UNDERSTANDING HOW INDUCED LUTEOLYSIS IN DAIRY CATTLE AFFECT CORPORA LUTEA CHARACTERISTICS AND FERTILITY

By

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ABSTRACT

UNDERSTANDING HOW INDUCED LUTEOLYSIS IN DAIRY CATTLE AFFECT CORPORA LUTEA CHARACTERISTICS AND FERTILITY

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Efficient manipulation of estrous cycle via exogenous treatments is critical for fertility in dairy cows following timed artificial insemination (TAI) programs. Luteolysis is a key event that warrants reproductive success in manipulated estrous cycles. The main objective of this thesis was to characterize luteolysis following different doses of cloprostenol sodium (CLO) and its effects on luteal blood flow (LBF) and fertility of lactating dairy cows. The first objective was to determine the effect of luteolytic and sub-luteolytic doses of CLO in dairy heifers with an early and mid-cycle corpus luteum (CL). Measurement of LBF was indicative of complete, and partial luteolysis following various doses of CLO. Heifers receiving multiple doses of CLO had complete disappearance of LBF 4 d post-treatment. The second objective was to assess the effects of different CLO dose strategies during TAI programs on pregnancy rates per AI (PR/AI) in lactating dairy cows. We also aimed to verify the association between LBF around TAI with PR/AI in cows that had a d 7 and 14 CL. Cows were treated with a single full dose, two full doses 24 h apart or a double dose of CLO. There was no evidence of differences in PR/AI between CLO doses. But, 3rd+ parity cows treated with a single full dose of CLO had greater pregnancy loss form d 24 to 34 post-AI. Treatment with double dose of CLO resulted in similar PR/AI independent on synchronization status. Amount of LBF at 2 and 4 d post-treatment was a predictor of PR/AI 34 d post-AI. Cows with decreased LBF of both d 7 and 14 CL below a median cutoff had greater PR/AI 24 d post-AI. Multiparous cows treated with double doses of CLO avoided lower fertility in non-synchronized cows and greater pregnancy losses compared to cows treated with a single full dose.

Someone once told me he would teach me all about dairy cattle.
Here I am trying to learn everything you could not teach me.
I dedicate this thesis for you, Ivan.

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At a random day of 2012 I went to work as an intern of Embryolab. I thought would be just another ordinary day of my life. There was this fuzz about a very important man coming to visit the laboratory. “The creator of Ovsynch”, they called him. I was at my second semester of college and the estrous cycle of cows was something I could not understand just yet. I had no idea what Ovsynch was. So, the name “Pursley” just went over my head. This important man was very kind with everyone and even asked my name and how to spell it. Neither one of us could imagine that was the first out of countless times he would be saying “Thainá”. It turned out that day was not as ordinary as I expected. It was the day I first met my future mentor, Dr. Pursley. I am grateful for coincidences and unexpected events. I am grateful that I got to have you as my advisor years after first meeting you. Thank you for being so supportive, patient and kind. You made this possible.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

KEY TO ABBREVIATIONS

AI artificial insemination

Ang II angiotensin II

ANPT-1 angiopoietin-1

ANPT-2 angiopoietin-2

bFGF basic fibroblast growth factor

CD color Doppler

CI confidence interval

CL corpus luteum

CLO cloprostenol sodium

CV coefficient of variation

d day/days

D diameter

DF dominant follicle

DIM days in milk

DIN dinoprost tromethamine

DO double-Ovsynch

E_2 estrogen

EDN1 endothelin 1

ELISA enzyme-linked immunosorbent assay

ER estrogen receptor

FGF fibroblast growth factor

FSH follicle stimulating hormone

G gonadotropin releasing hormone

GnRH gonadotropin releasing hormone

h hour/hours

hCG human chorionic gonadotropin

IFNγ interferon gamma

LBF luteal blood flow

LH luteinizing hormone

LV luteal volume

mg milligram

µg micrograms

mL milliliter

mm millimeters

µm micrometers

mm^3 millimeters cubed

mRNA messanger RNA

NC negative control

ng nanogram

NO nitric oxide

OXTR oxytocin receptor

P_4 progesterone

PC positive control

PG prostaglandin-$F_{2\alpha}$

PGE_2 prostaglandin-E_2

$PGF_{2\alpha}$ prostaglandin-$F_{2\alpha}$

PGFM prostaglandin-$F_{2\alpha}$ metabolite

PR progesterone receptor

PR/AI pregnancy rates per artificial insemination

PSPB pregnancy specific protein B

R radius

RIA radioimmunoassay

SEM standard error of the mean

SNAP S-Nitroso-N-acetyl-DL-penicillamine

TAI timed artificial insemination

TMR total mixed ration

TNFα tumor necrosis factor alpha

US ultrasound

V volume

VEGF vascular endothelial growth factor

vs versus

CHAPTER 1

REVIEW: BLOOD FLOW IN THE BOVINE CORPUS LUTEUM BEFORE AND DURING LUTEAL REGRESSION

INTRODUCTION

Physiological events in cattle reproduction are synchronously coordinated with emergence and disappearance of two main structures, the follicle, and the corpus luteum (CL). These structures are dynamically orchestrated via hypothalamic-pituitary axis, gonads, and uteri of cattle (Moore and Price, 1932; Hansel et al., 1975; Fink, 1979; McCracken, 1980; Schallenberger et al., 1985). Follicles emerge and grow in a wave-like pattern until ovulation and release of the oocyte (Savio et al., 1988; Ginther et al., 1989). A transient secretory gland forms from the remaining cells of the ovulated follicle (Reynolds et al., 1994). A combination of tissue remodeling, cell differentiation and intense angiogenesis during and after ovulation establish the CL (Acosta et al., 2003; Miyamoto et al., 2009). Follicles and CL interact via hormonal positive and negative feedbacks (Wathes et al., 1996; Robinson et al., 2001). The respective products from their secretion are estrogen (E_2) and progesterone (P_4), both derived from cholesterol (Veler et al., 1930; Slotta et al., 1934; Bert and Schrader, 1976). Ovulation precedes, and is requisite for, CL formation. Whereas CL regression, or luteolysis, precedes ovulation. These two main events occur cyclically. Granulosa and theca interna cells that secrete E_2 and androgens in the follicle will differentiate into large and small luteal cells and secrete P_4 once the CL forms (Meidan et al., 1990; Reynolds et al., 1994). This "cellular recycling" occurs every ~21 d in non-pregnant cycling cattle and it is named the estrous cycle. This chapter begins with a brief overview of the estrous cycle in the dairy cow. It will focus on physiology and morphology of the CL throughout the cycle. The role of luteal blood flow (LBF) as a marker of CL function is also described in detail.

INTERACTION BETWEEN NERVOUS AND REPRODUCTIVE SYSTEMS

The cow's estrous cycle is controlled via hormonal interactions. There are three sources of stimuli that dictate the fate of follicles and CL. Hormones secreted by the hypothalamus-pituitary

axis, gonads and uteri of cows interact via positive and negative feedbacks (Moore and Price, 1932; Hansel et al., 1975; Fink, 1979; McCracken, 1980; Schallenberger et al., 1985). These interactions lead into physiological events such as follicular waves, follicle development, estrus, ovulation, CL formation and luteolysis (Schams et al., 1977; Mccracken et al., 1999; Mihm et al., 2002; Boer et al., 2010).

The primary stimulus is the release of gonadotropin releasing hormone (GnRH or G) from the hypothalamus (Fink, 1979). Gonadotropin releasing hormone is a decapeptide hormone that stimulates the release of follicle stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary (Kaltenbach et al., 1974). Low frequency GnRH pulses are responsible for FSH secretion (Schallenberger et al., 1985; McNeilly, 1988; Bernard et al., 2010). High frequency pulses of GnRH are responsible for LH secretion (Rahe et al., 1980; Schallenberger et al., 1985). Follicle stimulating hormone and LH are also defined as gonadotrophic hormones, and therefore act as stimulators of the gonads (ovaries). Follicle stimulating hormone mediates the recruitment, growth, and maturation of primary follicles into secondary antral follicles (Greenwald, 1973; Mihm et al., 2002). Growth of antral follicles are dependent on FSH before acquisition of LH receptors during deviation of the dominant follicle (Ginther, 2000; Sartori et al., 2001). Low amplitude pulses of LH are the primary driver of follicular growth post-deviation (Sartori et al., 2001; Mihm et al., 2006). Luteinizing hormone mediates final maturation and growth of pre-ovulatory follicles (Ginther et al., 1998; Wiltbank et al., 2011a). A fully developed follicle may or may not ovulate depending on stage of the estrous cycle (Rahe et al., 1980; Walters and Schallenberger, 1984; Schallenberger et al., 1985).

Steroid hormones secreted in the ovaries (E_2 and P_4) are the second source of hormonal control of the estrous cycle. Steroid hormones are only secreted when the primary gonadotrophic

stimulus successfully induced follicular development and consequently the presence of a CL. Steroid hormones can both suppress or enhance hormonal release from the hypothalamus-pituitary axis depending on stage of estrous cycle (Beck et al., 1976; Baird and McNeilly, 1981; Ireland and Roche, 1982). Developing follicles secrete E_2 and inhibin that proportionally increase in circulation as follicular size increases (Hopko Ireland and Ireland, 1994; Bernard et al., 2010). In combination, E_2 and inhibin have a negative feedback on FSH release from the pituitary (McNeilly, 1988; Savio et al., 1988; Lucy et al., 1992). Subordinate follicles become atretic and a follicular wave does not emerge without a new surge of FSH (Pursley et al., 1993). The negative feedback of E_2 and inhibin persists until atresia or ovulation of the dominant follicle (Savio et al., 1993; Hopko Ireland and Ireland, 1994). A high amplitude LH surge causes ovulation near the onset of estrus (Chenault et al., 1975; Schallenberger et al., 1984, 1985; Walters and Schallenberger, 1984). But this event is dependent on certain hormonal scenarios. In non-manipulated estrous cycle, the LH surge occurs following a peak in circulating E_2 (Chenault et al., 1975; Schams et al., 1977; Schallenberger et al., 1985). Both the LH surge and E_2 peak only occur under low levels of P_4 (Louis et al., 1972; Chenault et al., 1975; Walters and Schallenberger, 1984). The CL develops rapidly post-ovulation and acquires P_4 secretory abilities around d 2 or 3 of the cycle concomitantly with a new wave of follicles (Miyamoto et al., 2009). Progesterone negative feedback occurs in the hypothalamic level by decreasing the amplitude of GnRH pulses (Roberson et al., 1989). The ultimate effect of high P_4 during the cycle is the decrease of LH pulsatility (Beck et al., 1976; Ireland and Roche, 1982; Roberson et al., 1989). Progesterone increases its circulating levels up until d 14 of the cycle in non-pregnant cows (Schams et al., 1977; Schallenberger et al., 1985; Sartori et al., 2004; Skarzynski et al., 2013). Luteolysis occurs in response to the third level of hormonal control in the cow: the uterus (Nancarrow et al., 1973).

Luteolysis is a programmed event that occurs in non-pregnant cows around d 17 – 18 of the cycle (Mcracken et al., 1999). It is induced via prostaglandin-$F_{2\alpha}$ ($PGF_{2\alpha}$ or PG) either from the endometrium or in an exogenous form (McCracken et al., 1970; Hansel et al., 1975; Peterson et al., 1975; Shemesh and Hansel, 1975). The primary stimulus for $PGF_{2\alpha}$ release from the endometrium is the local binding of oxytocin to its receptor (OXTR; Armstrong and Hansel, 1959; McCracken, 1980; Walters and Schallenberger, 1984). In the sheep, mechanical stimulation of uterus caused a greater release of $PGF_{2\alpha}$ at d 14 of the cycle in comparison to d 3, 8, and 11 through 13. Release of $PGF_{2\alpha}$ coincided with OXTR expression, whereas non-detectable $PGF_{2\alpha}$ coincided with the lack of OXTR expression (McCracken, 1980). Steroid hormones are indirect regulators of $PGF_{2\alpha}$ release by altering OXTR expression in the endometrium. Binding of P_4 to its receptor (PR) inhibits the expression of endometrium OXTR (Grazzini et al., 1998). On the contrary, binding of E_2 to its receptor (ER) in the uterine endometrium upregulates the expression of OXTR (Mccracken et al., 1999; Robinson et al., 2001). Expression of ER and PR are upregulated in response to E_2. Contrarily, ER in the uterus are downregulated by P_4 (Wathes et al., 1996). Additionally, long exposure to high P_4 levels eventually leads to downregulation of endometrium PR expression (Milgrom et al., 1973; Garret et al., 1988; Wathes et al., 1996). The downregulation of PR along with increasing levels of E_2 during late cycle culminates in ER upregulation (Leavitt et al., 1985; Okumu et al., 2010). Consequently, OXTR is expressed in the uterus resulting in $PGF_{2\alpha}$ release (Armstrong and Hansel, 1959; McCracken et al., 1973). Around d 18 of the cycle $PGF_{2\alpha}$ is released in 4 to 5 peaks that start the luteolytic cascade (Nancarrow et al., 1973; Peterson et al., 1975; Kindahl et al., 1976).

This series of synchronous hormonal interactions lead to cyclic events in cows. Commonly, the “classical” length is defined as a 21-d estrous cycle. In fact, some early studies reported an

exact average cycle of 21 d (Garret et al., 1988; Sirois and Fortune, 1990). However, more recent studies showed an increased average of 22.9 d in non-pregnant lactating dairy cows and 22 d in dairy heifers (Sartori et al., 2004). Each level of reproductive control has an impact onto the next level creating interdependent mechanisms. The clarification of such complex interactions allowed for exogenous control of the estrous cycle. Elucidation of main events occurring during the estrous cycle were the basis to develop every assisted reproduction technology in cattle (Thatcher, 2017). Causing ovulation and timely regressing CL are the main strategies for external estrous cycle manipulation (Stevenson, 2016). Chapter 3 will discuss both strategies in detail. In advance, each of these approaches are time sensitive, mostly when it comes to manipulation of CL lifespan.

CORPUS LUTEUM DEVELOPMENT DURING THE ESTROUS CYCLE OF COWS

The CL can be defined as a fast-developing transitory gland with high metabolic rate (Wiltbank et al., 1988). In fact, it develops in comparable rates of the most fast-growing tumors (Reynolds et al., 1994). Its aggressive development is accompanied by acquisition of secretory properties early following ovulation (Miyamoto et al., 2009). Luteal tissue is structured with steroidogenic and non-steroidogenic cells (O'Shea et al., 1979; Alila and Hansel, 1984). Steroidogenic cells are classified accordingly with their size within small (< 20 μm) and large luteal cells (20 – 30 μm) (O'Shea et al., 1979; Alila and Hansel, 1984; Farin et al., 1986). As mentioned, steroidogenic luteal cells are follicular-derived cells that were luteinized during and after ovulation. Small luteal cells derive from theca interna and large luteal cells origin from granulosa cells (Meidan et al., 1990; Reynolds et al., 1994). Small luteal cells increase their number and maintain constant size during the estrous cycle. Large luteal cells keep their number constant but reduce their size as the cycle advances (Farin et al., 1986). The majority of cell proliferation in the CL is from vascular origin (50 – 85%). Non-steroidogenic cells represent 50 – 70% of all

cells in a mature CL (Farin et al., 1986). Endothelial cells and pericytes constitute the CL vascular bedframe (O'Shea et al., 1979). Active endothelial cell proliferation is present in around 40% of all luteal microvessels (Augustin, 2005). Each luteal cell is in contact with one or more capillaries (Wiltbank et al., 1988). Successful establishment of luteal vascularization plays an important role in secreting P_4. Blood inflow delivers hormonal precursors and gonadotropins that are essential for CL functionality whereas blood outflow delivers P_4 into the main circulation (Janson et al., 1981; Meidan et al., 2005; Shirasuna et al., 2010).

The establishment of LBF occurs in response to angiogenic and vasoactive factors secreted locally (Miyamoto et al., 2009). An intense angiogenic process takes place post-ovulation (Acosta et al., 2003). In fact, many regulators of vascular function produced in the early CL are already being produced around ovulation (Berisha et al., 2016). Vascular endothelial growth factors (VEGFs) stimulate mitogenic activity of endothelial cells, regulate angiogenesis process and vascular permeability (Berisha et al., 2002). Fibroblast growth factors (FGFs), mostly basic FGF (bFGF), work as inducers of cellular growth, migration, and differentiation (Stirling et al., 1991; Augustin, 2000; Okada-Ban et al., 2000). Vascular endothelial growth factors and bFGF seem to act synergistically to construct the CL vascular system (Reynolds et al., 2000; Miyamoto et al., 2009). Angiopoietin-1 and -2 (ANPT-1 and ANPT-2) are involved with vascular stability (Yancopoulos et al., 2000). Angiopoietin-1 grants stability to formed blood vessels. Angiopoietin-2 antagonizes ANPT-1 by inducing active remodeling of endothelial cells and consequently destabilization of CL vascular system (Yancopoulos et al., 2000). Destabilization of blood vessels is a pre-requirement for either their formation or regression (Hanahan, 1997; Miyamoto et al., 2009). However, the fate of destabilized vascular network depends on VEGFs levels. When VEGF levels are high in the presence of destabilized blood vessels the result is formation of new vascular

network. In similar conditions, but under low levels of VEGF the result is regression of that vascular system (Hanahan, 1997).

Vasoactive substances play important role in controlling LBF dynamics (Miyamoto et al., 2009; Berisha et al., 2016). Endothelin 1 (EDN1) induces vasoconstriction or vasodilation depending in which receptor it activates (Berisha et al., 2002). Angiotensin II (Ang II) is another powerful vasoconstrictor (Berisha et al., 2002; Miyamoto et al., 2009). Opposing EDN1 and Ang II, nitric oxide (NO) induces vasodilation within the CL (Miyamoto et al., 2009). Finally, prostaglandins ($PGF_{2\alpha}$ and PGE_2) also incite hemodynamic changes within the CL. Prostaglandin-$F_{2\alpha}$ is produced in the uterus or within the CL, whereas PGE_2 is released by the CL (Milvae and Hansel, 1983; Miyamoto et al., 1993).

There are differences in expression and response to angiogenic and vasoactive factors in early, mid, and late cycle CL (Reynolds et al., 2000; Miyamoto et al., 2009; Berisha et al., 2016). Yet, LBF development results from their combined expressions and interactions (Klagsbrun and D'Amore, 1991). As a transitory gland, the CL rises up to its top secretory activity just before undergoing regression (Kindahl et al., 1976; Skarzynski et al., 2013). Luteolysis depends on synergic action of the same factors that create the CL and its vascular network. These factors also are involved with P_4 secretion along with their role in establishing a vascular bedframe within the CL.

In the early stages of luteal development VEGF and bFGF are abundant (Zheng et al., 1993; Reynolds and Redmer, 1998; Berisha et al., 2000, 2016). There is evidence that VEGF and bFGF combined stimulate P_4 secretion (Miyamoto et al., 1992; Kobayashi et al., 2001). Luteal tissue development measured as luteal volume (LV) and luteal function (P_4 levels) decreased when antibodies for VEGF and bFGF were directly injected in early-cycle CL (Yamashita et al., 2008).

This treatment also decreased mRNA for VEGF and bFGF and ANPT-1 and -2. These findings suggest that VEGF, bFGF and ANPTs modulate their expression and act together to develop the CL, but also to regulate P_4 secretion. Prostaglandin-$F_{2\alpha}$ expression peaks during the early stages of CL development (Milvae and Hansel, 1983). Levels of $PGF_{2\alpha}$ are higher early in comparison to mid-cycle micro-dialyzed CL (Kobayashi et al., 2001). Progesterone release was enhanced in $PGF_{2\alpha}$ treated early-cycle CL and luteinized granulosa cells (Conley and Ford, 1989; Miyamoto et al., 1993; Okuda et al., 1998; Kobayashi et al., 2001). Controversially, the main inducer of luteolysis does play important role in P_4 release during early cycle. Consequently, the early-cycle CL (d 0 to 4 of the estrous cycle) is refractory to exogenous $PGF_{2\alpha}$ luteolytic effects (Tsai and Wiltbank, 1998). Angiogenesis and the understanding of blood flow in the CL played a critical role in Chapter 2 regarding the use of color Doppler ultrasound to characterize outcomes of treating heifers that had two distinctly different levels of CL maturity with various doses of $PGF_{2\alpha}$.

