sangsritavong et al., 2002), and the metabolism of both E2 and P4 occurred at a greater rate (2.3 fold-increase) in lactating than in non-lactating cows (Sangsritavong et al., 2002). Thus, the lower steroid concentrations in higher producing cows might be caused by their continuous higher plane of nutrition, which increases the hepatic portal vein flow and the liver oxygen consumption (Parr et al., 1993), accelerating the liver metabolism in comparison to non-lactating cows (Wiltbank et al., 2006). In this regard, heifers had approximately twice as much E2 (6.5 vs. 3.2 pg/mL) and P4 (1.0 vs. 0.5 ng/mL) concentrations per mm^3 of follicular and luteal tissue volumes, respectively, compared to lactating cows (Sartori et al., 2004).

The reduced peripheral E2 and P4 in lactating cows might alter the mechanisms controlling reproductive cycles. Although follicles can grow at a regular rate prior to the pre-ovulatory stage (when they are FSH dependent), the elevated metabolic clearance of E2, reducing its peripheral concentrations, might force the follicles to grow to larger sizes to produce sufficient E2 to induce the GnRH/LH surge (Wiltbank et al., 2006). The delay in reaching E2 production peak that induces ovulation causes the pre-ovulatory follicle to be over-exposed to the LH pulses frequency (which are not affected by the metabolic rate) (Wiltbank et al., 2006). In addition, the lower circulating E2 links to the low duration of estrus expression in modern dairy cows is also related to milk production level (Lopez et al., 2004; Diskin, 2008; Roelofs, 2010). These processes contribute to delaying the follicular deviation and increasing the subordinate and dominant follicle size at deviation (Sartori et al., 2004), which, besides modifying LH pulses, contributes to ovulation failures, and therefore increases the risk of ovarian cysts (Wiltbank et al., 2000; 2002) and ultimately poor fertility.

In the following diestrus, follicular waves occurring under an environment with lower P4 and increased LH and FSH near time of follicular selection may be responsible (Wiltbank et al., 2006) for the much greater incidence of codominant follicles and multiple ovulations in lactating cows when compared to heifers (36 vs. 4% and 18 vs. 2%, respectively; Sartori et al., 2004). The lower circulating P4 might be responsible for a delay in FSH nadir and in increased LH pulses near follicle deviation, causing a selection of more than one dominant follicles (Wiltbank et al., 2014). In addition, the reduced rate of P4 rise following ovulation (at metestrus and early diestrus) could also be associated with reduced fertility (Folman et al., 1973). After ovulation, an optimal P4 rise is required for adequate establishment of pregnancy (Inskeep, 2004), and

increasing circulating P4 after AI is essential to promote uterine secretions of proteins and growth factors (Garrett et al., 1988), crucial for embryo development. Thus, the rate of P4 rise after ovulation is associated with oocyte quality and subsequent early embryo development (Mann et al., 1999).

Several studies have investigated the influence of circulating P4 concentrations before, during, and following time of ovulation with fertility at AI, but results were often inconsistent (Colazo et al., 2013; Monteiro et al., 2014; Bisinotto et al., 2015a). Lower circulating P4 during the spontaneous estrous cycle preceding AI was seen to reduce fertility at AI (Folman et al., 1973, Meisterling and Dailey, 1987), which is likely caused by higher LH pulses, lower follicular IGF-1 concentrations, and reduced luteal response to PGF (Cerri et al., 2011a; b). It was seen that supplementation of exogenous P4 (1.55 g) through an intra-vaginal device during the 7 d of follicular growth preceding luteolysis (during a timed AI synchronization protocol) increased conception rates (Colazo et al., 2017). Similarly, another study evaluated greater dose (2.76 g) of exogenous P4 supplementation during a timed AI synchronization protocol and reported improved conception rates (Bisinotto et al., 2013). However, the benefits in conception rates were only observed in cows that lacked a CL preceding AI comparing to cows that had the ovulatory follicle growing under basal P4 concentrations (Bisinotto et al., 2013). Both first and second wave follicles were more fertile when supplemented with P4 during follicular growth (Denicol et al., 2012). Also, cows that had greater circulating P4 at the initiation of a timed AI protocol (Dirandeh et al., 2015) or that started the protocol during diestrus (Cunha et al., 2008; meaning that follicle would grow under high P4) had lower risk of losing the pregnancy after AI compared to cows with lower circulating P4. These findings indicate that the increased metabolic clearance rate and consequently reduced circulating P4 in the estrous cycle preceding AI in lactating cows might be one underlying cause of decreased fertility in modern lactating dairy cows.

On the other hand, during estrus (around time of AI), a lower concentration of P4 is apparently beneficial to fertility (Brusveen et al., 2009; Ambrose et al., 2015; Colazo et al., 2017; Gobikrushanth et al., 2017). Increased P4 concentrations around the time of ovulation might reduce uterine or oviductal contractility, potentially impairing gamete transport and fertilization (Hunter, 2005). Blastocyst formation in vitro was reduced in high P4 media (Silva and Knight, 2000), and slight elevations in peripheral P4 concentrations were associated with reduced

endometrial thickness, which has been associated with reduced fertility (Souza et al., 2011). Several studies reported decreased proportions of cows pregnant after AI following timed AI protocols when circulating P4 concentrations were increased near time of AI (Wiltbank et al., 2014; Ambrose et al., 2015; Colazo et al., 2017). Specifically, pregnancy per AI was dramatically decreased if mean peripheral P4 concentration was above 0.4 ng/mL 16 h before AI (Wiltbank et al., 2014). Similarly, it has been reported that the optimal P4 concentration at time of AI was ≤ 0.5 ng/mL, and that P4 concentrations above 0.5 ng/mL reduced probability of pregnancy (Wilsdorf et al., 2016; Colazo et al., 2017). The reason for high circulating P4 concentrations near the time of AI is likely an incomplete luteolysis (Wiltbank et al., 2014). Although a partial luteolysis would still result in decreased circulating P4, P4 concentrations would be slightly higher compared to cases in which a complete luteolysis occurred. Numerous studies evaluating timed AI protocols reported that 5 to 30% of cows do not undergo a complete luteal regression after exogenous administration of PGF (Brusveen et al., 2009; Martins et al., 2011; Giordano et al., 2012b). In this regard, an incomplete luteolysis might have occurred if PGF was administered following a period with lower P4 concentrations, consequently reducing fertility (Martins et al., 2011).

Some of these studies (Martins et al., 2011, Giordano et al., 2012) reported lower rates of PGF-induced luteolysis in multiparous than in primiparous cows (e.g. 84 vs. 90%, $P = 0.03$; Giordano et al., 2012). Greater occurrence of incomplete luteolysis in multiparous than in primiparous cows could be a contributing factor to slightly elevated P4 near the time of AI and consequently, lower pregnancy per AI in multiparous than in primiparous cows (Tenhagen et al., 2004b; Santos and Rutigliano, 2009; Garcia-Ispierto and López-Gatius, 2016).

Following AI, the need of a CL and increasing P4 concentration for a successful pregnancy is unquestionable (Inskeep, 2004). Clemente et al. (2009) observed that in vitro P4 supplementation after fertilization had improved blastocyst yield, likely because P4 might have a direct effect on embryo development through early embryonic P4 receptors. Thus, P4 likely promotes advancement in conceptus elongation through favourable changes in the uterine environment (Clemente et al., 2009). Although effects of exogenous P4 supplementation on embryonic length on 13 and 16 d after AI was observed (Carter et al., 2008), reports on the degree of which P4 concentrations can affect fertility has been inconsistent. Some studies did not report relationships between level of P4 post-AI and fertility (Bulman and Lamming, 1978;

Mann and Lamming, 1999). Stronge et al. (2005) reported that circulating P4 levels at d 5, 6, and 7 post-AI were associated with increased pregnancy per AI, and that 60 to 85% of dairy cows evaluated had sub-optimal circulating P4 during early diestrus. Interestingly, Cummins et al. (2012) reported that cows with high genetic merit for high fertility had greater circulating P4 (34% higher concentrations) than cows with low genetic merit for fertility. Cows that successfully conceived at AI had greater plasma P4 at d 5, 6, 7 (Stronge et al., 2005), and 18 (Green et al., 2010) post-AI. Kenyon et al. (2013) reported that cows receiving embryo transfer at d 7 of the estrous cycle that had lower plasma P4 (< 5 ng/mL) on d 14 had greater odds of losing the pregnancy beyond d 28. The same study concluded that a faster rise in P4 levels during metestrus and early diestrus was positively associated with successful pregnancy establishment after embryo transfer.

The elevated metabolic clearance rate of P4 appears to have dramatic effects on fertility due to different mechanisms before and after AI. Reduced peripheral P4 in the estrous cycle preceding AI, slightly elevated P4 near the time of AI, and slow or delayed rise in P4 after AI might explain some of the high-incidence of reproductive failures frequently reported in the modern lactating dairy cow (Wiltbank et al., 2014).

2.5. REPRODUCTIVE MANAGEMENT TOOLS

Aiming to improve AI submission rates, accuracy of the timing of AI, and obtain greater pregnancy rates, several technologies and strategies for reproductive management have been developed over the years (Bisinotto et al., 2014; Chebel and Ribeiro, 2016). Such tools aim to manage or monitor a variety of time-dependent components of the estrous cycle to control the breeding program (Stevenson, 2001). Prostaglandin $F_{2\alpha}$ (i.e. PGF) and its analogues as luteolytic agents were developed in the 1970s as one of the first strategies to achieve targeted breeding by synchronizing time of estrus, resulting in considerable advancements to manage reproduction in dairy herds (Stevenson et al., 2001; Lauderdale, 2009; Chebel and Ribeiro, 2016). As PGF induces luteolysis in cows with a mature CL, exogenous administration of PGF allows to synchronize the time of luteolysis and, consequently, the time of estrus. A synchronization strategy for lactating cows with two injections of PGF given 11 to 14 d apart was one of the first commonly used methods on dairy farms in the USA (Nebel and Jobst, 1998; Stevenson et al.,

2001). This simple protocol addresses two scenarios: (1) cows with a mature CL at the time of the first PGF will have their CL regressed, come to estrus and ovulate, and consequently a mature and responsive CL will be present at the time of the second PGF; or (2) cows lacking a CL at the time of the first PGF will not respond, but will likely have a responsive CL (developed following a spontaneous ovulation) at the time of the second PGF. For both scenarios, cows are expected to come to estrus 2 to 3 d after the second PGF administration (Stevenson, 2001), depending on the follicular growth; thus, cows should be watched for estrus in order to receive AI.

In the late 1990s, protocols to synchronize ovulation (*Ovsynch*) using exogenous GnRH were developed (Macmillan and Thatcher, 1991; Twagiramungu et al., 1992; Pursley et al., 1995), which allows to inseminate cows at a fixed-time in relation to the hormones administration. In the Ovsynch protocol, a first GnRH injection is given at a random stage of the estrous cycle, aiming to induce a potential dominant follicle to ovulate and to trigger new follicular growth. Then, PGF is given 7 d later, inducing the regression of the newly formed CL (if the first GnRH induced an ovulation) or inducing the regression of an older CL (if a CL was already present at the time of first GnRH). Aiming to induce a pre-ovulatory LH surge, the second GnRH injection is given approximately 2 d after the PGF. Then, fixed-time AI is performed 16 to 24 h after the second GnRH (Pursley et al., 1995). The implementation of the Ovsynch protocol has become widely popular in the dairy industry as it was shown to enhance the reproductive performance and the overall profit per cow (LeBlanc, 2007), and reduce the cost per pregnancy when compared with AI based on detection of estrus alone (De Vries, 2011). Also, numerous variations to the Ovsynch protocol have been developed over the years, such as increasing PGF dose, shortening interval from first GnRH to PGF, and including supplementation of P4 during the protocol, with varying degrees of success in terms of efficiency (Bisinotto et al., 2014; Stevenson, 2016).

One of the most important aspects to optimize the efficiency and profitability of a reproductive program is the identification of non-pregnant cows early after AI (Chebel and Ribeiro, 2016). However, for optimal results, reproductive programs based exclusively on Ovsynch protocols require that non-pregnant cows are identified as soon as possible before starting the next protocol. Thus, a combination of both timed-AI program (i.e. Ovsynch) and estrus detection might provide the optimal efficiency (Galvao et al., 2013). However, the ability

of herd managers to accurately detect cows in estrus is a major factor influencing the success of combining timed-AI with estrus detection. For instance, Tenhagen et al. (2004a) reported that when the conception rate is adequate (35–40%) and the estrus detection rate is acceptable (approximately 55%), herds should rely on estrus detection rather than on timed-AI. However, if the estrus detection rate is low (< 30%), it might be more profitable to use only timed-AI protocols (Tenhagen et al., 2004a). The ability to detect cows in estrus and having higher proportion of cows receiving AI on detection of estrus is a major factor that increases profitability (Tenhagen et al., 2004a, Galvao et al., 2013). Although the implementation of synchronization protocols is usually seen to improve reproductive efficiency in dairy herds, important aspects that are often overlooked when discussing reproductive management strategies, are labor force (de Koning, 2010), facility design, and adaptation to the technology by each individual farm (Chebel and Ribeiro, 2016).

2.5.1. Precision technologies

A variety of new technologies have been developed in the dairy industry aiming to optimize the management efficiency and the labor need (de Koning, 2010; Rutten et al., 2013; Barkema et al., 2015). An increasing number of automated technologies that measure real-time physiological, behavioural, and production parameters such as temperature and rumen pH, estrus mounting and activity, lying behaviour, 3-D imaging for automated body condition scoring, milk P4 levels, mastitis and ketosis metabolites, and milk yield and components have been available for what is called “Precision Dairy Farming” (Bewley, 2010; Rutten et al., 2013; Barkema et al., 2015; Mottram, 2015). Eastwood et al. (2004) defined Precision Dairy Farming as “the use of information technologies for assessment of fine-scale animal and physical resource variability aimed at improved management strategies for optimizing economic, social, and environmental farm performance”. In addition to precision technologies to monitor real-time parameters of individual cows, the availability and adoption of automated management systems, such as calf feeder and robotic milking systems, is accelerating in dairy farms (de Koning, 2010; Barkema et al., 2015). The increasing adoption of automated and precision technologies offers a tremendous potential to improve health and welfare (e.g. detection of lameness through pedometers, detection of mastitis and ketosis through monitoring of metabolites in milk, improvement of calf

management and health through automated feeders), reproductive performance (e.g. detection of estrus through activity monitors and ovarian function through monitoring P4 in milk) (Bewley, 2010; Rutten et al., 2013), and enhance research advancements through the availability of large amounts of data in whole populations of cows in a short period of time.

Automated technologies allow identifying metabolic or physiological conditions (e.g. health or reproduction) in a more timely manner than is possible with only human observation (Svensson and Jensen, 2007; Rutten et al., 2013). The frequent and real-time monitoring of various parameters offers the opportunity to maximize individual animal potential, detect diseases early, and minimize the use of hormones and medications through preventive management (Bewley, 2010). However, the field of precision dairy technology is in its infancy. Most automated systems obtain almost continuous real-time information of every animal, generating an amount of data and actions lists largely unrealized (Barkema et al., 2015), and difficult for dairy managers to make optimal decisions (Steeneveld et al., 2011). Thus, research must accompany the development of these technologies to develop validated interpretive cut-points, algorithms, and generate practical and useful decision making strategies (Bewley, 2010; Rutten et al., 2013; Barkema et al., 2015). Data generated by these technologies should also be explored and incorporated into genetic evaluations for desirable traits (Bewley, 2010).

Precision technologies to assist reproductive management by detecting estrus have been evaluated and reviewed by several studies (Schofield et al., 1991; Roelofs et al., 2010; Saint-Dizier and Chastant-Maillard, 2012). Pedometers and activity monitors have shown high accuracy (70 to 100%) to predict estrus (Roelofs et al., 2010; Saint-Dizier and Chastant-Maillard, 2012), comparable to that of mount detection devices (Valenza et al., 2012). However, it is obvious that estrus detection systems will only detect cows that display estrus, and cows not displaying estrus (e.g. cows in anestrus) will be ignored by the system (Valenza et al., 2012; Stevenson et al., 2014). In this regard, a fully automated in-line milk P4 analyzer has recently become commercially available to monitor real-time ovarian activity, in addition to estimating time of estrus in cycling cows and pregnancy, based on milk P4 profiles (Friggens et al., 2008; Saint-Dizier and Chastant-Maillard, 2012; Mottram, 2014; Yu and Maeda, 2017).

2.5.2. In-line milk analysis system

An in-line milk analysis system (Herd Navigator™, DeLaval International, Tumba, Sweden & Lattec I/S, Hillerød, Denmark) has been marketed in Denmark since 2009, in the rest of Europe since 2010 **(**Saint-Dizier and Chastant-Maillard, 2012), and in Canada since 2014 (addressed in Chapters 3, 4, and 5**)**. The Herd Navigator™ system can be installed in either automated (robotic) milking systems or parlour milking systems. The system offers monitoring of reproduction, health, and metabolism indicators by frequently sampling milk and analyzing levels of P4 (indicator of ovarian function), lactate-dehydrogenase (indicator of mastitis), BHBA (indicator of ketosis), and urea (indicator of protein metabolism) (Saint-Dizier and Chastant-Maillard, 2012). In terms of ovarian function monitoring, the system takes a representative sample (approximately 1L) of milk from individual cows during milking and submits a homogenized sub-sample to an analyzer unit. The analyzer unit measures P4 using a dry-stick biosensor technique based on an immunoassay (Pemberton et al., 1998), and the colour intensity of dry-sticks is determined by an optical reader to quantify P4 values. After that, the analyzer unit sends the P4 values data to an on-farm software and P4 profiles from individual cows become readily accessible to the farm manager (Yu and Maeda, 2017).

The default Herd Navigator™ bio-model initiates sampling at approximately 21 days after calving and repeats at pre-determined modulated intervals based on fluctuations in P4 curves that indicate the phase of the estrous cycle (Friggens and Chagunda, 2005). On average, the system repeats sampling and assays P4 every 2 d (Tenghe et al., 2015) from first postpartum sample (approximately 21 days postpartum) until the cow is determined to be pregnant based on continuous high milk P4 profiles after AI. Six to 7 P4 records are expected per estrous cycle (Friggens and Chagunda, 2005). Actual P4 values (i.e. raw concentrations) obtained by the system are transformed to adjusted P4 values based on algorithms that control for outlier values. This is done to reduce random noise (Friggens and Chagunda, 2005) caused by surrounding humidity/temperature and differences between batches of dry-sticks (Jørgensen et al., 2016), that could under- or over-estimate fluctuations in P4 levels. This adjustment allows the bio-model algorithms to distinguish between the presence or the absence of luteal activity based on fluctuations in P4 levels around the standard 5 ng/mL threshold (Saint-Dizier and Chastant-Maillard, 2012). Based on the milk P4 curves, the Herd Navigator™ bio-model can monitor the

resumption of postpartum ovarian activity (i.e. cessation of anestrus period), length and shape of estrous cycles once the ovarian activity has initiated (Tenghe et al., 2015), estimate time of estrus based on decline in P4 levels (Friggens et al., 2008), and estimate early non-pregnancy or pregnancy statuses based on P4 profiles after AI (Friggens and Chagunda, 2005; Friggens et al., 2008; Saint-Dizier and Chastant-Maillard, 2012).

Once the P4 values decline from above to below the threshold, an alarm is triggered by the software to notify the farm manager (Friggens and Chagunda, 2005). By testing this bio-model for purpose of validation to detect estrus in an experimental herd, in-line milk P4 profiles detected 99% of the confirmed estruses, with 93.3% sensitivity and 93.7% specificity when comparing P4 profiles to that of confirmed estruses (Friggens et al., 2008). The assessment of in-line milk P4 profiles allows real-time monitoring of anestrus, estrous cycle phases, estrus, and pregnancy, in addition to P4 concentrations at various time points related to each of these events in individual cows in whole populations (i.e. entire herds). Thus, it offers the opportunity to explore a variety of components of luteal activity during the postpartum period that could not be studied previously.

The evaluation of P4 concentrations in milk samples have been widely used to assess factors of luteal activity, such as prevalence of anestrus in dairy herds (4) and occurrence of abnormal estrous cycles (1, 2, 3, 8, 9). However, such evaluations often relied on manual collection of milk samples (4, 8, 9), which is less practical and might restrict the size of the sampled population and sampling frequency. The restriction in sample size might limit the ability to assess epidemiological aspects of luteal activity in a large and homogeneous population of cows (i.e. whole herds in a short period of time). Also, studies that used manually collected data often evaluated data from a same herd over several years (Lamming and Darwash, 1998; Blavy et al., 2016), possibly representing different generation of cows with different genetic merit.

Previous studies have often classified cows for luteal activity parameters (i.e. interval from calving to resumption of ovarian activity or abnormal luteal activity lengths) based on population standards (e.g. population mean value ± standard deviations; Lamming and Darwash, 1988), or arbitrarily based on previous reports (Opsomer et al., 1998; Shrestha et al., 2004). By using in-line milk P4 data, it becomes possible to evaluate complete postpartum P4 profiles and to

characterize (i.e. define classifications for) several components of spontaneous luteal activity associated with infertility in individual cows from entire modern Holstein herds. For instance, factors such as delayed resumption of ovarian activity (Santos and Rutigliano, 2009; Ranasinghe et al., 2011; Ribeiro et al., 2016), occurrence of abnormal estrous cycles (Lamming and Darwash, 1998; Ranasinghe et al., 2011), and lack of a luteal phase preceding AI (Bisinotto et al., 2013), are known to reduce fertility at AI. However, specific conditions and cut-off values of luteal activity before and after AI (i.e. length of the luteal phase preceding estrus, time from cessation of luteal activity to AI, and P4 concentrations at various time points) that might be associated with infertility is yet to be characterized.

A research group in the Netherlands studied dairy herds monitoring in-line milk P4 profiles and explored genetic parameters luteal activity traits, such as commencement of postpartum luteal activity and first estrous cycle length (Tenghe et al., 2015, 2016). However, no known research has evaluated in-line milk P4 data to explore associations between components of luteal activity (i.e. during the early postpartum period and before and after AI) and fertility. Such evaluation could greatly contribute to filling knowledge gaps on spontaneous ovarian activity conditions associated with reduced fertility. Towards this general objective, three studies were conducted to investigate: (1) relationships among patterns of early postpartum luteal activity, (2) dynamics of pre- and post-insemination P4 profiles, and (3) luteal activity parameters early postpartum and before and after AI, that were associated with reduced fertility in commercial Holstein herds.

CHAPTER 3

RELATIONSHIPS AMONG EARLY POSTPARTUM LUTEAL ACTIVITY, PARITY, AND INSEMINATION OUTCOMES BASED ON IN-LINE MILK PROGESTERONE PROFILES IN CANADIAN HOLSTEIN COWS

The objectives of this retrospective study were to use in-line milk progesterone (mP4) data to investigate relationships of (1) commencement of luteal activity (CLA), and (2) luteal phase (LP) length and frequency preceding first postpartum AI, with parity and AI outcomes in Canadian Holstein cows. Starting 21 ± 1 days postpartum (DPP), levels of mP4 were assessed every 2.2 ± 2.0 d through an automated in-line milk analysis system (Herd Navigator™, DeLaval International, Tumba, Sweden) until ~55 d after first or second AI in 748 Holstein cows from two herds. The CLA was defined as the DPP of the first of at least two consecutive samples with mP4 ≥5 ng/mL, and the period with elevated mP4 (≥5 ng/mL) was defined as the LP. Cows were categorized by CLA [earlier (≤) or later (>) than 28, 35, 42, 49, 56 and 63 DPP], and by the pattern of LP frequency preceding first AI as having or not: (1) one or more normal LP (LP length ≥7 and ≤19 d); (2) one or more abnormal LP (LP length <7 or >19 d, or interluteal period ≥12 d); and (3) two or more LP (either normal or abnormal). Outcomes of first or second AI were determined by the interval between AI and cessation of the ensuing LP as: non-pregnant (mP4-decline ≤30 d), presumed-pregnant (no mP4-decline until 55 d), or presumed-pregnancy loss (mP4-decline between 31 and 55 d). The odds of pregnancy per AI (P/AI) at 55 d and pregnancy loss were evaluated using generalized linear mixed models. Primiparous cows had lower odds of having CLA ≤28 DPP [Odds ratio (OR) = 0.58, P = 0.002] and one or more abnormal LP (OR = 0.73, P = 0.04) than multiparous cows. In multiparous cows, CLA ≤28 DPP decreased pregnancy loss (OR = 0.48, P = 0.05) and CLA ≤56 DPP increased P/AI (OR = 4.69, P < 0.01) compared to a later CLA. Primiparous and multiparous cows that had one or more normal LP before first AI had increased P/AI (OR = 3.85 and 3.45, respectively, P < 0.01) and reduced pregnancy loss (OR = 0.26 and 0.27, respectively, P < 0.01) than cows without a normal LP. Primiparous cows that had one or more abnormal LP had decreased P/AI (OR = 0.62, P = 0.04) and increased pregnancy loss (OR = 1.64, P = 0.04) compared to those without an abnormal LP. In summary, AI outcomes were improved in multiparous cows that had early CLA

and in cows of both parity groups that had at least one normal LP before first AI. However, primiparous cows that had at least one abnormal LP had reduced AI outcomes. Relationships between early postpartum luteal activity and AI outcomes were inconsistent between primiparous and multiparous cows.

3.1. INTRODUCTION

The assessment of milk progesterone (mP4) concentrations has been widely used to classify luteal phases (i.e. normal vs. abnormal) (Lamming and Darwash, 1998; Opsomer et al., 2000; Blavy et al., 2016), benchmark reproductive status (Ambrose and Colazo, 2007), determine estrus (Friggens et al., 2008) and pregnancy (Bulman and Lamming, 1978), and estimate endocrine traits (Tenghe et al., 2015). However, the evaluation of abnormal ovarian activities, such as anestrus and prolonged cycles and their effects on fertility, often rely on manual collection of milk samples (Ambrose and Colazo, 2007; Gautam et al., 2010; Ranasinghe et al., 2011). Manual sampling makes it less practical, often limiting the size of the sampled population and frequency of sampling. An automated in-line milk analysis system (Herd Navigator™, DeLaval International, Tumba, Sweden) is available in Europe and Canada as a reproductive management tool, which analyzes mP4 at frequent, algorithm-driven intervals, starting approximately 3 wk after parturition. Towards the anticipated end of the luteal phase (LP), samples are taken at least once daily to determine estrus (Friggens et al., 2008) or pregnancy if a previous artificial insemination (AI) was performed. Thus, in-line mP4 data allows the assessment of early postpartum reproductive status at a whole herd level.

While transitioning from late gestation to early postpartum, high-producing dairy cows experience a number of profound metabolic changes that involve regulation of energy status, liver function, and mammary gland demand for glucose as required for lactation (Drackley, 1999). These metabolic challenges are often linked to abnormal ovarian processes associated with poor reproductive performance, such as prolonged anestrus, short estrus expression, and delayed ovulation (Wiltbank et al., 2006). A prolonged anestrus (Thatcher and Wilcox, 1973; Ribeiro et al., 2016a) and the presence of atypical estrous cycles early postpartum (Lamming and Darwash, 1998) are factors associated with reduced fertility (Santos and Rutigliano, 2009; Garcia-Ispierto and López-Gatius, 2016). Therefore, the evaluation of ovarian function based on

mP4 profiles may enhance the understanding of the declining trend in fertility in the high-producing dairy cow (Norman et al., 2009).

Metabolic challenges during the early postpartum period related to high milk yield and feed intake, such as negative energy balance (Beam and Butler, 1999) and high metabolic clearance rate of steroid hormones (Wiltbank et al., 2006), are known factors associated with altered ovarian function. For instance, circulating progesterone (P4) and estradiol concentrations are lower in lactating than in non-lactating cows (Sartori et al., 2004; Sangsritavong et al., 2002), likely due to differences in milk yield. In addition, induced-luteal regression occurs at a lower rate in multiparous than in primiparous cows (Brusveen et al., 2009; Giordano et al., 2012b). These factors may, at least in part, explain the greater fertility often reported in primiparous than in multiparous cows (Santos and Rutigliano, 2009). Nonetheless, the mechanisms by which early postpartum luteal activity may differentially influence the establishment and maintenance of pregnancy in primiparous and multiparous cows are still unclear. The assessment of mP4 profiles using the in-line milk analysis system offers a unique approach to continuously monitor P4 profiles in individual cows over several weeks, and to our knowledge, no such report exists from North American dairy herds.