Exogenous or endogenous $PGF_{2\alpha}$ may start the luteolytic cascade later than d 4 of the cycle and lead to functional and morphological regression (Wiltbank et al., 1995; Tsai and Wiltbank, 1998). The mid-cycle CL is a stable construct of steroidogenic and non-steroidogenic cells (Alila and Hansel, 1984). Angiogenic factors are downregulated and there is few, or none tissue remodeling (Berisha et al., 2000; Nio-Kobayashi et al., 2016). The mid-cycle (d 10 to 14) period coincides with the highest release of P_4 (Sartori et al., 2004). Mid-cycle LBF around the CL is intense and highly correlated with P_4 (Herzog et al., 2010). In point of fact, a mature CL is one of the most irrigated organs, with the greatest blood flow per tissue unit (Janson and Albrecht, 1975). High circulating P_4 levels secretion precedes the onset of luteolysis around d 17 – 18 (Schams et al., 1977). The luteolytic cascade is a complex process with changes in expression and interaction

of vasoactive substances. The final outcome of luteolysis is functional and morphological regression of the CL (Mccracken et al., 1999).

LUTEOLYSIS: A BLOOD FLOW MEDIATED EVENT

During non-manipulated estrous cycle innumerous conditions and factors act synergistically to cause luteal regression. Efficient induction of luteolysis via exogenous $PGF_{2\alpha}$ depends mostly on d of the estrous cycle. The CL becomes responsive to $PGF_{2\alpha}$ around d 5 of the estrous cycle (Wiltbank et al., 1995; Tsai and Wiltbank, 1998). Ginther et al., (2009) also demonstrated that occurrence of physiological complete luteal regression requires a pulsatile pattern of $PGF_{2\alpha}$ treatments. Luteal blood flow function becomes opposite during late cycle in comparison to early cycle. Luteal blood flow enhances cellular development and functionality in the early-cycle CL, but contributes to luteolysis in the late cycle CL. Changes in luteal hemodynamics and expression of vasoactive substances are primary markers of luteolysis (Miyamoto and Shirasuna, 2009). During endogenous luteolysis there was drastic decrease in ovarian blood flow ipsilateral to the CL. This decrease was accompanied with simultaneous decreasing levels of P_4 (Niswender et al., 1976; Ford and Chenault, 1981).

However, an acute peripheral increase in LBF was identified at the onset of luteolysis (Acosta et al., 2002; Miyamoto et al., 2005; Ginther et al., 2007). The increase in LBF was verified 0.5 to 2 h after exogenous $PGF_{2\alpha}$ administration and restricted to the periphery of the CL (Acosta et al., 2002). During non-manipulated cycles, LBF increased simultaneously with prostaglandin-$F_{2\alpha}$ metabolite (PGFM) peaks (Miyamoto et al., 2005; Ginther et al., 2007). Increase in LBF occurred in mid-cycle CL (d 10), but not in the early-cycle CL (d 4) (Acosta et al., 2002). Similar results were obtained during the experiment described in Chapter 2. Prostaglandin-$F_{2\alpha}$ induced an increase in LBF in the mid-cycle but not the early-cycle CL measured 1-h post-treatment. This

increase was induced following three different doses of $PGF_{2\alpha}$. Many studies focused on NO as the causing substance of LBF acute increase in the mature CL (Shirasuna et al., 2008). Nitric oxide induces apoptosis of bovine luteal cells and decreases P_4 secretion *in vitro* (Skarzynski et al., 2000, 2003). Local administration of a NO donor, S-nitroso-N-acetyl-DL-penicillamine (SNAP), induced a similar increase in LBF (Skarzynski et al., 2000). Treatment with SNAP also resulted in luteolysis-like effects in the CL. Cows that received a NO donor decreased P_4 levels below 1 ng/mL 3 d earlier in comparison to control cows. Luteal volume was reduced and inter-estrus intervals was shortened (Skarzynski et al., 2000). The inhibition of NO synthesis had CL protective effects. Disruption of NO pathway countered $PGF_{2\alpha}$-induced luteolysis and prolonged CL lifespan (Skarzynski et al., 2003). It was then proposed that the LBF increase mediated via NO is one of the luteolysis bottlenecks (Miyamoto et al., 2009).

Exogenous treatment or endometrium $PGF_{2\alpha}$ release changes the role of vasoactive substances in the CL. The combined action of $PGF_{2\alpha}$ and other substances creates pathways that lead to apoptosis and angiolysis in the regressing CL (Miyamoto et al., 2009). Such substances are produced in the CL, but also in endothelial cells that compose CL's vast vascular bedframe. In fact, endothelial cells are the first cells to go through apoptosis during luteolysis (Vonnahme et al., 2006). Vasoconstrictor factors such as EDN1 and Ang II are upregulated upon $PGF_{2\alpha}$ stimulus *in vivo* and *in vitro* (Girsh et al., 1996; Miyamoto et al., 1997; Ohtani et al., 1998; Hayashi and Miyamoto, 1999). Individually, EDN1 and Ang II also inhibit P_4 release from cultured luteal cells (Stirling et al., 1990; Miyamoto et al., 1997). In response to $PGF_{2\alpha}$ EDN1 and Ang II increase their expression within the CL periphery (Miyamoto et al., 2009). Endothelin 1 and Ang II positively feedback into luteal $PGF_{2\alpha}$ release (Kobayashi et al., 2001; Miceli et al., 2015). The primary stimulus of exogenous or endometrium $PGF_{2\alpha}$ commences a cycle of positive feedback between

luteal $PGF_{2\alpha}$, EDN-1 and Ang II within the CL (Shirasuna et al., 2004). The result is decrease in LBF via vasoconstriction of periphery blood vessels (Miyamoto et al., 2009). Apoptosis pathways are induced by $PGF_{2\alpha}$, but not luteal oxytocin (Shaw and Britt, 2000). Prostaglandin-$F_{2\alpha}$ activates protein kinase C and Ca^{+2} influx leading to luteal cells apoptosis (Wiltbank et al., 1989, 1991; Hansel et al., 1991; Wegner et al., 1991). Another effect of $PGF_{2\alpha}$ is the inhibition of VEGFs (Neuvians et al., 2004a). As mentioned previously, protein levels of VEGFs coordinate the fate of blood vessels in the presence of ANTPs. Protein levels of ANTP-2 remain constant throughout the cycle, whereas ANTP-1 decreases in the regressing CL (Tanaka et al., 2004). With the concomitantly decrease in ANTP-1 and VEGFs, ANTP-2 effects are no longer opposed or counteracted. Blood vessels undergo angiolysis leading to low luteal blood supply during regression (Miyamoto et al., 2009).

Other systems also regulate pathways that lead to functional and structural luteolysis. The immunological compartment of the CL also plays important roles during luteolysis. The presence of immune cells in luteal cells culture enhanced $PGF_{2\alpha}$ luteolytic properties, measured as decrease in P_4 levels (Liptak et al., 2005; Korzekwa et al., 2008b). Many studies investigated the role of T lymphocytes, monocytes/macrophages, neutrophils, and dendritic cells during luteolysis (Pepperell et al., 1992; Brännström et al., 1994; Penny et al., 1998; Spanel-Borowski, 2011; Shirasuna et al., 2012). Luteal T lymphocytes population changes their cell types, characteristics and increase their numbers during luteal regression (Poole and Pate, 2012). Immune cells increase their number towards late cycle via increased infiltration of the luteal tissue and local proliferation (Bauer et al., 2001). They are involved with tissue remodeling, phagocytosis, and secretion of secondary apoptosis mediators during luteolysis (Pavoola, 1979; Pate, 1995, 2003). Secretion of cytokines tumor necrosis factor alpha (TNFα) and interferon gamma (IFNγ) induce apoptosis of

cultured luteal cells (rev. Pate, 2003). mRNA for IFNγ, TNFα and its receptor increased during luteolysis (Neuvians et al., 2004b; Korzekwa et al., 2008a). Besides signaling cell death pathways, IFNγ and TNFα also stimulated intraluteal $PGF_{2\alpha}$ release (Fairchild and Pate, 1991; Fukuoka et al., 1992; Townson and Pate, 1996). The increase of $PGF_{2\alpha}$ feeds back into other suppressors of luteal function such as EDN-1 and Ang II (Miyamoto et al., 2009). Synthesis of intraluteal $PGF_{2\alpha}$ is essential for carrying out the luteolytic cascade and resulting in tissue involution. Intraluteal inhibition of $PGF_{2\alpha}$ synthesis had effects on maintenance of luteal tissue volume (Niswender et al., 2007). Local inhibition of cytokines synthesis utilizing ciclosporin-A delayed luteal tissue involution (Pate et al., 2012). CL that receive a luteolytic stimulus but do not regress have lower expression of genes that are related with prostaglandin synthesis and immune response (Atli et al., 2012). Pate et al. (2012) proposed that the resistance to $PGF_{2\alpha}$-induced luteolysis in the early-cycle CL may be due to different immune cell populations, incompatible with the luteolysis process.

All events occurring during CL lifespan are somewhat associated with its vascular network. Luteal blood flow enables interactions that lead to acquisition of secretory properties as well as complete tissue involution. In experimental settings, elaborated designs and techniques can be utilized to assess luteal function and LBF (Janson and Albrecht, 1975). Many of the studies cited so far utilized accurate techniques, such as CL microdialysis, protein and gene expression measurements. Data collected in such studies provided extensive data on substances that regulate CL function and how they interact. *In vitro* studies also helped to identify important factors and their effects on CL function.

COLOR DOPPLER ULTRASOUND AS A TOOL TO UNDERSTAND LUTEOLYSIS

Niswender et al. (1976) implanted Doppler transducers in ovarian arteries to measure changes in ovarian blood flow during estrous cycle. In the same study, ewes were infused with

various sizes of radioactive microspheres to estimate the locations where blood inflow was more prevalent in the ovary. However accurate, such techniques are reserved for small studies due to their labor-intensive and complex execution. Even mRNA, protein and hormonal quantification techniques are time-consuming and increase the cost of studies. In studies with large sample sizes, applying such methodologies would be unviable due to labor, time and the high cost associated with it. Therefore, in recent years simpler techniques to measure blood flow were refined and adapted for the CL (Miyazaki et al., 1998; Lüttgenau and Bollwein, 2014). Color Doppler ultrasonography enables the measurement of velocity, direction, and quantity of blood flow in a single vein or artery. The blood flow signals appear in red and blue colors depending on their direction in relation to the ultrasound transducer. Doppler signals going towards the transducer are shown in red, whereas signals going away from the transducer are shown in blue (Feliciano et al., 2003). The technique and calculations applied to measure blood flow in large blood vessels cannot be used in the CL. The CL vascular network is formed with several microcapillaries. It makes impossible to determine the velocity, direction, or quantity of blood flow because it captures signals of several capillaries at the same time. Therefore, this technique was adapted to estimate LBF area utilizing different measurements. The LBF signals are located in CL's periphery (Acosta et al., 2002; Miyamoto et al., 2005). Photos or videos of the greatest LBF area can be analyzed in automated software and provide quantitative data. Some studies reported LBF %, LBF area or the ratio between LBF/LV (Acosta et al., 2002; Ginther et al., 2007; Siqueira et al., 2019). Subjective scores are employed with certain accuracy and can be used as on-farm assessments (Guimarães et al., 2015; Andrade et al., 2019; Palhão et al., 2020).

The vascular network of a functional CL is distinct from a regressing/regressed CL. Luteal blood flow has been characterized during early cycle and luteolysis (Acosta et al., 2002, 2003).

Circulating P_4 levels, LV, and LBF were measured every 24 h from 1 d to 5 d after GnRH injection. All three variables increased synchronously during the first 5 d of CL development (Acosta et al., 2003). Luteal blood flow changes during $PGF_{2\alpha}$-induced and endogenous luteolysis were also described. As mentioned previously, a transitory increase in LBF was detected utilizing color Doppler following $PGF_{2\alpha}$ treatment in mid-cycle CL (Acosta et al., 2002). A bolus treatment of $PGF_{2\alpha}$ analogue also induces a transitory increase in circulating P_4 levels (Shrestha et al., 2010). During luteolysis, LBF seems to decrease in similar rates to circulating P_4 levels (Miyamoto et al., 2005). In the same study, decrease in LV (associated with tissue remodeling and involution) appears to be slightly delayed in relation to decrease in P_4. In fact, area of LBF was a better predictor of P_4 levels > 1 ng/mL in comparison to LV (Herzog et al., 2010). Milk or blood P_4 levels determined with enzyme-linked immunosorbent assay (ELISA) or radioimmunoassay (RIA) are still the golden standard to determine luteal function. However, the loss of the most sensitive P_4 assay from the market (Coat-a-Count) left the industry with assay kits that lack sensitivity to determine small changes in P_4 levels. A general cutoff used to confirm CL functionality is P_4 > 1 ng/mL (Ginther et al., 2009; Herzog et al., 2010). The cutoff of < 15 mm in diameter is well accepted when utilizing CL size to determine CL regression. There is no consensus regarding to a cutoff for LBF area where luteolysis occurred. That may be due to different methodologies and machines used to estimate LBF with color Doppler. Chapter 2 describes our laboratory's effort on defining guidelines to assess luteolysis based on LBF.

CHAPTER 2

EFFECT OF CLOPROSTENOL DOSE ON LUTEAL BLOOD FLOW AND LUTEAL VOLUME MEASUREMENTS IN EARLY AND MID-CYCLE CORPORA LUTEA

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INTRODUCTION

A complex angiogenic process takes place during and after ovulation followed by a gradual increase in both LV and LBF (Reynolds et al., 2000; Acosta et al., 2003; Miyamoto et al., 2009; Berisha et al., 2016). This allows the early CL to produce and release P_4 as part of its normal development (Acosta et al., 2003). Luteal function can be assessed utilizing RIA and ELISA hormonal assays for P_4. Yet, sensitivity of current P_4 assays lack the ability to assess complete luteolysis. Currently available kits are not accurate enough to detect subtle changes in P_4 levels when concentration is under 1 ng/mL.

Ultrasound with color Doppler can estimate LBF and LV as a real time assessment of CL function and may provide an alternative way of assessing luteolysis. These parameters are highly correlated with P_4 concentrations during the estrous cycle. However, LBF appears to be a better predictor of $P_4 > 1.0$ ng/mL in comparison to LV. Luteal volume had to exceed 60% of the maximal values, whereas LBF only exceeded 35% of the maximal values to reliably indicate $P_4 > 1.0$ ng/mL (Herzog et al., 2010).

Establishment of a vascular system within the CL is essential for acquisition of secretory properties, but also for luteolysis occurrence. Blood flow delivers steroid precursors and gonadotropins to the CL that allow for P_4 synthesis (Niswender et al., 1976; Janson et al., 1981). Luteal blood flow decreases rapidly during luteolysis and is followed by decreasing levels of P_4 (Niswender et al., 1976). An acute and transitory increase of blood flow occurs after a $PGF_{2\alpha}$ bolus treatment in mid-cycle, but not early-cycle CL. Luteal blood flow decreased significantly 2 d after treatment in mid-cycle CL but increased in early-cycle CL (Acosta et al., 2002). Studies verified that P_4 levels decreased synchronously with LBF in both spontaneous and $PGF_{2\alpha}$ induced luteolysis (Miyamoto et al., 2005; Lüttgenau et al., 2011).

Treatment with analogue drugs of $PGF_{2\alpha}$ result in luteolysis when administered after d 5 of the estrous cycle (Wiltbank et al., 1995; Tsai and Wiltbank, 1998). The early-cycle CL does not undergo luteolysis with a single treatment of $PGF_{2\alpha}$ (Tsai and Wiltbank, 1998). Simultaneous presence of $PGF_{2\alpha}$ responsive and resistant CL provides a scenario to better understand the interactions between these two distinct structures during $PGF_{2\alpha}$-induced luteolysis. Moreover, when concomitantly present a regressing CL may induce regression of refractory CL following exogenous treatment with $PGF_{2\alpha}$ (Howard and Britt, 1990; Stevenson, 2016).

We designed a non-invasive bioassay to establish the use of real time color Doppler as an indicator of luteolysis using varying doses of cloprostenol sodium (CLO) in early (d 4 CL) and mid-cycle CL (d 10 CL). It was hypothesized that LV and LBF may act as good predictors of luteal function, able to identify different response patterns to CLO treatment. It is also hypothesized that different doses of CLO will differentially impact luteal dynamics in mid-cycle CL, but not in early-cycle CL. Our overall objective was to establish guidelines to assess luteolysis with color Doppler based on control groups outcomes. A secondary objective was to describe long-term effects of the combination of a $PGF_{2\alpha}$ responsive and a $PGF_{2\alpha}$ refractory CL at the time of treatment.

MATERIALS AND METHODS

Experimental Units

This project was conducted from August 2017 to January 2018 on a commercial dairy herd, (Nobis Dairy Farm, St. Johns, Michigan, USA). The farm milked approximately 1,000 lactating cows and raised all replacement heifers. This trial utilized n = 37 11 to 12-month old Holstein heifers in n = 4 cohorts. All heifers were monitored with an activity monitoring system (SCR Heatime Pro+ System, Madison, Wisconsin, USA). They were fed a total mixed ration (TMR) once a d with free access to feed and water and were confined in a free-stall barn. The TMR

consisted of corn, wheat, and alfalfa silages, and corn-soybean meal-based concentrates formulated to meet nutrient recommendations for breeding age heifers (NRC, 2001). The Institutional Animal Care and Use Committee at Michigan State University approved all animal handling and procedures.

Treatments

All heifers received a combination of CLO (estroPLAN, Parnell, Overland Park, Kansas, USA) and GnRH (GONAbreed, Parnell, Overland Park, Kansas, USA) to induce the presence of early (d 4) and mid-cycle (d 10) CL on d of treatment (d 0; Figure 2.1).

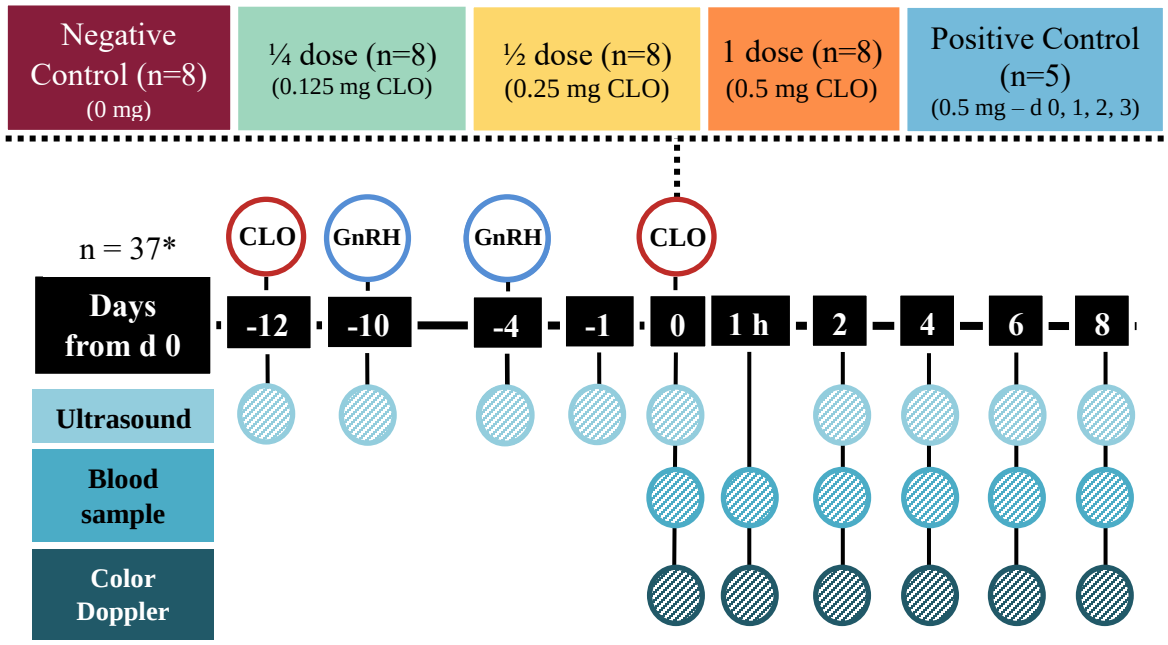

Figure 2.1. Description of the synchronization method utilizing cloprostenol sodium (CLO) and gonadotropin releasing hormone (GnRH) to ensure the presence of an early (d 4) and mid-cycle (d 10) CL at time of treatments (d 0) with different doses of CLO to allow for variation in luteolytic responses. *Thirty-seven heifers responded to the GnRH administration and had a d 4 and a d 10 CL at d 0. Those heifers were then randomly assigned to one of the 5 treatment groups at d 0.

Criteria based on activity monitoring and ultrasonography data was used to determine responsiveness to hormonal treatments. Only heifers that met the following criteria were included in the study: 1) Estrus on d –10 according to peak activity detected by the activity monitor system;

2) Presence of a dominant follicle (DF) > 10 mm and CL < 15 mm in diameter on d –10; 3) Ovulation to first GnRH on d –4 confirmed by a new CL in same ovary and position as the DF measured on d –10; 4) Presence of a new DF > 10 mm on d –4; and 5) Newly formed CL and the absence of a DF 3 d after last GnRH (d –1). 6) Heifers with only single ovulations were included in the study.

On d of treatment, heifers that met the above criteria were randomly assigned to receive one of five treatments: Negative Control (NC) that consisted of no treatment with CLO (n = 8); ¼ dose CLO (0.125 mg; n = 8); ½ dose CLO (0.25 mg; n = 8); 1 dose of CLO (0.5 mg; n = 8); or Positive Control (PC) that consisted of the repeated administration of 0.5 mg of CLO four times at 24 h intervals (n = 5; Figure 2.1).

Ultrasonography – ovulation and CL volume determination

Ultrasonography (SonoSite MicroMaxx, 10-5 MHz linear array probe, Bothell, Washington, USA) was used to map ovaries and measure ovarian structures ≥ 4 mm as described in Figure 2.1. Ovaries were scanned three times using the Cine memory function. The Cine memory clip allowed for frame by frame visualization to determine the greatest diameter of any given structure. Diameters was determined utilizing built-in calipers. The first diameter measurement for each structure was horizontal and the second was perpendicular to the 1st measurement. Luteal volume was calculated in mm^3 using the formula $V = (4/3)\,\pi\, R^3$. Radius (R) was calculated using the formula $R = (D^a/2 + D^b/2)/2$. D^a was the horizontal diameter of the CL and D^b was the perpendicular diameter to D^a. Volume of CL with cavities were calculated with the same formula but with the volume of the cavity subtracted from the total volume of the CL.

Ultrasonography – luteal blood flow determination

Assessment utilizing color Doppler was performed at specific times (Figure 2.1) to determine the effect of treatment on LBF of early and mid-cycle CL (Figure 2.1). If a spontaneous ovulation occurred following treatment the new CL, LBF was also recorded. Different sections of each CL were examined utilizing color Doppler. At least three Cine memory frames showing the greatest LBF area were saved for further analyses. Luteal Blood flow area was measured in number of colored pixels. An image editing software (Adobe Photoshop CS3, San Jose, California, USA) was used to select the desired area with a color range selection tool. The method consisted of using an eyedropper tool to select and delete only black, white, and gray tone pixels. The remaining colored pixel area was selected with the same tool and pixel count was determined by the software. The analyses utilized an average of three pictures from the same CL at each assessment (Figure 2.1).

Progesterone Analyses

Blood samples for P_4 were collected on all heifers on d 0 (before and 1 h after treatment), 2, 4, 6, and 8. D 2 samples were missing for one heifer on the 1 dose group, and for two heifers in the NC group. The 1-h sample was missing on one heifer of the PC group. Serum concentrations of P_4 were analyzed with a radioactive immunoassay validated by Engel et al., (2008). All sample concentrations were determined in one assay. The interassay CV was 8.15%, the intraassay CV was 4.84% and the sensitivity was 0.2 ng/mL.

Statistical Analyses

All statistical analyses were performed using SAS (version 9.4, SAS Institute Inc., Cary, North Carolina, USA). Serum P_4 concentrations, LV and LBF measurements are reported as mean ± SEM. Luteal volume and LBF data are reported in three different ways: INDIVIDUAL and

COMBINED. INDIVIDUAL refers to the individual measurement of LV or LBF of either the d 4 or 10 CL. COMBINED refers to the sum of LV or LBF in d 4 and 10 CL.

Analysis of residuals from continuous variables was utilized to assess normality with PROC UNIVARIATE and SGPLOT. Graphical methods such as scatter plot of predicted × residual values, boxplots, and histograms of residuals were used for normality and outlier checks. Shapiro-Wilk was utilized as a normality test. A Box-Cox transformation analysis was employed utilizing PROC TRANSREG on variables that presented a non-normal distribution to establish the value of the λ-exponent. The recommended λ, or a λ value within the 95% confidence interval (CI) was used to transform the non-normal distributed variables. Serum P_4 data presented a non-normal distribution and was log-transformed. Further analyses were performed with the transformed variable.

Differences in the proportion of heifers in which LBF decreased to zero were analyzed by chi-square test using the FREQ procedure. Analyses were performed for d 4 and d 10 CL separately. Treatment groups (¼, ½ and 1 dose of CLO) and control (NC and PC) heifers were included in the analyses.

Serum P_4 levels, LV and LBF were analyzed using PROC MIXED with the REPEATED statement to account for measurements over time (d 0/0 h, 0/1 h, 2, 4, 6 and 8). All analyses included treatment (¼, ½ and 1 dose of CLO) and control (NC and PC) heifers. The statistical model included the fixed effects of treatment, time, and their interaction. A first-order autoregressive [AR(1)] covariance structure was applied based on the lower Bayesian information criterion (BIC) score. Multiple comparisons between treatment groups were used to account for simple effects of treatment across time. Comparisons between different doses of CLO (¼, ½ and 1 dose), NC (no luteolysis) and PC (luteolysis) were used to interpret the effect of treatment on

CL regression. Significant interactions were sliced with PROC PLM using an output from PROC MIXED. Multiple comparisons within the interaction of treatment*time were performed between treatment groups and sliced by time. Multiple comparison *P*-values were adjusted with Tukey-Kramer adjustment method.

Heifers were separated into an additional group for INDIVIDUAL LV and LBF analyses of early and mid-cycle CL. Heifers were classified into two groups based on LBF regression of d 10 CL following treatment. Within each treatment group heifers were separated into complete regression of d 10 CL, defined as LBF = 0 at any time following treatment; and maintenance of LBF, which included heifers that maintain d 10 LBF > 0 until d 8 post-treatment. This separation was kept for all INDIVIDUAL analyses of LV and LBF of early and mid-cycle CL. Statistical significance was determined based on a $P < 0.05$.

RESULTS

The study design included two control groups to simulate different physiological scenarios that occur during normal luteal development. Data from untreated NC heifers provides parameters of physiological CL development, from d 4 to 12 (early to mid-cycle) and from d 10 to 18 of the estrous cycle (mid to late cycle). In contrast, PC heifers data describe the effect of treating multiple doses of CLO to induce luteolysis of both early and mid-cycle CL. Physiological parameters were determined in terms of serum P_4 levels, LV and LBF for heifers with combination of a main (d 10 CL) and accessory CL (d 4 CL; Table 2.1). For descriptive purposes, serum P_4 levels, INDIVIDUAL LV and LBF were reported for early and mid-cycle CL of NC and PC heifers (Figure 2.2).

	Negative controls (NC; n = 8)		
Day after treatment	COMBINED LV[1] mean ± SEM (range)	COMBINED LBF[2] mean ± SEM (range)	Serum P_4 levels[3] mean ± SEM (range)
0/0 h	10,595 ± 982 (6807 – 14,896)	5,971 ± 667 (4,157 – 8,366)	5.3 ± 0.4 (3.1 – 6.7)
0/1 h	-	6,304 ± 545 (4,247 – 8,817)	5.3 ± 0.4 (3.1 – 7.0)
2	10,863 ± 992 (7202 – 15,514)	7,947 ± 527 (5,386 – 9,707)	6.2 ± 0.7 (4.9 – 8.7)
4	12,376 ± 1,101 (8331 – 15,824)	8,291 ± 450 (6,497 – 9,962)	6.5 ± 0.6 (4.5 – 9.9)
6	10,522 ± 739 (6651 – 13,312)	8,594 ± 969 (4,933 – 11,945)	7.0 ± 0.9 (5.1 – 13.2)
8	8,446 ± 760 (4828 – 11,056)	8,613 ± 1,105 (3,292 – 12,443)	5.9 ± 0.8 (1.4 – 8.8)
	Positive controls (PC; n = 5)		
Day after treatment	COMBINED LV mean ± SEM (range)	COMBINED LBF mean ± SEM (range)	Serum P_4 levels mean ± SEM (range)
0/0 h	8,061 ± 1,427 (5,401 – 13,036)	4,625 ± 406 (3,506 – 5,513)	7.0 ± 0.6 (5.7 – 8.7)
0/1 h	-	8,488 ± 293 (7,515 – 9,229)	4.7 ± 0.8 (3.5 – 7.0)
2	4,409 ± 895 (2,022 – 6,603)	2,533 ± 377 (1,048 – 3,173)	1.5 ± 0.2 (1.0 – 2.3)
4	1,401 ± 309 (754 – 2,513)	0	0.87 ± 0.07 (0.7 – 1.1)
6	463 ± 65 (268 – 674)	0	0.81 ± 0.1 (0.6 – 1.1)
8	281 ± 39 (195 – 419)	0	2.4 ± 0.32[4] (1.6 – 3.2)

[1] COMBINED LV was measured as mm^3, and combines early and mid-cycle CL LV.
[2] COMBINED LBF was measured as pixel number and combines early and mid-cycle CL LBF.
[3] Serum P_4 levels were measured as ng/mL, and it is the result of early and mid-cycle CL.
[4] On d 8 all heifers in the PC group had ovulated and the P_4 levels can be attributed to new developing CL.

Table 2.1. COMBINED LV, LBF and serum P_4 levels from negative and positive controls describe physiological parameters of two different scenarios that occur during normal CL development. Data was collected from heifer with the simultaneous presence of an accessory and main CL (early and mid-cycle CL). Negative controls data is equivalent of normal luteal development of early and mid-cycle CL combination. During the assessment period (d 0 to 8 after treatment) the early-cycle CL developed from d 4 to d 12 of the estrous cycle. The mid-cycle CL developed from d 10 to d 18 of the cycle. The positive control data describes simultaneous luteolysis of early and mid-cycle CL.

Complete luteal regression determination

The PC treatment regimen included 4 doses of 0.5 mg of CLO 24 h between each dose (d 0, 1, 2 and 3). Treatment with multiple doses of CLO induced drastic decrease in LV and LBF disappearance in both CL. No colored pixels were detected from 2 to 4 d after the first CLO injection in both CL. The disappearance of LBF coincided with the decrease of serum P_4 levels to under < 1 ng/mL at d 4 after the first injection (0.87 ± 0.07 ng/mL). All heifers in the PC group had spontaneous ovulation following treatment. Two heifers had ovulations detected at d 2, and three heifers at d 4 following treatment.

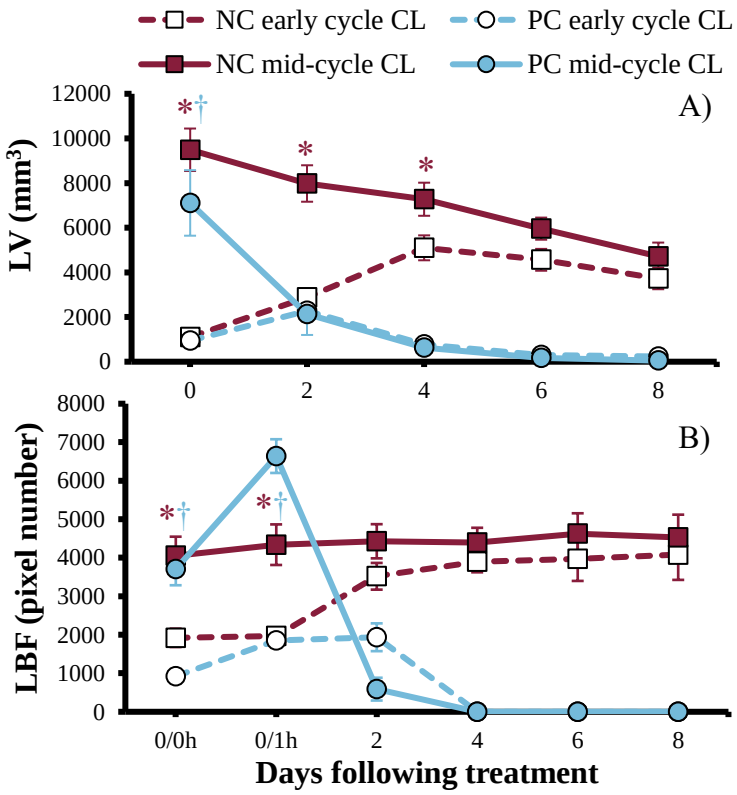

Figure 2.2. Negative and positive controls (A) INDIVIDUAL luteal volume (LV) and (B) luteal blood flow (LBF) for early and mid-cycle CL. The early-cycle CL developed from d 4 to 12 whereas the mid-cycle developed from d 10 to 18 of the estrous cycle. The symbols represent statistical differences between early and mid-cycle CL. Each comparison was estimated within d and its respective control group. The * symbol describes comparisons within NC; whereas the † describes comparisons within PC heifers ($P < 0.05$; Tukey-Kramer).

On the contrary, all NC heifers maintained LBF of early and mid-cycle CL, from d 0 to d 8 post-treatment. Early-cycle LBF, measured as colored pixel count, ranged from 819 to 6,937 including all d of assessment. The mid-cycle LBF ranged from 1,727 to 6,849 including all d of assessment. Therefore, disappearance of LBF was considered as a clear marker of complete luteal regression in both CL.

Sub-luteolytic doses of CLO (¼ and ½ dose) did not induce LBF disappearance of early-cycle CL measured 8 d after treatment. A full dose of CLO effectively regressed the d 4 LBF in 1 out of 8 heifers. Yet, this heifer did not have complete CL regression until 8 d following treatment (Table 2.2).

Complete LBF disappearance (n/n)	Days following treatment				
Treatment	0	2	4	6	8
Negative Control	0/8	0/8	0/8	0/8	0/8
¼ dose of CLO	0/8	0/8	0/8	0/8	0/8
½ dose of CLO	0/8	0/8	0/8	0/8	0/8
1 dose of CLO	0/8	0/8	0/8	0/8	1/8
Positive Control	0/5	0/5	5/5	5/5	5/5

Table 2.2. Effect of cloprostenol dose in 4 d CL on cumulative number of breeding age Holstein heifers in which LBF measured as colored pixel number decreased to 0 during the 8-d period following treatment ($P < 0.01$).

Complete LBF disappearance (n/n)	Days following treatment				
Treatment (n/n)	0	2	4	6	8
Negative Control	0/8	0/8	0/8	0/8	0/8
¼ dose of CLO	0/8	0/8	0/8	1/8	4/8
½ dose of CLO	0/8	0/8	3/8	3/8	5/8
1 dose of CLO	0/8	2/8	7/8	8/8	8/8
Positive Control	0/5	2/5	5/5	5/5	5/5

Table 2.3. Effect of cloprostenol dose in 10 d CL on cumulative number of breeding age Holstein heifers in which LBF measured as colored pixel number decreased to 0 during the 8-d period following treatment ($P < 0.01$).

The mid-cycle CL was not surprisingly more sensitive to CLO administration compared to the early CL (Table 2.3). However, ¼ and ½ dose of CLO were insufficient to induce complete LBF regression in all heifers. Time to complete LBF disappearance was prolonged as dose of CLO decreased. One full dose of CLO induced complete regression of d 10 LBF in all heifers from d 4 to 6 post-treatment. Further INDIVIDUAL analyses will describe results separately between heifers with LBF maintenance and LBF regression of the d 10 CL.

Control groups: INDIVIDUAL LV and LBF parameters

Negative controls. In NC heifers early-cycle INDIVIDUAL LV increased from d 0 to d 8 after treatment (or from d 4 to d 12 of the estrous cycle). The early-cycle LV peaked at d 4 with a slight decrease up until d 8 after treatment. The early-cycle CL maintained lower LV in comparison to mid-cycle CL when in the same d of the estrous cycle. early-cycle LV averaged 4,562.9 whereas mid-cycle LV averaged 9,491.7 mm^3, both at d 10 of the estrous cycle. A downward trend in mid-cycle LV was present in untreated NC heifers. Average LV decreased from d 0 to 8 (or from d 10 to d 18 of the estrous cycle). Early-cycle LBF increased from d 0 to d 8 after treatment, reaching similar average LBF in comparison with the main CL. Mid-cycle LBF maintained a plateau throughout the study.

Positive controls. In PC heifers, treatment with 4 doses of CLO 24 h apart impaired normal development of early CL, measured as average LV. PC heifers average LV peaked at d 2, after 2 injections of CLO, with no differences in comparison with NC heifers. From d 4 to 8 post-treatment LV was decreased in PC in comparison with NC heifers. Mid-cycle LV decreased drastically from d 0 to d 8 following PC treatment regimen. Similar to LV in the early-cycle CL, LBF increased from d 0 to d 2 post-treatment. Yet, PC treatment resulted in early-cycle LBF disappearance from d 2 to d 4 post-treatment. In mid-cycle CL average LBF decreased drastically from d 0 to d 2 post-

treatment and reached zero 4 d after the first treatment. Comparisons with NC and PC heifers were utilized to interpret the effect of different doses of CLO on serum P_4 levels, LV and LBF. All figures include NC and PC data for comparison.