The objectives of this retrospective study were to use in-line mP4 data to investigate relationships of (1) commencement of luteal activity (CLA), and (2) LP length and frequency preceding first AI, with parity and AI outcomes in Canadian Holstein cows. Considering the greater fertility reported in primiparous cows, and the lower fertility associated with anestrus, and abnormal cycles, we specifically tested the hypotheses that primiparous cows have higher odds of earlier CLA and lower odds of having abnormal LP before first AI than multiparous cows. We also hypothesized that an earlier CLA, the presence of one or more normal LP, the absence of abnormal LP, and the presence of two or more LP (either normal of abnormal) preceding first AI are associated with increased odds of pregnancy and reduced odds of pregnancy loss in both parity groups.

3.2. MATERIALS AND METHODS

3.2.1. In-line milk analysis system and records description

Records of mP4 concentrations analyzed through an in-line milk analysis system (Herd Navigator™) were accessed using a herd management software (AlPro™, DeLaval International, Tumba, Sweden). The Herd Navigator™ is an electronic management tool that, based on a bio-model (Friggens and Chagunda, 2005), automatically takes milk samples, quantifies P4 through a dry-stick enzyme immunoassay technique (Pemberton et al., 1998), and stores records of both raw (actual) and adjusted (smoothed) mP4 values, at algorithm-driven intervals after parturition. Smoothed mP4 values are based on a local linear growth model that controls for outlier values in time series analyses to reduce random noise (Friggens and Chagunda, 2005) due to surrounding humidity/temperature and differences between batches of dry-sticks (Jørgensen et al., 2016). This adjustment allows algorithms to distinguish between presence or absence of luteal activity using an mP4 threshold of 5 ng/mL. Using this bio-model, Friggens et al. (2008) reported a 93.3% sensitivity and a 93.7% specificity for detection of estrus.

In the data used, milk sampling started 21 ± 1 DPP (Mean ± SD) and repeated at a frequency of 4.4 ± 2.0 d until CLA was determined (i.e. at least two consecutive mP4 samples 5 ng/mL); then, samples were collected every 2.2 ± 2.0 d. Once a decline (<5 ng/mL) in mP4 was determined (hereafter referred to as mP4-decline) after CLA, four subsequent samples were taken approximately 7, 12, 16 and 20 d later, and thereafter, samples were taken at least once daily in order to detect the next mP4-decline. If a cow was eligible for AI (after the elective waiting period), the mP4-decline event was immediately flagged as a “heat alarm”, and AI recommended about 36 h later. Following AI, if the mP4 increased to 5 ng/mL and remained 5 ng/mL for approximately 30 d post-AI, a potential pregnancy was declared by the system and sampling continued every 3 d until either mP4-decline or until approximately 55 d when sampling stopped and pregnancy was declared.

3.2.2. Demographics and management of herds

In-line mP4 records from the years 2014–2016 were obtained from two commercial dairy herds (Herds A and B) located in Alberta, Canada, milking approximately 420 and 350 Holstein cows, respectively. Both herds had been using the Herd Navigator™ system as the main tool for making reproductive decisions and inseminating cows based on mP4 curves (see **Section 3.2.1**), for approximately 18 months prior to the evaluation of data. Based on herd records, first AI occurred at 68 ± 13 and 71 ± 20 DPP (Mean ± SD), and average calving interval was 389 ± 54 and 398 ± 53 d in Herds A and B, respectively. After evaluating individual mP4 profiles (as described in **Section 3.2.3**), data from 350 primiparous (154 and 196 from Herds A and B, respectively) and 398 multiparous cows (204 and 194 from Herds A and B, respectively) that calved between June 2014 and December 2015 were used. Overall daily milk yield (kg/d) during first 60 DPP averaged 31.4 ± 4.3 and 29.7 ± 5.1 for primiparous cows and 45.0 ± 6.6 and 43.8 ± 6.3 for multiparous cows in Herds A and B, respectively. Cows in both herds were housed in free-stall barns, milked thrice daily through a parlor system, and fed a total mixed ration prepared in accordance with NRC (2001) guidelines. Rations included alfalfa, barley and/or corn silage, alfalfa or grass hay, and concentrates (barley grain, commercial mix and minerals) as major ingredients. All AI were performed by experienced technicians using frozen-thawed commercial semen.

3.2.3. Enrolment criteria and classifications of luteal activity from parturition to first AI

To be enrolled in the study, all 748 cows had to meet the following criteria: (1) first mP4 record collected before 28 DPP; (2) CLA occurred before 150 DPP; (3) neither subjected to induced luteolysis nor induced-ovulation treatments from first mP4 record until evaluated AI; (4) AI occurred later than 40 DPP and after the CLA; (5) AI occurred within 4 d following mP4-decline identified through the in-line milk analysis system; (6) increase in mP4 (≥5 ng/mL) occurred within 14 d post-AI; and (7) interval between two consecutive mP4 records was no longer than 8 d.

Profiles of mP4 were evaluated between first postpartum sample (21 ± 1 DPP) and first AI (71 ± 16 DPP) to determine CLA and LP length and frequency. The CLA was defined as the

DPP of the first of at least two consecutive samples with mP4 5 ng/mL after parturition. All cows were classified as having CLA earlier or later than each of six weekly interval-classes: 28, 35, 42, 49, 56, and 63 DPP. The length of LP (number of days between first mP4 ≥5 ng/mL and subsequent mP4-decline of same LP) and interluteal interval (number of days between mP4-decline of previous LP and first subsequent mP4 ≥5 ng/mL indicative of next LP) were used to characterize LP. A LP length of 7 and 19 d following an interluteal interval of <12 d was considered normal, whereas a LP length of <7 or >19 d, or an interluteal interval of 12 d (Lamming and Darwash, 1998), was considered abnormal. Based on those criteria, cows were classified as having or not having one or more normal LP, one or more abnormal LP, and two or more LP (either normal or abnormal).

3.2.4. AI outcomes

The assessment of pregnancy status was based on the interval between AI and subsequent mP4-decline (i.e. interval between AI and cessation of subsequent LP), regardless of the Herd Navigator™ alarms. Cows that had mP4-decline ≤30 d post-AI were declared non-pregnant, and cows having an uninterrupted LP (no mP4- decline) until 55 d post-AI (last sample obtained for that lactation) were presumed-pregnant (considered as "pregnant" in this manuscript for evaluations of P/AI). Cows that had mP4-decline between 31 and 55 d post-AI were presumed to have suffered pregnancy loss (consider as "pregnancy loss" in this manuscript for evaluations of the proportion of pregnancy loss). The odds of P/AI (pregnant vs. non-pregnant at 55 d) and proportion of P/AI ($\frac{Pregnant}{Pregnant\ +\ non-prenant\ at\ 55\ d}$; %), and the odds of pregnancy loss (pregnancy loss vs. pregnant) and proportion of pregnancy loss ($\frac{Pregnancy\ loss}{Pregnancy\ loss\ +\ pregnant\ at\ 55\ d}$; %), were determined for first AI outcomes. In addition, odds of cumulative pregnancy up to second postpartum AI (P/2ndAI; pregnant vs. non-pregnant at 55 d after first and second AI) and proportion of P/2ndAI ($\frac{Pregnant\ after\ first\ or\ second\ AI}{Pregnant\ +\ non-prenant\ at\ 55\ d\ after\ first\ and\ second\ AI}$; %), were determined.

3.2.5. Statistical analyses

All analyses were performed using SAS® 9.4 (Studio 3.5 platform, SAS Institute Inc., Cary, NC, USA), considering cow as the experimental unit. Probability values 0.05 were considered significant, and values between 0.051 and 0.10 as trends. All cowswere categorized into CLA earlier or later than each of the six classes: 28, 35, 42, 49, 56 and 63 DPP, and categorized as having (yes) or not having (no) each of the three patterns of LP frequency based on the length: one or more normal LP, one or more abnormal LP, and two or more LP (either normal or abnormal).

Using the GLIMMIX procedures for logistic regression, each class of CLA (early vs. late) was modelled against parity (primiparous vs. multiparous), including herd as a random effect. Similarly, each class of LP frequency (yes vs. no) was modelled against parity, including CLA nested within herd as random effects. For primiparous and multiparous cows, each class of CLA and LP frequency was modelled against each AI outcome (P/AI, pregnancy loss, and P/2ndAI), including DPP at first AI as fixed and herd as random effects. If DPP resulted in $P \leq 0.15$, it was also included as a random effect. For the comparisons of AI outcomes between parity groups within each CLA and LP frequency category, parity was modelled against AI outcomes (P/AI and pregnancy loss), including herd and DPP (when $P \leq 0.15$) as random effects. All cows were included in all analyses except when evaluating pregnancy loss, where cows declared non-pregnant at 30 d post-AI (n = 420) were not considered.

The FREQ procedure was used to obtain proportions of cows within each category of CLA and LP frequency, P/AI, pregnancy loss, and P/2ndAI, and results reported using least square means (LSM), odds ratio (OR) and 95% confidence limits. Probability values presented are those relative to the OR and 95% confidence intervals (CI) of logistic regression models.

3.3. RESULTS

3.3.1. Categorization of cows by commencement of luteal activity, luteal phase frequency, and AI outcomes

Overall, the percentages of cows categorized as having CLA earlier than 28, 35, 42, 49, 56, and 63 DPP were 26.5, 52.9, 67.9, 77.4, 83.8, and 89.4%, respectively. The proportions of cows categorized as having one or more normal LP, one or more abnormal LP, and two or more LP (either normal of abnormal) were 73.3, 50.9, and 58.0%, respectively. After first AI, 45.2% (338/748) of cows had mP4 ≥5 ng/ mL beyond 30 d, 30.2% (226/748) had mP4 ≥5 ng/mL until 55 d (overall P/AI), and 33.1% (112/338) had mP4-decline between 31 and 55 d (overall pregnancy loss). The cumulative P/2ndAI was 47.3% (354/748).

In primiparous cows, 49.4% (173/350) had mP4 ≥5 ng/mL beyond 30 d, 35.1% (123/350) had mP4 ≥5 ng/mL until 55 d (P/AI), and 28.9% (50/173) had mP4-decline between 31 and 55 d (pregnancy loss). Cumulative P/2ndAI in primiparous cows was 49.7% (174/ 350).

In multiparous cows, 41.5% (165/398) had mP4 ≥5 ng/mL beyond 30 d, 25.9% (103/398) had mP4 >5 ng/mL until 55 d (P/AI), and 37.6% (62/165) had mP4-decline between 31 and 55 d (pregnancy loss). Cumulative P/2ndAI in multiparous cows was 45.2% (180/ 398).

3.3.2. Associations of commencement of luteal activity and luteal phase length and frequency with parity and AI outcomes

Frequencies and odds ratio analyses of CLA and LP frequency classes between primiparous and multiparous cows are presented in Table 3.1.

Frequencies and logistic regression analyses outcomes of CLA classes on P/AI and pregnancy loss for primiparous and multiparous cows are presented in Table 3.2. In primiparous cows, there was no significant effect of CLA classes on P/AI or pregnancy loss. In multiparous cows, CLA earlier than 28 DPP reduced pregnancy loss, and CLA earlier than 56 DPP increased P/AI and reduced pregnancy loss (Table 3.2).

Table 3.3 presents outcomes of the analyses of LP frequency on P/AI and pregnancy loss for primiparous and multiparous cows. Primiparous cows that had one or more normal LP or two or more LP before first AI had increased P/AI and reduced pregnancy loss, while primiparous cows that had one or more abnormal LP had reduced P/AI and increased pregnancy loss. Multiparous cows that had one or more normal LP or two or more LP (either normal of abnormal) had increased P/AI and reduced pregnancy loss (Table 3.3).

The cumulative P/2ndAI in primiparous cows was associated with the same classes of LP frequency that affected pregnancy at first AI. The cumulative P/2ndAI in multiparous cows was positively associated with CLA earlier than 56 DPP, having one or more normal LP, or having two or more LP (Table 3.4).

Direct comparisons of AI outcomes between primiparous and multiparous cows within same categories of CLA and LP frequencies are presented in Figs. 1–4. Primiparous cows had greater P/AI than multiparous cows within all classes of CLA (Fig. 3.1) and LP frequency (Fig. 3.2). Multiparous cows had greater pregnancy loss than primiparous cows when CLA occurred later than 28 and 56 DPP (Fig. 3.3), and among those without an abnormal LP until first AI (Fig. 3.4).

3.4. DISCUSSION

3.4.1. Evaluation of AI outcomes based on in-line milk progesterone profiles

Peripheral concentrations of P4 can stay elevated up to 9 d after embryonic death in cases where embryonic death preceded a luteolysis (Ryan et al., 1992; Giordano et al., 2012a). Thus, the frequent determination of mP4 allowed us to indirectly estimate embryonic losses (i.e. occurring from 22 to 30 d post-AI with high P4 levels maintained beyond 31 d). At least partially for this reason, we may have estimated greater pregnancy loss (33.1%) than other studies (Szenci et al., 1998: 8.6%; Santos and Rutigliano, 2009: 13.2%) that evaluated pregnancy losses at similar post-AI period (from 30 to 58 d) and used traditional diagnostic methods, such as palpation per rectum or transrectal ultrasonography.

As determination of pregnancy loss was based exclusively on mP4 profiles post-AI, we could not differentiate cows with prolonged LP post-AI from those undergoing luteolysis caused

by a true embryonic loss, which could have contributed to the high incidence of pregnancy loss reported here. It was previously reported that 95% of the prolonged LP (≥20 d in length) in the early postpartum period occurred in the first two estrous cycles (Ranasinghe et al., 2011). Similarly, a recent study in a large population of dairy cows reported that prolonged LP (≥23 d in length in that study) occurred mostly in the first postpartum estrous cycle (Blavy et al., 2016). Thus, it is likely that the incidence of prolonged LP that occurred following AI in our study would have been small, as we only evaluated AI that occurred after the CLA (i.e. after the first postpartum estrous cycle).

3.4.2. Associations among commencement of luteal activity, parity, and AI outcomes

In most cows (76.0% of primiparous and 78.6% of multiparous cows), CLA occurred earlier than 49 DPP, whereas in approximately 50% of cows CLA occurred by 35 DPP. Similarly, other studies using mP4 data reported CLA occurring earlier than 50 DPP in 74–79% of the cows (Opsomer et al., 2000; Ambrose and Colazo, 2007; Gautam et al., 2010), but that 65% of cows had CLA occurring earlier than 35 DPP (Gautam et al., 2010). Previously, the threshold for early and late CLA using mP4 data was often chosen based on population standards [i.e. one standard deviation above population mean of days to CLA (Lamming and Darwash, 1998)] or arbitrarily [referring to antecedent studies (Opsomer et al., 1998; Shrestha et al., 2004), in order to enable objective comparisons of mP4 profiles. We evaluated six classes for CLA to investigate to what extent the time to CLA affects AI outcomes, separately in primiparous and multiparous cows. A greater proportion of primiparous than multiparous cows had CLA occurring later than 28 DPP (79.1 vs. 68.6%) and later than 63 DPP (13.1 vs. 8.3%), which agrees with other reports of a later onset of postpartum ovarian activity in primiparous than in multiparous cows (Bulman and Lamming, 1978; Santos and Rutigliano, 2009; Vercouteren et al., 2015). Although the average daily milk yield of the evaluated herds was greater in multiparous than in primiparous cows, the additional energy demand for growth in primiparous cows might have led to reduced partitioning of nutrients for other bio- logical events [(Bulman and Lamming, 1978; Santos and Rutigliano, 2009; Wathes et al., 2007). In addition to being more sensitive to metabolic changes (e.g. negative energy balance) in the early postpartum period than multiparous cows (Santos and Rutigliano, 2009; Wathes et al., 2007), primiparous cows are more

likely to develop uterine diseases (Vercouteren et al., 2015; Goshen and Shpigel, 2006), which are known factors affecting resumption of postpartum cyclicity (Beam and Butler, 1999).

As an indicator of metabolic changes in the early postpartum period, a negative energy status is associated with reduced luteinizing hormone (LH) pulse-frequency, impairing dominant follicle growth, thereby extending the interval from parturition to CLA (Beam and Butler, 1997). In addition to the negative energy balance (Vercouteren et al., 2015; Taylor et al., 2003), several other factors have been associated with delayed first ovulation, such as: (1) lower milk production during first 90 DPP (Santos and Rutigliano, 2009), (2) parturition in winter or spring months (Vercouteren e tal., 2015), (3) presence of clinical and metabolic postpartum diseases (e.g. mastitis, uterine diseases, digestive or respiratory problems, ketosis) (Ribeiro et al., 2016a; Vercouteren et al., 2015), and (4) lower body condition score at parturition or at AI (Santos and Rutigliano, 2009).

An early resumption of cyclicity after parturition is one of the major contributors for improved fertility later in the same lactation (Thatcher and Wilcox, 1973; Ribeiro et al., 2016a). The earliest threshold of interval from parturition to CLA to significantly affect P/AI was 56 DPP in multiparous cows, while primiparous cows with CLA later than 28 DPP tended to have decreased P/AI. A reduced P/AI in cows having delayed CLA was in accordance with the findings of Bittar et al. (2014), where cows that ovulated by 24 DPP (regardless of whether ovulation was gonadotropin-releasing hormone-induced or spontaneous) had increased risk (hazard ratio = 1.4) of pregnancy by 300 DPP compared to cows that failed to ovulate by 24 DPP. Gautam et al. (2010) also evaluated different interval-classes of CLA, reporting reduced fertility when CLA occurred later than 35 DPP. However, neither of the above studies evaluated the influence of parity on CLA and subsequent fertility.

Although we found that CLA earlier than 28 DPP reduced pregnancy loss only in multiparous cows, induced early first ovulation reduced the risk of pregnancy losses in other studies, regardless of parity (Santos and Rutigliano, 2009; Bitar et al., 2014). Similarly, delayed first ovulation was associated with increase embryonic mortality (Santos et al., 2004). In contrast, Ranasinghe et al. (2010) reported that CLA earlier than 28 DPP increased the occurrence of prolonged LP early postpartum, which negatively affected fertility. In this regard, the occurrence of metabolic and uterine disorders reduces conception and increases embryo

mortality (Ribeiro et al., 2016a), and is also associated with late resumption of cyclicity (Vercouteren et al., 2015; Goshen and Shpigel, 2006). The earlier CLA associated with increased P/AI and reduced pregnancy loss in multiparous cows observed in the present study is likely because an earlier CLA led to a greater number of cycles until first AI, increasing uterine exposure to P4 and benefiting fertility outcomes as previously suggested (Thatcher and Wilcox, 1973). This supports our findings that greater frequency of luteal activity (one or more normal LP or two or more LP e either normal or abnormal) improved AI outcomes.

3.4.3. Associations among abnormal luteal activity, parity, and AI outcomes

The two main factors underlying atypical physiological patterns in ovarian cycles are negative energy status (Beam and Butler, 1997; Staples et al., 1990) and increased feed intake and milk yield (Wiltbank et al., 2006; Sangsritavong et al., 2002). The increased feed intake and milk yield increases the hepatic blood flow and metabolic clearance rate of estradiol and P4, reducing their circulating concentrations (Wiltbank et al., 2006; Sangsritavong et al., 2002). The reduced estradiol might delay LH peak release (Wiltbank et al., 2006), while the reduced P4 might increase LH pulse frequency (Stock and Fortune, 1993), overexposing the pre-ovulatory follicle to lower- intensity LH pulses (Wiltbank et al., 2006). This overexposure to LH pulses may mature the oocytes earlier than the optimum, compromising the oocyte quality and delaying ovulatory events in the early post- partum period (Wiltbank et al., 2006). As a cause of delayed ovulation and prolonged period of dominance, large ovulatory follicles were more frequent in multiparous than in primiparous cows and associated with increased probability of pregnancy loss following that ovulation (Colazo et al., 2015).

That over 50% of the cows had one or more abnormal LP was greater than (Blavy et al., 2016; Royal et al., 2000) or similar to (Lamming and Darwash, 1998; Opsomer et al., 1998; Shrestha et al., 2004) previous reports evaluating LP based on mP4. An increase of more than 10% in the incidence of abnormal P4 from the late 1970's to late 1990's has been reported (Royal et al., 2000), which is likely related to the increasing milk yield and feed intake (Lucy, 2001). In addition, a recent study evaluating a population of Holstein, Danish Red, and Jersey cows from a single experimental herd (Blavy et al., 2016) reported an increased variability of LP length. In that study, the threshold for abnormal (prolonged) LP was estimated to be 23 d, while previously

it was estimated to be 19 d (Lamming and Darwash, 1998). This would indicate that common evaluations of abnormal LP using priori set of classifications (Lamming and Darwah, 1998; Ranasinghe et al., 2011; Opsomer et al., 1998; Shrestha et al., 2004) similar to the ones used in the present study, should be carefully evaluated as estimators of true abnormal LP.

In the current study, the odds of having one or more abnormal LP was lower in primiparous than in multiparous cows, as previously reported (Opsomer et al., 2000; Ranasinghe et al., 2011). However, having one or more abnormal LP was significantly associated with reduced P/AI and increased pregnancy loss only in primiparous cows. As primiparous cows also had later CLA than multiparous cows possibly due to greater metabolic or uterine problems, it is possible that part of the abnormal LP from primiparous cows was associated with health disorders (Opsomer et al., 2000; Ranasinghe et al., 2011) affecting fertility. Lamming and Darwash (1998) reported that cows having abnormal ovarian activity had lower conception rates, increased days open, higher embryonic mortality, and required more veterinary interventions (suggesting more health or reproductive disorders) than those having normal activity. Apparently, there is possibly a link between health or metabolic disorders (Santos and Rutigliano, 2009; Shrestha et al., 2004), compromised ovarian activity (Opsomer et al., 2000; Bittar et al., 2014), and subsequent fertility in primiparous cows.

The greater occurrence of one or more abnormal LP seen in multiparous cows could have been due to the increased metabolic clearance rate of steroids (Sangsritavong et al., 2002), altering the ovarian processes (Wiltbank et al., 2006) but not significantly affecting AI outcomes as noticed in primiparous cows. In addition, lower responses to induced-luteolysis has been reported in multiparous cows compared to primiparous cows (Giordano et al., 2013a; Martins et al., 2011), which might indicate greater occurrence of incomplete luteolysis (i.e. prolonged LP) in multiparous cows. The different AI outcomes noticed in primiparous and multiparous cows having one or more abnormal LP, suggests that most of the inconsistencies in luteal activity factors reported in previous studies were likely because parity groups were not evaluated separately. In addition, the equal criteria used to classify normal vs. abnormal ovarian activity in both parity groups might be not accounting for their expected differences (related to milk yield, feed intake, and luteolysis rate) in ovarian activity.

3.4.4. Parity effects on AI outcomes by each category of luteal activity

It has been often reported that primiparous cows have greater fertility than multiparous cows (Santos and Rutigliano, 2009; Garcia-Ispierto and López-Gatius, 2016; Tenhagen et al., 2004b). However, effects of parity on fertility are usually influenced by several factors such as: (1) anestrus (Bittar et al., 2014), (2) dystocia (Taylor et al., 2004; Gröhn and Rajala-Schultz, 2000), (3) health disorders (Huszenicza et al., 1987), (4) synchronization protocols used (Astiz and Fargas, 2013), (5) mP4 concentrations around time of AI (Chapter 4), and as observed in the current study, (6) early postpartum luteal activity.

Greater P/AI were evident in primiparous cows in all categories of earlier CLA (Fig. 3.1), with the greatest difference in those with CLA earlier than 28 DPP (46.6% in primiparous vs. 28.8% in multiparous cows). In a large study, Santos and Rutigliano (2009) reported a greater incidence of pregnancy loss in multiparous (15.6%) than in primiparous cows (8.6%). In the present study, when CLA occurred later than 56 DPP, multiparous cows had dramatically increased pregnancy loss compared to an earlier CLA (69.3 vs. 34.9%), unlike primiparous cows (21.7 vs. 30.0%; Table 3.2). However, it is important to note that only a small number of cows had CLA later than 56 DPP for the evaluation of pregnancy loss. Apparently, in both primiparous and multiparous cows, the major factors that negatively affect P/AI are the absence of normal LP and the occurrence of at least one abnormal LP before first AI (Fig. 3.2). Furthermore, the absence of normal LP before first AI resulted in the greatest proportion of pregnancy losses in cows from both parity groups (Fig. 3.4).

3.5. Conclusion

Inconsistencies observed in AI outcomes between parity groups having similar CLA and LP frequency indicate that fertility in primiparous and multiparous cows are differently influenced by early postpartum ovarian activity. Regardless, a high frequency of LP preceding first AI, including at least one normal LP, is the major factor benefiting AI outcomes. The increased variability in the LP length recently reported in literature (Blavy et al., 2016) and the high occurrence of abnormal LP found in the present study could be contributing factors to the poor reproductive performance prevalent in dairy herds. Data from the in-line mP4 analysis

allow the identification of several luteal activity components, and can be a valuable tool for future research to characterize abnormal cycles impacting fertility in modern dairy cows, and to develop strategies to improve fertility.

Table 3.1. Frequencies and odds ratio (OR) analyses of the six classes of commencement of luteal activity (CLA) and three classes of patterns of luteal phase (LP) frequency between primiparous (n = 350) and multiparous (n = 398) cows.

Factor	Class	Category	Primiparous, n (%)	Multiparous, n (%)	OR[3]	95% CI	*P*[4]
CLA, DPP	1	≤ 28	73 (20.9)	125 (31.4)	0.58	0.42-0.81	0.002
		> 28	277 (79.1)	273 (68.6)	Ref.	–	–
	2	≤ 35	174 (49.7)	222 (55.8)	0.79	0.59-1.06	0.12
		> 35	176 (50.3)	176 (44.2)	Ref.	–	–
	3	≤ 42	238 (68)	270 (67.8)	1.02	0.75-1.39	0.89
		> 42	112 (32)	128 (32.2)	Ref.	–	–
	4	≤ 49	266 (76)	313 (78.6)	0.88	0.62-1.24	0.45
		> 49	84 (24)	85 (21.4)	Ref.	–	–
	5	≤ 56	284 (81.1)	343 (86.2)	0.71	0.48-1.05	0.09
		> 56	66 (18.9)	55 (13.8)	Ref.	–	–
	6	≤ 63	304 (86.9)	365 (91.7)	0.62	0.39-1.01	0.05
		> 63	46 (13.1)	33 (8.3)	Ref.	–	–
LP frequency	1	Absence of normal LP	86 (24.6)	114 (28.6)	Ref.	–	–
		One or more normal LP	264 (75.4)	284 (71.4)	1.32	1.82-0.93	0.11
	2	Absence of abnormal LP	185 (52.9)	182 (45.7)	Ref.	–	–
		One or more abnormal LP	165 (47.1)	216 (54.3)	0.73	0.54-0.98	0.04
	3	Less than two LP	162 (46.3)	152 (38.2)	Ref.	–	–
		Two or more LP	188 (53.7)	246 (61.8)	0.74	0.55-0.99	0.05

All cows were classified in each of six binomial classes [12 categories of early (≤) and late (>)] of CLA.

[b] All cows were classified in each of three binomial classes (6 categories) of LP frequency.

[c] Odds ratio referring to primiparous.

[d] P-value of comparison between two categories within each CLA or LP frequency class relative to logistic regression models, OR and 95% confidence intervals (CI).

DPP = Days postpartum.

Table 3.2. Frequencies and odds ratio (OR) analyses for pregnancy per AI (P/AI) and pregnancy loss at first AI in primiparous and multiparous cows for each class of commencement of luteal activity (CLA; Early vs. Late).