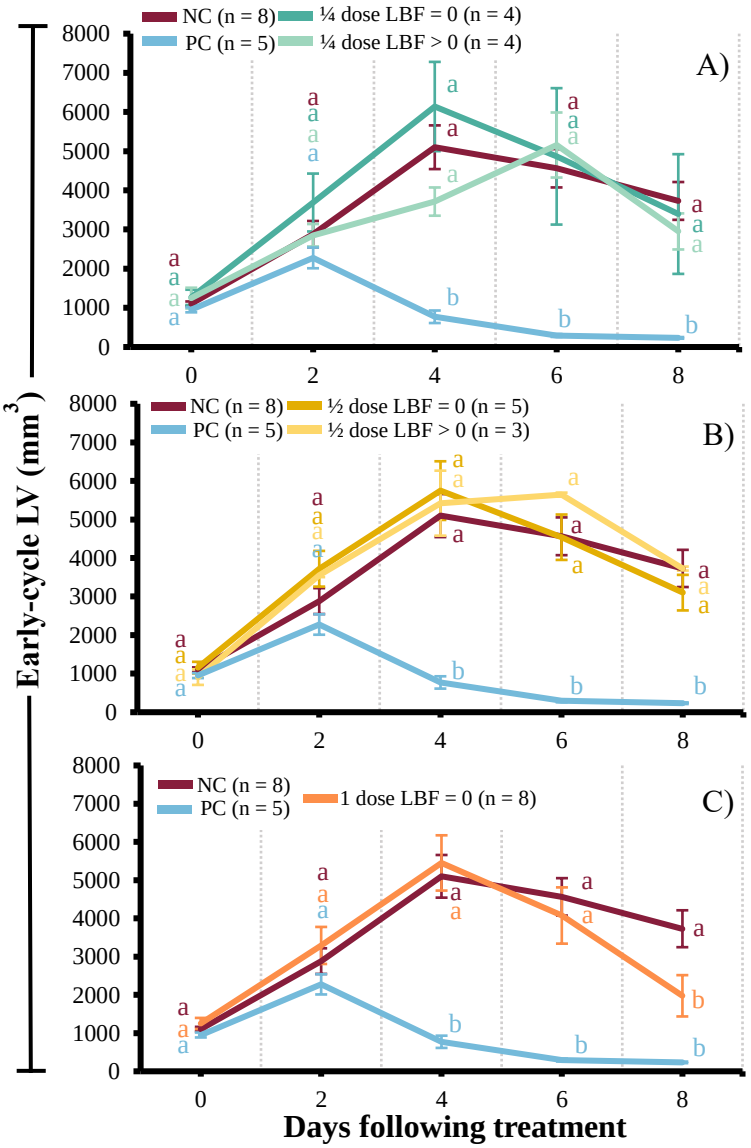

Figure 2.3. The effect of ¼ (A), ½ (B) and full (C) doses of cloprostenol sodium (CLO) INDIVIDUAL LV (mm^3) in breeding age Holstein heifers compared to negative (no CLO) and positive (4 doses 0.5 mg CLO 24 h between each dose) controls. Heifers shown separately based on outcome of treatment on d 10 CL. Treatments sharing different letter superscripts inside the dotted lines differed significantly within that d ($P < 0.05$; Tukey-Kramer).

Early-cycle CL: effect of CLO dose on LV and LBF

Treating heifers with ¼ and ½ doses of CLO did not attenuate growth rate of d 4 CL measured as LV (Figure 2.3 A and B, respectively). Complete LBF regression of the d 10 CL did not impact average LV following ¼ and ½ doses of CLO. Early-cycle CL development occurred normally following 1 dose of CLO until d 6. Yet, a full dose of CLO decreased average LV from d 6 to 8 in comparison with NC on the same d (Figure 2.3 C).

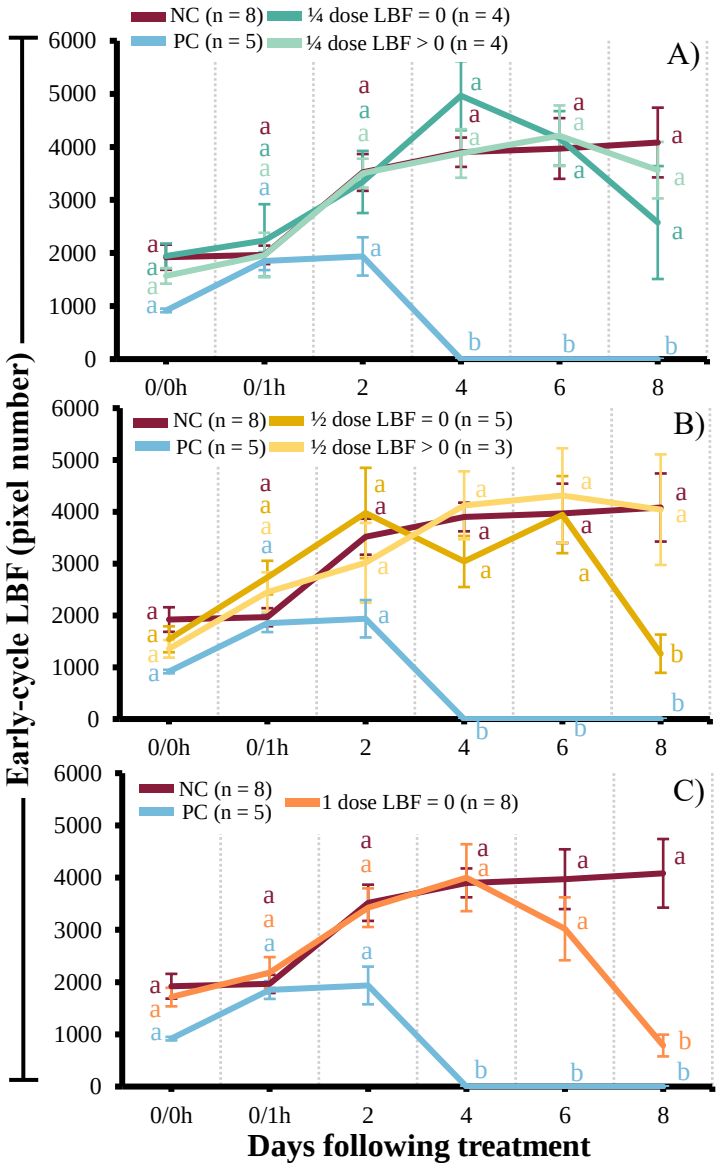

Figure 2.4. The effect of ¼ (A), ½ (B) and full (C) doses of cloprostenol sodium (CLO) on early-cycle (d 4) INDIVIDUAL LBF (pixel number) in breeding age Holstein heifers compared to

negative (no CLO) and positive (four doses 0.5 mg CLO 24 h between each dose) controls. Heifers shown separately based on outcome of treatment on d 10 CL. Treatments sharing different letter superscripts inside the dotted lines differed significantly within that d ($P < 0.05$; Tukey-Kramer).

Luteal blood flow of the d 4 CL seemed unaffected following treatment with ¼ dose of CLO (Figure 2.4 A). Luteal blood flow was not different from non-treated controls, regardless classification between d 10 CL regression or maintenance of LBF. Heifers receiving ½ dose of CLO and classified within LBF regression of the d 10 CL eventually decreased d 4 LBF (Figure 2.4 B). This decrease was detected at d 8 post-treatment in comparison to ½ dose heifers that did not have d 10 CL regression and NC heifers. Heifers treated with a full dose of CLO decreased early-cycle LBF from d 6 to 8. Average LBF was lower in comparison with NC at d 8. All heifers in this treatment also had complete LBF regression of the d 10 CL (Figure 2.4 C).

Mid-cycle CL: effect of CLO dose on LV and LBF

Treating CLO to heifers with d 10 CL resulted in decreased LV post-treatment. The response pattern to CLO depended on dose and classification into regression or maintenance of d 10 LBF. All tested doses of CLO (¼, ½ and 1 dose) induced a reduction of d 10 LV at d 2 and 4 post-treatment in comparison with NC (Figure 2.5 A, B and C). However, heifers classified as maintaining LBF > 0 in the ¼ and ½ dose groups had intermediate LV on d 6 and 8. Heifers that received a full dose of CLO presented a similar LV reduction rate in comparison with PC treated heifers (Figure 2.5 C).

The effect of treatment on LBF dynamics varied according to CLO dose. Besides the dose effect, classification between complete LBF regression or maintenance also influenced response following CLO treatment. All groups that received CLO at d 0 showed an acute increase in LBF in comparison to untreated heifers, 1 h after treatment (Figure 2.6 A, B and C). However, heifers that received ¼ dose and were classified within maintaining LBF > 0 had intermediate LBF 1 h

post-treatment. There were no differences amongst this group and any other treatment group 1 h after treatment (Figure 2.6 A).

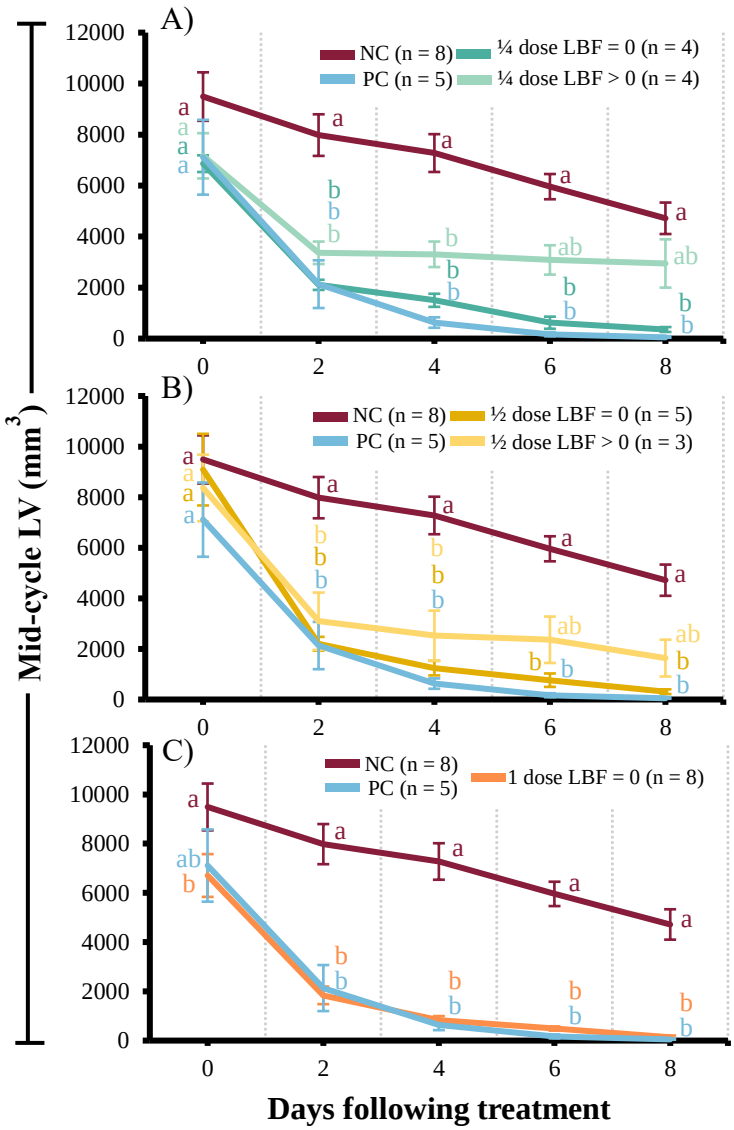

Figure 2.5. The effect of ¼ (A), ½ (B) and full (C) doses of cloprostenol sodium (CLO) on mid-cycle (d 10) INDIVIDUAL LV (mm^3) in breeding age Holstein heifers compared to negative (no CLO) and positive (four doses 0.5 mg CLO 24 h between each dose) controls. Heifers shown separately based on outcome of treatment on d 10 CL. Treatments sharing different letter superscripts inside the dotted lines differed significantly within that d ($P < 0.05$; Tukey-Kramer).

Some heifers that received sub-luteolytic doses of CLO (¼ and ½ dose) still regressed LBF completely. Yet, the time to complete LBF disappearance was not the same between ¼ and ½ dose of CLO as described previously (Table 2.3).

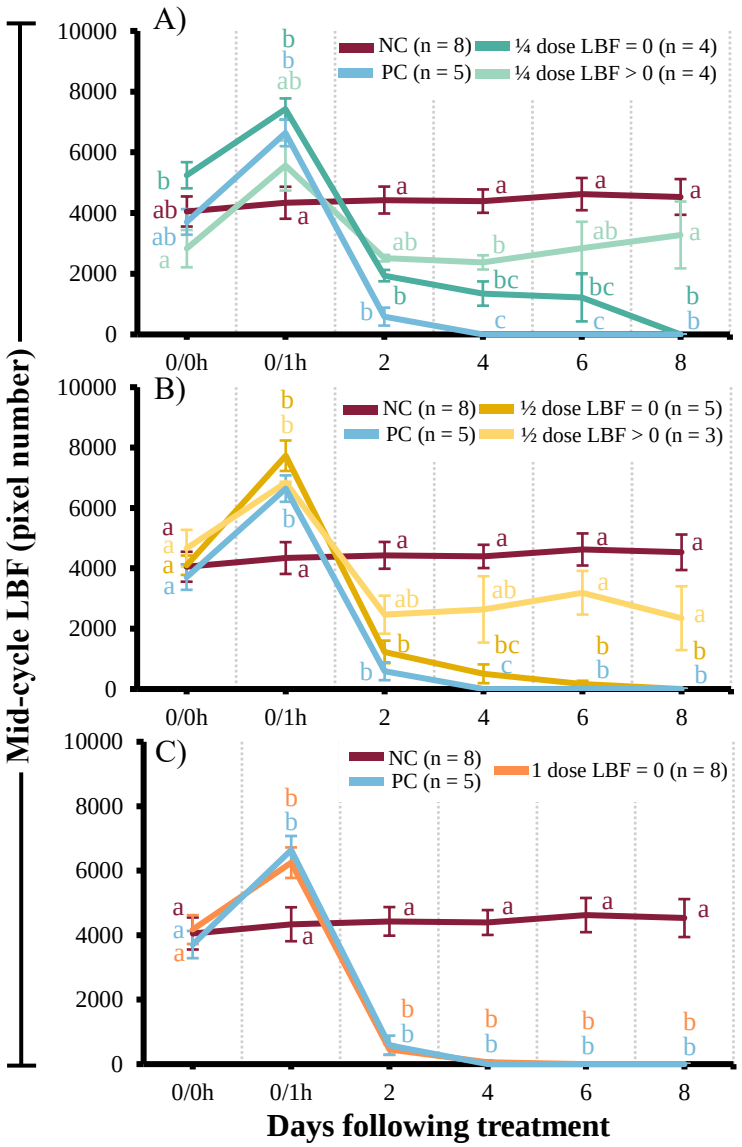

Figure 2.6. The effect of ¼ (A), ½ (B) and full (C) doses of cloprostenol sodium (CLO) on mid-cycle (d 10) INDIVIDUAL LBF (pixel number) in breeding age Holstein heifers compared to negative (no CLO) and positive (four doses 0.5 mg CLO 24 h between each dose) controls. Heifers shown separately based on outcome of treatment on d 10 CL. Treatments sharing different letter superscripts inside the dotted lines differed significantly within that d ($P < 0.05$; Tukey-Kramer).

Heifers that maintained LBF in the ¼ and ½ dose groups showed a slight decrease in LBF followed by an upwards trend, with no differences in comparison to NC d 8 after treatment. Heifers that received 1 dose of CLO were as efficient as PC in reducing LBF. No differences on LBF were observed between these two groups from d 0 to d 8 following treatment.

Treatment effects on serum P_4 levels

There were early effects of CLO treatment on serum P_4 levels in ¼, ½, 1 dose and PC heifers. Two d after CLO treatment, serum P_4 levels in those groups decreased in comparison with NC. At 4 d post-treatment serum P_4 levels increased on ¼, ½ and 1 dose groups, but not on PC group. However, treating CLO to heifers with a d 4 and a d 10 CL seemed to induce long terms effects on serum P_4 levels.

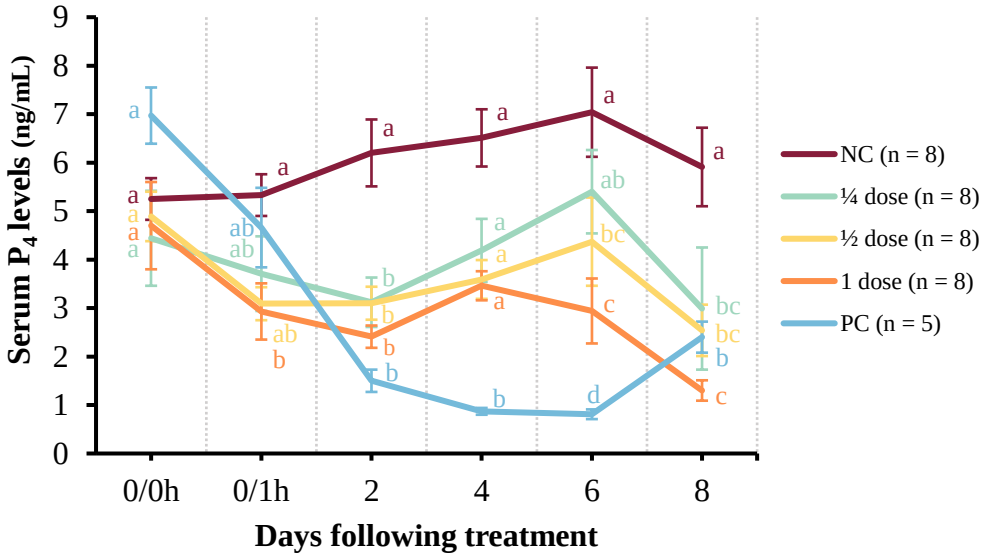

Figure 2.7. The effect of treating breeding age Holstein heifers with early (d 4) and mid-cycle (d 10) CL with different luteolytic doses of cloprostenol sodium (CLO) compared to negative (no CLO) and positive (four doses 0.5 mg CLO 24 h between each dose) controls on circulating P_4 concentrations (ng/mL). Letter superscripts refer to multiple comparisons between treatments and within d following treatment. Treatments sharing different letter superscripts inside the dotted lines differed significantly within that d ($P < 0.05$; Tukey-Kramer).

A reduction in serum P_4 levels was detected 8 d after treatment, and it seemed independent on CLO dose. All groups that received CLO on d 0 had lower P_4 levels in comparison with NC d

8 after treatment. Comparisons with PC on d 6 and 8 are confounded. All heifers in this group had spontaneous ovulation and the present P_4 is secreted by a newly formed CL (Figure 2.7).

DISCUSSION

Our intention on treating different doses of CLO was to create partial and complete luteolysis scenarios. Partial or incomplete luteolysis is observed in 10 to 20% of lactating dairy cows that receive a single dose of $PGF_{2\alpha}$ during timed artificial insemination (TAI) programs (Brusveen et al., 2009; Martins et al., 2011a; Wiltbank et al., 2015). Treatment with sub-luteolytic doses of CLO intended to induce similar scenarios that occur in lactating in dairy cows, but rarely occur in heifers (Sartori et al., 2004). Other studies succeeded in inducing partial luteolysis in cows, and heifers utilizing intrauterine or intramuscular decreased doses of $PGF_{2\alpha}$ (Ginther et al., 2009; Trevisol et al., 2015). Measurement of LBF was sensitive enough to detect a slight decrease followed by an increase in heifers that received sub-luteolytic doses of CLO. Trevisol et al. (2015) also reported a decrease followed by a rebound in both LV and LBF in cows treated with a sub-luteolytic dose of $PGF_{2\alpha}$, measured in a 48-h period. Surprisingly, ¼ and ½ dose of CLO still induced complete luteal regression of mid-cycle CL in a proportion of heifers. However, time to complete luteal regression of the d 10 CL was prolonged in those heifers in comparison with 1 dose and PC heifers.

An acute increase in d 10 CL LBF was present 1 h after CLO treatment, but not in the d 4 CL. These findings agree with another study where CLO was treated to heifers with either an early or a mid-cycle CL (Acosta et al., 2002). This increase was independent on CLO dose. Yet, heifers that received ¼ dose of CLO and also maintained LBF had an intermediate increase. An acute increase in LBF within a mid-cycle CL from 0.5 to 2 h post-treatment was reported before (Acosta et al., 2002; Miyamoto et al., 2005; Ginther et al., 2009). Moreover, it was suggested as one of the

first and necessary events to start the luteolytic cascade (Miyamoto et al., 2005, 2009). To our knowledge, this is the first report where low doses of CLO also resulted in a transient LBF increase. In the present study, increase in LBF 1 h post-treatment not necessarily resulted in complete luteal regression. Heifers that received ¼ and ½ dose of CLO and experienced this an increase in LBF were still able to maintain LBF after treatment during the 8-d assessment period. At the last d of assessment there were no differences in LBF between untreated NC and groups that received sub-luteolytic doses of CLO.

With the exception of the PC group, it was only expected to induce luteal regression of the mid-cycle CL. One of our objectives was to understand luteal dynamics in the early-cycle CL when in the presence of a mid-cycle CL that either underwent complete luteal regression or did not. The early-cycle CL was refractory to $PGF_{2\alpha}$-induced luteolysis at the time of treatment. Many studies have reported a period during early CL development where $PGF_{2\alpha}$ does not induce luteolysis (Wiltbank et al., 1995; Tsai and Wiltbank, 1998; Acosta et al., 2002). This period extends from d 0 to d 5 of the estrous cycle and luteal development is not affected by $PGF_{2\alpha}$ administration. In fact, $PGF_{2\alpha}$ along with VEGF and bFGF are stimulators of CL development early in the estrous cycle (Miyamoto et al., 2009).

Acosta et al. (2002) treated a full dose of CLO to heifers with a d 4 CL. The early CL normally developed 2 d after treatment. The early-cycle CL increased serum P_4 levels, LV and LBF post-treatment. In the present study, follow-up period was extended up to 8 d to verify if long-term impacts of CLO and/or regression of the mid-cycle CL were present. Sub-luteolytic doses of CLO did not seem to impair early-cycle CL development in comparison to NC. Yet, heifers that received ½ dose of CLO with simultaneous mid-cycle LBF regression had lower early-cycle LBF 8 d after treatment. A more evident decrease was identified 8 d after treatment in both LV and LBF

in heifers that received a full dose of CLO. All heifers in that group also had d 10 CL complete LBF regression. Eight d after treatment are equivalent to d 12 of the estrous cycle for the early-cycle. Luteal regression at this time is not expected to occur.

The premature decrease in early-cycle LBF could be an effect of simultaneous regression of the mid-cycle CL. Howard and Britt (1990) reported premature regression of a human chorionic gonadotropin (hCG) induced CL 2 d after ovulation, when in the presence of a regressing mature CL. Another study reported that cows with a combination of an older and new CL had greater luteolysis rates in comparison with cows that only had a new CL (Stevenson, 2016). However, the same study defined a new CL as a 7-d old accessory CL, which is inconsistent with the characteristics of the accessory CL in the present data (4-d old CL).

The early effects on serum P_4 levels in CLO treated groups was most certainly due to the regression of the d 10 CL, susceptible to CLO. Multiple treatments with CLO impaired P_4 secretion in PC heifers. However, heifers treated with ¼, ½, and 1 dose of CLO increased serum P_4 levels up to d 4 post-treatment. Luteal function was maintained regardless of mid-cycle CL regression. Thus, early-cycle CL acquired and maintained endocrine function, regardless of CLO dose. Yet, treating CLO to heifers with a d 4 and d 10 CL seemed to induce long terms effects on serum P_4 levels. A reduction in serum P_4 levels was detected from d 6 to 8 after treatment. Serum P_4 levels reduction seemed independent on CLO dose. This effect could be attributed solely to regression of the d 10 CL. However, INDIVIDUAL analyses showed that the early-cycle CL was also impacted following 1 dose (decrease in LV and LBF) and ½ dose of CLO (decrease in LBF). Both groups also had complete regression of d 10 CL. The long-term effects on serum P_4 reduction are most likely due to the interaction between the regressing mid-cycle CL and early-cycle CL. Heifers with mid-cycle CL complete regression were more likely to have low levels of P_4 in comparison

to heifers that maintained a functional mid-cycle CL. Serum P_4 levels could have been insufficient to exert its negative feedback blocking OXTR and ER expression in the uterus (Grazzini et al., 1998; Robinson et al., 2001). The lack of P_4 negative feedback could trigger intrinsic luteolytic mechanisms from d 10 to 12 of the estrous cycle (d 6 to 8 post-treatment) thus resulting in the premature luteolysis of the early-cycle CL.

The inclusion of control groups provided data to identify different response patterns following treatment with various doses of CLO. Through multiple comparisons it was possible to identify the presence, or the lack of, statistical differences between treatments and controls. However, that may not be the case in other experimental settings. Data described in Table 2.1 may provide guidelines for future applications of ultrasonography as a tool to assess luteolysis in contrast with normal CL development. We advocate that these parameters may vary when using different ultrasound machines and methodologies.

Several studies have demonstrated the relationship between LBF and circulating P_4 levels (Niswender et al., 1976; Janson et al., 1981; Reynolds et al., 2000; Acosta et al., 2002, 2003). Non-steroidogenic and steroidogenic cellular differentiation and migration takes place during the first d of luteal development (Alila and Hansel, 1984; Reynolds et al., 2000). Pericytes and endothelial cells establish a vast vascular bedframe within the CL in response to angiogenic and vasoactive factors (Reynolds et al., 2000; Miyamoto et al., 2009; Berisha et al., 2016). Each luteal cell is in contact with one or more capillaries (Wiltbank et al., 1988). During luteolysis there is a drastic decrease in ovarian blood inflow followed by decreasing levels of P_4 (Niswender et al., 1976). Blood flow within the CL decreases along with serum P_4 levels following $PGF_{2\alpha}$ administration (Miyamoto et al., 2005; Lüttgenau et al., 2011). We acknowledge that decreased or intermediate LBF may also be associated with CL regression. However, the absence of LBF was a clearer

marker of luteal regression in PC heifers. The time to complete disappearance of LBF laid in between 2 to 4 d after the first CLO administration.

In contrast, determining luteal regression based on LV alone could be misleading. It is well established that increase in LV is accompanied by increasing levels of P_4 (Acosta et al., 2002; Sartori et al., 2004). That is particularly evident from early to mid-cycle. The mid-cycle CL is characterized for its stable and compact luteal tissue (Farin et al., 1986). This period of the estrous cycle coincides with the highest production of P_4 (Sartori et al., 2004). Yet, a continuous decrease in mid-cycle LV was observed in untreated NC heifers. A decrease in LV was reported somewhere else, starting at d 12 of the estrous cycle (Pate et al., 2012). A lack of dose response in CLO treated heifers is another downfall of utilizing LV as a luteolysis indicator. Average mid-cycle LV decreased in comparable rates between CLO treated groups, regardless of dose.

This trial was our laboratory's effort to define guidelines on assessing luteal function via ultrasonography. Based on control groups outcomes substantial information was gathered on both development and regression of CL. Treatment with various doses of CLO successfully induced response patterns that may occur in lactating dairy cows during TAI programs.

In summary, luteal function measured with color Doppler allowed for identification of dose-response patterns during CLO induced luteolysis. Treatment with ¼, ½, and a full dose of CLO induced an acute increase in LBF measured 1 h after treatment. However, the increase in LBF did not necessarily result in complete mid-cycle CL regression. The presence of a regressing mid-cycle CL induced premature luteolysis of early-cycle CL from d 10 to 12 of the estrous cycle. Multiple doses of CLO resulted in LBF disappearance between d 2 and 4 after the first injection. Further studies are warranted to investigate the timing of LBF disappearance following final $PGF_{2\alpha}$ of Ovsynch and its effects on pregnancy rates per artificial insemination (PR/AI).

CHAPTER 3

REVIEW: CONTROLLING HORMONAL INTERACTIONS TO ENHANCE FERTILITY OF LACTATING DAIRY COWS

INTRODUCTION

Early studies that identified the uterus as the origin of luteolytic stimuli in cattle were key in the development of pharmacological products to control CL functionality. Luteal lifespan was prolonged in cows that had undergone complete hysterectomy (Wiltbank and Casida, 1956; Copelin et al., 1987) leading to the understanding that the uterus was involved in controlling the lifespan of the CL. Unilateral hysterectomy decreased probability of regression of contralateral CL (Moor and Rowson, 1966) indicating that the horn ipsilateral to the CL was where the luteolytic stimuli was being produced. Purified suspensions of cow's endometrium were able to decrease CL volume and circulating P_4 in hysterectomized hamsters (Lukaszewaska and Hansel, 1970). However, this last study failed to isolate the endometrium luteolysin nor could describe how it reached the CL. Later, many studies identified and described $PGF_{2\alpha}$ as a main inducer of luteolysis in cattle (Nancarrow et al., 1973; Peterson et al., 1975; Shemesh and Hansel, 1975; Kindahl et al., 1976). Around the same time it was elucidated that $PGF_{2\alpha}$ was secreted at the ipsilateral horn to the CL and reached the ovary via a countercurrent process (Ginther and Del Campo, 1973).

Release of endogenous $PGF_{2\alpha}$ depends on P_4 and E_2 hormonal interactions and their feedback on endometrium OXTR expression, as discussed in Chapter 1. Interestingly, E_2 and P_4's role in either stimulating or inhibiting OXTR seems to depend on their simultaneous exposure. Estrogen upregulates endometrium OXTR expression and consequently increases $PGF_{2\alpha}$ release (McCracken, 1980). However, the level of effectiveness of this mechanism seems to depend on preceding endometrium exposure to P_4. Continuous infusion of P_4 (500 µg/h) blocked the effects of continuously infusing E_2 (0.5 µg/h) on oxytocin-mediated release of $PGF_{2\alpha}$ at d 2 and 6 of infusion. Following 10 d of infusion the inhibitory action of P_4 ceased and E_2 positive feedback on oxytocin-mediated release of $PGF_{2\alpha}$ was restored (McCracken, 1980). Moreover, the effect of

oxytocin was 100-fold greater after 10 d of P_4 infusion in comparison to absence of P_4 exposure (McCracken, 1980). Another study reported increased expression of E_2 and oxytocin receptors following termination of a 5-d infusion with P_4 (500 µg/h; Leavitt et al., 1985). More recently, Shimizu et al. (2010) proposed mechanisms in which P_4 and E_2 interact to induce changes in endometrium transcriptome. Based on cluster analysis they identify different patterns of gene upregulation/downregulation of endometrium exposed to either E_2 or P_4, or a combination of E_2 and P_4. Estrogen response was amplified in some clusters when P_4 was administered in combination with E_2. Other genes required P_4 priming to respond to E_2. Clusters of genes that responded to P_4 alone were unchanged with latter E_2 exposure. Finally, E_2 exposure countered P_4 response within some of the clusters. This data endorses the importance of steroid hormones interactions and their interdependence during the estrous cycle.

Manipulation of estrous cycle via endocrine treatments can increase fertility in dairy cows (Bisinotto and Santos, 2012). Taking advantage of hormonal interactions that control events during estrous cycle allows for fine control of luteal and follicular development. Currently, estrous cycle manipulation in the USA relies in induced luteolysis and via ovulations commercially available GnRH and $PGF_{2\alpha}$, respectively. This chapter discusses current strategies of estrous cycle manipulation that rely on controlling time of luteolysis, ovulation and artificial insemination (AI). It also describes underlying physiology of such strategies and their relationship with fertility in lactating dairy cows.

HISTORY OF EXOGENOUS $PGF_{2\alpha}$ USAGE IN ESTROUS CYCLE MANIPULATION

The death of the CL is a programmed event that occurs around d 18 of the estrous cycle in cycling cows. Characterization of luteolysis key factors allowed for external control of CL lifespan. A programmed event then became passive of alterations via exogenous treatments.

Use of $PGF_{2\alpha}$ pharmaceuticals was first regulated by FDA in 1979 for dinoprost tromethamine (DIN; 25 µg dose) and later in 1983 for cloprostenol sodium (CLO; 500 µg dose). Dinoprost tromethamine is a biological analogue, whereas CLO is a synthetic analogue of $PGF_{2\alpha}$. Dinoprost tromethamine is metabolized at very similar rates in comparison to endogenous released $PGF_{2\alpha}$. One dose of DIN is almost completely metabolized during its first passage through the lungs (Mccracken et al., 1999). Thus, DIN has a short half-life of ~8 minutes (Kindahl et al., 1976). Cloprostenol sodium is a more stable molecule with greater resistance to lung metabolization (Bourne et al., 1980). Comparatively, CLO has a longer half-life of ~3 h (Reeves, 1978).

Induced luteolysis with DIN or CLO did not alter the proportion of cows with $P_4 < 0.5$ ng/mL measured daily during a 4-d period. In the same study there was only a trend for greater PR/AI in cows treated with CLO in comparison to DIN (Martins et al., 2011b). However, complementary data demonstrated that CLO induced a more rapid decrease of P_4 levels in the first 12 h post-treatment (Martins et al., 2011a). Faster decrease in P_4 following CLO yielded greater circulating levels of E_2 48 h after treatment. A more abrupt decrease in P_4 results in increased LH pulsatility and greater E_2 levels (Bergfeld et al., 1996).

In fact, the use of exogenous $PGF_{2\alpha}$ alone was one of the first strategies that attempted to manipulate estrous cycle in cattle. Treatment with $PGF_{2\alpha}$ intends to cause CL regression and concentrate time of estrus in treated cows. Yet, variation in d to standing estrus based on random $PGF_{2\alpha}$ treatment is considerably high. Cows show signs of estrus anytime from 1 up to 5 d, with a greater concentration of estrus at d 3 and 4 post-$PGF_{2\alpha}$ (Pursley et al., 2012). Estrus expression following $PGF_{2\alpha}$ depended on d of cycle treatment was given. Cows were detected in estrus following treatment with $PGF_{2\alpha}$ after, but not before d 5 of the estrous cycle (Louis et al., 1972).

Artificial insemination following $PGF_{2\alpha}$-induced estrus resulted in similar fertility in comparison to AI after natural estrus detection. Estrus detection following $PGF_{2\alpha}$ improved 21-d service rates and reduced d to conception in comparison to estrus detection alone (Stevenson and Pursley, 1994). A large field study utilized either CLO or DIN to induce luteolysis and bred cows after estrus detection. Treatment with CLO tended to increase estrus detection rates. Yet, within 1st parity cows CLO significantly enhanced estrus detection rates, and consequently service rate. Cows that received CLO to induce estrus had greater PR/AI and pregnancy rate. The increase in pregnancy rate was restricted to 1st parity cows that received CLO, once service rate was enhanced only for those cows (Pursley et al., 2012).

Similar fertility and greater service rates are two reasons why this strategy is still utilized in dairy operations. However, its main pitfall is that randomly inducing luteolysis does not concentrate estrus in a predetermined d. Therefore, treatment with $PGF_{2\alpha}$ alone still relies on intensive estrus detection to identify eligible cows for AI. Larson and Ball said in 1992 “a major problem is lack of a consistent, precise time of estrus in relation to the appropriate $PGF_{2\alpha}$ injection, rather than an inherent infertility”. The end goal of estrous cycle manipulation was to control time of ovulation and predetermine time of AI.

The leading step to upgrade estrous synchronization strategies came along with the advent of ultrasonography (Fricke, 2002). Until early 1980’s study of ovarian structures was restricted to terminal studies or rectal palpation. Collection of accurate follow-up and real-time data was very limited in those given circumstances. Some of the first published reports of using ultrasound to assess ovarian structures was on horses (Ginther and Pierson, 1984). Later in that year the same authors reported detailed description of ovarian structures assessed with ultrasound in cattle (Pierson and Ginther, 1984). Having available a non-invasive, real-time technique that could

describe ovarian dynamics throughout the estrous cycle was revolutionary. Therefore, ultrasonography mediated the development of new strategies to synchronize estrous cycle in cows.

SYNCHRONIZATION OF OVULATION – OVSYNCH

Ovsynch (Figure 3.1) was the pioneer strategy on combining induced ovulations and timely induced luteolysis (Pursley et al., 1995). Moreover, it overcame the biggest pitfall of simply inducing CL regression with $PGF_{2\alpha}$: estrus detection was not necessary during Ovsynch. Ovsynch was designed to exogenously induce an ovulation with GnRH in a fixed time following $PGF_{2\alpha}$. Controlling time of ovulation also meant predetermining AI time. That was the debut of TAI terminology. Ovsynch was a "natural consequence of a great deal of previous research on follicular waves, use of GnRH, and use of $PGF_{2\alpha}$ in cattle", defined by the scientists that developed it (Wiltbank and Pursley, 2014).

The underlying physiology of Ovsynch is theoretically simple. It starts with a GnRH treatment that induces an LH surge, ovulation, formation of a new CL and emergence of a new follicular wave (Figure 3.1). Seven d later, in the presence of a responsive CL, $PGF_{2\alpha}$ is given to induce luteolysis and decrease P_4 levels. Forty-eight h later another GnRH treatment is given to induce an LH surge and ovulation of the dominant follicle prior to TAI (Pursley et al., 1995). Extending the period from $PGF_{2\alpha}$ to second GnRH from 48 to 56 h increased ovulation rates. This modification was implemented into the original protocol (Brusveen et al., 2008). Follow-up research determined that TAI should be performed 16 h following GnRH (Pursley et al., 1998). TAI 16 h after the last GnRH of Ovsynch is the current industry standard.

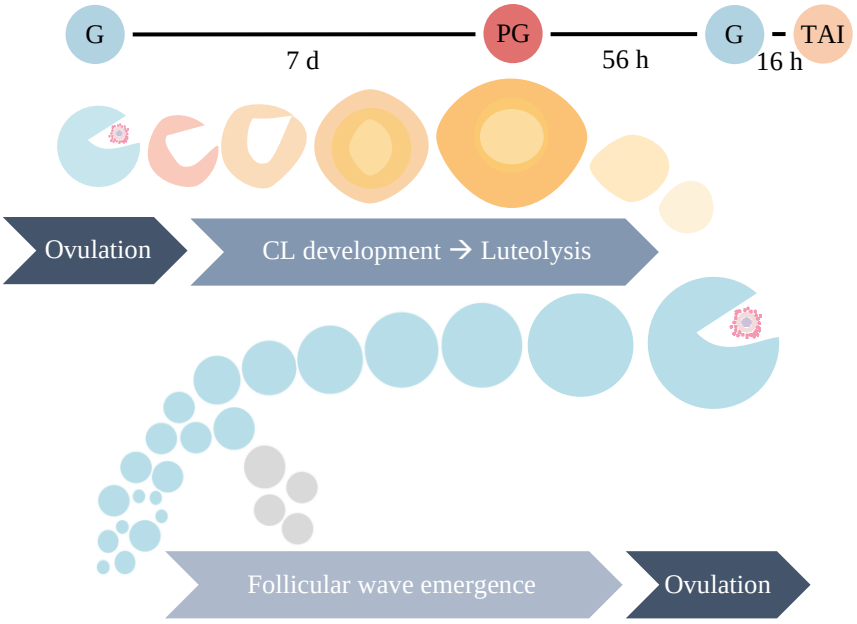

Figure 3.1. Description of synchronization of ovulation (Ovsynch) in dairy cattle including how GnRH (G in blue) causes ovulation to form a new CL, when luteolysis occurs, and how a final GnRH causes ovulation. A new follicular wave emerges following every ovulation.

Cows inseminated after Ovsynch had similar PR/AI compared to cows inseminated following estrus detection (Pursley et al., 1997b). Ovsynch did not improve reproduction performance in terms of PR/AI, but rather increased service rates (Pursley et al., 1997a). In an Ovsynch program 100% of open cows receive AI, whereas estrus detection rates are limited to ~50% (Washburn et al., 2002; Lopez et al., 2004).