CLA class[a], DPP	**P/AI (%), OR and 95% CI**					**Pregnancy loss (%), OR and 95% CI**				
	Early, n (%)	Late, n (%)	OR[b]	95% CI	*P*[c]	Early, n (%)	Late, n (%)	OR[b]	95% CI	*P*[c]
Primiparous cows (n = 350)										
28	34/73 (46.6)	89/277 (32.1)	1.59	0.93-2.72	0.09	12/46 (26.1)	38/127 (29.9)	0.85	0.39-1.84	0.68
35	67/174 (38.5)	56/176 (31.8)	1.14	0.72-1.80	0.57	27/94 (28.7)	23/79 (29.1)	0.95	0.48-1.89	0.88
42	90/238 (37.8)	33/112 (29.5)	1.19	0.71-1.98	0.52	37/127 (29.1)	13/46 (28.3)	1.19	0.54-2.62	0.67
49	101/266 (38.0)	22/84 (26.2)	1.34	0.73-2.44	0.35	42/143 (29.4)	8/30 (26.7)	1.49	0.56-3.97	0.42
56	105/284 (37.0)	18/66 (27.3)	1.05	0.53-2.09	0.89	45/150 (30)	5/23 (21.7)	2.87	0.80-10.28	0.10
63	113/304 (37.2)	10/46 (21.7)	1.28	0.52-3.12	0.59	47/160 (29.4)	3/13 (23.1)	3.93	0.69-22.36	0.12
Multiparous cows (n = 398)										
28	36/125 (28.8)	67/273 (24.5)	1.27	0.78-2.07	0.34	13/49 (26.5)	49/116 (42.2)	0.48	0.23-1.01	0.05
35	58/222 (26.1)	45/176 (25.6)	1.03	0.65-1.64	0.91	39/97 (40.2)	23/68 (33.8)	1.33	0.69-2.56	0.39
42	74/270 (27.4)	29/128 (22.7)	1.24	0.75-2.05	0.41	46/120 (38.3)	16/45 (35.6)	1.12	0.55-2.30	0.76
49	88/313 (28.1)	15/85 (17.7)	1.70	0.91-3.18	0.10	51/139 (36.7)	11/26 (42.3)	0.82	0.35-1.94	0.65
56	99/343 (28.9)	4/55 (7.3)	4.69	1.63-13.51	<0.01	53/152 (34.9)	9/13 (69.3)	0.24	0.07-0.82	0.02
63	101/365 (27.7)	2/33 (6.1)	4.93	1.13-21.48	0.03	57/158 (36.1)	5/7 (71.4)	0.23	0.04-1.23	0.09

[a] All cows of each parity group were classified in each of six binomial classes (total = 12 categories) of CLA.

[b] Odds ratio referring to Early CLA.

[c] P-value of comparison between two categories (Early vs. Late) relative to logistic regression models, OR and 95% confidence intervals (CI);

DPP = Days postpartum.

Table 3.3. Frequencies and odds ratio (OR) analyses for pregnancy per AI (P/AI) and pregnancy loss at first AI in primiparous and multiparous cows for each pattern of luteal phase (LP) frequency class.

Class[a]	P/AI (%), OR and 95% CI					Pregnancy loss (%), OR and 95% CI				
	Yes, n (%)	No, n (%)	OR[b]	95% CI	*P*[c]	Yes, n (%)	No, n (%)	OR[b]	95% CI	*P*[c]
Primiparous cows (n = 350)										
One or more normal LP	110/264 (41.7)	13/86 (15.1)	3.85	2.00-7.14	<.001	35/145 (24.1)	15/28 (53.6)	0.26	0.14-0.50	<.001
One or more abnormal LP	49/165 (29.7)	74/185 (40)	0.62	0.39-0.97	0.04	28/77 (36.4)	22/96 (22.9)	1.64	1.03-2.56	0.04
Two or more LP	86/188 (45.7)	37/162 (22.8)	2.50	1.56-4.17	<.001	24/110 (21.8)	26/63 (41.3)	0.40	0.24-0.64	<.001
Multiparous cows (n = 398)										
One or more normal LP	90/284 (31.7)	13/114 (11.4)	3.45	1.79-6.67	<.001	42/132 (31.8)	20/33 (60.6)	0.27	0.12-0.61	0.002
One or more abnormal LP	56/216 (24.5)	50/182 (27.5)	0.78	0.49-1.25	0.30	33/86 (38.4)	29/79 (36.7)	1.08	0.56-2.10	0.81
Two or more LP	80/246 (32.5)	23/152 (15.3)	2.94	1.59-5.26	<.001	39/119 (32.7)	23/46 (50.0)	0.35	0.15-0.81	0.01

[a] All cows of each parity group were classified in each of three classes (total = 6 categories) of LP frequency.

[b] Odds ratio referring to having the pattern of LP frequency (Yes).

[c] P-value of comparison between two categories (Yes vs. No) relative to logistic regression models, OR and 95% confidence intervals (CI).

Table 3.4. Frequencies and odds ratio (OR) analyses for cumulative pregnancy up to second AI (P/2ndAI) in primiparous and multiparous cows for each class of commencement of luteal activity (CLA; Early vs. Late) and luteal phase (LP) frequency (Yes vs. No).

Class[a]	**Primiparous cows (n = 350)**					**Multiparous cows (n = 398)**				
CLA, DPP	Early, n (%)	Late, n (%)	OR[b]	95% CI	*P*[c]	Early, n (%)	Late, n (%)	OR[b]	95% CI	*P*
28	42/73 (57.5)	132/277 (47.7)	1.36	0.80-2.31	0.26	63/125 (50.4)	117/273 (42.9)	1.40	0.91-2.15	0.13
35	91/174 (52.3)	83/176 (47.2)	1.15	0.75-1.76	0.52	105/222 (47.3)	75/176 (42.6)	1.20	0.81-1.80	0.36
42	121/238 (50.8)	53/112 (47.3)	1.07	0.68-1.69	0.77	128/270 (47.4)	52/128 (40.6)	1.32	0.86-2.03	0.21
49	138/266 (51.9)	36/84 (42.9)	1.34	0.81-2.22	0.25	148/313 (47.3)	32/85 (37.7)	1.47	0.89-2.41	0.13
56	147/284 (51.8)	27/66 (40.9)	1.40	0.81-2.44	0.23	164/343 (47.8)	16/55 (29.1)	2.12	1.14-3.96	0.02
63	156/304 (51.3)	18/46 (39.1)	1.38	0.72-2.65	0.33	168/365 (46.0)	12/33 (36.4)	1.36	0.64-2.87	0.43
LP frequency	Yes, n (%)	No, n (%)	OR[d]	95% CI	*P*	Yes, n (%)	No, n (%)	OR[d]	95% CI	*P*
One or more normal LP	151/264 (57.2)	23/86 (26.7)	3.57	2.04-5.88	<.001	141/284 (49.7)	39/114 (34.2)	1.85	1.16-2.94	<0.01
One or more abnormal LP	71/165 (43.0)	103/185 (55.7)	0.59	0.38-0.90	0.02	96/216 (44.4)	84/182 (46.2)	0.91	0.61-1.37	0.66
Two or more LP	114/188 (60.6)	60/162 (37.0)	2.70	1.67-4.35	<.001	131/246 (53.3)	49/152 (32.2)	2.22	1.43-3.45	<.001

[a] All cows from each parity group were classified into each binomial class of CLA (total = 12 categories) and LP frequency (total = 6 categories).

[b] Odds ratio of P/2AI referring to Early CLA.

[c] P-value of comparison between two categories (Early vs. Late or Yes vs. No) relative to logistic regression models, OR and 95% confidence intervals (CI).

[d] Odds ratio of P/2AI referring to having the pattern of LP frequency (Yes);

DPP = Days postpartum.

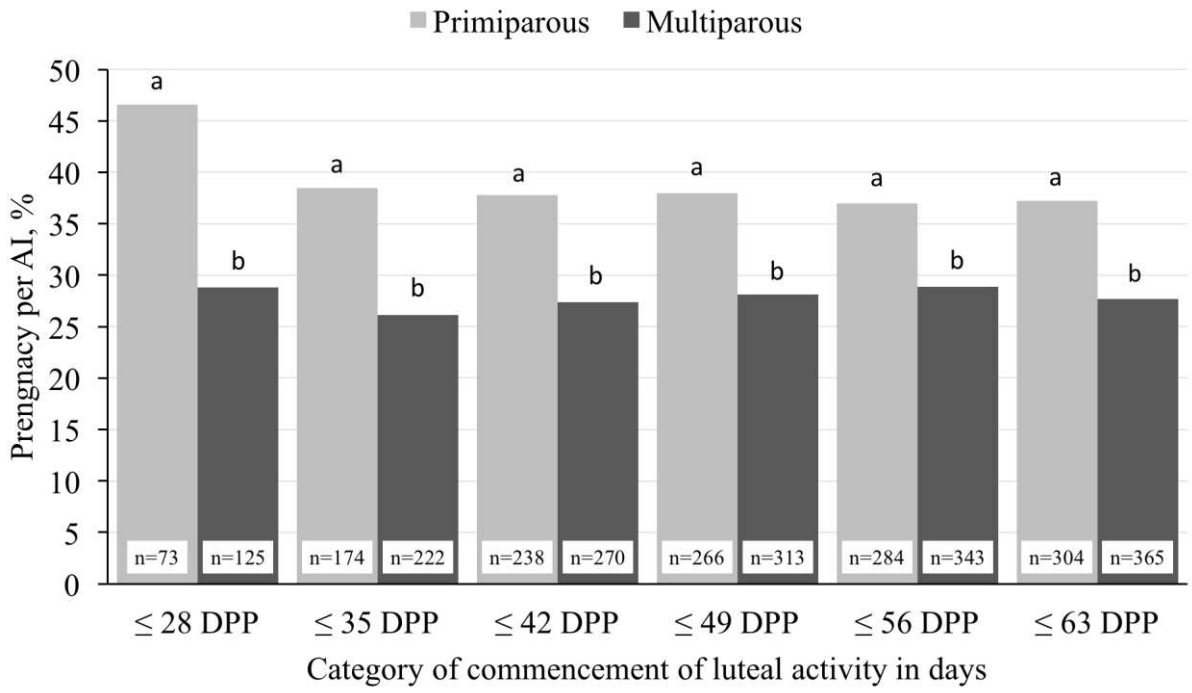

Fig. 3.1. Pregnancy per AI (%) at first AI in primiparous and multiparous cows within each category of early commencement of luteal activity. DPP = days postpartum; n = total number of cows per category; [a,b] $P \leq 0.05$ between primiparous and multiparous cows within same category.

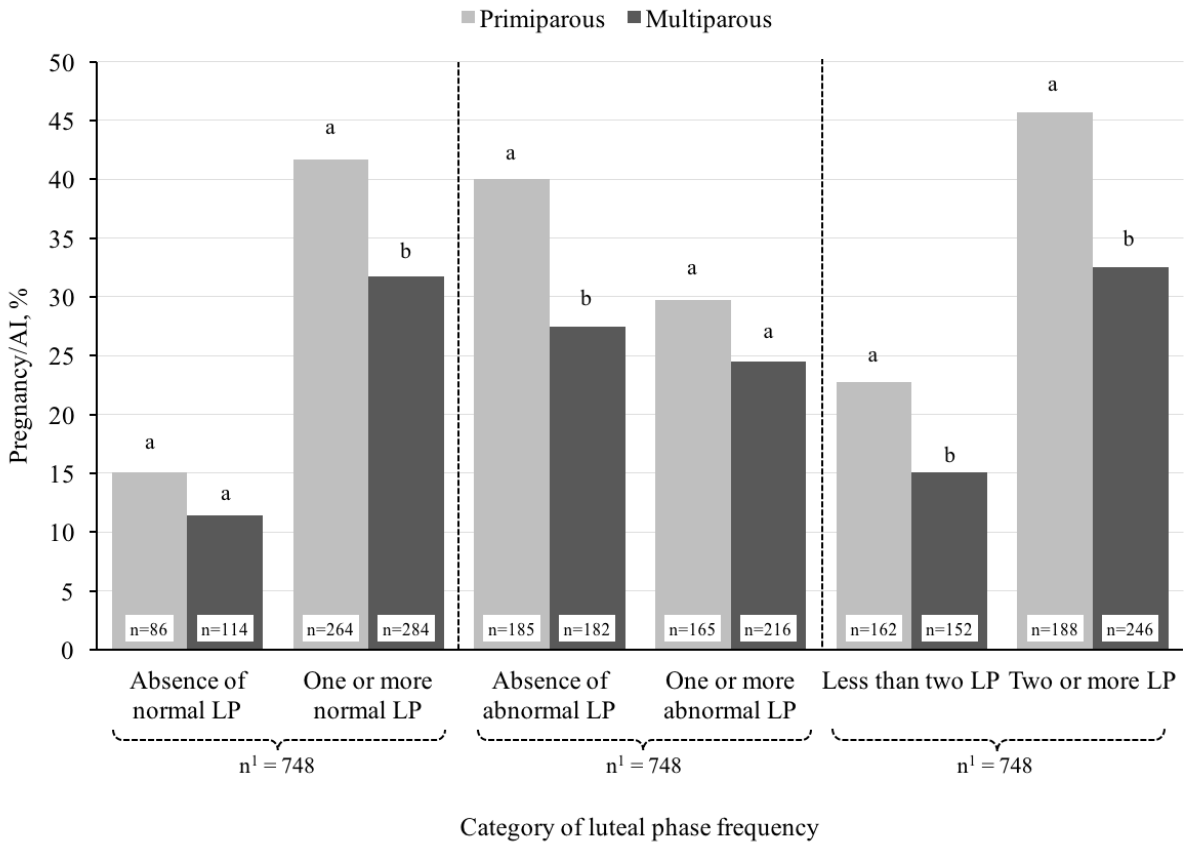

Fig. 3.2. Pregnancy per AI (%) at first AI between primiparous and multiparous cows within each category of luteal phase (LP) frequency before first AI. n = total number of cows per category; n^1 = all cows were included in each of the three classes of LP frequency; ^{a,b} $P \leq 0.05$ between primiparous and multiparous cows within same category.

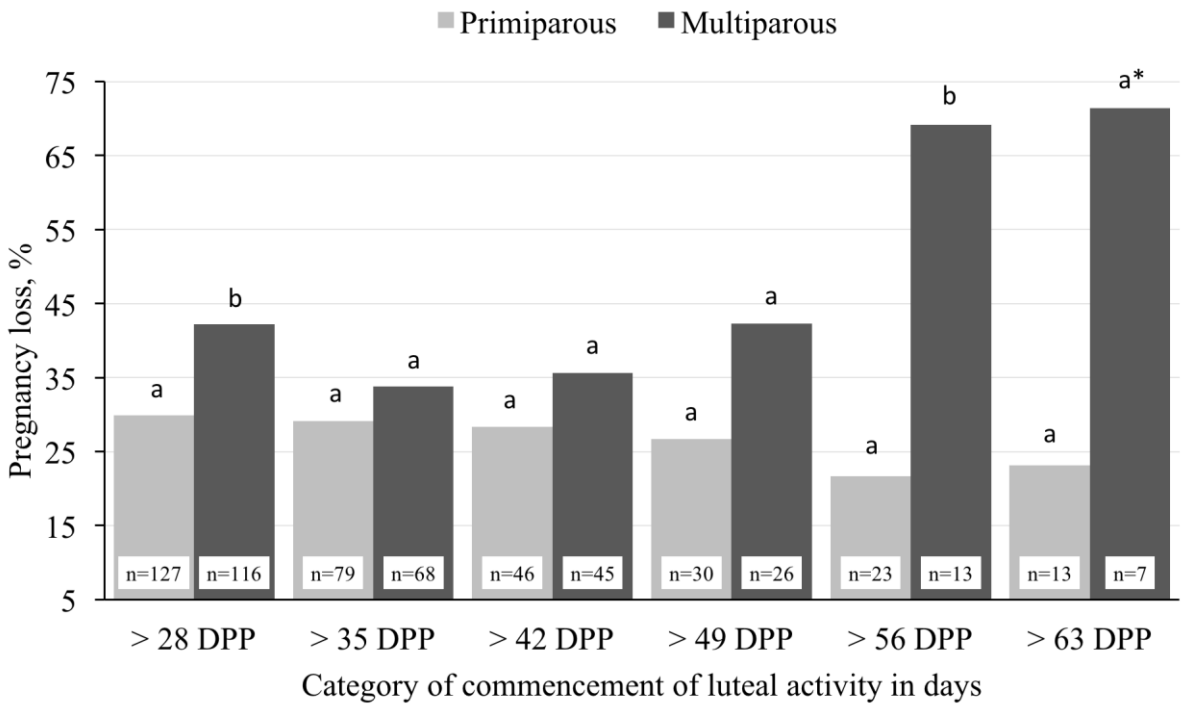

Fig. 3.3. Pregnancy loss (%) at first AI in primiparous and multiparous cows within each category of late commencement of luteal activity. DPP = days postpartum; n = total number of cows with mP4 ≤ 5 ng/mL beyond 30 d post-AI used to calculate proportion of pregnancy loss per category; **[a,b]** P ≤ 0.05 between primiparous and multiparous cows within same category; *P = 0.06 between parity groups within same category.

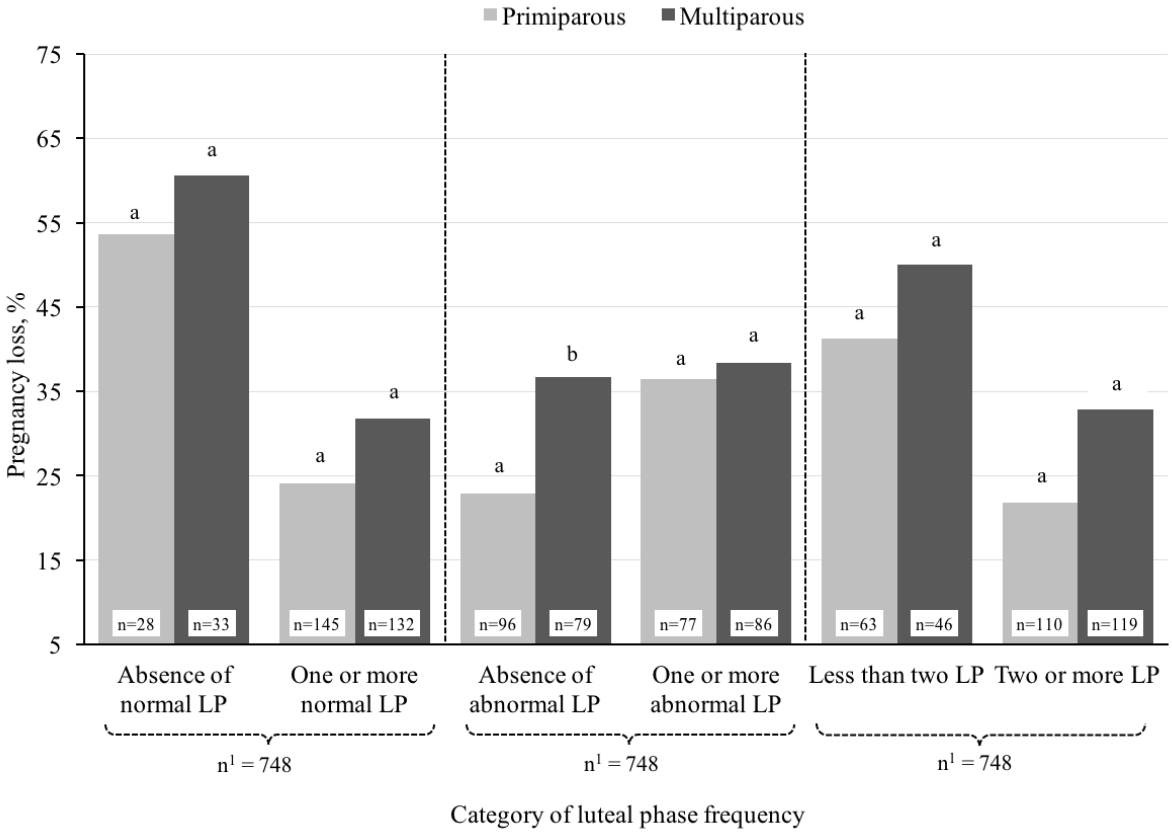

Fig. 3.4. Pregnancy loss (%) at first AI in primiparous and multiparous cows within each category of luteal phase (LP) frequency occurring before first AI. n = total number of cows with mP4 ≤ 5 ng/mL beyond 30 d post-AI used to calculate proportion of pregnancy loss per category; n^1 = all cows were included in each of the three classes of LP frequency. **[a,b]** P ≤ 0.05 between primiparous and multiparous cows within same category.

CHAPTER 4

DYNAMICS OF PRE- AND POST-INSEMINATION PROGESTERONE PROFILES AND INSEMINATION OUTCOMES DETERMINED BY AN IN-LINE MILK ANALYSIS SYSTEM IN PRIMIPAROUS AND MULTIPAROUS CANADIAN HOLSTEIN COWS

The objective was to evaluate in-line milk progesterone (mP4) data to determine dynamics of pre- and post-insemination mP4 profiles and their associations with parity and outcomes of artificial insemination (AI) in Holstein cows. Milk progesterone concentrations (ng/mL) were quantified at pre-determined time points before and after AI through an automated in-line milk analysis system (Herd Navigator™, DeLaval International, Tumba, Sweden). Only AI (~d0; n = 605) preceded by an mP4-decline (at least two samples of mP4 ≥5 ng/mL followed by at least one sample <5 ng/mL; d–2) were evaluated. Maximum mP4 attained between d–15 and d–2 (PrePeak), d–2, d5, d10, d14, maximum mP4 attained within 21d post-AI (PostPeak), and rate-of-daily-change between mP4 time points (ng/mL/d) were analyzed. Primiparous and multiparous cows were classified by AI outcomes based on post-AI mP4 profiles into three groups: (1) non-pregnant (OPEN; mP4-decline 30d post-AI), (2) presumed-pregnant (PREG; no mP4-decline until 55d post-AI), and (3) presumed-pregnancy loss (P-LOSS; mP4-decline between 31 and 55d post-AI). For profile comparisons, smoothed mP4 data were analyzed using mixed linear models. Primiparous cows had greater ($P < 0.01$) mP4 than multiparous cows at d5 (4.6 ± 0.2 vs. 2.8 ± 0.1), 10 (11.1 ± 0.4 vs. 7.6 ± 0.2), 14 (19.7 ± 0.4 vs. 16.1 ± 0.3) and PostPeak (23.5 ± 0.3 vs. 21.7 ± 0.2). The rate-of-daily-change was greater ($P < 0.01$) in primiparous than in multiparous cows from d–2 to 5 (+0.2 ± 0.03 vs. 0.1 ± 0.02) and from d5 to 10 (+1.2 ± 0.05 vs. +0.9 ± 0.03), but lesser ($P < 0.01$) from d14 to PostPeak (+0.9 ± 0.09 vs. +1.3 ± 0.06). In primiparous cows, mP4 in PREG was greater at d10 and PostPeak than OPEN (11.1 ± 0.5 and 24.2 ± 0.5 vs. 9.6 ± 0.4 and 22.3 ± 0.4, respectively, $P < 0.04$), but lesser at d5 than P-LOSS (4.4 ± 0.3 vs. 5.7 ± 0.4, $P = 0.04$). In multiparous cows, mP4 at d–2 was lesser in PREG than OPEN and P-LOSS (3.2 ± 0.1 vs. 3.4 ± 0.04 and 3.5 ± 0.1, respectively, $P \leq 0.03$), but greater at d10, d14 and PostPeak in PREG than in OPEN (8.2 ± 0.4, 16.8 ± 0.5 and 22.7 ± 0.4 vs. 6.9 ± 0.3, 14.8 ± 0.3 and 19.7 ± 0.2, respectively, $P < 0.01$). Multiparous PREG cows had greater rate-of-daily-

change in mP4 than OPEN cows from d5 to 10 and from d10 to 14 (+1.0 ± 0.06 and +2.2 ± 0.11 vs. +0.8 ± 0.04 and +1.9 ± 0.08, respectively, $P < 0.03$). Overall post-AI mP4 increased faster and were greater in primiparous than in multiparous cows. Based on in-line mP4 profiles, greater mP4 levels near time of AI (d–2 in multiparous and d5 in primiparous cows) and lesser mP4 beyond d10 were negatively associated with a successful pregnancy.

4.1. INTRODUCTION

Milk progesterone (mP4) data have been widely used to characterize ovarian activity (Bulman and Lamming, 1978; Lamming and Darwash, 1998; Ambrose and Colazo, 2007; Gautam et al., 2010; Blavy et al., 2016), pregnancy status (Bulman and Lamming, 1978; Gorzecka et al., 2011) and to evaluate associations between progesterone (P4) levels around time of artificial insemination (AI) and pregnancy (Stronge et al., 2005; Gorzecka et al., 2011). However, characterizing complete P4 profiles from early postpartum period until pregnancy establishment in lactating dairy cows through manual milk sampling is labor-intensive; hence rarely done. The in-line milk analysis system (IMAS; Herd Navigator™, DeLaval International, Tumba, Sweden) is a relatively new herd management tool that allows evaluation of postpartum mP4 profiles both in individual cows and in whole herds from about 3 wk postpartum until pregnancy establishment (Chapter 3). The IMAS uses a bio-model (Friggens and Chagunda, 2005) that drives automatic milk sampling in every cow and measures mP4 at frequent intervals to estimate luteal function, estrus, time to AI and AI outcomes (non-pregnancy, pregnancy, pregnancy loss) (Mottram, 2015). In addition to assisting with reproductive management decisions, the assessment of frequent mP4 data generated by the IMAS gives a new opportunity to evaluate parameters of luteal activity (Mayo and Lucy, 2016; Tenghe et al., 2015; Chapter 3), such as mP4 levels at specific time points, and their associations with fertility. Given the considerable variability in luteal phase length in the modern dairy cow (Blavy et al., 2016; Chapter 3), evaluating characteristics of luteal activity through mP4 profiles in cows that conceived and maintained a pregnancy would enhance the understanding of the dairy cow fertility.

The IMAS (Herd Navigator™) has been commercially available since 2008 in Europe and since 2011 in Canada. In addition to monitoring reproductive events, it is designed for early

detection of ketosis, mastitis, and to monitor milk urea profiles. Although data from European herds using the IMAS have been used to establish benchmarks of luteal activity (Mayo and Lucy, 2016) and endocrine fertility traits (Tenghe et al., 2015, 2016), no report exists on evaluating mP4 profiles in relation to fertility. The IMAS bio-model offers a novel approach to monitor cyclic status (i.e. commencement of postpartum luteal activity) (Mayo and Lucy, 2016; Chapter 3), abnormal luteal cycles (Chapter 3), and detection of estrus (Friggens et al., 2008).

After ovulation, an optimal P4 environment is required for establishment of pregnancy (Inskeep, 2004), and increasing concentrations of P4 following AI support embryo development through uterine secretions of proteins and growth factors (Garrett et al., 1988). To understand the influence of P4 on fertility, recent studies have investigated the effects of P4 supplementation on pregnancy outcomes during or following synchronization protocols (Colazo et al., 2013; Monteiro et al., 2014; Bisinotto et al., 2015a) with inconsistent results in post-AI evaluations. These inconsistencies could be at least partially explained by confounding effects on luteal activity parameters which are still poorly characterized, such as parity, as the levels of feed intake and milk production (expected to be lower in primiparous than in multiparous cows) affect P4 concentrations (Sangsritavong et al., 2002). While it is frequently reported that pregnancy per AI is greater in primiparous than in multiparous cows (Tenhagen et al., 2004b; Balendran et al., 2008; Norman et al., 2009; Garcia-Ispierto and López-Gatius, 2016), the underlying factors contributing to the increased fertility in primiparous cows are not fully understood. Recent reports using IMAS in commercial dairy herds (Mayo and Lucy, 2016; Chapter 3) or continuous manual milk sampling for mP4 determination in a research herd (Blavy et al., 2016) indicate that differences in ovarian function exist between primiparous and multiparous cows, such as in characteristics of luteal cycles early postpartum (Blavy et al., 2016; Mayo and Lucy, 2016; Chapter 3). Frequent automated mP4 sampling can help identify characteristics of P4 profiles associated with successful pregnancies, pregnancy losses, and potential differences in P4 dynamics among parity groups, which have not been studied previously.