Yet, the proportion of cows that respond to each injection of the Ovsynch (synchronization rate) is not as perfect as its theory. The "randomness factor" of Ovsynch plays against its synchronization success. Ovulation following the first GnRH injection may or may not occur depending on the d of the cycle and the presence of a responsive dominant follicle. Vasconcelos et al. (1999) tested initiating Ovsynch in different periods of the estrous cycle. Cows received the first GnRH injection either 1 – 4, 5 – 9, 10 – 16, or 17 – 21 d of estrous cycle. GnRH treatment at 5 – 9 d of the estrous cycle yielded the greatest ovulation rate (96%) whereas, starting Ovsynch at

1 – 4 d of the estrous cycle resulted in the lowest ovulation rates (23%). Bello et al. (2006) tested initiating Ovsynch on d 4, 5, and 6 of the estrous cycle. GnRH treatment on d 6 resulted in greater ovulation rates in comparison to starting Ovsynch on a random d of the cycle (85 vs. 54%, respectively). Initiating Ovsynch on d 6 also increased the proportion of synchronized cows in comparison to a random Ovsynch (92 vs. 69%, respectively).

Perhaps the key to synchronization success during Ovsynch is ovulation to the first GnRH. Starting the protocol when ovulation is more likely to occur eliminates Ovsynch random factor and improves fertility of dairy cows (Moreira et al., 2000; Wiltbank and Pursley, 2014). Just as Ovsynch was described as a natural outcome of previous research, Ovsynch itself became the framework to develop future synchronization programs. This program opened a path to achieve better understanding of estrous manipulation, physiology, and fertility of dairy cows (Thatcher, 2017). Follow-up research focused on optimizing ovulation to GnRH treatments and warranting complete luteal regression prior to TAI.

THE NEXT LEVEL OF OVSYNCH: PRE-SYNCHRONIZATION PROGRAMS

A solution to maximize synchronization following Ovsynch was to pre-synchronize cows to a certain stage of the estrous cycle. Pre-Ovsynch programs increased the proportion of cows on an ideal d to induce ovulation after the first GnRH (Wiltbank and Pursley, 2014). Presynch-11 and Double-Ovsynch (DO) are typically utilized as pre-synchronization programs and manipulate estrous cycle via distinct mechanisms (Moreira et al., 2001; Galvão et al., 2007; Souza et al., 2008).

Pre-synchronization with DO consists of two Ovsynch programs 7 d apart (Souza et al., 2008). The second GnRH of the first Ovsynch induces ovulation 7 d prior starting the breeding portion Ovsynch. In that manner, cows are at d 7 of the estrous cycle when the first GnRH of Ovsynch is administered. The study that developed this program compared DO with Presynch-11.

Ovulation to the first GnRH of Ovsynch did not differ between the two pre-synchronization protocols (71.8 vs 66.7%, respectively). However, treatment with DO impacted circulating P_4 levels during the program. DO decreased the proportion of cows with low P_4 at the first GnRH of Ovsynch and increased the proportion of cows with high P_4 at final $PGF_{2\alpha}$. Moreover, cows synchronized with DO achieved greater PR/AI in comparison with Presynch-11 (Souza et al., 2008). The authors attributed such improve in fertility to resolution of anovular conditions via GnRH administration during DO. Causing ovulations with exogenously induced LH surge is one approach to induce cyclicity in anestrus cows (Wiltbank et al., 2002). Inducing cyclicity prior to 1st service improved fertility (Herlihy et al., 2012).

Pre-Ovsynch programs are commonly utilized to maximize PR/AI at 1st service (Caraviello et al., 2006; Wiltbank and Pursley, 2014). They are long protocols that start early post-partum to guarantee 1st service conception at a target range of days in milk (DIM), usually ~75 to 81. Utilizing such long protocols for 2nd+ services would increase d to conception and calving intervals. This scenario is undesirable in dairy operations from a profitability standpoint and subsequent fertility (Plaizier et al., 1997; Groenendaal et al., 2004; Middleton et al., 2019). Therefore, resynchronization programs are often shorter and scheduled along with pregnancy diagnosis (Fricke, 2002; Fricke et al., 2016). Ovsynch is commonly used as a resynch strategy due to its short duration. GGPG is also an option to resynchronize cows. GGPG is an Ovsynch preceded by an extra GnRH 7 d prior to Ovsynch initiation. Treatment with GnRH prior to the first GnRH of Ovsynch improved synchronization rates and PR/AI in comparison to a random Ovsynch (Lopes et al., 2013).

The pre-synchronization portion of fertility programs results in a main CL that forms prior to Ovsynch initiation. For example, cows synchronized with DO or GGPG are at d 7 of the estrous

cycle at fist GnRH of Ovsynch. That means they have a CL and a dominant follicle at d 7 of development. Consequently, a secondary luteal structure forms, termed accessory CL. Cows that respond to GnRH injections in a fertility program have a d 14 (main CL) and a 7 CL (accessory CL) at the time of $PGF_{2\alpha}$. The combination of a main and an accessory CL results in 50% increase in P_4 levels in comparison to only a main CL (Pursley and Martins, 2012). A new follicular wave emerges following ovulation to the first GnRH of Ovsynch. The selected dominant follicle within that wave develops for 7 d under high P_4 until induced luteolysis.

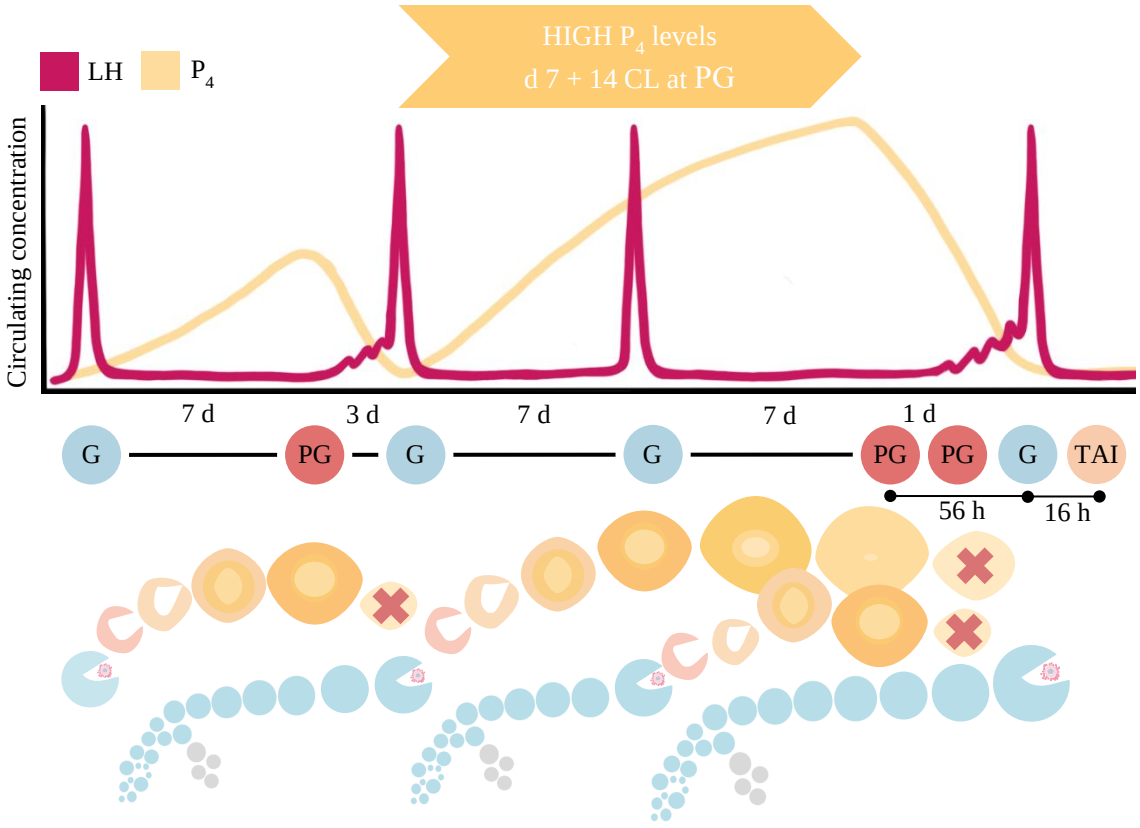

Figure 3.2. Description of ovarian structures dynamics and hormonal concentrations of LH and P_4 in cows that respond to DO program. Combination of GnRH (G in blue) and $PGF_{2\alpha}$ (PG in red) treatments result in a more refined control of ovarian structures and levels of P_4 in comparison to a random Ovsynch (Figure 3.1). GnRH induces ovulation via an exogenous LH surge. Second and third GnRH treated 7 d apart result in the presence of a d 7 and 14 CL at the time of final $PGF_{2\alpha}$. Treatment with $PGF_{2\alpha}$ causes luteolysis and decrease in P_4 levels. As circulating P_4 levels decrease LH pulsatility increases.

Figure 3.2 shows a schematic representation of how follicular and luteal dynamics change in response to GnRH and $PGF_{2\alpha}$ treatments during a DO program. Within cows displaying similar ovarian structures at the final $PGF_{2\alpha}$ 96% had complete regression following treatment. Cows in that stage of the estrous cycle have 92% synchronized ovulations to the last GnRH of Ovsynch (Bello et al., 2006).

Pre-Ovsynch programs are often called fertility programs. They increase PR/AI through different mechanisms. (Bisinotto and Santos, 2012; Pursley and Martins, 2012; Thatcher, 2017). Yet, the concept is similar: creating ovulations, starting a new follicular wave and a new CL.

STRATEGIES TO ENHANCE PR/AI IN LACTATING DAIRY COWS RECEIVING TAI

Perhaps cows that respond to every injection of a pre-synchronization and Ovsynch are programmed to achieve great fertility for two different reasons. 1) Controlled development of preovulatory follicle through high P_4 and decreased dominance phase. 2) Induction of high P_4 levels from the first GnRH of Ovsynch up until $PGF_{2\alpha}$-induced luteolysis.

The negative feedback that high P_4 exerts in the central nervous system blocks ovulation but also modulates follicle growth. This negative feedback becomes more accentuated as P_4 levels increase (Roberson et al., 1989). Manipulating levels of P_4 is a valuable tool to either enhance or withdraw P_4's negative feedback (Cerri et al., 2011). Dominant follicle's growth is stimulated when LH pulses are more frequent (Ginther, 2000; Wiltbank et al., 2011b). Consequently, the resulting larger follicle secretes greater E_2 levels (Butler et al., 2008). Increasing levels of E_2 feeds back into LH release, also increasing its pulsatility (Schams et al., 1977). This scenario may result in overly large follicles. In experimental settings, low P_4 resulted in greater follicle size and increased basal LH concentrations in comparison to cows with a main and accessory CL (Cerri et al., 2011). Preovulatory follicle size has a quadratic effect on PR/AI. Ovulation of overly small (<

~12 mm) or overly large (> ~19 mm) preovulatory follicles resulted in decreased PR/AI. Cows that ovulated follicles around 16 mm have greater PR/AI. Ovulation to the first GnRH of Ovsynch, and creation of an accessory CL also decreased the variability in preovulatory follicle size (Bello et al., 2006). Thus, regulating follicular size via P_4 concentrations seems imperative to enhance fertility.

The final GnRH of Ovsynch induces the LH surge before it occurs naturally in most cows. Time from follicular recruitment until ovulation is shortened during TAI. Restricting the dominance period resulted in greater PR/AI and greater proportion of high-grade embryos (Cerri et al., 2009; Santos et al., 2010). This effect is probably related with controlling the age of the dominant follicle and consequently the oocyte age (Revah and Butler, 1996).

Another aspect of low P_4 concentration during follicular development is the recruitment of two dominant follicles and hence, double ovulations. Cows with high P_4 during pre- and post-dominance phase had fewer double ovulations and ovulated smaller follicles at final GnRH. In fact, creating a low P_4 environment pre- and post- dominance was a great model to induce double ovulations (49%) and large follicles (19.6 mm). Cows that double ovulate had greater PR/AI in comparison to single ovulators. However, twinning increased the chances of pregnancy loss (Martins et al., 2018).

Another important hormonal interaction between P_4 and E_2 levels occurs following $PGF_{2\alpha}$-induced luteolysis. As mentioned previously, a more rapid decrease in P_4 levels following $PGF_{2\alpha}$ resulted in greater E_2 levels. The withdraw of P_4's negative feedback on LH pulsatility is essential for final follicular growth (Mihm et al., 2006). A peak of E_2 precedes and causes the pre-ovulatory LH surge in the absence of P_4's negative feedback (Chenault et al., 1975). There is also evidence that levels of E_2 and estrus expression prior to AI increase fertility and alter endometrium

transcriptome (Martins et al., 2011b; Davoodi et al., 2016; de Sá Filho et al., 2017). Levels of E_2 at the time of final ovulation of Ovsynch was a predictor of PR/AI. Pregnant cows had greater E_2 levels 56 h after induced luteolysis in comparison to non-pregnant cows (Martins et al., 2011b). Exogenous estradiol administration around proestrus upregulated expression of genes involved with uterine tissue proliferation (de Sá Filho et al., 2017). Behavioral estrus in cows is directly correlated with E_2 levels (Perry et al., 2014). Presence of estrus behavior around AI caused changes in gene expression in the endometrium and CL. Moreover, estrus expression around AI yielded longer embryos, which is correlated with greater embryo survival (Davoodi et al., 2016). One way of increasing E_2 levels prior to TAI is to induce complete luteal regression following the $PGF_{2\alpha}$ injection.

Complete luteal regression is one critical event that must occur prior to TAI. The presence of a d 7 and 14 CL increases chances of complete luteal regression in comparison to only having a 7-d CL (Stevenson, 2016). Interestingly, cows with greater P_4 at the time of $PGF_{2\alpha}$ had greater luteolysis rates. Moreover, PR/AI increased in cows with higher P_4 at the time of $PGF_{2\alpha}$ (Bello et al., 2006; Martins et al., 2011b). Yet, increasing P_4 levels via induced ovulations and CL formation does not solve the issue of incomplete luteal regression prior to TAI. During a TAI program around 5 – 20% of cows experience incomplete luteal regression (Moreira et al., 2000; Gümen et al., 2003; Brusveen et al., 2009; Martins et al., 2011b). Cows without complete luteal regression have reduced or even null chances of becoming pregnant following TAI (Souza et al., 2007; Martins et al., 2011b). Even decimal point increases in P_4 concentration lead to drastic decrease in PR/AI.

STRATEGIES TO IMPROVE LUTEOLYSIS RATES

Strategies to overcome incomplete luteal regression during TAI programs were tested in experimental and field studies. Addition of a second $PGF_{2\alpha}$ 24 h apart from the first $PGF_{2\alpha}$

treatment was the pioneer strategy to improve luteolysis in a random Ovsynch program. The addition of a second $PGF_{2\alpha}$ successfully increased complete luteolysis in ~11 percentage points in comparison to a single treatment. This study failed to detect increases in PR/AI (Brusveen et al., 2009). A follow-up field study tested this hypothesis in multiple herds. Treatment with 2 doses of $PGF_{2\alpha}$ 24 h apart increased PR/AI in 13% during an Ovsynch program. Treatment with two doses of $PGF_{2\alpha}$ had a linear effect on complete luteolysis and PR/AI as P_4 levels increased at treatment in cows synchronized with DO (Wiltbank et al., 2015). A recently published metanalysis demonstrated a positive effect of adding a second $PGF_{2\alpha}$ in both luteolysis rates and PR/AI (Borchardt et al., 2018).

The next question was if an increased dose instead of increased frequency of $PGF_{2\alpha}$ would result in similar/not inferior luteolysis rates and fertility. Adding injections to a program may decrease its compliance (Stevenson, 2012). Therefore, an increased dose of $PGF_{2\alpha}$ in a single injection would be more practical and efficient than two separate injections. Giordano et al. (2013) increased 50% of CLO dose (0.75 mg vs. 0.5 mg) in a DO program. Treatment with an increased dose of CLO enhanced luteolytic properties and increased PR/AI in multiparous cows. Another study attempted to double the dose of DIN during an Ovsynch program (Barletta et al., 2018). Controversially, doubling the dose of DIN had no effect on luteal regression nor PR/AI. It is possible that the lack of a dose-response was due to DIN short half-life. Even a double dose of DIN would increase its action duration by one half-life (another ~8 minutes). A question that was often asked in both academia and industry was if double dose of CLO could lead to similar/not inferior luteolysis rates and fertility in comparison to two doses of CLO 24 h apart. Cloprostenol sodium has a longer half-life than DIN and therefore may enhance luteolysis when administered in higher doses. Chapter 4 describes a complete randomized study where cows were treated one

full dose, two full doses 24 apart and one double dose of CLO during TAI programs. The effects of CLO dose strategy were assessed on PR/AI and luteal function assessed with ultrasonography.

CHAPTER 4

THE EFFECT OF A DOUBLE DOSE OF CLOPROSTENOL SODIUM ON LUTEAL BLOOD FLOW AND PREGNANCY RATES PER AI IN LACTATING DAIRY COWS

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INTRODUCTION

Insufficient luteolysis with a single dose of $PGF_{2\alpha}$ during Ovsynch limits the potential of fertility programs. Inadequate luteolysis is likely due to the d 7 accessory CL created in these programs to enhance circulating P_4 (Stevenson, 2016). The CL acquires luteolytic capacity around d 6 of the estrous cycle (Nascimento et al., 2014). Many of these young CL created during TAI programs may be too immature to completely regress to a single dose of $PGF_{2\alpha}$. The luteolytic process can be accelerated by administering multiple $PGF_{2\alpha}$ injections (Brusveen et al., 2009; Ginther et al., 2009; Giordano et al., 2013). Combined data from recent studies indicate that insufficient luteal regression during Ovsynch programs is associated with very low PR/AI (Borchardt et al., 2018). Failure of luteolysis occurs in at least 20% of multiparous cows treated with one dose of $PGF_{2\alpha}$ (Martins et al., 2011b). Thus, amplification of the $PGF_{2\alpha}$-induced luteolysis in Ovsynch programs appears critical to improve fertility in cows subjected to fertility programs.

There are two $PGF_{2\alpha}$ products approved for use in the U.S. for fertility programs, CLO, and DIN. Cloprostenol sodium has a half-life of approximately 3 h compared to 8 minutes for DIN (Kindahl et al., 1976; Reeves, 1978). Dinoprost is more susceptible to lung metabolization whereas CLO, due to its molecular structure, is more resistant to metabolization (Bourne et al., 1980). Cloprostenol sodium may be more advantageous to increase dose due to potential increased time in circulation that can be attained. A single treatment of an increased dose of DIN did not increase luteolysis rates or fertility during resynchronization with Ovsynch (Barletta et al., 2018). Treatment with double dose of DIN increases its action period by one half-life, or only by ~8 minutes. Increasing the dose of CLO from 0.5 to 0.75 mg, enhanced luteolytic properties and increased P/AI in multiparous cows (Giordano et al., 2013). Double dosing CLO will allow for 0.25 mg to still be in circulation 6 h after treatment. Increasing the time of CL exposure to CLO

may result in enhanced luteolytic properties. Cloprostenol sodium enhanced rate of P_4 decrease following a single dose treatment in lactating dairy cows (Martins et al., 2011a).

Two doses of $PGF_{2\alpha}$ 24 h apart increased the percent of cows with complete luteolysis prior TAI in comparison to a single dose of $PGF_{2\alpha}$ (Brusveen et al., 2009). This second dose of $PGF_{2\alpha}$ was broadly implemented in the dairy industry. However, increasing the number of management actions in fertility programs increases labor costs and can result in non-compliance issues (Stevenson, 2012).

Ultrasound measurements have been reported as good predictors of luteal function. Determination of LV and LBF had high positive correlations with P_4 levels during the estrous cycle (Acosta et al., 2002; Herzog et al., 2010). Complete and partial luteolysis were identified with both ultrasound and P_4 assays (Ginther et al., 2007, 2009; Trevisol et al., 2015). However, there is no current evidence that LV or LBF around TAI could act as predictors of pregnancy.

The hypothesis of this study was a double dose of CLO (1.0 mg) would not result in different PR/AI compared to two 0.5 mg doses 24 h apart but would have greater PR/AI compared to a single dose (0.5 mg) in a TAI program. This study also hypothesized that increased (1.0 mg) and more frequent (two 0.5 mg 24 h apart) treatment with CLO would result in a greater reduction in LV and LBF in comparison to a single dose of CLO (0.5 mg). An objective was to determine if LBF measurements before and after treatment can be utilized to predict PR/AI and pregnancy loss.

MATERIALS AND METHODS

Experimental Units

This project was conducted from October 2018 to June 2019 on a commercial dairy herd, (Nobis Dairy Farm, St. Johns, Michigan, USA). During the study period n = 1,051 cows were available for enrollment in the trial. Cows with health problems were not enrolled (n = 62). Thus,

n = 989 cows were enrolled in the study and assigned to a treatment. After enrollment and treatment, n = 25 were removed from the study. These cows did not receive AI in compliance with the protocol or were removed from the herd prior to pregnancy diagnosis. Outcomes from treatments were collected from n = 964 lactating Holstein cows. They were fed a TMR once a day with free access to feed and water and were confined in a free-stall barn. The TMR consisted of corn, wheat, and alfalfa silages, and corn-soybean meal-based concentrates formulated to meet nutrient recommendations for high producing lactating dairy cows (NRC, 2001). The Institutional Animal Care and Use Committee at Michigan State University approved all animal handling and procedures.

Treatments

Cows ranging from 1st to 7th parity and receiving 1st, 2nd or 3rd service were included in the study (Figure 4.1). Treatments were randomly assigned within 1st (n = 330), 2nd (n = 312) and 3rd+ (n = 322) parities, TAI program (DO for 1st AI; n = 554) or (GGPG for 2nd and 3rd AI; n = 410), and synchronization status (ovulation to both G2 and G3). At d -1 cows were allocated to receive one of the three treatments at d 0: 0.5 mg of CLO (FULL; estroPLAN, Parnell, Overland Park, Kansas, USA; n = 343), the 1st of two 0.5 mg of CLO 24 h apart (TWO/24; n = 316), or 1.0 mg CLO (DOUBLE; n = 305). Cloprostenol and GnRH (GONAbreed, Parnell, Overland Park, Kansas, USA) were administered intramuscularly in either the semitendinosus or semimembranosus muscles. Ovulation to the 2nd and 3rd GnRH injections of Double Ovsynch (G2 and G3) were verified on all cows using ultrasound (MyLab DeltaVET, 10-5 MHz linear array probe, Esaote, Genoa, Italy). Cows were classified into "synchronization" categories (Figure 1) based on ovulation outcomes in the 2nd Ovsynch of Double Ovsynch. Cows that ovulated to both G2 and G3 of Double Ovsynch (n = 689) were considered "synchronized" and cows that ovulated

to only one, or none, of these GnRH injections were considered “non-synchronized” (n = 275). Cows that ovulated to G2 and G3 had at least one d 7 and one d 14 CL on d of treatment (d 0). Cows that ovulated to only G2 or G3 had either a d 14 or 7 CL.

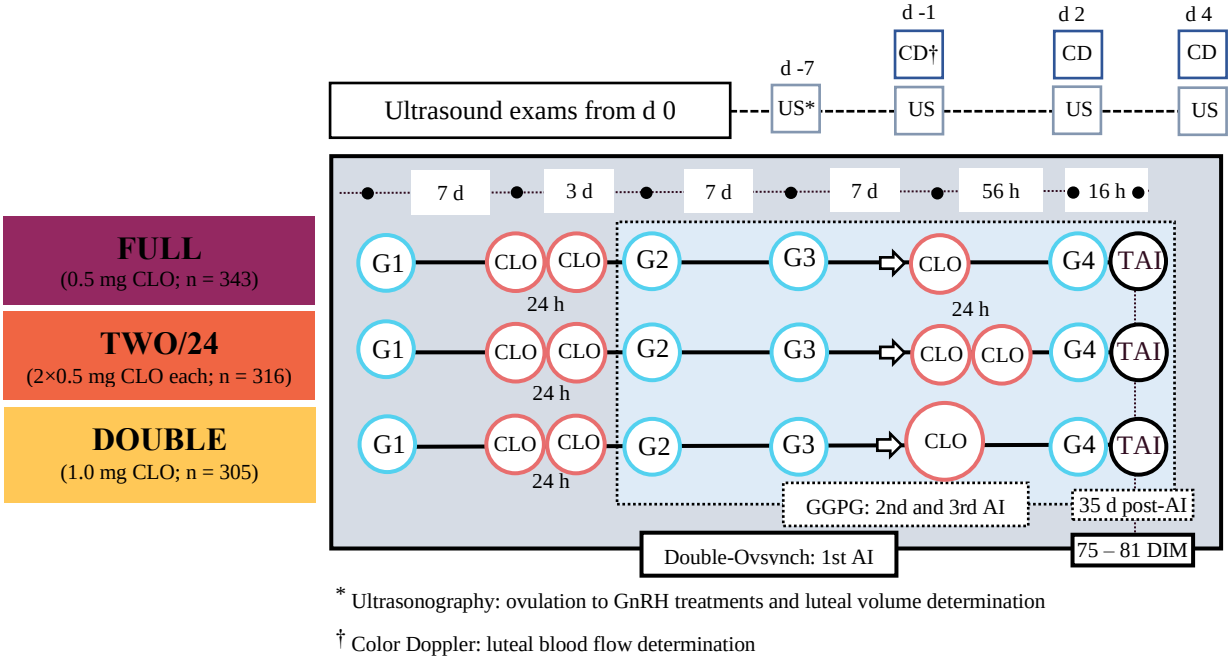

Figure 4.1. Schematic diagram of administered treatments, TAI programs schedule, and data collection. Lactating dairy cows received 1st AI from 75 – 81 DIM following Double-Ovsynch program. Non-pregnant cows were resynchronized with GGPG program and received 2nd and 3rd AI 35 d after the previous AI. At d 0 cows were randomly assigned to receive one out of three treatments at d 0: FULL (0.5 mg of CLO, n = 343), TWO/24 (2 doses of 0.5 mg of CLO 24 h apart, n = 316) or DOUBLE (1.0 mg of CLO, n = 305) dose of cloprostenol sodium. Ultrasound exams were performed on d -7, -1, 2 and 4 in relation to d of treatment. Ovulation to GnRH treatments and luteal volume measurement were determined on those days. Color Doppler assessment was utilized to determine LBF dynamics before (d -1), and 2 and 4 d after CLO treatment.

Pregnancy Diagnoses

Pregnancy was diagnosed on d 24 (Middleton and Pursley, 2019). Blood samples were collected on d 17 and 24 post-AI from the coccygeal vein or artery with serum separation tubes (Venous Blood Collection Tubes: SST, BD Vacutainer, Franklin Lakes, NJ). All samples were stored at 4°C until processing at d 25 post-AI. Samples were centrifuged at 3,000 RPM for 20 minutes to allow serum separation. Weekly ELISA assays (bioPRYN, BioTracking, Moscow,

Idaho, USA) were utilized to measure increase of pregnancy specific protein B (PSPB) optical density (OD) levels from d 17 to 24 post-AI. Intra- and inter-assay CV was 4.9 and 5.8 %.

Pregnant cows at d 24 were rechecked by the herd veterinarian on d 34 and 62 post-AI. Pregnancy was diagnosed via transrectal ultrasonography each time. Pregnancy loss was categorized in the following periods: between 24 to 34, 34 to 62, and 62 to 184 d post-AI. Total pregnancy loss included cows that experienced pregnancy loss at any time following the d 24 diagnosis.

Ultrasonography – ovulation, follicle size and luteal volume determination

Linear array ultrasonography was used to map ovaries and describe ovarian dynamics. Each ovary was evaluated three times per session. Ovarian maps were drawn on paper for each session. An electronic video file of each ovarian scan was also saved. Videos were utilized for retrospective consulting and a more reliable evaluation of ovarian changes throughout the pre-treatment and post-treatment periods. The frame-by-frame feature allowed to determine the greatest diameter of any given structure. All measurements were determined using built-in calipers. The first diameter measurement for each structure was horizontal and the second was perpendicular to the 1st measurement.

Ovulation to G2, G3 and G4 was confirmed at d -7, -1 and 4, respectively. Ovulation to G2 was defined as the presence of at least one functional d 7 CL and a DF $\geq$ 8 mm average diameter on d -7. Corpora lutea present on d -7 were re-examined on d -1. Ovulation to G3 was confirmed on d -1 as the presence of a newly formed CL at same ovary and position as the DF measured on d -7. Cows that ovulated to G2 and G3 had at one d 7 and one d 14 CL at treatment with CLO (d 0).

Assessment of follicle size was performed at time of G4. The ovulatory follicle size was calculated as the average of the horizontal diameter and the perpendicular diameter. When more than one follicle ovulated following G4 final follicle size was calculated as the average of both follicles.

A large subset of cows that ovulated to both G2 and G3 were utilized to assess the effects of treatment on luteal volume and blood flow before and after treatment (n = 607). Luteal volume was assessed on all d 7 and 14 CL before treatment (d -1), 2 and 4 after treatment. LV was calculated in mm^3 using the formula $V = (4/3)\ \pi\ R^3$. Radius (R) was calculated using the formula $R = (D^a /2 + D^b /2)/2$. D^a was the horizontal diameter of the CL and D^b was the perpendicular diameter to D^a. CL with a fluid cavity had the cavity volume subtracted from total LV. Cavity volume was calculated with the same formula used for LV. Individual LV was calculated separately for d 7 and d 14 CL. Final d 14 LV was calculated as the average of all CL formed after G2. Final d 7 LV was calculated as the average of all CL formed after G3.

Ultrasonography – luteal blood flow determination

Luteal blood flow from d 7 and 14 CL was assessed before (d -1), and 2 and 4 d after, treatment. Each CL was evaluated utilizing color Doppler. Images showing the greatest LBF concomitantly with the greatest CL diameter and with the least artifacts were used to select the colored pixel area. Luteal blood flow area was determined as colored pixel area. An image editing software (Adobe Photoshop CS3, San Jose, California, USA) was used to select the desired area with a color range selection tool. The method consisted of using an eyedropper tool to select and delete only black, white, and gray tone pixels. The remaining colored pixel area was selected with the same tool. Pixel count was determined by the software output. Luteal blood flow was calculated as the average of 2 pictures from the same CL at each assessment. Luteal blood flow was calculated

for d 7 and d 14 CL individually. Luteal blood flow of cows with more than two d 7 or 14 CL at time of treatment were averaged and the average was reported as one d 7 or 14 CL.

Statistical Analyses

All statistical analyses were performed using SAS (version 9.4, SAS Institute Inc., Cary, North Carolina, USA). Luteal volume and LBF measurements are reported as mean ± SEM. Luteal volume and LBF data are reported in two different ways: individual and combined. Individual refers to the individual measurement of LV or LBF of either the d 7 or 14 CL. Combined refers to the sum of LV or LBF in d 7 and 14 CL.

Binomial variables (PR/AI and pregnancy loss) were analyzed with a generalized linear mixed model fitted with PROC GLIMMIX. Differences in proportions were determined using the chi square test of independence. Pregnancy rates per AI were calculated at d 24, 34 and 62 post-AI in three groups of cows. Analyses were performed in all cows to calculate overall PR/AI. Pregnancy rates per AI were also analyzed in cows that ovulated to both G2 and G3 (synchronized cows) and cows that ovulated to either G2 or G3 (non-synchronized cows). Dose of CLO, parity, TAI program and the interaction of TAI program and parity were included in all models. The model utilized to test differences in synchronized cows PR/AI at d 34 post-AI also included the interaction of CLO treatment and TAI program. Interactions were only included when Type III fixed effects *P*-values were < 0.2. Weekly cohorts, cow, AI technician and sire were considered for inclusion as random effects. Random effects integrated the model when covariance parameter estimate was greater than 0. The LSMEANS statement was utilized to slice fixed effects. PROC PLM was used to slice significant interactions.

Differences in proportions were estimated with PROC FREQ. The tables options were utilized to obtain proportions estimates. Mantel-Haenszel Chi-Square was used to test differences in proportions. Descriptive statistics utilized features of PROC FREQ and PROC MEANS.

Combined LV and LBF were analyzed using PROC MIXED at days -1, 2 and 4. The statistical model included the fixed effects of treatment, parity and TAI protocol and the interaction of treatment*parity. The LSMEANS statement was utilized to slice fixed effects. Significant interactions between treatment and parity were sliced with PROC PLM using an output from PROC MIXED. The interactions were sliced between treatments and within parity. Significance level was set at $P < 0.05$ and adjusted with Tukey-Kramer test for multiplicity.

Predicted probabilities of PR/AI and pregnancy loss was calculated in relation to combined LV and combined LBF before treatment (d -1), 2 and 4 d post-treatment. PSPB OD levels were evaluated on d 24 post-AI as a predictor of pregnancy loss. Wald chi-square was utilized to test the maximum likelihood of pregnancy and pregnancy loss using PROC LOGIT.

RESULTS

Main effects of treatment on pregnancy rate per AI and pregnancy losses

Table 4.1 describes the overall effect of a double dose of cloprostenol sodium (1.0 mg) on PR/AI in lactating dairy cows (n = 964) treated for 1st AI with DO or 2nd and 3rd AI using GGPG. There was no evidence of an effect of CLO dose in PR/AI at 24, 34 or 62 d post-AI in 1st or 2nd and 3rd AI.

There was no evidence of an overall effect of treatment on pregnancy losses at 34, 62, and from 62 d post- AI to parturition (Table 4.2). However, 3rd+ parity cows treated with FULL had greater pregnancy loss in comparison with TWO/24 and DOUBLE (Figure 4.2).

	TREATMENT			
Overall PR/AI % (n/n) at:	FULL	TWO/24	DOUBLE	*P*-value
24 d post-AI	54.8 (188/343)	49.7 (157/316)	53.8 (164/305)	0.32
34 d post-AI	47.9 (164/342)	46.5 (147/316)	50.8 (155/305)	0.59
62 d post-AI	44.8 (151/337)	43.37 (134/309)	46.7 (142/304)	0.69

Table 4.1. Effect of treating lactating dairy cows with either with FULL (0.5 mg), TWO/24 (2 injections of 0.5 mg 24 h apart), or DOUBLE (1.0 mg) dose of cloprostenol sodium at final $PGF_{2\alpha}$ of Double Ovsynch for 1st AI (n = 554) or GGPG for 2nd and 3rd AI (n = 410) on overall pregnancy rates per AI at 24, 34, and 62 d post-AI. *P*-value refers to the fixed effect of treatment.

	TREATMENT			
	FULL	TWO/24	DOUBLE	*P*-value
Pregnancy loss 24 to 34 days post-AI % (n/n)	12.3^{a} (23/187)	6.4^{ab} (10/157)	5.5^{b} (9/164)	0.07
Pregnancy loss 34 to 62 days post-AI % (n/n)	5.6^{a} (9/160)	4.9^{a} (7/142)	7.8^{a} (12/153)	0.74
Pregnancy loss 62 days post-AI to parturition % (n/n)	3.4^{a} (5/146)	4.0^{a} (5/126)	2.3^{a} (3/129)	0.56

Table 4.2. Effect of treating lactating dairy cows with either with FULL (0.5 mg), TWO/24 (2 injections of 0.5 mg 24 h apart), or DOUBLE (1.0 mg) of cloprostenol sodium at final $PGF_{2\alpha}$ of Double Ovsynch for 1st AI and GGPG for 2nd and 3rd AI combined (n = 964) on pregnancy losses at 34, and 62 post-AI and 62 d post-AI to parturition. The *P*-value refers to the fixed effect of treatment. Values sharing different letter superscripts differed significantly between treatment and within period of pregnancy loss ($P < 0.05$).

Table 4.3 describes the effect of treatment on PR/AI within each parity group (1st, 2nd, and 3rd+). There was no effect of treatment within each parity. Although, 1st and 2nd parity cows had greater overall PR/AI at each diagnosis compared to 3rd+ parity cows ($P = 0.02$). There were no significant interactions between parity and treatment.

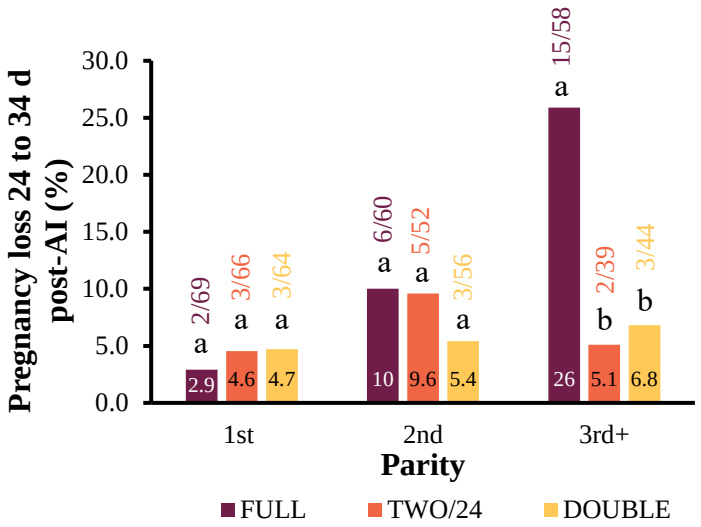

Figure 4.2. Effect of treatment with FULL (0.5 mg), TWO/24 (2 injections of 0.5 mg 24 h apart), or DOUBLE (1.0 mg) dose of cloprostenol sodium at final $PGF_{2\alpha}$ of Double-Ovsynch and GGPG on pregnancy loss from 24 to 34 d post-AI in lactating dairy cows within 1st (n = 199), 2nd (n = 168), and 3rd+ (n = 141) parities (n = 508). Bars sharing different letter superscripts differed significantly within parity ($P < 0.05$).

Table 4.4 describes the effect of treatment in cows that "synchronized" thus having at least one d 7 and one d 14 CL at time of treatment. Cows with 7 and 14 d CL accounted for 71% of all cows in the study. Within non-synchronized cows (did not have both a d 7 and 14 CL at treatment) n = 130 had only d 7 CL, n = 106 had only d 14 CL, and n = 16 cows with neither a 7 or a 14 d CL. Synchronized cows treated with FULL had greater PR/AI in comparison to TWO/24 and DOUBLE 24 d post-AI. At 34 and 62 d post-AI there were no differences in synchronized cows PR/AI regardless of treatment. No effect of treatment was observed in non-synchronized cows at 24, 34 or 62 d post-AI.

Synchronized cows had overall greater PR/AI at 24, 34 and 62 d post-AI in comparison to non-synchronized cows. Ovulation to G2 and G3 increased PR/AI 35% (42 to 57%) at d 24 post-AI. Cows treated with DOUBLE (1.0 mg) had similar PR/AI in both synchronized and non-synchronized groups in contrast to FULL and TWO/24 h treatments that had greater PR/AI in synchronized vs. non-synchronized groups (Table 4.4).

PR/AI % (n/n) at:	FULL	TWO/24	DOUBLE	*P*-value	Overall PR/AI PARITY
	1st PARITY				
24 d post-AI	61.1 (69/113)	61.0 (66/109)	59.3 (64/108)	0.97	60.3
34 d post-AI	59.3 (67/113)	57.8 (63/109)	56.5 (61/108)	0.91	57.9
62 d post-AI	55.5 (61/110)	55.6 (60/108)	52.8 (57/108)	0.90	54.6
	2nd PARITY				
24 d post-AI	56.6 (60/106)	50.5 (52/103)	54.4 (56/103)	0.67	53.9
34 d post-AI	50.9 (54/106)	45.6 (47/103)	51.5 (53/103)	0.66	49.4
62 d post-AI	47.1 (49/104)	43.0 (43/100)	46.1 (47/102)	0.83	45.4
	3rd+ PARITY				
24 d post-AI	47.6 (59/124)	37.5 (39/104)	47.0 (44/94)	0.26	44.1*
34 d post-AI	35.0 (43/123)	35.6 (37/104)	43.6 (41/94)	0.37	37.7*
62 d post-AI	33.3 (41/123)	30.7 (31/101)	40.4 (38/94)	0.34	34.6*

Table 4.3. Effect of treating 1st (n = 330) 2nd (n = 312) or 3rd+ (n = 322) parity lactating dairy cows with either FULL (0.5 mg), TWO/24 (2 injections of 0.5 mg 24 h apart), or DOUBLE (1.0 mg) dose of cloprostenol sodium at final $PGF_{2\alpha}$ of Double Ovsynch for 1st AI and GGPG for 2nd and 3rd AI combined on pregnancy rates per AI at 24, 34, and 62 d post-AI. *P*-value refers to proportion of PR/AI between CLO doses within parity. Overall PR/AI of 3rd+ parity cows marked with * differed from 1st and 2nd parity cows at 24, 34 and 62 d post-AI.