Therefore, a retrospective study was conducted using data generated through an automated IMAS to determine the dynamics of pre- and post-AI mP4 profiles and their associations with parity (primiparous, multiparous) and AI outcomes (non-pregnant, pregnant, pregnancy loss) in Canadian Holstein cows. As milk yield affects P4 metabolism (Sangsritavong et al., 2002) and

elevated P4 post-AI is essential for pregnancy (Inskeep, 2004), we tested the hypothesis that mP4 levels before and after AI and the rate-of-daily-change in mP4 post-AI are greater (more rapid increase) in primiparous than in multiparous cows and in cows that become pregnant than in cows with other AI outcomes.

4.2. MATERIALS AND METHODS

4.2.1. Herds and management

Data relating to 605 AI of 115 primiparous (1st lactation) and 249 multiparous ($2nd^{+}$ lactation) Holstein cows that calved between June 2014 and December 2015, had not been subjected to reproductive hormone interventions during the luteal phases evaluated (see Section 4.2.3), and had been inseminated beyond 40 d postpartum, were obtained from two dairies located in Alberta, Canada using the IMAS (Herd Navigator™). Records were accessed through a dairy-management software program (AlPro™, DeLaval International). Both herds used the IMAS as their main reproductive management tool and inseminated cows based on mP4 curves (as described in Section 4.2.2). Artificial inseminations were performed by experienced and trained technicians using frozen-thawed commercial semen. Based on herd records, Herds A and B milked approximately 420 and 350 Holstein cows, and calving intervals averaged 393 (range 324–545) and 372 (range 322–482) d, respectively.

Overall daily milk yield during first 60 d postpartum for Herds A and B averaged 31.4 ± 4.3 and 29.7 ± 5.1 kg for primiparous cows and 45.0 ± 6.6 and 43.8 ± 6.3 kg for multiparous cows, respectively. Cows from both herds were milked thrice daily through a parlor system, housed in free-stall barns, and fed a total mixed ration in accordance with NRC (2001) guidelines, using ingredients typical for high-producing herds in Alberta. Major ingredients were silage (alfalfa, barley and/or corn), hay (alfalfa or grass) and concentrates (barley grain, commercial mix, and minerals).

4.2.2. In-line milk progesterone analysis system

The Herd Navigator™ is programmed to collect milk samples automatically and analyze mP4 in individual cows through a dry-stick biosensor technology and enzyme immunoassay (Pemberton et al., 1998), based on a bio-model that establishes frequency and quantification of mP4 samples (Friggens and Chagunda, 2005). To reduce the random variation associated with the testing environment (temperature/humidity) and differences in batches of sticks and reagents, raw (actual) mP4 concentrations are adjusted to smoothed values (i.e. levels) based on a standardized method to control for outliers expected in the serial sampling system, as described by Friggens and Chagunda (Friggens and Chagunda, 2005). The bio-model was validated to detect estrus based on the pattern of sample frequency to detect the mP4-decline at the end of a luteal phase (period of high to low mP4; indicating estrus will occur), with 93.3% of sensitivity and 93.7% of specificity (Friggens et al., 2008).

In brief, milk samples started at 21 d postpartum and were taken, on average, every 2 d until a pregnancy was declared based on mP4 profiles post-AI. Luteal phase was considered to have initiated at the first of two consecutive samples with mP4 ≥5 ng/ mL following a sample <5 ng/mL, and the end of a luteal phase was determined when two consecutive mP4 readings of ≥5 ng/mL were followed by at least one reading <5 ng/mL (referred hereinafter as mP4-decline). If the cow was eligible to be inseminated, the mP4-decline was immediately notified in the dairy-management software as a "heat alarm" and AI recommended ~36 h later. After mP4-decline, programmed sampling occurred approximately at 7 ± 1, 12 ± 1, 16 ± 1 and 20 ± 1 days later, followed by samples at least once daily aiming to detect the next mP4-decline. If the luteal phase continued with no mP4-decline until ~30 d post-AI, a potential pregnancy was declared by the system. Then, samples were taken, on average, every 3 d until ~55 d post-AI, when the cow was declared pregnant if the luteal phase remained uninterrupted (no mP4-decline).

4.2.3. Data description

Six-hundred-five mP4-decline events that were followed by an AI (364 first and 241 s postpartum AI) within 48 h, and a subsequent initiation of luteal phase within 16 d, were assessed. Based on smoothed mP4 values, mP4-decline events were used as the reference time

points and set as d 2 relative to approximate time of AI (d 0). Thus, mP4 records at the highest level between d–15 and d–2 (PrePeak), at d 2, 5, 10, 14, and at the highest level between d 10 and d 21 (PostPeak) were evaluated. The rate-of-daily-change in mP4 (ng/mL/d) was calculated between all intervals as the difference between two time points of mP4, divided by the interval in days between same two time points (e.g. rate-of-daily-change from d 5 to d 10: mP4 at d10 e mP4 at d5). By manually assessing each mP4 profile post-AI, the period between AI and mP4-decline (period between AI and initiation of luteal phase plus period of uninterrupted luteal phase) was determined to identify three groups of AI outcomes (as in Chapter 3): non-pregnant (mP4-decline 30 d post-AI; designated as "OPEN" in this manuscript); presumed-pregnant (no mP4-decline until 55 d post-AI; designated as "PREG" in this manuscript); and presumed-pregnancy loss (mP4-decline between 31 and 55 d post-AI; designated as "P-LOSS" in this manuscript). Thus, all AI outcomes (OPEN, PREG, P-LOSS) were determined based on post-AI mP4 profiles.

4.2.4. Statistical analyses

Analyses were performed with SAS® 9.4 (Studio 3.5 platform, SAS Institute Inc., Cary, NC, USA), considering AI as the experimental unit and cow as a repeated measure subject using variance components structure. Probability values 0.05 were considered significant. First, an exploratory analysis was performed using the MEANS and UNIVARIATE procedures with Extreme Observations selection method. Continuous variable with skewness < 1 or >1 were considered abnormal and adjusted with outlier removal until a maximum range of 2 to 2 for analyses.

Aiming to evaluate the effects of parity and AI outcomes on each time point of mP4 levels and rate-of-daily-change, dependent variables [six time points of mP4 (PrePeak, d–2, 5, 10, 14 and PostPeak) and eight rate-of-daily-change variables (PrePeak to d–2, d–2 to 5, d 5 to 10, d 5 to 14, d 5 to PostPeak, d 10 to 14, d 10 to PostPeak and d 14 to PostPeak)] were first separately modelled against the fixed effects of herd (A or B), AI number (first or second AI) and season at AI [warm (May to August) or cold (September to April)] to evaluate the need to include these variables as random effects (included when $P \geq 0.15$), using the MIXED procedure. Then, for the evaluation of parity effects on mP4 and rate-of-daily-change, models had parity (primiparous,

multiparous) included as fixed effect with AI outcome (OPEN, PREG, P-LOSS) in the random statement.

To assess the effects of AI outcome on each time point of mP4 and rate-of-daily-change variables for primiparous and multiparous cows, the interaction of AI outcomes with parity was included as fixed effect to evaluate multiple comparisons and differences within each parity group. Because the effects of time points on mP4 levels were not of interest, they were not included in the analyses. Models used REML estimation, Satterthwaite approximation and Tukey's method for multiple comparisons. Summary statistic reports were presented as Mean ± SD and the analyzed dependent variables were reported as least square means (LSM) ± SEM.

4.3. RESULTS

Summary statistics for mP4 levels and rate-of-daily-change between time points, after adjusting for outlier exclusion (stated within the missing values category), are presented in Table 4.1. First and second AI occurred at 70 ± 17 (range 42–170) and 99 ± 20 (range 62–180) d postpartum, respectively. Mean PrePeak mP4 was 19.4 ng/mL with a minimum value of 5.5 ng/mL. The mean (±SD) interval between PrePeak and AI was 6.6 ± 2.7 d, and between AI and PostPeak was 17.4 ± 2.3 d. At mP4-decline (d–2), mean mP4 was 3.40 ng/mL with minimum and maximum values of 0.48 and 4.96 ng/mL, respectively. Mean mP4 at d 14 was 17.13 ng/mL with a minimum value of 5.06 ng/mL.

In four of the six time points, primiparous cows had significantly greater mP4 levels than multiparous cows (Fig. 4.1). Levels of mP4 (ng/mL) were greater in primiparous than in multiparous cows at d 5 (4.6 ± 0.2 vs. 2.8 ± 0.1, $P < 0.001$), d 10 (11.1 ± 0.4 vs. 7.6 ± 0.2, $P < 0.001$), d 14 (19.7 ± 0.4 vs. 16.1 ± 0.3, $P < 0.001$) and at PostPeak (23.5 ± 0.3 vs. 21.7 ± 0.2, $P < 0.01$).

While the rate-of-daily-change in mP4 levels from PrePeak to d–2 did not differ between the parity groups, the rate-of-daily-change from d–2 to 5 was greater in primiparous than in multiparous cows, being positive (daily increase) in 54.4% of primiparous and negative (daily decrease) in 75.1% of multiparous cows. Primiparous cows had a greater rate-of-daily-change

from d 5 to 10 and a lower rate-of-daily-change from d 14 to PostPeak than multiparous cows (Table 4.2).

Overall, the distribution of cows into OPEN, PREG and P-LOSS groups were 59.0, 28.1 and 12.9%, respectively. Among primiparous cows, 50.0% were classified in OPEN, 36.0% in PREG and 14.0% in P-LOSS groups, while multiparous cows were 62.6% in OPEN, 24.9% in PREG and 12.5% in P-LOSS groups.

Primiparous PREG cows had greater ($P < 0.04$) mP4 at d 10 and at PostPeak than those OPEN (11.1 ± 0.5 and 24.2 ± 0.5 vs. 9.6 ± 0.4 and 22.3 ± 0.4 ng/mL, respectively). Primiparous P-LOSS cows had greater (P 1⁄4 0.04) mP4 at d 5 (5.7 ± 0.4 vs. 4.4 ± 0.3 ng/mL) than PREG cows (Fig. 4.2).

Multiparous PREG cows had greater mP4 (ng/mL) than those OPEN at d 10 (8.2 ± 0.4 vs. 6.9 ± 0.3, $P < 0.01$), at d 14 (16.8 ± 0.5 vs. 14.8 ± 0.3, $P < 0.001$) and at PostPeak (22.7 ± 0.4 vs. 19.7 ± 0.2, $P < 0.001$) (Fig. 4.3). Multiparous PREG cows had lower ($P = 0.03$) mP4 than those in OPEN and P-LOSS groups at d–2 (3.2 ± 0.1 vs. 3.4 ± 0.04 and vs. 3.5 ± 0.1, respectively) (Fig. 4.3 inset). No significant differences were observed between AI outcome groups in primiparous and multiparous cows at other time points of mP4.

The rate-of-daily-change in mP4 did not differ between primiparous PREG and OPEN cows. However, primiparous PREG cows had lower rate-of-daily-change from d–2 to 5 than primiparous P-LOSS cows. Multiparous PREG cows had greater rate-of-daily-change in mP4 from d 5 to 10, d 5 to 14, d 5 to PostPeak and d 10 to 14 than those OPEN (Table 4.3).

4.4. DISCUSSION

Primiparous cows had greater post-AI mP4 levels than multiparous cows, and greater rate-of-daily-change in mP4 from d −2 to 5 and d 5 to 10, supporting our hypothesis that mP4 levels and their rate-of-daily-change are greater post-AI, at least for most time points, in primiparous cows. The greater mP4 levels in primiparous than in multiparous cows was expected as a higher milk yield in multiparous cows would be associated with greater dry matter intake and, consequently, accelerated metabolic clearance of P4, decreasing its peripheral concentrations (Sangsritavong et al., 2002). Furthermore, different aspects related to ovarian function were

reported to differ between parity groups, such as interval between calving and commencement of luteal activity (Chapter 3; Mayo and Lucy, 2016), length of luteal phases (Blavy et al., 2016), and luteal responses to induced-luteolysis (Martins et al., 2011). Multiparous cows reportedly required greater doses of exogenous PGF than primiparous cows to achieve similar luteolysis rate (Giordano et al., 2013b), suggesting potential differences in corpus luteum (CL) responses to PGF (greater occurrence of incomplete CL regression in multiparous than in primiparous cows).

We evaluated only cows that had a decline in mP4 below the IMAS threshold of 5 ng/mL, so cows with partially regressed CL (luteolysis initiated, but mP4 not declined below 5 ng/mL) would have been automatically excluded from the study. However, the negative rate-of-daily-change from d–2 to 5 observed in most multiparous cows (~75%) suggests that even though cows had an mP4-decline (below 5 ng/mL), some cows failed to have completed CL regression at d–2 and continued the decline in mP4 levels until d 5. This would indicate that the algorithms and threshold based on smoothed values used by the IMAS bio-model may not be sensitive enough to detect CL regression in all cases. In this regard, a previous study evaluating the same bio-model reported ~4% of cows not dropping mP4 below the smoothed threshold, despite having mP4 curves shaped similar to that of cows in estrus (Friggens et al., 2008). Another possibility is that the IMAS is not always precisely identifying the timing of CL regression: as the bio-model is programmed to take a sample at ~ d 16 and the following one at ~ d 20 post-AI (and start the daily sampling beyond ~ d 20), cows having an early mP4-decline between d 16 and 20 will be flagged late (i.e. only at the d 20 sample). Multiparous cows had a greater rate-of-daily-change in mP4 than primiparous cows from d 14 to PostPeak dispelling compromised CL quality and function in multiparous than in primiparous cows, at least during the late luteal phase.

The three groups of AI outcomes (OPEN, PREG, P-LOSS) had similar PrePeak mP4 levels, regardless of parity. In contrast, past studies reported reduced fertility in cows having lower P4 levels during the estrous cycle preceding AI (Folman et al., 1973; Meisterling and Dailey, 1987). The presence of high P4 during the pre-ovulatory follicular growth is associated with lower LH pulses, greater concentration of follicular insulin-growth factor-I (Cerri et al., 2011b), improved luteolytic responses (Cerri et al., 2011a), and subsequent fertility (Inskeep, 2004). Colazo et al. (2013) reported that supplementation of exogenous P4 (1.55 g) during the first 7 d of a timed AI synchronization protocol increased the proportion of cows responding to

the protocol and improved conception rates in non-synchronized cows. Interestingly, Bisinotto et al. (2013) used a greater exogenous P4 dose (2.76 g) during a timed AI synchronization protocol and reported that only cows that did not have a functional CL benefited from the treatment, achieving similar conception rates as those with a functional CL.

Collectively, these findings suggest that the presence of high P4 levels during the pre-ovulatory follicular development is essential for better fertility. However, by evaluating a single sample referring to the highest smoothed value of mP4 in the 13 d preceding mP4-decline, we were not able to detect such an association in the current study. Although the PrePeak mP4 in the P-LOSS group was not different than in the PREG group in our study, decreased risk of losing the pregnancy was reported when cows started a synchronization protocol with higher circulating P4 (Dirandeh et al., 2015) or during the diestrus (Cunha et al., 2008), indicating that a greater period of high P4 preceding AI benefits pregnancy maintenance in synchronized cows.

Multiparous PREG cows had significantly lower mP4 at d −2 than OPEN and P-LOSS (3.2 vs. 3.4 and vs. 3.5 ng/mL, respectively). Because more than 20% of cows might have a delayed or incomplete regression of the CL in the early postpartum period (Wiltbank et al., 2012b), and multiparous cows often required a higher dose or repeated administrations of PGF to achieve CL regression (Giordano et al., 2013b), a possible incomplete CL regression could result in slightly greater mP4 around the time of mP4-decline (i.e. d −2), as we noticed in multiparous OPEN and P-LOSS cows. This greater mP4 at d −2 and near time of AI could be lowering the chances of conception and increasing chances of pregnancy losses in cows that conceived. Recent studies reported that a delayed or incomplete CL regression was responsible for elevations in circulating P4 near time of AI, which reduced fertility (Brusveen et al., 2009). Post-AI, primiparous PREG cows had greater mP4 at d 10 and at PostPeak than those OPEN, while multiparous PREG cows had consistently greater mP4 beyond d 10 than those OPEN. In accordance with previous studies evaluating effects of natural (Stronge et al., 2005) or supplemented (Mann and Lamming, 1999; Yan et al., 2016) P4 on fertility, findings support our hypothesis that cows that conceive have higher mP4 levels post-AI than cows with other outcomes, irrespective of parity differences.

Primiparous P-LOSS cows had greater rate-of-daily-change in mP4 from d–2 to 5 and greater mP4 at d5 and 10 than those OPEN and PREG, suggesting that a more rapid increase or higher P4 levels around the fertilization period could be affecting embryo development,

compromising pregnancy maintenance. Thus, our hypothesis that pregnant cows would have greater mP4 and greater rate-of-daily-change post-AI than cows with other outcomes was not supported when comparing to those suffering pregnancy loss. Primiparous cows having greater rate-of-daily-change from d–2 to 5 and greater mP4 at d 5, and multiparous cows having greater mP4 at d–2, likely had greater P4 near time of AI. This might have been detrimental to pregnancy establishment through either reduced uterine or oviductal contractility, negatively affecting gamete transport (Hunter, 2005), or by impairing blastocyst formation (Silva and Knight, 2000).

It is important to note that slight differences, albeit significant, within individual time points of mP4 levels, should be cautiously interpreted, as the current study based the comparisons of mP4 profiles on smoothed values to address our objective of comparing profile differences amongst parity and AI outcome groups. Notwithstanding, Ambrose et al. (2015) reported that cows subjected to a timed AI synchronization protocol that did not conceive had higher plasma P4 concentrations at day of AI than cows that conceived, which could be associated with a delayed or incomplete CL regression (i.e. asynchrony) preceding AI in cows that failed to conceive. Increased fertility in cows receiving low-dose PGF at the time of AI was also reported (Ambrose et al., 2015), which could have benefitted cows that had a delayed or incomplete CL regression at time of administration. These findings support ours that higher mP4 at d–2 (multiparous cows), at d 5 (primiparous cows) and a greater rate-of-daily-change from d–2 to 5 (primiparous cows) could have been associated with higher mP4 at time of AI, caused by asynchrony of ovulation and/or affecting gamete transport and fertilization.

Primiparous P-LOSS cows had a more rapid increase (greater rate-of-daily-change) in mP4 from d–2 to 5 and greater mP4 at d 5 than other groups, which indicates either a shorter period between AI and ovulation (represented by the earlier increase in mP4), or AI occurring after ovulation. When evaluating AI occurring at different time points relative to GnRH-induced ovulation, Pursley et al. (1998) reported that cows inseminated at the later time (32 h post-GnRH, meaning 0–12 h after ovulation occurred) had the lowest calving rate and tended to have the greatest pregnancy loss rate comparing to AI occurring earlier (8–24 h post-GnRH). The shorter period between AI and ovulation also reduced embryo quality (Saacke, 2008). In this regard, the rapid elevation in P4 levels in the early stages post-AI (potentially associated to AI

occurring later than the optimum) could have had a detrimental effect on uterine environment or embryo quality, compromising pregnancy maintenance.

While some studies have reported no significant association between P4 concentration post-AI and pregnancy outcomes (Bulman and Lamming, 1978; Monteiro et al., 2015), others reported a positive effect of increased P4 on pregnancy (Mann and Lamming, 1999; Carter et al., 2008; O'Hara et al., 2014), such as greater mP4 at days 5, 6 and 7 post-AI increasing odds of embryo survival (Stronge et al., 2005). Based on our results, a potential reason for inconsistencies among previous reports could be because parity groups were not evaluated separately, especially for P4 concentrations early post-AI. To understand the influence of P4 on fertility and potentially develop practical recommendations, various studies evaluated the effect of exogenous supplementation of P4 post-AI on fertility (Yan et al., 2016), with indications of positive (Garcia-Ispierto and López-Gatius, 2016; Carter et al., 2008; Clemente et al., 2009), negative (Van Cleeff et al., 1996; Parr et al., 2014), or no (Monteiro et al., 2015; Colazo et al., 2013; Monteiro et al., 2014) effects. The discrepancies among different studies may be attributable to differences in breed, dose, and stage of estrous cycle during which treatments occurred. However, in support of our results that primiparous P-LOSS cows had greater mP4 at d 5, Monteiro et al. (2015) reported decreased pregnancy rates in embryo-recipient cows induced to have greater P4 concentrations by exogenous supplementation from 4 to 8 d after estrus, suggesting that embryos are less likely to survive when exposed to high P4 during very early stages of development.

In contrast, Kenyon et al. (2013), reported that a faster rise in P4 from d 0 to 7 was associated with improved pregnancy maintenance. In addition, higher P4 after d 4 relative to AI was associated with greater trophoblast length at later stages (Clemente et al., 2009; Mann et al., 2006), greater uterine concentrations of interferon-tau (Mann et al., 2006), and reduced pregnancy losses (Colazo et al., 2013), potentially indicating a direct effect of P4 on early embryonic development or an indirect effect via a conducive uterine environment. Our results suggest that improved conception is associated with high P4 beyond d 10 regardless of whether the pregnancy was sustained or lost.

In conclusion, primiparous cows had distinctly different mP4 profiles (greater levels and more rapid increase early post-AI) than multiparous cows. In both parity groups, cows that were

not pregnant at 55 d post-AI (OPEN or P-LOSS) had higher mP4 near the time of AI (d–2 in multiparous and d 5 in primiparous cows). Beyond d 10, PREG cows had higher mP4 profiles than OPEN cows, but not different than P-LOSS cows. We believe that additional samples taken by the IMAS to quantify mP4 closer to the time of AI (that is, two to three samples within the 48 h following mP4-decline) will improve the precision of the timing of AI.

Using IMAS data, the present study has demonstrated significant differences in mP4 profiles between primiparous and multiparous cows and among cows with different AI outcomes. A wider adoption of this precision technology will undoubtedly improve our understanding of the factors affecting reproductive physiology of the modern dairy cow, facilitating informed decision making to enhance fertility in dairy herds.

Table 4.1. Overall mean, standard deviation (SD), minimum (Min) and maximum (Max) values respective to milk progesterone levels (mP4; ng/mL) and rate-of-daily-change be- tween time points (ng/mL/d); number of observations (n) after outlier exclusion, skewness (Skew), and missing and excluded values (ME).

Variable	Mean	SD	Min	Max	n	Skew	ME
Time points of mP4							
PrePeak[a]	19.38	5.05	5.52	27.80	593	–0.87	12
d –2	3.40	0.72	0.48	4.96	599	0.09	6
d 5	3.24	2.08	0.87	10.99	575	1.90	30
d 10	8.26	4.36	0.99	24.25	576	0.94	29
d 14	17.13	4.59	5.06	26.08	521	–0.60	84
PostPeak[b]	21.55	4.00	8.66	27.80	588	–1.36	17
Rate-of-daily-change							
PrePeak to d –2	–4.37	2.82	–15.12	–0.04	595	–0.92	10
d –2 to 5	–0.02	0.35	–2.39	+1.22	582	0.93	23
d 5 to 10	+0.93	0.59	–1.27	+3.34	565	–0.53	40
d 5 to 14	+1.45	0.57	–0.91	+2.49	529	–0.86	76
d 5 to PostPeak	+1.34	0.41	0.00	+2.32	590	–0.85	15
d 10 to 14	+2.06	1.11	–2.81	+5.07	517	–0.52	88
d 10 to PostPeak	+1.63	0.68	0.00	+4.36	568	0.10	37
d 14 to PostPeak	+1.19	0.95	–2.49[c]	+4.95	507	0.82	98

[a]PrePeak = highest mP4 level at d –6.6 ± 2.7 (mean ± SD).

[b]PostPeak = highest mP4 level at d 17.4 ± 2.3 (mean ± SD).

[c]Four OPEN cows attained PostPeak level before d 14.

Table 4.2. Rate-of-daily-change in milk progesterone between time points (ng/mL/d; LSM ± SEM) in primiparous and multiparous cows.

Rate-of-daily-change, ng/mL/d	Primiparous (n = 172)	Multiparous (n = 433)	P-value[1]
PrePeak to d –2	–4.57 ± 0.25	–4.25 ± 0.17	0.47
d –2 to 5	+0.19 ± 0.03	–0.07 ± 0.02	<.0001
d 5 to 10	+1.18 ± 0.05	+0.91 ± 0.03	<.0001
d 5 to 14	+1.60 ± 0.05	+1.44 ± 0.03	0.07
d 5 to PostPeak	+1.40 ± 0.04	+1.35 ± 0.02	0.29
d 10 to 14	+2.13 ± 0.10	+2.11 ± 0.07	0.90
d 10 to PostPeak	+1.53 ± 0.06	+1.65 ± 0.04	0.10
d 14 to PostPeak	+0.93 ± 0.09	+1.28 ± 0.06	<0.01

[a]$P \leq 0.05$ indicates differences between primiparous and multiparous cows.

Table 4.3. Rate-of-daily-change (ng/mL/d) between milk progesterone time points (ng/mL/d; LSM ± SEM) by parity in non-pregnant (OPEN), presumed-pregnant (PREG), and presumed- pregnancy loss (P-LOSS) groups.

Rate-of-daily-change	Primiparous (n = 172)					Multiparous (n = 433)				
	Insemination outcome			P-values[1]		Insemination outcome			P-values[1]	
	OPEN (n = 86)	PREG (n = 62)	P-LOSS (n = 24)	OPEN vs. PREG	PREG vs. P-LOSS	OPEN (n = 271)	PREG (n = 108)	P-LOSS (n = 54)	OPEN vs. PREG	PREG vs. P-LOSS
PrePeak to d –2	–4.53 ± 0.30	–4.96 ± 0.36	–4.23 ± 0.58	0.54	0.48	–4.30 ± 0.17	–4.09 ± 0.28	–4.34 ± 0.39	0.64	0.69
d –2 to 5	+0.06 ± 0.04	+0.15 ± 0.07	+0.40 ± 0.07	0.19	0.05	–0.08 ± 0.02	–0.06 ± 0.03	–0.08 ± 0.05	0.63	0.79
d 5 to 10	+1.04 ± 0.06	+1.19 ± 0.07	+1.31 ± 0.12	0.12	0.36	+0.76 ± 0.04	+1.03 ± 0.06	+0.96 ± 0.08	<0.001	0.47
d 5 to 14	+1.60 ± 0.06	+1.56 ± 0.07	+1.65 ± 0.12	0.75	0.59	+1.30 ± 0.04	+1.51 ± 0.06	+1.52 ± 0.08	<0.05	0.92
d 5 to PostPeak	+1.44 ± 0.04	+1.42 ± 0.05[a]	+1.35 ± 0.08	0.81	0.53	+1.26 ± 0.02	+1.40 ± 0.04	+1.40 ± 0.06	0.03	0.98
d 10 to 14	+2.30 ± 0.12	+2.08 ± 0.14	+2.00 ± 0.23	0.27	0.75	+1.88 ± 0.08	+2.18 ± 0.11	+2.28 ± 0.16	0.03	0.59
d 10 to PostPeak	+1.72 ± 0.08	+1.57 ± 0.09	+1.29 ± 0.14	0.20	0.10	+1.62 ± 0.04	+1.64 ± 0.07	+1.69 ± 0.09	0.81	0.69
d 14 to PostPeak	+1.06 ± 0.11	+1.10 ± 0.12	+0.73 ± 0.21	0.38	0.13	+1.27 ± 0.07	+1.33 ± 0.09	+1.23 ± 0.13	0.64	0.55

[1]Comparisons between OPEN and PREG and between PREG and P-LOSS groups in primiparous and multiparous cows.

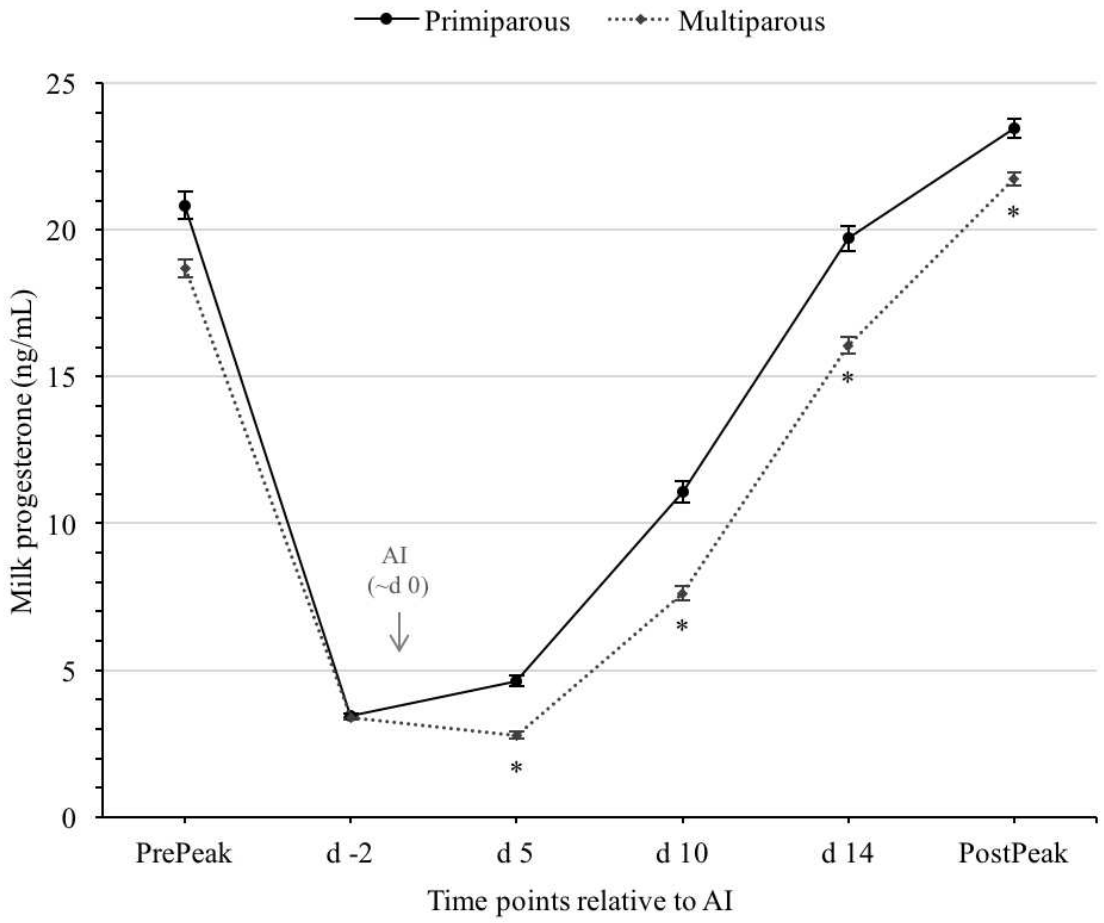

Fig. 4.1. Comparison of milk progesterone levels pre- and post-AI between primiparous and multiparous cows. *****Primiparous > Multiparous, $P \leq 0.01$.

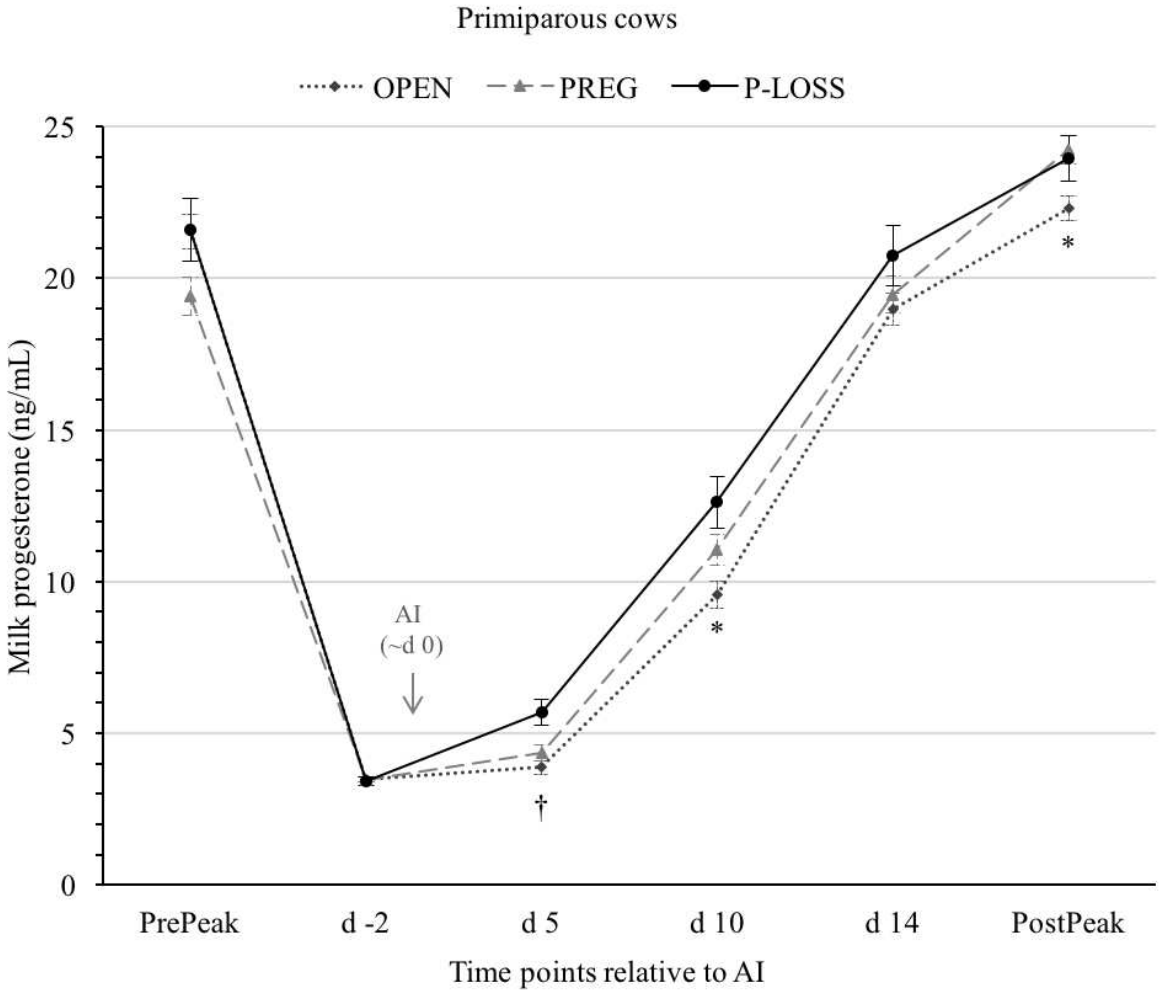

Fig. 4.2. Comparison of milk progesterone levels pre- and post-AI in primiparous cows among non-pregnant (OPEN), presumed-pregnant (PREG), and presumed-pregnancy loss (P-LOSS) groups. *PREG > OPEN, $P \leq 0.05$. †P-LOSS > PREG, $P \leq 0.05$.

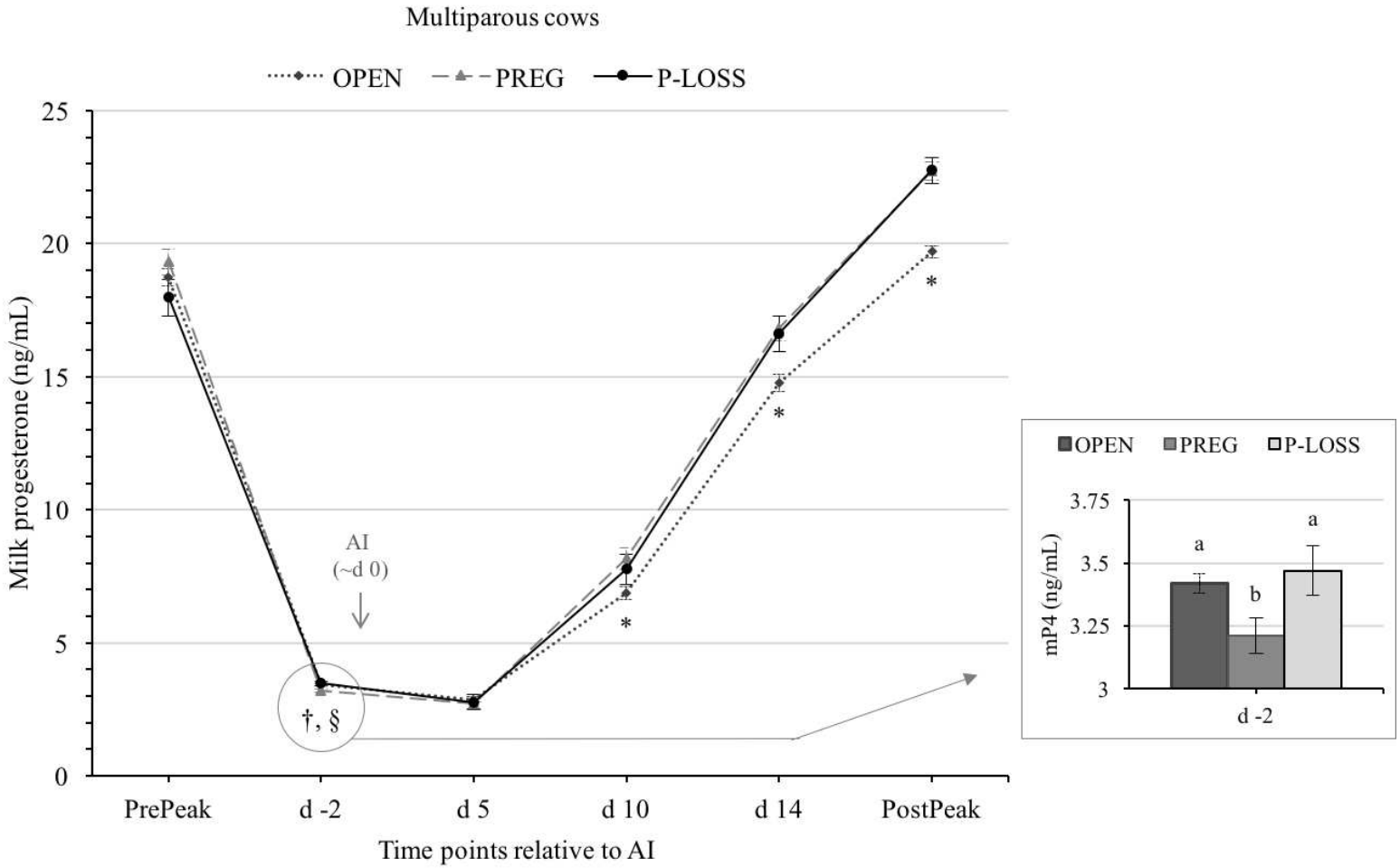

Fig. 4.3. Comparison of milk progesterone (mP4) levels pre- and post-AI in multiparous cows among non-pregnant (OPEN), presumed-pregnant (PREG), and presumed-pregnancy loss (P-LOSS) groups. *PREG > OPEN, $P \leq 0.05$. †P-LOSS > PREG, $P \leq 0.05$. §OPEN > PREG, $P \leq 0.05$. a,bDifferent superscripts indicate significant differences, $P \leq 0.05$.

CHAPTER 5

USING IN-LINE MILK PROGESTERONE DATA TO CHARACTERIZE PARAMETERS OF LUTEAL ACTIVITY ASSOCIATED WITH REDUCED FERTILITY IN HOLSTEIN COWS

Our objectives were to use in-line milk progesterone (P4) data to characterize specific parameters of luteal activity in the early postpartum period, and before and after AI, associated with reduced pregnancy per AI (P/AI) in dairy herds. Records of AI (n = 4,674) and of milk P4 (n = 158,961) obtained through an in-line milk analysis system (Herd Navigator™, DeLaval, Tumba Sweden) from 2014 to 2016 for 1,437 lactations of 941 Holstein cows in 4 commercial herds were evaluated. On average, P4 records were obtained every 2 d between 23 and 210 days in milk (DIM). Fluctuations in milk P4 values (ng/mL) were based to determine luteal activity (at least two P4 ≥ 5ng/mL after at least one P4 < 5ng/mL), luteal phases (uninterrupted period of at least two P4 ≥ 5ng/mL), mP4-decline (first P4 decline to < 5ng/mL after a luteal phase), and pregnancy (uninterrupted luteal phase until 55 d post-AI). Early postpartum luteal activity parameters were commencement of luteal activity (CLA; first postpartum luteal activity) and the number of postpartum cycle in which first AI occurred. Parameters before AI were: (1) length of the luteal phase preceding AI, (2) peak P4 in luteal phase preceding AI, (3) P4 concentration at mP4-decline, and (4) interval from mP4-decline to AI. Parameters after AI were: (1) interval from AI to luteal activity, (2) P4 concentrations at d4, (3) at d10, and (4) at d14. We used logistic regression models to evaluate the quadratic effects of parameters on P/AI, and identified cut-off values of parameter to predict P/AI using receiver operating characteristic (ROC) or through quantile comparisons. Overall P/AI was 25.1%. Cows with CLA later than 50 DIM had reduced P/AI compared to an earlier CLA. Before AI, significant cut-off values (area under the curve > 0.50; P ≤ 0.05) associated with reduced P/AI were (1) first AI occurring before 2nd cycle, (2) luteal phase preceding AI ≥ 14.4 d long, (3) peak P4 preceding AI < 24.7ng/mL, (4) P4 concentration at mP4-decline ≤ 0.5 ng/mL, (5) interval from mP4-decline to AI of ≥ 1.6 d. After AI, an interval from AI to luteal activity of ≤ 7.5 or > 13 d, and P4 concentration at d4 < 0.7 or > 3.4ng/mL, decreased P/AI. Significant cut-off values of parameters after AI associated with reduced P/AI were P4 concentration at d10 ≤ 12.4ng/mL and at d14 ≤ 22.7ng/mL. Additional parameters that negatively affected fertility were milk yield within 60 DIM ≥ 36.2kg/d, milk

yield at time of AI ≤ 31.5kg/d for first lactating cows, and infrequent Herd Navigator™ sampling (of ≥ 1 d interval between consecutives samples) among the three last P4 samples preceding mP4-decline. Using in-line milk P4 data, we identified a high prevalence of luteal activity conditions associated with reduced fertility. The characterization of the conditions presented here can be used to benchmark ovarian function in modern dairy herds monitoring real-time in-line milk P4 profiles and develop recommendations to improve reproductive performance.

5.1. INTRODUCTION

Reproductive efficiency is one of the main aspects influencing the profitability of dairy operations (Ribeiro et al., 2012). An increased rate of cows becoming pregnant at artificial insemination (AI) after the postpartum elective waiting period will increase the proportion of cows in the high productive stage of lactation (i.e. less cows in the late, low-producing stage of lactation) and, consequently, increase herd yearly milk yield. Although the genetic merit for milk production has dramatically increased in the dairy cow over the past decades, the reproductive performance of dairy herds has concomitantly decreased (Lucy, 2001; Norman et al., 2009). For instance, while the milk yield per lactation increased from 6,500 to 9,000 kg between 1970's and 2000's, the 21-d pregnancy rates decreased from 22 to 12 % (Vries and Risco, 2005), and the number of AI required to achieve a pregnancy almost doubled (Lucy, 2001). Although increased milk production per se is not necessarily detrimental to fertility, it is known that the postpartum negative energy balance, often associated with high milk production, affects the hypothalamus-ovarian axis and delays resumption of postpartum cyclicity (Beam and Butler, 1999). In addition, the increased feed intake in high producing cows increases the metabolic clearance rate of estradiol and progesterone (P4), reducing their peripheral concentrations (Sangsritavong et al., 2002) and altering ovarian function (Wiltbank et al., 2006). In association with lower peripheral estradiol concentrations, high producing cows show less overt estrus behavior than low producing cows (Lopez et al., 2004). These factors may, at least partially, explain the increased challenge of having a high proportion of cows submitted to AI after the elective waiting period and at the optimal timing related to estrus, fertilization, and ovulation.

Aiming to improve AI submission rates and accuracy of timing of AI in relation to estrus and ovulation, several reproductive strategies and technologies have been developed, such as

protocols for synchronization of ovulation (Pursley et al., 1997; Wiltbank and Pursley, 2014), estrus activity monitors (Løvendahl and Chagunda, 2010; Valenza et al., 2012), and more recently, an in-line milk P4 analysis system (Friggens et al., 2008; Chapters 3 and 4). The in-line milk analysis system (Herd Navigator™, DeLaval International, Tumba, Sweden) is an automated tool that samples and analyzes milk and measures P4 levels approximately every 2 d. Based on alterations in milk P4 levels, the in-line milk analysis system detects both the onset and end of a luteal phase, and determines estrus as well as pregnancy. Although the Herd Navigator™ is mainly designed to detect time of estrus (Friggens et al., 2008), the frequent sampling starting approximately 3 wk postpartum until the AI outcome is known allows the monitoring of postpartum ovarian activity (i.e. luteal activity) in individual cows and at the whole herd level based on P4 profiles.

Milk P4 profiles have been extensively used to evaluate luteal cycles (Bulman and Lamming, 1978; Lamming and Darwash, 1998; Blavy et al., 2016; Chapter 3) and pregnancy status (Bulman and Lamming, 1978; Gorzecka et al., 2011; Chapters 3 and 4). However, the restriction in sample size due to manual collection of samples as in the past studies might limit the ability to evaluate epidemiological aspects of ovarian activity conditions associated with fertility in a large and homogeneous population of cows (i.e. whole herds). The assessment of data from herds using the Herd Navigator™ offers the opportunity to better understand several components of spontaneous luteal activity and its associations with fertility. Using such data, recent studies have explored genetic parameters of endocrine fertility traits (Tenghe et al., 2015, 2016), associations between early postpartum luteal activity and fertility (Chapter 3), and differences in P4 dynamics between pregnant and not pregnant cows (Chapter 4). Although a delayed resumption of luteal activity and the occurrence of abnormal luteal phases preceding first AI is known to reduce fertility (Lamming and Darwash, 1998; Ranasinghe et al., 2011; Chapter 3), specific parameters of the luteal function preceding and following AI (i.e. length of the luteal phase preceding estrus, time from P4 decline to AI, and P4 concentrations at various time points) that might increase or decrease the chances of pregnancy in Holstein herds is not known.

Therefore, the objective of our study was to use in-line milk P4 data to characterize specific parameters of luteal activity before and after AI associated with reduced fertility in Holstein herds. Specifically, we aimed to (1) evaluate early postpartum parameters associated

with reduced chances of pregnancy at first AI, and (2) define optimal cut-off values of luteal phase length, interval between P4 decline and AI, and P4 concentrations before and after AI that were associated with reduced chances of pregnancy. We hypothesized that there are cut-off values for: (1) a delayed commencement of luteal activity and lower frequency of cycles preceding first AI, (2) a prolonged luteal phase preceding AI, (3) lower P4 concentrations in the luteal phase preceding AI, (4) a prolonged interval from decline in milk P4 levels and AI, (5) higher P4 concentrations around time of AI, (6) either a short or a long interval from AI to luteal activity, and (7) lower P4 concentrations after d 10 post-AI, associated with reduced fertility at AI.

5.2. MATERIALS AND METHODS

5.2.1. In-line Milk P4 Analysis and Records Description

Milk P4 records (n = 195,931) sampled and analyzed through an automated in-line milk analysis system (Herd Navigator™, DeLaval International, Tumba, Sweden) were obtained. The Herd Navigator™ is an electronic dairy herd management tool that automatically samples milk in-line, at frequent intervals in every milking cow and, using a bio-sensor technique (Pemberton et al., 1998), immediately quantifies and stores P4 concentration values. By default, sampling starts at approximately 3 wk postpartum and repeats at algorithm-driven intervals, on average every 2 d, based on a bio-model described in detail by Friggens and Chagunda (2005) and presented elsewhere (Tenghe et al., 2015; Chapters 3 and 4). The bio-model sampling frequency aims to estimate the time of estrus based on the decline in milk P4 levels (expected after a spontaneous luteolysis). Thus, raw P4 values (i.e. concentrations) are adjusted to smoothed P4 values (i.e. levels), based on a local linear growth model that controls for random noise expected in time series analyses [as described by Friggens and Chagunda (2005)], due to surrounding differences between batches of dry-sticks and in temperature/humidity (Jørgensen et al., 2016). This adjustment allows the bio-model to more accurately distinguish between high and low P4 phases (using a 5 ng/mL threshold for smoothed values), indicating the reproductive events (i.e. luteolysis and initiation of luteal activity). By evaluating this bio-model, Friggens et al. (2008) reported 93.3% sensitivity and 93.7% specificity for detection of estrus when comparing to P4 profiles of confirmed estruses.

Records of milk P4 values of 2,264 Holstein cows (3,693 lactations) from four commercial dairy herds in Alberta, Canada, were obtained from Lattec I/S (Hillarød, Denmark) as Excel files (Microsoft Corp., Richmond, WA) for the period March 2014 to December 2016. Herd demographics such as number of cows, AI records, and P4 sampling range in days in milk (DIM) used for analyses after filtering criteria (as described in Section 5.2.2) are presented in Table 5.1. Each milk P4 record contained corresponding information of herd, cow, parity, sampling date and time, DIM, P4 concentration, P4 level, and P4 level slope. In addition, data files containing records of calving date, AI date (n = 8,008), and milking date, time and yield (n = 888,535), were obtained and matched with corresponding P4 records.

By evaluating the time-series data, chronological events were coded using Excel algorithms to identify each record with new variables of interest, such as (1) first record of the lactation, (2) last record of the lactation, (3) gap between consecutives samples, and (4) events related to fluctuation in milk P4 levels. Fluctuations in milk P4 levels identified periods of low vs. high milk P4 phases using the default 5 ng/mL threshold. An increase in milk P4 levels from at least one sample < 5 ng/mL to at least two samples ≥ 5 ng/mL was set as a luteal activity. The decrease in milk P4 levels from at least two samples ≥ 5ng/mL to at least one sample < 5 ng/mL was set as a milk P4 decline (referred from now on as mP4-decline). A luteal phase was defined as the period of uninterrupted high milk P4 (interval from luteal activity to mP4-decline), and an inter-luteal phase was defined as the period of low milk P4 (between a mP4-decline and a subsequent luteal activity).

5.2.2. Filtering Criteria

Sets of filtering criteria were applied to the milk P4 data both at a lactation-level and at a variable-level to exclude periods of non-sampling (i.e. gaps) that would result in inconsistencies and bias estimation of events (i.e. inaccurate estimation of time of luteal activity or mP4-decline). Initially, all lactations that had the first P4 record obtained later than 50 DIM were excluded. For the evaluation of early postpartum parameters and first AI, only lactations that had first P4 record obtained ≤ 25 DIM were retained. At a variable-level, (1) if there was a gap > 15 d between two consecutive samples between the first postpartum record and the first postpartum luteal activity, all records from that period were excluded (Tenghe et al., 2015); (2) if there was a

gap > 8 d during a luteal phase, or a gap > 4 d in the last three samples preceding a mP4-decline, all records from that luteal phase were excluded; (3) if there was a gap > 8 d during an inter-luteal phase, all records from that inter-luteal phase were excluded; and (4) if a luteal phase or an inter-luteal phase had less than two sample records, all records from those phases were excluded.

For the AI data, the following filtering criteria were applied: (1) if an AI occurred during a luteal phase, or if there were two consecutive AI occurring within the same inter-luteal phase period, those AI records were excluded; and (2) if an AI was not preceded by a mP4-decline within 5 d, or not followed by a luteal activity, that AI record was excluded. Milk records consisted of date, time, and yield of each milking. For the evaluation of early postpartum parameters and first AI, the total daily milk yield was used to obtain the average daily yield between 10 and 60 DIM for lactations that had a minimum of 10 daily milk records within that range. In addition, the average daily milk yield between 4 d before and 4 d after each AI record (total = 8 daily milk records) was obtained for all AI that had a minimum of 4 daily records within that range. After filtering, 158,961 P4 and 5,049 AI records from 1,529 lactations were used for statistical analyses to evaluate parameters before and after AI.

5.2.3. Description of Luteal Activity Parameters

For the evaluation of early postpartum parameters and first AI, only lactations that had first postpartum P4 record obtained ≤ 25 DIM were retained. Because the initiation of postpartum P4 sampling was set to 40 DIM in one herd (Herd D), only the three other herds (A, B, and C) were evaluated. The following variables were defined: (1) **Commencement of luteal activity (CLA),** referring to the interval from calving to the initiation of postpartum luteal activity, represented by an increase in P4 levels from at least one record < 5 ng/mL to at least two consecutives records ≥ 5 ng/mL. If the first P4 record had P4 ≥ 5 ng/mL, the time of the first P4 record was defined as CLA; and (2) **Cycle number**, referring to the number of the postpartum cycle at which first AI occurred, considering the day of mP4-decline as the initiation of a new cycle. In addition, variables respective to **DIM** at which first AI occurred and **milk yield ≤ 60 DIM** (average daily yield between 10 and 60 DIM) were evaluated.

For the evaluation of parameters of luteal activity before and after AI, the following variables were considered:

Pre-AI Luteal Phase Length. The pre-AI luteal phase length was defined as the period, in days, from the day of luteal activity (first record of P4 level ≥ 5 ng/mL of that luteal phase) until the day of mP4-decline that preceded AI.

Pre-AI Peak P4. The pre-AI peak P4 was defined as the maximum P4 concentration (i.e. highest raw P4 value) recorded in the last 8 d of the luteal phase preceding the mP4-decline. Within that 8 d range, 5.6 ± 2.1 records (Mean ± SD) were available and average sampling gap was 1.3 ± 0.6 d.

P4 at mP4-decline. The P4 at time of mP4-decline was defined as the P4 concentration at the day of first record of P4 < 5 ng/mL representing the cessation of the luteal phase that preceded an AI.

Slope at mP4-decline. The slope at mP4-decline was defined as the rate of daily change (ng/mL/d) in P4 levels obtained by the Herd Navigator™ among the last three records preceding a mP4-decline event, including the P4 record at time of mP4-decline.

mP4-decline to AI Interval. The interval, in days, between the time of mP4-decline that preceded AI and the time of AI, excluding AI that occurred later than 5 d following mP4-decline. All P4 records had information of date and time of the day of which sample occurred. However, only information of date was available for AI records; thus, the time of AI was set as 12 pm, considering that all farms inseminated cows within a range from 8 am to 4 pm.

AI to Luteal Activity Interval. The interval, in days, between day of AI (d 0) and the subsequent luteal activity, represented by the first record of P4 level ≥ 5 ng/mL after AI indicating the initiation of the luteal phase.

P4 at d 4. Referred to the P4 concentration at the first P4 record after the mP4-decline, that occurred between 3 and 6 (4.4 ± 0.6) d post-AI.

P4 at d 10. Referred to the P4 concentration at the P4 record after a mP4-decline that occurred between 9 and 12 (10.1 ± 0.5) d post-AI.

Slope at d 10. Referred to the rate of daily change (ng/mL/d) in P4 levels among the last three records preceding d 10 (i.e. among records at mP4-decline, d 4, and d 10).

P4 at d 14. Referred to the P4 concentration at the P4 record after a mP4-decline that occurred between 12 and 15 (14.1 ± 0.6) d post-AI.

Slope at d 14. Defined as the rate of daily change (ng/mL/d) in P4 levels among the last three records preceding d 14 (i.e. among records at d 4, d 10, and d 14).

In addition to the parameters of luteal activity, the following variables were defined for each AI: (1) **Cycle number** (referred to the number of postpartum cycle at which AI occurred, considering the day of mP4-decline as the initiation of a new cycle); (2) **Milk yield at AI** (average daily yield between 4 d before and 4 d after AI); and (3) **Gap to mP4-decline,** referred to the average interval between consecutive samples (in days) among the last three samples preceding the mP4-decline.

As reported previously in our first two studies evaluating post-AI milk P4 profiles (Chapters 3 and 4), the definition of AI outcome for each AI (i.e. resulting in non-pregnancy or pregnancy) was based on the length of the luteal phase post-AI. In this regard, two possible scenarios occurred: (1) **Non-pregnancy**, defined as when the luteal phase post-AI had a mP4-decline (decrease in P4 levels to < 5 ng/mL) before 50 d post-AI. The mean (± SD) length of luteal phase post-AI in situations which a non-pregnancy was determined was 14 ± 7 (range, 2 to 44) d; (2) **Pregnancy**, defined as when the luteal phase post-AI was uninterrupted (no mP4-decline) until the last sample obtained for that luteal phase. The bio-model is programmed to stop sampling at approximately 55 d post-AI when a luteal phase remained uninterrupted. The mean interval between AI and last sample obtained in situations which a pregnancy was determined was 56 ± 6 (range, 50 to 117) d. Thus, the minimum interval from AI to the last sample of an uninterrupted luteal phase post-AI to define a pregnancy was 50 d, and pregnancies had an uninterrupted luteal phase of 46 ± 7 (range, 33 to 107) d. Pregnancy per AI (P/AI) was defined as the proportion of AI among all AI that resulted in a pregnancy.

5.2.4. Statistical Analysis

All analyses were performed with SAS 9.4 (SAS Institute Inc., Cary, NC, USA), considering each AI as the experimental unit. The MEANS and UNIVARIATE procedures were used to obtain descriptive statistics, such as demographics of the herds, sampling frequency and gaps, and variables used for analyses. For continuous variables defined based on the interval between consecutive events (i.e. luteal phase length, interval from mP4-decline to AI, and from AI to luteal activity), those with skewness values outside the range of ± 2 had their extreme 1%

values excluded as outliers. Because our objectives included characterizing optimal P4 concentrations associated with P/AI (regardless how values ranged among their distribution), no values respective to P4 concentrations were excluded as outliers. After removing outliers, 4,674 AI records respective to 1,437 lactations of 941 cows were retained. Frequencies of occurrences of categorical data were obtained using the FREQ procedure.

The main outcome of interest was P/AI, which was analyzed as a binary distribution (pregnant vs. not pregnant) using a generalized linear mixed models procedure (PROC GLIMMIX). Because the initiation of P4 sampling occurred at 42 DIM (rather than 21 DIM) in all cows of one herd (Herd D), evaluations of early postpartum parameters and first AI outcome only used data from Herds A, B, and C. Fixed effects initially included in a multivariable model were herd (A, B, C), year (2014, 2015, 2016), season at calving [winter (December, January, February), spring (March, April, May), summer (June, July, August), fall (September, October, November)], season at AI, parity (1^{st}, 2^{nd}, 3^{rd+}), cycle number (1 to 4) when AI occurred, and milk yield ≤ 60 DIM. As there was a significant effect of herd, subsequent models included herd as a random intercept with unstructured covariance matrices. Similarly, initial analysis for parameters before and after overall AI included were herd, year, season at AI, parity, cycle number at AI, and milk yield at AI. As there were repeated AI from the same lactation and repeated lactations from the same cow, parity (1 to 8) was included as a random effect, and cow as a repeated subject. Contrast estimates exponentials and confidence limits of multiple comparisons of categorical variables were assessed as further described. As the initial model showed significant effects of herd and cycle number (1^{st} vs. 2^{nd+}), subsequent analyses included herd as a random intercept with unstructured covariance matrices, and cycle number (1^{st} vs. 2^{nd+}), nested within parity, as a random effect. Other independent variables were kept as fixed effects for the secondary models if their effect on the model estimates was greater than 10%.

Continuous variables respective to the parameters of interest were included in the secondary models as linear effects. Variables were individually included because the variable-level filtering criteria applied to individual variables caused imbalance in their number of observations. As differences in luteal activity parameters between primiparous and multiparous cows have been reported in our previous studies (Chapters 3 and 4), individual models also included the interaction between each luteal activity parameter and parity. If the interaction was significant, that variable was further investigated by parity groups. Parameters that showed

significant effects in the secondary analyses were then separately modelled with the effect of a quadratic or linear (if no quadratic effect was evident) structure to generate predicted values for probability of P/AI. Models included cycle number, nested within parity, as a random effect, with cow as the repeated subject. Because the dispersion of values within a same continuous variable varied, odds ratio and confidence limits estimates were assessed using one standard deviation unit offset from the mean value for each variable. Probability mean values were computed using predictors of the random effect (BLUP and ILINK options). Predicted probability curves were plotted using the SGSCATTER procedure.

To identify optimal cut-off values of continuous variables of interest that had a significant quadratic or linear effect on the outcome of P/AI, receiver operating characteristic (ROC) curve analyses were performed using the LOGISTIC procedure. The ROC analysis generates a curve that accounts for the sensitivity (proportion of experimental units with the condition that is above a threshold) and specificity (proportion of experimental units that did not have the condition and was below a threshold) to identify the optimal threshold of a continuous variable that predicts the occurrence of a condition (i.e. pregnancy vs non-pregnancy). The Youden index (i.e. the point in the ROC curve that had the highest combined sensitivity and 1 – specificity) and the area under the curve were obtained for each variable. Variables that had a cut-off point that significantly predicted P/AI were stratified into two groups (≤ and > the threshold).

Therefore, multivariable logistic regression models were built including those independent variables from the initial analysis plus each individual stratified variable, with herd as a random intercept and cycle number, nested within parity, as a random effect. Variables with no significant cut-off point to predict P/AI through ROC curve were divided into four quantiles based on the percentiles of distribution (≤ 25^{th}, between 25^{th} and median, between median and 75^{th}, and > 75^{th}). As an exception, the interval between AI and luteal activity was grouped based on the frequency of sampling after AI. This criterion was chosen because the pre-determined frequency of sampling following a mP4-decline is less frequent (i.e. samples occur at ~ 4, 10, 14 d post-AI), and the sampling interval could influence this parameter rather than the true physiological interval that luteal activity occurred post-AI. Thus, this variable was grouped as occurring ≤ 7.5 d, between 7.5 and 13 d, or > 13 d post-AI. For all comparisons, probability values ≤ 0.05 were considered significant, while values between 0.051 and 0.10 were considered tendencies.

5.3. RESULTS AND DISCUSSION

5.3.1. Demographics of Herds and Sampling Pattern

Herd demographics in relation to milking system, number of cows, and sampling frequency are presented in Table 5.1. Approximately 50% of all data (cows, parity, AI and P4 records) were from Herd B, and 41, 29, and 30% of the AI records evaluated were from 1st, 2nd, and 3rd+ lactation cows, respectively. The first and last P4 record occurred on average at 23 and 210 DIM, respectively. Descriptive statistics of herd performance in relation to DIM to first and second AI, interval between two successive AI, days to pregnancy, overall pregnancy per AI and milk yield up to 60 DIM are presented in Table 5.2. Overall, first postpartum AI occurred at 74 DIM. However, due to the prolonged elective waiting period in Herd D, the first AI occurred, on average, at 106 DIM in that herd. Overall average interval between consecutive AI records was 33 d and, on average, days to pregnancy was 118 DIM.

Although the Herd Navigator™ bio-model has been described (Friggens et al., 2008) and its data used to evaluate reproductive parameters in European dairy herds (Tenghe et al., 2015), no study described the P4 sampling frequency and patterns in commercial herds running the system. Thus, descriptive statistics of records intervals (gaps) overall and within each period (e.g. before CLA and during luteal and inter-luteal phases) observed in the present study are presented in Table 5.3. Overall sampling gap between consecutives P4 records was 2.2 d, with a greater gap between records within the period before the CLA (4.4 d gap), and a shorter gap between records among the last 3 sampling preceding a mP4-decline event (1.1 d gap). Gaps between AI and first, second, and third subsequent P4 records were 4.4, 10.1, and 14.1 d.

5.3.2. Early Postpartum Parameters and Pregnancy at First AI

For the evaluation of pregnancy at first AI (P/1stAI), initial models showed significant effects of herd (A = 32.6% P/1stAI, B = 16.3% P/1stAI, C = 21.1% P/1stAI; $P < 0.001$), year (2014 = 17.4% P/1stAI, 2015 = 25.5% P/1stAI, 2016 = 21.1% P/1stAI; $P = 0.04$), calving season (Winter = 27.7% P/1stAI, Spring = 23.3% P/1stAI, Summer = 22.4% P/1stAI, Fall = 16.9% P/1stAI; $P < 0.01$), and parity (1st = 28.2% P/1stAI, 2nd = 21.1% P/1stAI, 3rd+ = 15.8% P/1stAI; P <

0.001). As differences in reproductive performance among different herds were expected, the lower P/1stAI observed in year 2014 is likely because most of the AI evaluated in that year were from Herd B, which had the lowest P/1stAI. In accordance with a previous study, cows calving in fall had lower P/1stAI compared to cows calving in other seasons (Santos and Rutigliano, 2009), and 1st lactating cows had greater P/1stAI compared to 2^{nd+} lactating cows (Tenhagen et al., 2004b; Santos and Rutigliano, 2009).

Our initial objective was to characterize parameters of early postpartum luteal activity associated with P/1stAI. To better understand to what extend milk yield, cycle number, and CLA influenced first AI outcomes, our approach was to evaluate their quadratic effects on predicted probability of P/1stAI (Fig. 5.1). Descriptive statistics for variables used to evaluate early postpartum parameters are presented in Table 5.4.

There was an inverse quadratic effect of milk yield ($P = 0.01$) and CLA ($P < 0.01$) on P/1stAI. Evaluation of early postpartum parameters and P/1stAI categorized based on ROC cut-off values or quantile groups are presented in Table 5.5. The cut-off value of milk yield to significantly predict P/1stAI was 36.2 kg/d (se: 55%, sp: 57%, AUC: 0.55; $P = 0.01$). A greater milk yield could be associated with delayed resumption of cyclicity (i.e. CLA) in high yielding dairy cows (Butler, 2003), and an early resumption of cyclicity after parturition is a major factor benefiting fertility (Ribeiro et al., 2016a). However, previous studies evaluating CLA often defined "early" and "late" CLA groups based on population mean values (Lamming and Darwash, 1998) or arbitrarily (i.e. based on antecedent studies; Opsomer et al., 1998; Shrestha et al., 2004). Although there was no significant cut-off value of CLA to predict P/1stAI, cows with CLA between 35 and 50 DIM had the greatest P/1stAI compared to CLA ≤ 27 or ≥ 50 DIM. In this regard, (Ranasinghe et al., 2011) also reported that CLA < 28 DIM was associated with reduced fertility, through increased occurrence of prolonged luteal phases early postpartum. In contrast, other studies reported increased fertility when induced resumption of cyclicity (i.e. CLA) occurred by 21 DIM (Ambrose and Colazo, 2007) and by 24 DIM (Bittar et al., 2014). In support that a late CLA (≥ 50 DIM) reduced P/1stAI, our first study (Chapter 3) also documented increased odds of pregnancy loss in multiparous cows with late (> 56 DIM) CLA.

There was a positive quadratic effect of cycle number ($P < 0.001$), but no significant effect of DIM ($P = 0.29$) on P/1stAI. The cut-off value of cycle number that predicted P/1stAI with the

best combined sensitivity (se: 68%) and specificity (sp: 50%) was 2nd cycle (AUC: 0.60; P < 0.001), meaning that fertility was improved if cows had at least one completed estrous cycle before first AI. Similarly, previous studies reported that cows expressing estrus in the early postpartum period (up to 30 or 50 DIM) had increased fertility (Thatcher and Wilcox, 1973; Yániz et al., 2006; Ambrose and Colazo, 2007). These parameters might be inter-related as cows with delayed CLA will have fewer estrous cycles preceding first AI, and consequently reduced P/1stAI. We did not observe a significant effect of DIM on P/1stAI. Although it has been reported that AI occurring at fewer DIM can result in lower fertility (Tenhagen et al., 2003), it is possible that some cows from the present study were intentionally subjected to a late first AI due to a reproductive or health disorder (e.g. prolonged anestrous or postpartum diseases), which would affect fertility at AI (Ribeiro et al., 2016a) and null any benefits of inseminating at a later DIM.

5.3.3. Parameters Before and After AI and Overall Pregnancy per AI

Descriptive statistics for variables used to evaluate parameters of luteal activity before and after AI are presented in Table 5.6. To address our objectives of characterizing parameters of luteal activity before and after AI associated with P/AI, initial models showed significant effects of herd (A = 32.5% P/AI; B = 17.2% P/AI, C = 27.8% P/AI, D = 49.1% P/AI; P < 0.001), year (2014 = 26.6% P/AI, 2015 = 27.6% P/AI, 2016 = 21.8% P/AI; P < 0.001), and cycle number (< 2 = 17.3% P/AI, ≥ 2 = 26.7% P/AI; P < 0.001) and tendencies for the effects of season (Winter = 26.3% P/AI, Spring = 24.4% P/AI, Summer = 26.9% P/AI, Fall = 22.9% P/AI; P = 0.09) and parity (1st = 26.8% P/AI, 2nd = 26.2% P/AI, 3rd+ = 21.8% P/AI; P = 0.07). Comparisons among variables categorized based on cut-off values or quantiles groups are presented in Table 5.7.

5.3.3.1. Parameters Before AI Associated with Reduced Pregnancy per AI

For the characterization of parameters before AI associated with reduced fertility, we hypothesized that a prolonged pre-AI luteal phase, lower pre-AI peak P4, a prolonged interval from mP4-decline to AI, and high P4 at mP4-decline were associated with reduced P/AI. In support to our hypotheses, we observed inverse quadratic effects of pre-AI luteal phase length (P < 0.001), a positive quadratic effect of pre-AI peak P4 (P < 0.001), and inverse quadratic effects

of P4 at mP4-decline ($P < 0.001$) and of mP4-decline to AI interval ($P < 0.001$) on P/AI. In addition, there was a positive quadratic effect of gap to mP4-decline ($P < 0.001$) on P/AI. There was no significant effect of slope at mP4-decline on P/AI ($P = 0.39$). Predicted probability curves for the effects of pre-AI luteal phase length, pre-AI peak P4, P4 at mP4-decline, mP4-decline to AI interval, and gap to mP4-decline are presented in Fig. 5.2.

Pre-AI Luteal Phase Length. The cut-off value of pre-AI luteal phase length that predicted P/AI was 14.4 d (se: 0.74, sp: 0.33, AUC: 0.54; $P < 0.001$), and AI that occurred following a prolonged luteal phase (i.e. > 14.4 d long) had reduced chances of pregnancy. Evaluations of abnormal luteal phases (i.e. short or prolonged) in previous studies were often based on pre-determined classifications (Gorzecka et al., 2011; Meier et al., 2009) or based on general assumption of the bovine estrous cycle length i.e. 18 to 24 d long; (Savio et al., 1990; Forde et al., 2011), which often resulted in estimates of high prevalence of abnormal cycles (~ 50%) in the postpartum period (Opsomer et al., 1998; Shrestha et al., 2004). However, an increase in the estrous cycle length has been suggested in the modern dairy cow (Royal et al., 2000; Remnant et al., 2015; Blavy et al., 2016). This increased estrous cycle length could be caused by abnormal reproductive function in the modern dairy cows, that could be one underlying reason for the infertility seen over the past decades in dairy cows (Lucy, 2001). In this regard, we characterized the extent by which the duration of the luteal phase preceding AI affects the success at AI, based on milk P4 profiles. Based on the evidence gathered in the present study, it is likely that a luteal phase > 14.4 d represents an impaired or delayed spontaneous luteolysis and, by the time the delayed luteolysis eventually occurs the expected pre-ovulatory follicle might not be in the optimal stage or quality for ovulation and fertilization of the released oocyte if ovulation did occur.

Pre-AI Peak P4. The cut-off value of pre-AI peak P4 that predicted P/AI was 24.7 ng/mL (se: 0.59, sp: 0.47, AUC: 0.53; $P < 0.001$), and pregnancy was less likely to occur when the peak P4 was less than 24.7 ng/mL. Cows having low P4 levels during the 7 (Bisinotto et al., 2013) or 15 d preceding AI are less fertile than cows with greater P4 levels preceding AI (Folman et al., 1973). Also, cows starting a synchronization of ovulation protocol with lower circulating P4 had increased risk of losing the pregnancy (Dirandeh et al., 2015; Cunha et al., 2008). However, studies often included in their evaluations cows that lacked a corpus luteum (i.e. luteal phase) in the cycle prior to AI (Folman et al., 1973; Bisinotto et al., 2013; Dirandeh et al., 2015),

confirming the importance of the presence of P4 during the pre-ovulatory follicular growth on adequate LH pulses, follicular insulin-like growth factor-I, luteolytic responses, and subsequent fertility (Cerri et al., 2011a,b; Inskeep, 2004). In the present study, we only evaluated cows that had a mP4-decline (i.e. had a luteal phase) prior to AI because our objective was to characterize to what extent the P4 profiles in the luteal phase preceding AI would affect fertility. Although all luteal phases evaluated had milk P4 ≥ 5ng/mL, we observed 45% of those luteal phases with sub-optimal milk P4 peak (< 24.7 ng/mL) prior to AI, which was associated with decreased fertility at AI. In contrast, Bisinotto et al. (2015) evaluated only cows that had a corpus luteum (CL) prior to AI and did not observe improved fertility in those having higher plasma P4 through additional supplemental progesterone (7.4 ng/mL in supplemented cows vs. 6.2 ng/mL in control cows). However, it is likely that cows with lower peak P4 during the luteal phase preceding AI might have had reduced P/AI due to altered luteolytic signals (Shaham-Albalancy et al., 1997), shortened CL lifespan, lower intra-follicular IGF-1 and compromised embryo quality (Cerri et al., 2011a; b).

P4 at mP4-decline. The cut-off value of P4 concentration at mP4-decline that predicted P/AI was 0.50 ng/mL (se: 0.62, sp: 0.44, AUC: 0.53; P < 0.01), and AI that occured following a high P4 at mP4-decline (> 0.50 ng/mL) had decreased chances of pregnancy compared to those with lower P4. Although all evaluated AI were preceded by a mP4-decline below the threshold of 5 ng/mL, it is possible that an incomplete or inadequate luteolysis would result in a decrease in P4 below the threshold, but still a slightly high P4 concentration around the time of AI. In this regard, it has been reported that more than 20% of cows might experience an incomplete or inadequate luteolysis during synchronization protocols (Wiltbank et al., 2012b), which might cause elevated P4 levels near time of AI and consequently reduced fertility (Brusveen et al., 2009; Ambrose et al., 2015; Colazo et al., 2017). In the present study, 43% of AI was preceded by P4 concentrations at mP4-decline between 0.50 and 4.56 ng/mL, and associated with reduced P/AI. This indicates a high prevalence of inadequate decline in P4 levels after luteolysis even within naturally-cycling cows, which might be altering sperm or oocyte transport (Hunter, 2005) or affecting the timing of ovulation in relation to the AI potentially contributing to lower than optimal P/AI. In this regard, giving a luteolytic dose of PGF to cows with P4 > 0.50 ng/mL at P4 decline might be an intervention strategy to improve fertility. Alternatively, increasing the sampling frequency to quantify P4 at least one additional time 12 to 24 h after P4 decline was

first recorded would, in our opinion, improve the precision of AI with the potential to increase fertility.

mP4-decline to AI Interval. The cut-off value of interval to AI that predicted P/AI was 1.6 d (se: 0.37, sp: 0.74, AUC: 0.57; $P < 0.001$), and AI occurring beyond 1.6 d after mP4-decline decreased the chances of pregnancy. Although many studies have evaluated the optimal time of AI in relation to estrus activity (Foote et al., 1979; Stevenson et al., 2014) or to induced-ovulation under synchronization protocols (Pursley et al., 1998), no such report exists evaluating time of AI in relation to spontaneous cessation of luteal phase. In this regard, we believe that the optimal time of AI following mP4-decline might vary considerably among cycles, as the success at AI mainly depends on the time of AI in relation to the estrus activity (Stevenson et al., 2014; Bombardelli et al., 2016) and ovulation (Pursley et al., 1998). Pursley et al. (1998) observed increased P/AI when AI occurred between 8 and 24 h following GnRH, or approximately 32 to 72 h after PGF-induced luteolysis. Also, Bombardelli et al. (2016) reported 23 to 41 h as the optimal interval from onset of estrus activity to AI. However, these two studies evaluated cows that were subjected to protocols of synchronization of ovulation, so a new follicular wave was always expected to have been initiated ~ 7 d preceding the PGF-induced luteolysis. As we observed a large variation in the length of luteal phase preceding AI, the stage of the pre-ovulatory follicle after spontaneous mP4-decline might have varied in relation to the time of estrus behaviour, ovulation, and fertilization. Regardless, the standard recommendation by DeLaval for herds using the Herd Navigator™ is to inseminate cows between 36 and 48 h after a mP4-decline. Our findings indicate that this interval should not exceed 38 h to achieve improved proportion of pregnancies at AI with the highest probability of pregnancy attained when AI occurred within the first 24 h (Fig. 5.2 e).

Gap to mP4-decline. The cut-off value of the average interval among the last three samples preceding mP4-decline that predicted P/AI was 0.98 d (se: 0.66, sp: 0.44, AUC: 0.53; $P < 0.001$), and P/AI was reduced if the average sampling gap exceeded 0.98 d. Although this variable has no direct physiological bearing, this result indicates that less frequent samples at the end of the luteal phase is likely detecting the luteolysis (i.e. mP4-decline) too late compared to the true time of luteolysis. As all AI events occurred based on the mP4-decline detected by the system, a delayed detection of mP4-decline will also cause a delayed AI in relation to the optimal timing of AI. Although the sampling bio-model is designed to have a frequent sampling

(at least once daily) at the end of the luteal phase (starting ~ 18 d following a previous mP4-decline), we observed a high prevalence (41%) of AI being preceded by a lower sampling frequency preceding mP4-decline. This inconsistent sampling frequency at the end of the luteal phases could be caused by the increased incidence of abnormal cycles (i.e. either short or prolonged) in the modern dairy cow (Royal et al., 2000; Remnant et al., 2015; Blavy et al., 2016). In this regard, a short or prolonged luteal phase could be interfering with the bio-model sampling pattern.

5.3.3.2. Parameters After AI Associated with Reduced Pregnancy per AI

For the characterization of parameters after AI associated with reduced fertility, our hypotheses were that a short or prolonged interval from AI to luteal activity, higher P4 near time of AI (i.e. at d 4 post-AI), and lower P4 beyond d 10 post-AI, were factors associated with decreased P/AI. In support of our hypotheses, there were negative quadratic effects of AI to luteal activity interval ($P < 0.001$) and of P4 at d4 ($P < 0.001$), but positive quadratic effects of P4 at d10 ($P < 0.001$), slope at d10 ($P = 0.002$), P4 at d14 ($P < 0.001$), and slope at d14 ($P < 0.001$). There was an interaction between milk yield at AI and parity ($P = 0.01$), being significant only for 1st parity cows. Predicted probability curves for the effects of AI to luteal activity interval, P4 at d4, P4 at d10, slope at d10, P4 at d14, slope at d14, and milk yield at AI (1st parity cows) are presented in Fig. 5.3.

AI to Luteal Activity Interval. Because the first, second, and third P4 samples following AI occurred at 4 (range 3 – 6), 10 (range 9 – 12), and 14 (range 12 – 15) d post-AI, respectively, the interval between AI and subsequent luteal activity was categorized in 3 groups as occurring ≤ 7.5 d, between 7.5 and 13 d, or > 13 d post-AI. The greatest P/AI was observed when the luteal activity occurred between 7.5 and 13 d post-AI. An early (≤ 7.5 d) and a delayed (> 13 d) onset of luteal activity post-AI were prevalent in 17 and 16% of the AI evaluated, respectively, and both were associated with reduced P/AI. The factors often linked to impaired ovulation (i.e. onset of luteal activity) are the negative energy balance (Staples et al., 1990; Beam and Butler, 1997) and the increased metabolic clearance rate of estradiol and P4 in high producing cows (Wiltbank et al., 2006). Reduced estradiol might delay the time at which estradiol levels are sufficiently elevated to induce GnRH/LH surge (Stock and Fortune, 1993). In this case, the

ovulatory GnRH/LH surge would be triggered only when the pre-ovulatory follicle has reached an adequate size to produce enough estradiol, leading to delayed ovulation and the release of an aged oocyte (Revah and Butler, 1996; Wiltbank et al., 2006).

By analyzing P4 profiles in 1,682 dairy cows, Lamming and Darwash (1998) reported that 13% of cycles had a delayed ovulation (defined as an inter-luteal phase ≥ 12 d). Our study indicates an increase in the prevalence of delayed ovulation, as we observed a delayed interval from AI to luteal activity (> 13 d) in approximately 16% of the cycles evaluated, and being associated with reduced P/AI. Interestingly, a short interval (≤ 7.5 d) from AI to luteal activity also reduced P/AI. It is possible that the short interval to increase in P4 levels post-AI is caused by an incomplete luteolysis preceding mP4-decline, leading to high P4 near time of AI and more rapid increase in P4 early post-AI. Similar to a high P4 at mP4-decline, the rapid increase in P4 levels post-AI might have affected fertilization through impaired oviductal contractility and gamete transport (Hunter, 2005) or early embryo development including blastocyst formation (Silva and Knight, 2000). Another possibility is that some AI were performed too late relative to time of ovulation, which would have lowered the chances of fertilization and resulting in increased P4 at d4.

P4 at d4. Although there was no significant cut-off value of P4 concentration at d4 post-AI to predict P/AI (AUC = 0.47), comparisons among quantiles showed that concentrations between 0.68 and 3.38 ng/mL were associated with greater P/AI comparing to P4 concentrations < 0.68 or > 3.38 ng/mL. The P4 concentrations at d4 might be related to the interval between AI and luteal activity presented above. A short interval between AI and luteal activity will result in greater P4 concentration at d4, while a delayed interval between AI and luteal activity will result in lower P4 levels at d4. Both scenarios associated were with reduced P/AI.

P4 concentrations and slopes beyond d10. There were significant cut-off values to predict P/AI for P4 at d10 (cut-off: 12.4 ng/mL, se: 0.81, sp: 0.30, AUC: 0.56; $P < 0.001$), slope at d10 (cut-off: 0.39 ng/mL/d, se: 0.88, sp: 0.21, AUC: 0.53; $P < 0.01$), P4 at d14 (cut-off: 22.7 ng/mL, se: 0.56, sp: 0.54, AUC: 0.56; $P < 0.001$), and slope at d14 (cut-off: 4.7 ng/mL/d, se: 0.71, sp: 0.41, AUC: 0.58; $P < 0.001$). For all the above parameters, categories below the cut-off values had decreased P/AI (Table 5.7). Because increasing P4 levels following AI is essential to nourish the uterine environment and support embryo development (Garrett et al., 1988) and

pregnancy (Inskeep, 2004), lower P4 levels beyond d10 were expected to be associated with reduced fertility. However, several studies investigated the effects of supplementing exogenous P4 post-AI on fertility, and reported inconsistent associations with fertility [positive (Garcia-Ispierto and López-Gatius, 2016; Carter et al., 2008), negative (Van Cleeff et al., 1996; Parr et al., 2014), or no effects (Colazo et al., 2013; Monteiro et al., 2014)]. Although the conditions among studies varied (i.e. breed, P4 dose, estrous cycle stage), our results provide an insight that cows benefiting from P4 supplementation might be those having sub-optimal P4 concentrations (i.e. below the cut-off) at different time points relative to AI.

In addition to the luteal activity parameters, there was also a significant cut-off value of milk yield at AI that predicted P/AI in 1st parity cows (cut-off: 31.5 kg/d, se: 0.66, sp: 0.45, AUC: 0.57; $P < 0.001$). Interestingly, 1st parity cows yielding ≤ 31.5 kg/d at time of AI had decreased P/AI compared to those with greater yield (22.0 vs. 30.2%, respectively). It is expected that higher producing cows will be more likely to undergo negative energy status (Lee and Kim, 2006) or metabolic disorders (Fleischer et al., 2001) that would impair fertility. However, the severity of negative energy balance is less evident in 1st than in 2^{nd+} parity cows (Lee and Kim, 2006). Also, we evaluated AI occurring at any period postpartum (i.e. from 24 to 180 DIM), so level of milk yield at time of AI might be irrespective of the metabolic challenges undergone during the early postpartum period. Thus, it is likely that lower milk yield at AI was associated with the occurrence other health disorders near time of AI, that could have negatively affected both milk yield (Collier et al., 2017) and pregnancy per AI (Santos et al., 2010).

These conditions related to sub-optimal P4 concentrations associated with reduced P/AI were highly prevalent, ranging from 27% (d10) to 51% (d14) among all AI.

5.3.4. Prevalence of Conditions Associated with Reduced Fertility and Potential Strategies to Improve Fertility

We were surprised to see that most luteal activity parameters evaluated in the present study had significant effects on P/AI, and the categorized conditions associated with reduced fertility were highly prevalent among all AI (Fig. 5.4). For instance, 46% of cows received first AI before the 2nd postpartum cycle, and those AI resulted in much lower P/AI (16 vs 28%) than AI

occurring beyond the 2nd cycle. Cows receiving first AI before 2nd cycle are likely related to those having a delayed CLA, which also reduced fertility when occurring beyond 50 DIM, in 25% of the cows. These results suggest that, in herds monitoring real-time milk P4 profiles, avoiding insemination of cows in the first estrous cycle could result in improved pregnancy at first AI.

We observed that more than 30% of the AI were preceded by a prolonged (> 14.4 d) luteal phase, and among all the pre-AI luteal phase, 45% had a sub-optimal peak P4, both factors negatively affecting P/AI. Although we were not able to evaluate the specific mechanism(s) affecting P/AI in these categories (prolonged luteal phase and sub-optimal peak P4), an induced-interruption of luteal phases exceeding 14 d (i.e. through administration of exogenous PGF), or exogenous P4 supplementation in cycles that had not attained a peak P4 > 24 ng/mL could improve fertility at AI.

Approximately 43% of the AI were preceded by high P4 at mP4-decline (> 0.50 ng/mL), and 17% of AI were followed by a rapid increase in P4 levels, both factors likely related to incomplete luteolysis and associated with reduced P/AI. Because the Herd Navigator™ system immediately notifies the time of mP4-decline, we believe that an administration of exogenous PGF in cases where the P4 concentration at time of mP4-decline is > 0.50 ng/mL would reduce the proportion of incomplete luteolysis, potentially decreasing P4 concentrations around time of AI and improve fertility in these cows. In this regard, Ambrose et al. (2015) observed improved conception rates in cows receiving low-doses of PGF at time of AI, which could have benefited cows that had incomplete luteolysis and high P4 around time of AI. Also, in the present study, 16% of AI were followed by a delayed luteal activity (> 13 d), which likely caused sub-optimal P4 at d 4 being associated with reduced P/AI. In this regard, another strategy might be to administer exogenous GnRH at time of AI to reduce the incidence of delayed ovulations, consequently improving chances of pregnancy.

The prevalence of sub-optimal P4 concentrations at d10 and at d14 associated with reduced fertility was 27 and 51%, respectively. Inconsistent benefits on fertility have been observed among studies testing exogenous P4 supplementation at different time points, post-AI (Mann et al., 1999; Parr et al., 2014; Colazo et al., 2013; Monteiro et al., 2015). Thus, it is likely that cows benefiting from post-AI P4 supplementation would be those having a sub-optimal P4 post-AI. In

this regard, herds monitoring real-time P4 profiles have the opportunity to supplement P4 only in cows with sub-optimal P4 concentrations. However, future research is required to evaluate the benefits in terms of performance and costs associated with strategic hormonal intervention to manipulate and reduce luteal activity conditions associated with reduced fertility.

In conclusion, our results demonstrate that the modern dairy cow has a high prevalence of different conditions of luteal activity before and after AI associated with reduced fertility. The characterization of these various conditions can be used to benchmark ovarian function in herds monitoring real-time in-line milk P4 profiles. In such herds, there is a potential for strategic and targeted hormonal interventions before or after AI in cows presenting unfavourable luteal activity conditions. Further research is required to better understand factors associated with abnormal luteal activity conditions and to actively test potential strategic recommendations to improve success at AI.

Table 5.1. Herds demographics such as milking system, number of milking cows during the study period, Herd Navigator™ (HN) installation date, number of cows and lactations evaluated, number of AI and progesterone (P4) records used for analyses, and descriptive statistics of first and last HN record in the postpartum period.

		Herd A	Herd B	Herd C	Herd D	Overall
Milking system		Parlor	Parlor	Robot	Robot	
Milking cows	**n**	420	355	140	110	1025
HN installation date[a]		Oct-14	Apr-14	Jan-15	Mar-14	
Cows evaluated	**n**	197	482	139	123	941
Lactations evaluated	**n**	330	742	186	179	1437
AI records	**n**	1381	2391	583	316	4674
AI in 1st parity	**n**	570	1005	205	164	1,944
AI in 2nd parity	**n**	431	646	170	90	1,337
AI in 3rd+ parity	**n**	380	743	208	62	1,393
P4 records	**n**	56,888	64,242	17,189	20,642	158,961
First record[b]	**n**	1381	2394	583	316	4674
	mean	21.8	21.2	21.3	42.4	22.8
	min	20.2	20.1	20.1	42.0	20.1
	max	47.6	47.2	48.7	46.9	47.6
	sd	4.47	3.05	3.79	2.21	6.4
Last record[c]	**n**	1381	2394	583	316	4674
	mean	191.9	213.1	242.0	207.7	210.1
	min	58.2	61.2	42.4	118.7	42.4
	max	357.4	393.8	600.1	366.0	600.1
	sd	58.67	54.37	101.55	48.82	64.85

[a] Date (month-year) which the Herd Navigator™ was installed and P4 records started to be obtained.
[b] Days in milk which the first P4 record was obtained in the postpartum period.
[c] Days in milk which the last P4 record was obtained in the postpartum period.

Table 5.2. Descriptive statistics of herd performance in relation to days in milk (DIM) to first and second postpartum AI, interval between consecutive AI, days to pregnancy, overall pregnancy per AI, and milk yield within 60 DIM (average daily yield between 10 and 60 DIM).

		Herd A	Herd B	Herd C	Herd D	Overall
First AI (DIM)	n	498	742	186	179	1605
	mean	68.5	70.4	72.0	105.8	73.9
	min	23.5	33.5	29.5	63.5	23.5
	max	106.5	179.5	390.5	169.5	390.5
	sd	12.34	18.46	31.02	17.18	21.84
Second AI (DIM)	n	357	547	141	83	1128
	mean	95.1	98.8	101.3	137.8	100.8
	min	42.5	56.5	52.5	102.5	42.5
	max	165.5	220.5	175.5	181.5	220.5
	sd	18.98	22.98	24.47	18.97	24.16
AI Interval (d)	n	712	1566	386	124	2788
	mean	34.4	31.6	33.3	33.1	32.6
	min	16	15	18	18	15
	max	234	160	334	82	334
	sd	22.65	17.58	27.20	17.20	20.53
Days to pregnancy (DIM)	n	444	412	162	155	1173
	mean	104.7	118.9	139.9	127.4	117.5
	min	46.5	33.5	29.5	75.5	29.5
	max	294.5	275.5	390.5	243.5	390.5
	sd	41.64	51.79	72.29	34.76	51.16
Pregnancy per AI	%	32.2	17.2	27.8	49.1	25.1
Milk yield ≤ 60 DIM (kg/d)[a]	n	474	707	169	325[b]	1350
	mean	39.4	37.5	34.4	42.8	37.8
	min	21.9	12.3	16.2	19.7	12.3
	max	61.7	60.4	56.8	66.5	61.7
	sd	8.68	9.15	8.82	8.59	9.08

[a] Average daily milk yield between 10 and 60 DIM

[b] Herd D had P4 records available only starting from 42 DIM; thus, milk yield ≤ 60 DIM was not used in further statistical analysis for this herd. Milk yield data presented here for Herd D are respective to all milk records available, irrespective of matching the P4 records obtained.

Table 5.3. Descriptive statistics [observations (n), mean, minimum (Min), maximum (Max), standard deviation (SD), and percentiles (25^{th}, 50^{th}, and 75^{th})] of: progesterone (P4) sampling gaps (interval [d] between consecutive records) and frequency, gap between AI and following P4 records (d), and frequency of daily milk yield records within each classification used to obtain variables of interest.

Parameter	n	Mean	Min	Max	SD	25^{th}	50^{th}	75^{th}
Overall P4 sampling gap[a]	158,324	2.23	0.03	14.95	1.92	0.67	1.33	3.68
*P4 sampling gap from calving to CLA[b]	10,741	4.42	0.26	14.95	2.02	2.69	4.44	5.94
*P4 sampling gap from CLA to last sample[c]	139,071	2.08	0.03	8.00	1.83	0.67	1.14	3.37
P4 sampling gap during luteal phases	120,424	1.92	0.03	8.00	1.64	0.67	1.04	2.67
P4 sampling gap during inter-luteal phases	27,690	2.74	0.05	8.00	2.31	0.67	1.54	4.99
P4 sampling gap of last 3 records preceding mP4-decline	5,544	1.07	0.20	3.99	0.66	0.64	0.81	1.35
P4 sampling gap within last 8 d preceding mP4-decline[d]	4,668	1.27	0.31	3.99	0.63	0.80	1.06	1.66
Frequency of P4 records within -8 d from mP4-decline[e]	4,668	5.63	2.00	8.00	2.11	4.00	6.00	8.00
Gap between AI and first following P4 record	4,517	4.44	3.05	5.96	0.55	4.13	4.40	4.76
Gap between AI and second P4 record	4,264	10.12	9.06	11.98	0.49	9.74	10.11	10.27
Gap between AI and third P4 record	3,752	14.08	12.06	14.99	0.55	13.78	14.16	14.45
Frequency of milk records within ±4 d relative to AI	4,669	7.98	4.00	8.00	0.20	8.00	8.00	8.00

CLA = Commencement of luteal activity

[a] Interval between consecutive P4 records, including all records used for statistical analysis.

[b] Interval between consecutive P4 records, including only records before the CLA.

[c] Interval between consecutive P4 records, including only records after the CLA.

[d] Interval between consecutive P4 records, including only records obtained in the last 8 d preceding an mP4-decline.

[e] Number of P4 records available within the last 8 d preceding an mP4-decline.

*Because the initiation of P4 sampling occurred at 42 DIM in all cows of Herd D (rather than 21 DIM as in other three herds), evaluations of these parameters only included data from Herds A, B, and C.

Table 5.4. Descriptive statistics [observations (n), mean, minimum (Min), maximum (Max), standard deviation (SD), standard error (SE), skewness (Skew), and percentiles (25^{th}, 50^{th}, and 75^{th}) of early postpartum parameters used for the evaluation of pregnancy at first postpartum AI.

Parameter*	n	Mean	Min	Max	SD	SE	Skew	25^{th}	50^{th}	75^{th}
Milk yield ≤ 60 DIM (kg/d)[a]	1350	37.8	12.3	61.7	9.08	0.25	0.0	31.0	37.5	45.1
Commencement of luteal activity (d)[b]	1351	40.7	20.1	150.3	20.5	0.56	1.6	27.2	35.4	49.8
Cycle number at AI[c]	1354	1.6	1	4	0.63	0.02	0.7	1	2	2
DIM[d]	1354	69.6	23.5	179.5	16.94	0.46	1.4	59.5	66.5	76.5

[a] Average daily milk yield between 10 and 60 days in milk (DIM).
[b] Interval from calving to commencement of luteal activity (first mP4 values ≥ 5 ng/mL).
[c] Number of postpartum cycle which artificial insemination (AI) occurred.
[d] DIM of which AI occurred.
*Because the initiation of P4 sampling occurred at 42 DIM in all cows of Herd D (rather than 21 DIM as in other three herds), evaluations of these parameters only included data from Herds A, B, and C.

Table 5.5. Incidence and odds ratio analyses of early postpartum parameters categorized based on ROC[1] cut-off value or quantile groups on pregnancy at first AI (P/1^{st}AI).

Parameter[2]	Category	Incidence, %	P/1^{st}AI, %	P/1^{st}AI, n	Odds ratio	Lower	Upper	P
Cycle number at AI	< 2^{nd} cycle	45.6	15.6	96/614	0.48	0.36	0.64	<.0001
	≥ 2^{nd} cycle	54.4	28.1	206/733	Ref			
CLA[3] (d)	Q1[a]	25.2	23.2	71/349	1.54	1.02	2.31	0.04
	Q2[ab]	25.0	26.1	80/347	1.96	1.31	2.93	0.001
	Q3[b]	25.1	33.0	101/348	2.48	1.68	3.67	<.0001
	Q4[c]	24.7	17.7	54/343	Ref			
Milk yield ≤ 60 DIM[4] (kg/d)	< 36.2	45.5	26.7	168/630	1.83	1.40	2.38	<.0001
	≥ 36.2	54.6	18.1	137/756	Ref			

[1] ROC = receiver operating characteristic curve
[2] Only variables that showed significant quadratic effects (P ≤ 0.05) on the linear models are presented. Because the initiation of P4 sampling occurred at 42 DIM in all cows of Herd D (rather than 21 DIM as in other three herds), evaluations of these parameters only included data from Herds A, B, and C.
[3] CLA = Commencement of luteal activity. Q1 = below 25^{th} percentile; Q2 = between 25^{th} and 50^{th} percentiles; Q3 = between 50^{th} and 75^{th} percentiles; Q4 = above 75^{th} percentile.
[4] Average daily milk yield between 10 and 60 days in milk (DIM).
[a,b,c,d] Different superscripts mean significant differences (P ≤ 0.05) of multiple comparisons.

Table 5.6. Descriptive statistics [observations (n), mean, minimum (Min), maximum (Max), standard deviation (SD), standard error (SE), skewness (Skew), and percentiles (25th, 50th, and 75th) of parameters that had significant quadratic effects on pregnancy per AI.

Parameter	N	Mean	Min	Max	SD	SE	Skew	25th	50th	75th
Pre-AI Luteal Phase Length[a] (d)	4429	13.71	1.49	43.30	6.33	0.10	1.82	9.98	12.44	15.35
Pre-AI Peak P4[b] (ng/mL)	4674	23.95	5.00	27.99	3.59	0.05	-1.73	22.71	25.01	26.48
P4 at mP4-decline[c] (ng/mL)	4674	0.59	0.02	4.56	0.47	0.01	3.06	0.34	0.45	0.67
Interval to AI[d] (d)	4533	1.85	0.07	4.94	0.56	0.01	-0.15	1.55	1.87	2.27
AI to Luteal Activity Interval[e] (d)	4533	10.10	0.08	33.39	3.67	0.05	1.73	9.61	10.07	10.65
P4 at d4 (ng/mL)	4517	3.12	0.02	27.99	4.48	0.07	2.86	0.68	1.40	3.38
P4 at d10 (ng/mL)	4517	3.12	0.02	27.99	4.48	0.07	2.86	0.68	1.40	3.38
Slope at d10 (ng/mL/d)	4264	1.84	-4.67	8.68	1.66	0.03	0.25	0.79	1.96	2.72
P4 at d14 (ng/mL)	3752	20.70	0.10	27.99	5.97	0.10	-1.40	18.16	22.54	25.05
Slope at d14 (ng/mL/d)	3752	5.06	-4.21	9.97	2.20	0.04	-0.71	3.74	5.40	6.69
Milk Yield at AI[f] (kg/d)	4674	37.99	11.45	65.31	8.32	0.12	0.11	31.93	37.52	43.91
Gap to mP4-decline[g] (d)	4674	1.05	0.20	3.99	0.64	0.01	1.57	0.63	0.79	1.32

[a] Length of the luteal phase that preceded AI.
[b] Maximum P4 concentration recorded in the last 8 d of the luteal phase that preceded AI.
[c] P4 concentration at the day of first record < 5 ng/mL following a luteal phase.
[d] Interval between cessation of the luteal phase (mP4-decline) and AI.
[e] Interval between AI and the day which subsequent luteal activity (P4 values ≥ 5 ng/mL) occurred.
[f] Average daily milk yield between 4 d before and 4 d after AI.
[g] Average interval between consecutive P4 records among the last three records preceding mP4-decline.

Table 5.7. Incidence and odds ratio analyses of categorized parameters before and after AI on pregnancy per AI (P/AI).

Parameter[1]	Category	Incidence, %	P/AI, %	P/AI, n	OR	Lower	Upper	P
Pre-AI LP Length[2] (d)	≤ 14.4 d	68.6	27.7	840/3038	1.42	1.22	1.66	<.0001
	> 14.4 d	31.4	21.5	299/1391	Ref			
Pre-AI Peak P4[3] (ng/mL)	≤ 24.7 ng/mL	45.4	22.9	486/2123	0.84	0.73	0.96	0.014
	> 24.7 ng/mL	54.6	26.9	687/2551	Ref			
P4 at mP4-decline[4] (ng/mL)	≤ 0.5 ng/mL	57.5	27.0	726/2685	1.28	1.11	1.47	<.001
	> 0.5 ng/mL	42.6	22.5	447/1989	Ref			
Interval to AI[5] (d)	≤ 1.6 d	28.7	32.7	427/1302	1.18	1.01	1.38	0.042
	> 1.6 d	71.3	23.1	746/3231	Ref			
AI to LA Interval[6] (d)	≤ 7.5 d	17.3	20.8	163/782	0.64	0.53	0.78	<.0001
	7.5 to 13 d	67.1	29.0	883/3041	Ref			
	> 13 d	15.7	17.9	127/710	1.93	1.56	2.39	<.0001
P4 at d4 (ng/mL)	Q1[a]	25.1	17.8	201/1132	0.72	0.58	0.89	0.003
	Q2[b]	25.0	29.8	336/1129	1.48	1.22	1.80	<.0001
	Q3[b]	24.9	29.9	337/1126	1.40	1.15	1.70	0.001
	Q4[c]	23.2	23.2	262/1130	Ref			
P4 at d10 (ng/mL)	≤ 12.4 ng/mL	27.1	18.2	210/1156	0.53	0.45	0.63	<.0001
	> 12.4 ng/mL	72.9	28.6	888/3108	Ref			
Slope at d10 (ng/mL/d)	≤ 0.39 ng/mL/d	18.4	16.4	128/783	0.48	0.39	0.60	<.0001
	> 0.39 ng/mL/d	81.6	27.9	970/3481	Ref			
P4 at d14 (ng/mL)	≤ 22.7 ng/mL	51.4	22.8	440/1930	0.66	0.57	0.77	<.0001
	> 22.7 ng/mL	48.6	30.4	553/1822	Ref			
Slope at d14 (ng/mL/d)	≤ 4.7 ng/mL/d	37.9	20.5	291/1422	0.57	0.49	0.67	<.0001
	> 4.7 ng/mL/d	62.1	30.1	702/2330	Ref			
Milk yield at AI[7] (kg/d)	≤ 31.5 kg/d	41.7	22.0	178/811	0.80	0.64	1.00	0.052
	> 31.5 kg/d	58.3	30.2	342/1133	Ref			
Gap to mP4-decline[8] (d)	< 0.98 d	58.6	28.1	769/2741	1.36	1.18	1.57	<.0001
	≥ 0.98 d	41.4	20.9	404/1933	Ref			

[1]Only variables that showed significant quadratic effects (P ≤ 0.05) on the linear models are presented.
OR = Odds ratio.
[2] Length of the luteal phase (LP) that preceded AI.
[3] Maximum P4 concentration recorded in the last 8 d of the luteal phase that preceded AI.
[4] P4 concentration at the day of first record < 5 ng/mL following a luteal phase.
[5] Interval between cessation of the LP (mP4-decline) and AI.
[6] Interval between AI and the day which subsequent luteal activity (LA; P4 values ≥ 5 ng/mL) occurred.
[a,b,c,d] Different superscripts mean significant differences (P ≤ 0.05) of multiple comparisons.
[7]Average daily milk yield between 4 d before and 4 d after AI for 1st parity cows.
[8] Average interval between consecutive P4 records among the last three records preceding mP4-decline.

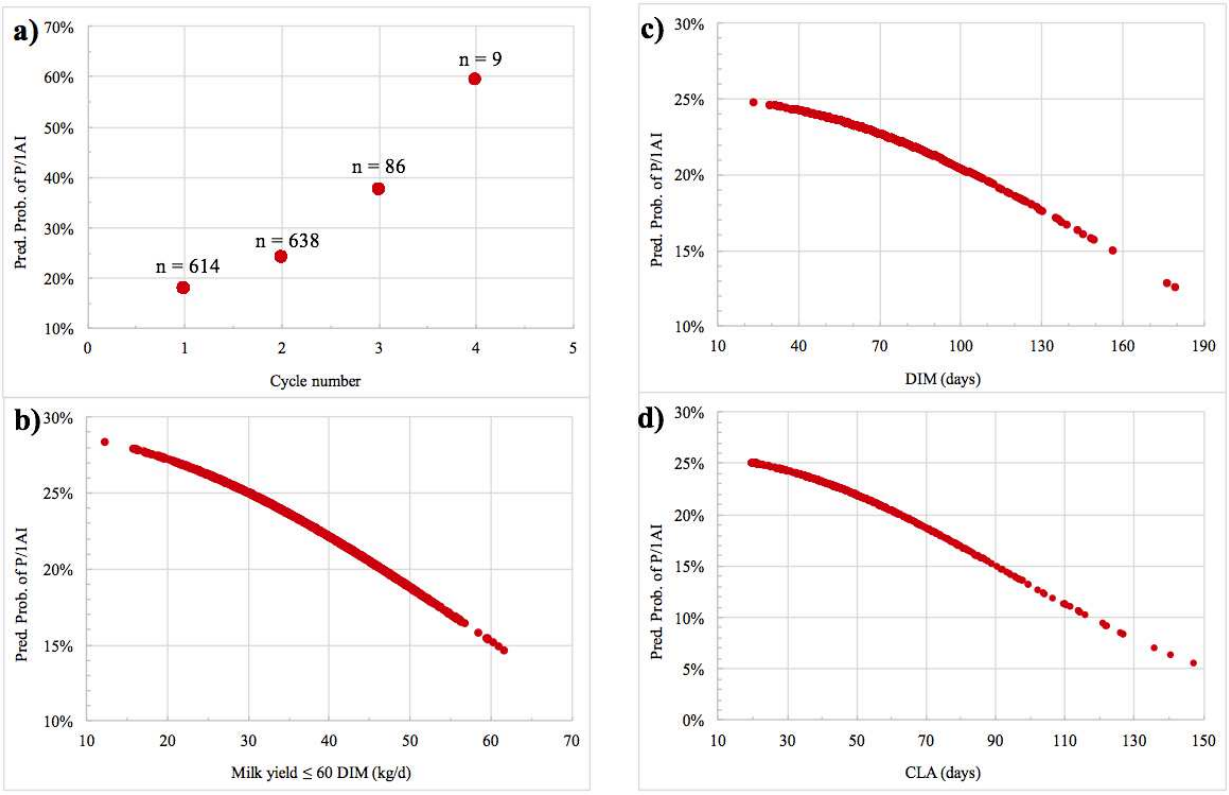

Fig. 5.1. Predicted probability of pregnancy per AI at first AI (P/1stAI) according to **(a)** number of postpartum cycle in which AI occurred, **(b)** average daily milk yield between 10 and 60 DIM, **(c)** days in milk (DIM) at which AI occurred, and **(d)** interval between calving and commencement of luteal activity (CLA). Data presented here represent only three herds (A, B, and C) because P4 sampling started at 42 DIM in Herd D (rather than at 21 DIM in other herds).

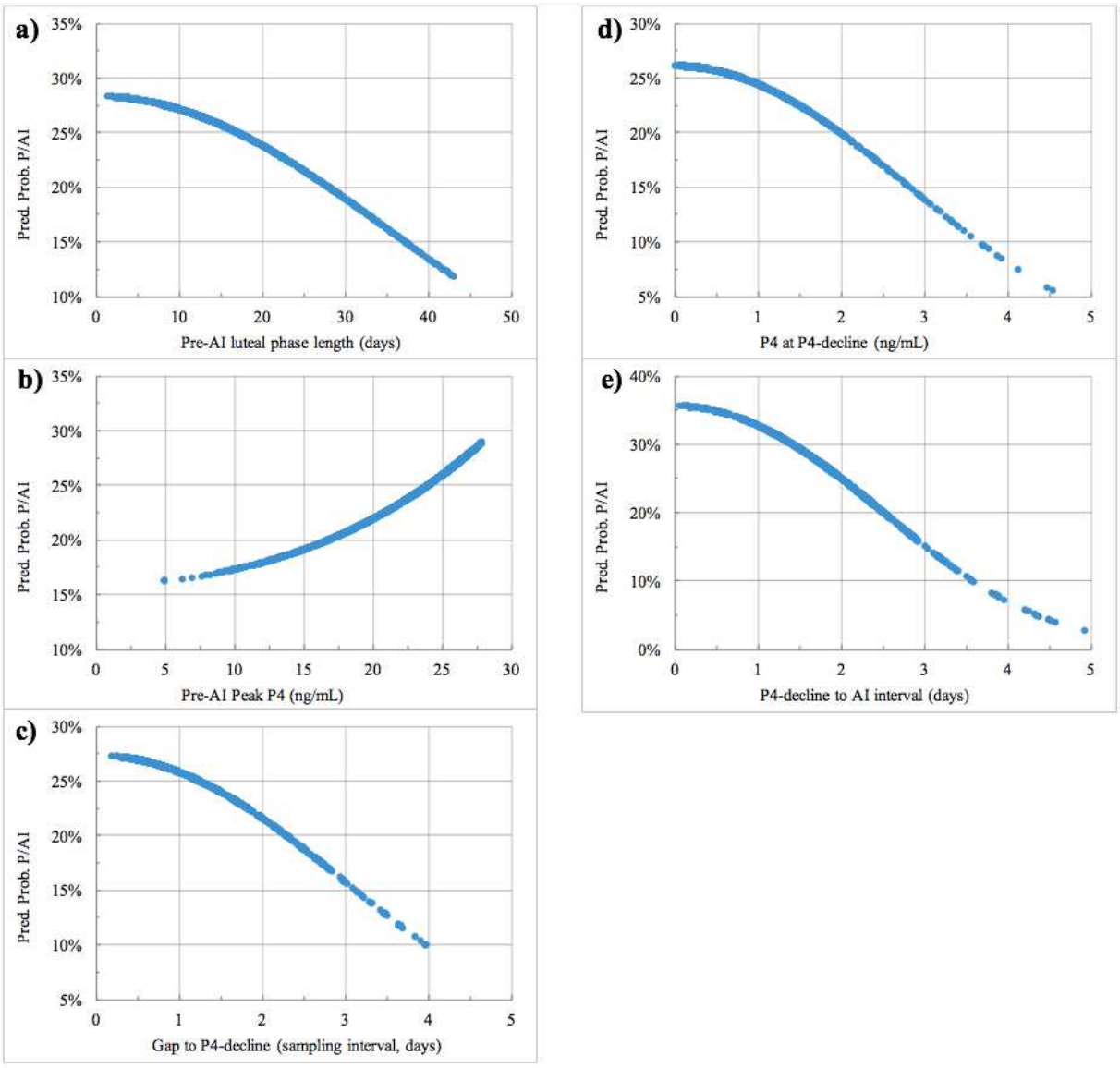

Fig. 5.2. Predicted probability of pregnancy per AI (P/AI) according to parameters before AI: (a) Pre-AI luteal phase length, (b) Pre-AI Peak P4 concentration, (c) average sampling interval (gap) in last 3 samples preceding mP4-decline, (d) P4 concentration at mP4-decline, and (e) days from mP4-decline to AI interval. Data presented include all four herds (A, B, C and D).

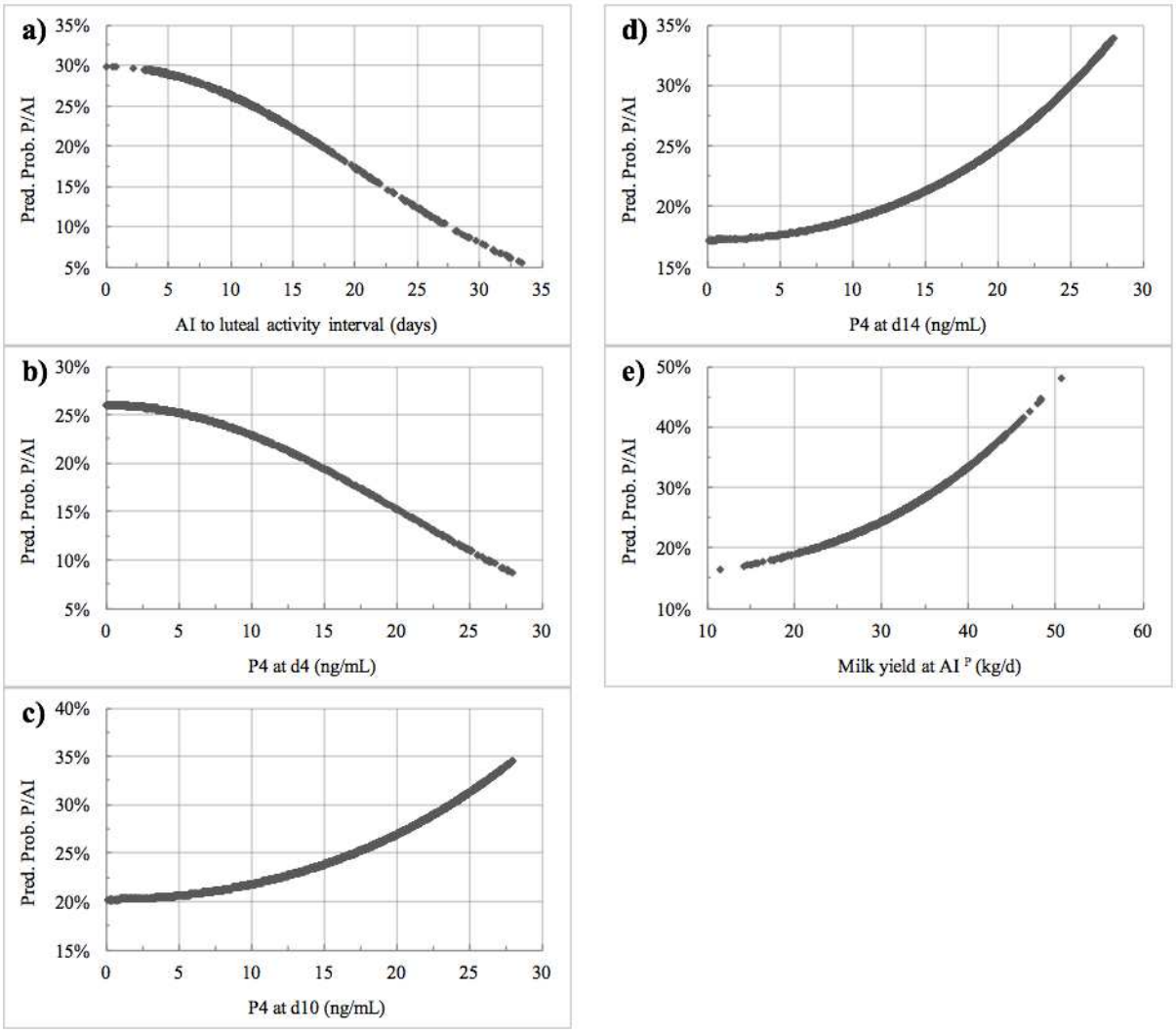

Fig. 5.3. Predicted probability of pregnancy per AI (P/AI) according to parameters after AI: (a) days from AI to luteal activity interval, (b) P4 concentration at d4, (c) P4 concentration at d10, (d) P4 concentration at d14, and (e) average daily milk yield within ± 4d relative to AI; [p] results shown only for 1st parity cows as the model for this parameter was not significant for multiparous cows. Data presented here include all four herds (A, B, C and D).

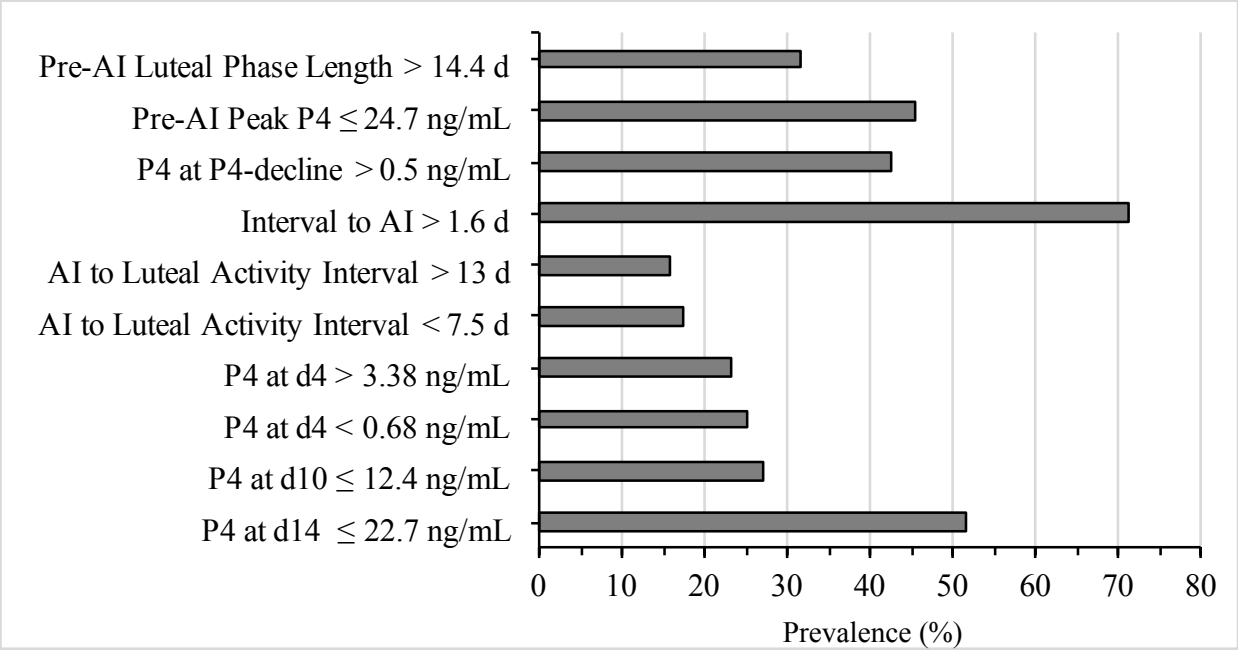

Fig. 5.4. Prevalence (%) of conditions associated with reduced overall pregnancy per AI according to categories of luteal activity parameters classified based on cut-off values that predicted pregnancy per AI or based on groups distribution (Table 5.7).

CHAPTER 6
GENERAL DISCUSSION, FUTURE DIRECTIONS, AND CONCLUSIONS

6.1. GENERAL DISCUSSION

Infertility is one of the major aspects affecting profitability of dairy operations (Ribeiro et al., 2012). A better understanding of the reproductive physiology of modern dairy cows and factors associated with fertility impairment is essential to maintain a sustainable industry. In this regard, the present thesis aimed to evaluate in-line milk P4 profiles in individual cows in Canadian Holstein herds and assess components of ovarian activity associated with fertility. The studies presented contributed to better understand conditions of ovarian activity associated with reduced fertility in modern dairy cows having implication to reproductive management of dairy herds monitoring in-line milk P4 profiles.

Three studies aiming to evaluate a variety of components of luteal activity associated with fertility were conducted, by retrospectively assessing in-line milk P4 profiles starting early postpartum until determination of AI outcomes in Holstein herds. The technology used to obtain in-line milk P4 data (Herd Navigator™) is relatively new. It has been available in Europe since 2009 (Saint-Dizier and Chastant-Maillard, 2012) and in Canada since 2011 (info provided by the DeLaval Canada). In 2015, when the present research was initiated, 4 herds in Alberta and 35 herds in Canada were using the Herd Navigator™ system. In 2017, the number of dairy herds using the Herd Navigator™ system has increased to 9 in Alberta and to approximately 60 in Canada. Although there is an increasing number of dairy herds adopting the Herd Navigator™ in Canada, to our knowledge, the system is yet to be approved for commercial applications in the USA. In Europe, researchers (Tenghe et al., 2015; 2016) evaluated in-line milk P4 data from the Herd Navigator™ in 11 dairy herds in the Netherlands to explore endocrine fertility traits and define their genetic associations. However, to our knowledge, the present study is the first to evaluate components of luteal activity and AI outcomes as direct indicators of fertility based on in-line milk P4 data, and the first to assess in-line milk P4 profiles from North American Holstein cows.

The first two studies presented here were conducted by evaluating milk P4 profiles of individual cows obtained through an on-farm herd management software in two commercial herds. In the first study (Chapter 2), our objectives were to investigate relationships of (1) commencement of luteal activity (CLA) and (2) luteal phase length and frequency preceding first AI, with parity and AI outcomes. The reason we aimed to evaluate the effects of parity (primiparous vs. multiparous) was because there are differences in postpartum energy metabolism (Wathes et al., 2007) and in luteolytic rates during synchronization protocols between different parities (Brusveen et al., 2009; Giordano et al., 2012b), which could be a confounding factor in evaluating CLA and luteal phases length. We hypothesized that primiparous cows had earlier CLA and less abnormal luteal phases preceding first AI than multiparous cows. Then, we hypothesized that early CLA, the absence of abnormal luteal phases, and a high frequency of luteal phases (either normal or abnormal) preceding first AI were associated with improved fertility.

To investigate to what extent the time from calving to CLA affected fertility, we categorized all cows into CLA earlier or later than each of six interval-classes (i.e. 28, 35, 42, 49, 56, and 63 DIM) and evaluated each class independently using mixed effects logistic regression models. We chose this approach because previous studies have often categorized the threshold of early vs. late CLA based on population standards (e.g. population mean ± standard deviations; Lamming and Darwash, 1998) or arbitrarily (based on previous studies; Opsomer et al., 1998; Shrestha et al., 2004). In contrast to our first hypothesis, we observed that the primiparous cows were more likely to have late CLA than multiparous cows. None of the six CLA categories affected fertility in primiparous cows, but multiparous cows had pregnancy per AI and pregnancy losses affected if CLA was delayed (> 56 DIM). Although it is well established that delayed resumption of postpartum cyclicity affects fertility at first AI (Thatcher and Wilcox, 1973; Gautam et al., 2010; Ribeiro et al., 2016a), other studies have not reported parity effects separately. We found that over 50% of cows had an abnormal luteal phase (i.e. shortened or prolonged) preceding first AI. Over the past decades, the incidence of abnormal luteal phases has increased in the modern dairy cow (Royal et al., 2000), which could be one contributing factor to reduced fertility (Lamming and Darwash, 1998; Lucy, 2001; Ranasinghe et al., 2011). We concluded that a high frequency of luteal phases preceding first AI, including at least one normal luteal phase, is a major factor benefiting fertility.

In the second study (Chapter 4), we aimed to determine the dynamics of pre- and post-AI milk P4 profiles and their associations with parity and AI outcomes. In this case, the evaluation of parity effects was important because multiparous cows have greater milk yield than primiparous cows, and milk yield is known to affect metabolic clearance rate of P4 (Sangsritavong et al., 2002). Thus, we first hypothesized that milk P4 levels were greater in primiparous than in multiparous cows. High P4 during the growth of the ovulatory follicle preceding AI (Bisinotto et al., 2013), and increasing P4 following ovulation (Inskeep, 2004) are factors associated with improved fertility; thus, our second hypothesis was that greater milk P4 levels pre- and post-AI were associated with improved AI outcomes. Our objectives were to compare differences in milk P4 profiles amongst parity and AI outcome groups; thus, we evaluated adjusted (i.e. smoothed) milk P4 values at different time points pre- and post-AI using mixed effects ANOVA models.

Primiparous cows had higher P4 profiles and a more rapid increase in milk P4 levels after AI than multiparous cows. Also, cows that became pregnant had greater milk P4 levels after AI than cows that were open. As open cows had lower milk P4 levels after AI than pregnant cows, the lower milk P4 after AI observed in multiparous relative to that in primiparous cows could be a contributing factor to the lower fertility often reported in multiparous than in primiparous cows (Tenhagen et al., 2004b; Santos and Rutigliano, 2009; Garcia-Ispierto and López-Gatius, 2016). An interesting finding of this study was that cows that were not pregnant after AI (open cows or cows suffering pregnancy loss) had greater milk P4 levels near the time of AI (at d –2 and d 5 relative to the day of AI) than cows that became pregnant. An incomplete regression of the CL could reflect in this slightly elevated P4 near the time of AI, which is known to negatively affect fertility (Wiltbank et al., 2014; Ambrose et al., 2015; Wilsdorf et al., 2016; Colazo et al., 2017)**.** In conclusion, lower milk P4 profiles after AI (i.e. following ovulation) and slightly elevated milk P4 levels near the time of AI (i.e. during estrus) observed in open compared to pregnant cows could be underlying factors contributing to infertility in modern dairy herds.

In the first two studies, we assessed in-line milk P4 profiles to explore associations between patterns of luteal activity and fertility, and between milk P4 profiles and fertility. The third study (Chapter 5) was designed to characterize those and additional parameters in the early postpartum period and before and after each AI with a different approach. Our approach aimed to benchmark cut-off values of each parameter to identify conditions associated with reduced

probability of pregnancy and assess their prevalence in a larger population. For this purpose, we obtained data files from the bio-model's database (Lattec I/S, Hillarød, Denmark) respective to all milk P4 and AI records available in four herds using the Herd Navigator™. After applying sets of filtering criteria to exclude periods of infrequent sampling, we defined parameters of luteal activity in relation to the early postpartum period (e.g. CLA and number of cycle of which first AI occurred), and before and after each AI throughout the postpartum period (e.g. length of luteal phase preceding AI, intervals between fluctuations in P4 curves and AI, and P4 concentrations at various time points before and after AI). Specifically, we aimed to potentially identify cut-off values to define: (1) delayed CLA, (2) sub-optimal number of cycles preceding first AI, (3) prolonged luteal phase preceding AI, (4) delayed interval from milk P4 decline and AI, and (5) sub-optimal P4 concentrations during the luteal phase preceding AI, at day of milk P4 decline, and at different time points after AI.

Similar to the first study (Chapter 3), the outcome of interest was pregnancy per AI (i.e. pregnant vs. not pregnant after AI) as a binomial variable, which was analyzed using mixed effects logistic regression models. However, in contrary to the first study where all explanatory variables were categorical and classified based on priori sets of rules (i.e. definitions of short or prolonged luteal phases based on previous studies), all the explanatory variables of interest in this study were analyzed as continuous distribution. The evaluation of continuous variables allowed us to determine their quadratic or linear effects on predicted values for probability of pregnancy. Then, to identify the optimal cut-off value of each continuous variable that would predict the response variable (i.e. pregnancy), we used receiver operating characteristic (ROC) curve analyses (https://support.sas.com/kb/25/018.html). The ROC is an objective method to calculate a cut-off value when a binary outcome (e.g. pregnant vs. not-pregnant) is dependent on a continuous variable (Sasse, 2002).

The ROC analysis has been extensively used in dairy science studies to evaluate cut-offs of physiological parameters associated with a binomial response variable, such as fertility outcomes (Cerri et al., 2009; Stevenson, 2016; Colazo et al., 2017; Hon Cheong et al., 2017), occurrence of diseases (Martinez et al., 2012; Suthar et al., 2013; Taponen et al., 2016), or to validate diagnostic tests for pregnancy (Faustini et al., 2007; Gábor et al., 2016; Wilsdorf et al., 2016; Mayo et al., 2016). The ROC analysis generates a curve that accounts for the estimated sensitivity (e.g. proportion of observations that was classified as pregnant and that was above a

cut-off value of the distribution) and specificity (e.g. proportion of observations that was classified as not-pregnant and that was below a cut-off value of the distribution) of a continuous variable. It considers all the sensitivity/specificity pairs possible by modifying the cut-off value in the whole range of observations. The optimal cut-off value of a continuous variable is the point in the ROC curve that has the highest combined sensitivity and false positive fraction (1 – specificity), called the Youden index. The ROC curve analysis plots the relation between sensitivity and 1 – specificity for the whole cut-offs values range, and the area under the ROC curve receives a value from 0 to 1 that indicates the power of the model to predict the response variable (Greiner et al., 2000)**.** The area under the curve generates a probability value indicating whether the cut-off is significant to predict the response variable. An area under the curve value of 0.5 means that a model has no predictive power, while a value of 1 indicates perfect predictive power (i.e. 100% sensitivity and 100% specificity) (Sasse, 2002).

We evaluated the cut-off values, area under the curve, and probability values for each parameter of interest. Variables that had a significant cut-off were stratified into two groups (below vs. above the cut-off). The reason we used ROC initially is because our main objective was to identify cut-off values associated the probability of pregnancy, and to assess the prevalence of conditions below or above the cut-off associated with reduced probability of pregnancy. However, variables that not had a significant ROC curve ($P > 0.05$) were stratified into four quantiles based on the values distribution: Q1 (below 25th percentile), Q2 (between 25th percentile and median), Q3 (between median and 75th percentile), and Q4 (above 75th percentile) to compare each quantile against the probability of pregnancy. Comparisons among different quantiles would allow the identification of meaningful differences in sub-groups of observations across the distribution. For instance, quantile comparisons would allow the identification of variables in which medium values (i.e. Q2–Q3) are positively associated with the response, but both low (i.e. Q1) and high (Q4) values are negatively associated with the response.

Among variables compared based on quantiles, both very early (< 28 DIM) and delayed (> 50 DIM) CLA were associated with reduced fertility, and cows having a moderate CLA (between 28 and 50 DIM) had the greatest probability of pregnancy. We expected that an early CLA would be associated with improved fertility (as documented in Chapter 3); however, Ranasinghe et al. (2011) reported that cows with a CLA < 28 DIM had increased prevalence of prolonged luteal phases, which reduced fertility. Thus, it is likely that by increasing the number

of cows and evaluating separate categories, we were able detect the trend of lower fertility at AI in some cows with a very early CLA (< 28 DIM). By dividing cows only into two CLA categories (i.e. early vs. late) as we did in the first study, we possibly included cows with both lower and higher probability of pregnancy in both groups.

We identified significant cut-off values associated with reduced probability of pregnancy for the following conditions before AI: (1) low number of cycles preceding first AI (when < 2), (2) prolonged luteal phase preceding AI (when > 14.4 d long), (3) sub-optimal peak P4 in the luteal phase preceding first AI (when < 24.7 ng/mL), (4) elevated P4 at day of milk P4 decline (when > 0.5 ng/mL) and (5) delayed AI after milk P4 decline (when ≥ 1.6 d). For parameters after AI, we identified cut-off values of sub-optimal P4 concentrations associated with reduced probability of pregnancy at d 10 (when ≤ 12.4 ng/mL) and d 14 (when ≤ 22.7 ng/mL) post-AI. For most parameters, we observed a high prevalence of those conditions associated with reduced fertility based on the cut-off values (Fig. 5.4). For instance, 46% of cows received first AI before the 2nd cycle, and those AI resulted in much lower pregnancy per AI (16 vs. 28%) than AI occurring beyond the 2nd cycle. Also, the proportion of AI preceded by a prolonged luteal phase was 30%, and 45% of the luteal phases preceding AI had sub-optimal peak P4. Potential intervention strategies to overcome those luteal activity conditions associated with reduced fertility should be investigated in future research.

6.2. LIMITATIONS

In the first study (Chapter 3), AI outcomes (i.e. pregnancy and pregnancy loss) were determined based exclusively on milk P4 profiles after AI, and we observed a very high incidence of pregnancy losses (33%) compared to most previous studies that documented incidences of less than 15% (Szenci et al., 1998; Chebel et al., 2004; Santos and Rutigliano, 2009; Ribeiro et al., 2014)**.** The unexpected high incidence of pregnancy loss observed in our study could have been due to cows that failed to conceive at AI (open cows), but coincidently had a prolonged luteal phase after AI. These cows would have had a late decline in milk P4 levels (i.e. beyond d 30 post-AI) and would have been erroneously considered as suffering a pregnancy loss. This would indicate a limitation in using in-line milk P4 profiles alone to diagnose early pregnancy (i.e. 30 d post-AI) or pregnancy losses (between 30 and 55 d post-AI),

as the variability in "normal" luteal phase length has been increasing in the modern dairy cow and a high incidence of long estrous cycles (i.e. classified as prolonged luteal phases) can be expected (Remnant et al., 2015, 2016; Blavy et al., 2016).

In the second study (Chapter 4), we observed that non-pregnant cows had elevated milk P4 near time of AI. Incomplete luteolysis could result in elevated P4 near time of AI and consequently lower fertility; however, it is possible that the sampling bio-model used by the Herd Navigator™ to detect fluctuations in milk P4 levels (i.e. to detect the day of milk P4 decline at the end of a luteal phase) may not be sensitive enough to detect complete luteolysis in all cases. A previous study that tested that bio-model reported that milk P4 levels in 4% of cows that were confirmed to be in estrus did not decline below the predetermined threshold of high vs. low P4 (Friggens et al., 2008).

In the third study (Chapter 5), a significant cut-off value for the Herd Navigator™ sampling frequency preceding the milk P4 decline was identified. We observed that if the average sampling interval in the last 3 samples prior to milk P4 decline was longer than 1 day between samples, the probability of pregnancy is reduced. This is likely because a lower frequency of sampling at the end of a luteal phase might be detecting the milk P4 decline too late in relation to the time of true luteolysis, and decisions regarding AI were exclusively based on milk P4 decline events. This finding supports our inference from the second study (Chapter 4) that not-pregnant cows could have had elevated P4 near the time of AI, at least partially, due to delayed detection of the timing of CL regression by the Herd Navigator™ sampling bio-model. After a CL regression (i.e. milk P4 decline), the bio-model is designed to take a sample at ~d 16 and the following one at ~d 20. Thus, cows having the next CL regression anytime between d 16 and d 20 will be flagged late (i.e. only at the d 20 sample).

One possible reason for inconsistency in sampling frequency prior to milk P4 decline might be the increased incidence of abnormal cycles (i.e. short or prolonged) recently reported in the modern dairy cow (Royal et al., 2000; Remnant et al., 2015; Blavy et al., 2016). The bio-model is designed to detect the end of a luteal phase through increased frequency of samples between approximately d 16 and 30 post-AI; thus, cows with short or long cycles might have luteolysis occuring earlier or later than the period of high sampling frequency. This would indicate a limitation in the current sampling bio-model to accurately detect luteolysis in luteal

phases with abnormal length. In order to more accurately detect the time of luteolysis (i.e. milk P4 decline) and of ovulation (i.e. luteal activity), an increase in the frequency of sampling and consequently reduced interval between samples would be required. Also, extra samples being taken between the time of milk P4 decline and the first subsequent sample (which presently occurs ~7 d after milk P4 decline) would allow more frequent monitoring of P4 concentrations closer to the time of AI. More frequent monitoring would provide a more accurate data to evaluate effects of P4 concentrations during the time of AI, estimating the probability of pregnancy after AI, and consequently improving precision of timing of AI.

6.3. RECOMMENDATIONS FOR FUTURE RESEARCH

It was not in the scope of our study to assess factors and mechanisms associated with or underlying each of those various conditions (i.e. above or below the cut-offs) associated with reduced fertility. However, opportunities for future research exist in exploring farm- and cow-level factors (such as managerial, nutritional, and metabolic parameters) associated with ovarian function. For instance, in-line milk measures of BHBA, lactate-dehydrogenase, and urea are also possible in herds using the Herd Navigator™. Studying such data for their association with luteal activity conditions would undoubtedly provide new evidences of relationships among postpartum metabolic status (i.e. energy balance through BHBA data), inflammatory processes (i.e. mastitis through lactate-dehydrogenase data), luteal activity, and fertility. Based exclusively on our results, recommendations for strategic interventions before, during, and after time of milk P4 decline or AI could be developed to overcome some of the luteal activity conditions associated with infertility. Potential interventions before AI include:

- **Administration of exogenous PGF in luteal phases exceeding 14 d in length.** We observed that AI occurring following cessation of prolonged luteal phases (characterized as > 14 d long) resulted in reduced probability of pregnancy. Thus, administration of PGF in non-inseminated cows having luteal phases > 14 d (determined based on milk P4 profiles) would induce a CL regression and consequently a precipitous decline of milk P4. In such a scenario, cows would not have a prolonged luteal phase prior to AI, possibly increasing the probability of pregnancy.

- **Exogenous supplementation of P4 in cows having luteal phases preceding AI of sub-optimal (< 24 ng/mL) P4 concentrations.** We observed that AI occurring after luteal phases that had not reached peak P4 concentration of 24 ng/mL resulted in reduced probability of pregnancy. In this regard, it is possible that exogenous P4 supplementation (e.g. the administration of an intra-vaginal P4 release device) in cows having sub-optimal P4 concentrations (i.e. not reaching 24 ng/mL) in the luteal phase preceding AI would enhance P4 concentrations and increase the probability of pregnancy.

The proportion of AI preceded by elevated P4 concentrations at time of milk P4 decline (i.e. near the time of AI) was 43%, and 17% of AI were followed by a rapid increase in P4 levels (resulting in elevated P4 at d 4 post-AI). Both factors are possibly related to an incomplete luteolysis where: (a) P4 would not drop to basal levels at time of milk P4 decline and (b) P4 would stay elevated near the time of AI and increase shortly after AI. Also, 16% of AI were followed by a slow increase in P4 levels, which indicates a delayed ovulation (resulting in lower P4 at d 4 and at d 10). Thus, potential interventions during the time of AI include:

- **Exogenous administration of PGF strategically in cows with elevated P4 (> 0.5 ng/mL) at the time of milk P4 decline.** We observed that cows that had P4 concentrations > 0.5 ng/mL at time of milk P4 decline (~ 2 d before AI) had reduced probability of pregnancy. It is possible that this slightly elevated P4 concentration (i.e. > 0.5 ng/mL) was caused, at least partially, by incomplete CL regression. Thus, administration of PGF strategically in cows with elevated P4 concentration will likely induce complete luteolysis, reducing P4 concentrations near the time of AI and possibly increasing the probability of pregnancy.

- **Exogenous administration of GnRH at time of AI to reduce the incidence of delayed ovulations.** We determined that 16% of the AI were followed by a delayed luteal activity. Thus, it is likely that the administration of GnRH at the time of AI would reduce the proportion of cows having a delayed luteal activity after AI. Based on our current results alone, it is not possible to predict which cows will have a delayed ovulation; thus, this

strategy would need to be applied to all cows receiving AI in order to document potential reduction in the prevalence of delayed ovulations.

Post-AI, the prevalence of sub-optimal P4 concentrations at d 10 and at d 14 observed in the third study (Chapter 5) was 27 and 51%, respectively. The benefits of supplementing exogenous P4 post-AI have been inconsistent among past studies (Mann and Lamming, 1999; Parr et al., 2014; Colazo et al., 2013; Monteiro et al., 2015). Thus, we can predict that cows benefiting from P4 supplementation are likely those having sub-optimal natural P4 levels. Future studies should evaluate risk factors that predict the occurrence of specific luteal activity conditions (e.g. delayed ovulation and sub-optimal P4 post-AI), which could enhance targeted recommendations to improve fertility, such as management strategies or strategic hormonal supplementation.

6.4. CONCLUSIONS

The results of the present thesis demonstrated that Holstein cows have a high prevalence of abnormal luteal activity associated with reduced probability of pregnancy at AI. Thus, abnormal luteal activity parameters could be underlying factors to the high incidence of infertility in modern dairy herds. We have provided novel information of specific cut-off values for various parameters of luteal activity associated with reduced probability of pregnancy at AI. These parameters are monitored in real-time and readily accessible in herds monitoring in-line milk P4 profiles (i.e. using the Herd Navigator™), and the cut-off values can be used as novel benchmarks of ovarian activity conditions. These benchmarks can be used by producers, herd managers, veterinarians and technical consultants using the system to assess and compare prevalence of various ovarian dysfunction conditions both at the individual cow and at the whole-herd levels. Further research is required to explore factors associated with the luteal activity conditions that result in lower probability of pregnancy at AI. In addition, the characterization of these conditions presented here can be used as points of reference for future research to determine if on-farm strategies for targeted decision making can be implemented to overcome sub-optimal luteal activity conditions associated with reduced fertility.

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