Ovulation to GnRH treatments during DO and GGPG

Cows receiving DO for 1st AI had 87% of cows considered synchronized with at least one d 7 and one d 14 CL at the time of treatment (484/554). Cows resynchronized with GGPG had 50% of cows classified as synchronized at the time of treatment (205/410). Individual ovulation rates were 96 and 66% after G2 and 92 and 81% after G3 for cows that received DO and GGPG, respectively.

PR/AI % (n/n) at:	TREATMENT			*P*-value line	Overall PR/AI
	FULL	TWO/24	DOUBLE		
24 d post-AI					
Synchronized	64.2 (131/204)	52.9 (109/206)	53.8 (106/197)	0.03	57.0
Non-Synchronized	38.6 (39/101)	39.8 (35/88)	48.8 (42/86)	0.39	42.2
P-value column	<0.01	0.03	0.31	-	<0.01
34 d post-AI					
Synchronized	56.2 (114/203)	51.0 (105/206)	50.8 (100/197)	0.16	52.5
Non-Synchronized	34.7 (35/101)	34.1 (30/88)	46.5 (40/86)	0.22	38.2
P-value column	<0.01	0.01	0.37	-	<0.01
62 d post-AI					
Synchronized	52.5 (105/200)	47.3 (96/203)	46.9 (92/196)	0.16	48.7
Non-Synchronized	34.0 (34/100)	30.6 (26/85)	41.9 (36/86)	0.35	35.4
P-value column	0.01	0.01	0.31	-	<0.01

Table 4.4. Effect of treating lactating dairy cows with either FULL (0.5 mg), TWO/24 (2 injections of 0.5 mg 24 h apart), or DOUBLE (1.0 mg) dose of cloprostenol sodium at final $PGF_{2\alpha}$ of Double-Ovsynch for 1st AI and GGPG for 2nd and 3rd AI that were synchronized with at least one d 7 and one d 14 CL at time of treatment, or not synchronized, on pregnancy rates per AI at 24, 34, and 62 d post-AI.

Overall ovulation rate to G4 was 97% (662/680) in synchronized cows. There was no effect of CLO dose on follicle diameter in cows at G4. Average follicle diameter was 15.9 ± 0.12, 16.1 ± 0.13 and 16.1 ± 0.14 mm in FULL, TWO/24 and DOUBLE at time of G4. Double ovulation rate totaled 7.4% (49/662) in all cows that ovulated to G4. There was no relationship between proportion of CLO dose and double ovulation. There was no difference in overall PR/AI in cows with double vs. single ovulations (69% vs. 58%). Yet, cows with double ovulations had greater total pregnancy loss compared to cows with single ovulations (29 vs. 14% respectively; $P = 0.02$).

Effect of treatment on combined LV reduction

There was no effect of treatment on -1 or 2 d after treatment in LV. Yet, cows treated with DOUBLE had significant lower LV in comparison to cows treated with FULL measured at d 4 (Figure 4.3). There were no differences in combined LV amongst 1st and 2nd parity cows at d 2 and 4, regardless of CLO dose. Cows in 3rd+ parity had reduced LV when treated with DOUBLE vs. FULL measured 4 d after treatment ($P < 0.05$).

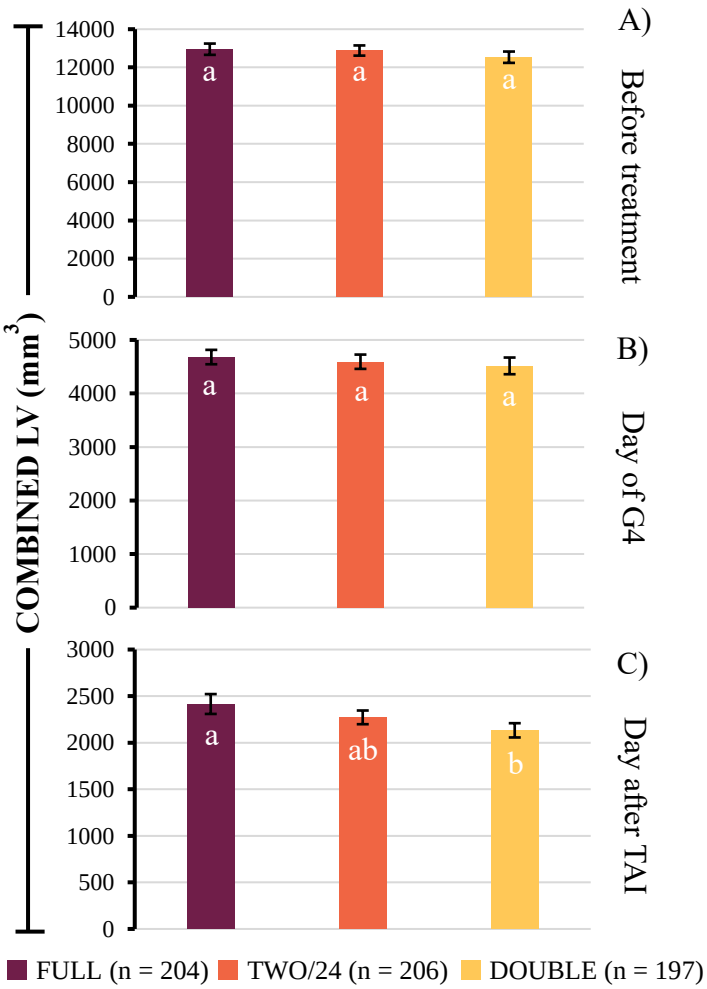

Figure 4.3. Effect of treatment with FULL (0.5 mg), TWO/24 (2 injections of 0.5 mg 24 h apart), or DOUBLE (1.0 mg) dose of cloprostenol sodium at final $PGF_{2\alpha}$ of Double-Ovsynch and GGPG on combined luteal volume (mm^3) of lactating dairy cows with a d 7 and d 14 CL. Bars sharing different letter superscripts differed significantly within day ($P < 0.05$).

Effect of treatment on combined LBF reduction

Figure 4.4 describes the effect of CLO dose on LBF reduction in cows with a d 7 and a d 14 CL combination. No differences in combined LBF were observed between CLO treatments before treatment. Cows at 1st AI had greater LBF before treatment in comparison to resynchronized cows. Cows that received DOUBLE had a greater reduction in LBF 2 d after treatment compared to cows treated with FULL. Only 12.3% (75/607) cows had disappearance of d 7 LBF and 4.3% (26/607) had completely regressed d 14 LBF 4 d after treatment.

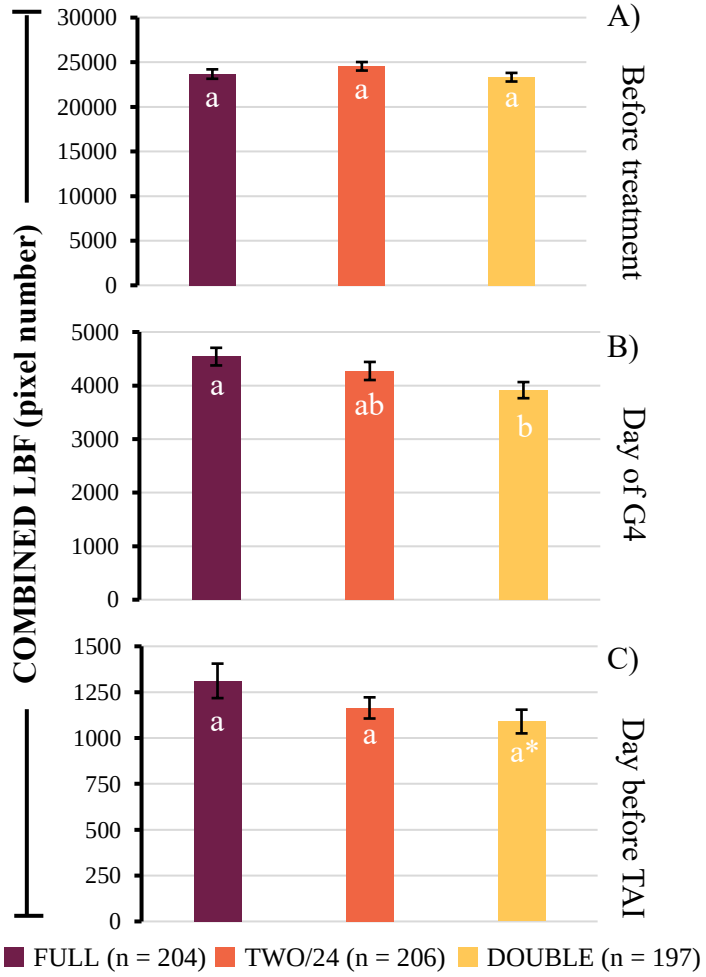

Figure 4.4. Effect of treatment with FULL (0.5 mg), TWO/24 (2 injections of 0.5 mg 24 h apart), or DOUBLE (1.0 mg) dose of cloprostenol sodium at final $PGF_{2\alpha}$ of Double-Ovsynch and GGPG on combined luteal blood flow (pixel number) of lactating dairy cows with a d 7 and d 14 CL at treatment. Bars sharing different letter superscripts differed significantly within day ($P < 0.05$). * Indicates a tendency for a significant difference between DOUBLE and FULL.

Treatment with increased or more frequent doses of CLO did not differ in LBF disappearance in 1st and 2nd parity cows. Yet, 3rd+ parity cows had greater disappearance of LBF when treated with DOUBLE compared to FULL, measured at 2 and 4 d after treatment ($P < 0.05$).

Luteal blood flow disappearance as a predictor of pregnancy

Total LBF amount measured before treatment was not a predictor of pregnancy on d 24, 34 or 62 post-AI. Combined LBF measured 2 d after treatment was a predictor of pregnancy at d 34, but not at 24 and 62 post-AI. The probability of pregnancy diagnosed at d 34 post-AI increased as LBF amount decreased ($P = 0.02$). Average combined LBF measured at d 4 was not a significant predictor of pregnancy on d 24 and 62 post-AI. Yet, Average LBF on d 4 after treatment predicted pregnancy 34 d post-AI. Pregnancies increased as LBF decreased ($P = 0.046$).

We classified cows according to amount of LBF present on d 4 into two groups: LBF above or below the median. The median was calculated separately for d 7 (median = 370 pixels) and d 14 CL (median = 494 pixels). Cows that had at least one CL (d 7 or d 14) with LBF above the median were classified as "above the median". Cows with LBF above the median were considered to fail to completely regress the CL. Cows with LBF of both CL below the median were considered as cows with complete CL regression, due it markedly low LBF. Only 263/607 cows had simultaneous regression of d 7 and d 14 LBF below the median. The proportion of CLO treatment that contributed with simultaneous regression of d 7 and 14 CL was not significantly different ($P = 0.3$). Cows with regression of both d 7 and d 14 CL had greater P/AI at d 24 post-AI in comparison to cows that did not met this criteria (62.7 vs. 52.6; $P = 0.01$). Simultaneous regression of d 7 and d 14 also resulted on greater levels of PSPB measured at d 24 post-AI ($P < 0.01$).

Luteal blood flow amount and PSPB levels as predictors of pregnancy loss

Combined LBF measured on d -1, 2 and 4 did not predict pregnancy loss diagnosed at any time during the study. However, PSPB levels at d 24 post-AI were predictive of early pregnancy loss. Pregnancy losses occurring from d 24 to 34 increased as PSPB levels decreased ($P < 0.01$). Pregnancy losses that occurred later compared to d 34 post-AI could not be predicted by PSPB levels at d 24 post-AI. Cows that maintained pregnancy had greater PSPB levels at d 24 post-AI in comparison to cows with pregnancy loss diagnosed at any time in the study (0.161 ± 0.002 vs. 0.145 ± 0.004, respectively; $P < 0.01$). Levels of PSPB measured at d 24 post-AI did not differed between CLO dose strategy ($P = 0.6$).

DISCUSSION

Outcomes from this study support the use of a double dose of CLO for luteolysis in lactating dairy cows treated with the fertility program DO and GGPG. This is based on three key results: 1) Overall PR/AI was not different in DOUBLE vs. TWO/24, 2) DOUBLE maintained levels of high fertility in both synchronized and non-synchronized cows compared to FULL in which cows had decreased PR/AI in non-synchronized subset, and 3) LV and LBF in 3^{rd}+ parity cows decreased to lower levels compared to FULL suggesting that luteolysis in older cows was enhanced with DOUBLE. Complete luteolysis is clearly a requirement for pregnancy in lactating dairy cows treated with fertility programs (Martins et al., 2011). Prostaglandin-$F_{2\alpha}$ products were primarily tested in cattle that would normally have a single CL (Lauderdale et al., 1974). Most data would suggest that luteolysis in beef cattle and dairy heifers was not problematic (Hittinger et al., 2004; Meneghetti et al., 2009). More recently, studies utilizing fertility programs in lactating dairy cows determined that chances of luteolysis appeared to be less and compromised chances for pregnancy (Brusveen et al., 2009; Martins et al., 2011b). The seminal paper from Brusveen et al.

(2009) determined that two doses $PGF_{2\alpha}$ 24 h apart increased the chances for luteolysis compared to a single dose. Evidence indicates that two doses $PGF_{2\alpha}$ 24 h apart is the prevailing choice for dairy producers and veterinarians utilizing fertility programs. Fertility programs are designed to control the onset of follicular waves via the ovulation of a dominant follicle (Wiltbank and Pursley, 2014). These ovulations result in accessory CL that increase P_4 during growth of the pre-ovulatory follicle and reduce the chances of double ovulations (Brusveen et al., 2009; Pursley and Martins, 2012; Martins et al., 2018). Yet, these accessory CL are generally 6 to 8 d old at time of last $PGF_{2\alpha}$ of Ovsynch (Pursley, et al., 1995) dependent on the type of fertility program that was utilized. It is likely the reason the second dose of $PGF_{2\alpha}$ in the Brusveen et al. (2009) study enhanced luteolysis was due to the effect on the less mature d 7 CL at time of $PGF_{2\alpha}$ in DO. Yet, the current study indicated there was no difference in decreases in LBF or LV in the d 7 CL compared to the d 14 CL.

The FULL dose outcomes in the current study were contrary to several other published reports in which two doses of $PGF_{2\alpha}$ outperformed a single dose in terms of fertility and luteolysis rates (Brusveen et al., 2009; Carvalho et al., 2015; Wiltbank et al., 2015; Heidari et al., 2017).

Pregnancy was first diagnosed 24 d post-AI with a novel method that utilizes within-cow differences in PSPB levels (Middleton and Pursley, 2019). Pregnancy loss from d 24 to 34 post-AI was associated with CLO dose and parity. Third-plus parity cows treated with 0.5 mg of CLO experienced greater pregnancy loss from 24 to 34 d post-AI. In the present study, diagnosing pregnancy prior to the usual ~30 d post-AI pregnancy diagnosis allowed for identification of early pregnancy losses that other studies could not detect. There are three pivotal periods of pregnancy loss in lactating dairy cattle. The first period and second period encompass the majority of pregnancy losses in lactating dairy cattle. Pregnancy loss was estimated at 20 – 50% for the first

period (first week of pregnancy) and 25 – 41% for the second period (between d 8 to 27 post-AI). Losses from d 28 to 60 d post-AI were less frequent, around 12% (Wiltbank et al., 2016). Thus, it is a reasonable assumption that multiparous cows treated with a single $PGF_{2\alpha}$ may have lower PR/AI due to greater early pregnancy losses rather than conception failure. Yet, in the present study multiparous cows (2^{nd} and 3^{rd}+ parity) had similar PR/AI between CLO dose strategy at d 24, 34 and 62 post-AI.

Greater pregnancy loss between d 24 and 34 post-AI in 3^{rd}+ parity cows treated with FULL could not be explained by luteal function following treatment. Amount of LBF present at d 4 was not a predictor of pregnancy loss diagnosed at any time during the study. Yet, 3^{rd}+ parity cows had reduced disappearance of LBF 2 and 4 d after treatment. If true, the connection between this outcome and pregnancy losses is not explainable with other data in the literature. But, a greater proportion of multiparous cows treated with a single dose of $PGF_{2\alpha}$ experienced incomplete luteal regression prior to TAI (Giordano et al., 2013; Wiltbank et al., 2015). A decrease in P_4 prior to TAI is imperative to allow rising E_2 levels and uterine priming (Cerri et al., 2011; Martins et al., 2011a; Northrop et al., 2018). Cows that underwent pregnancy loss between d 24 and 34 had a ≥ 10% increase of PSPB levels from d 17 to 24 post-AI. Presence of PSPB in the mother's circulation is indicative that embryonic attachment occurred (Wallace et al., 2015). We speculate that incomplete luteolysis may turn the uterine environment unsuitable for proper embryonic attachment and maintenance of pregnancy.

Ovulation to GnRH treatments during TAI programs impact overall fertility and luteolysis rates prior to TAI (Bello et al., 2006; Giordano et al., 2013). Based on other studies, ovulation failure to 1^{st} GnRH of Ovsynch (herein referred as G3) results in fewer cows with $P_4 > 1$ ng/mL at $PGF_{2\alpha}$ (Bello et al., 2006). In cows treated with a single dose of $PGF_{2\alpha}$, higher P_4 levels at the time

of treatment increased probability of complete luteolysis (Martins et al., 2011b). Synchronization with DO and GGPG resulted in high ovulation rates to G2 and G3. Aside from high individual ovulation rates, 71% of all cows in this study were synchronized to both G2 AND G3. Ovulation rates to both G2 and G3 were associated with greater fertility. Overall PR/AI at d 24 post-AI increased 35% in synchronized vs. non-synchronized cows, across all CLO dose. Giordano et al., (2013) reported a similar increase (35.6%) in PR/AI in cows that ovulated to G3 in a DO program. Thus, it is possible that the majority of cows in this study had both an optimal hormonal milieu and synchronized ovarian structures to achieve high fertility during DO and GGPG, regardless CLO treatment.

Luteal function was assessed before and after treatment via ultrasonography. The effects of treatment were only verified in synchronized cows that had both a d 7 and 14 CL at CLO treatment. There were no differences in combined LV and LBF before treatment. There was a marked decrease in both combined LV and LBF from d -1 to d following treatment with all CLO dose strategies. Circulating levels of P_4 decrease concomitantly with LV and LBF during $PGF_{2\alpha}$-induced luteolysis. Yet, Acosta et al. (2002) observed that decrease in LV following $PGF_{2\alpha}$ treatment was somewhat delayed in comparison to LBF and P_4 levels 2 d after treatment. The effect of treating various doses of CLO on combined LBF was detected at d 2 after treatment. Decrease in LBF involves a broad process of angiolysis but is also modulated almost instantly via vasoactive substances that induce increase/decrease in LBF (Reynolds et al., 2000; Miyamoto et al., 2009). Treatment with DOUBLE resulted in lower combined LBF in comparison to FULL. Different than our hypothesis, cows that received TWO/24 had intermediate LBF 2 d after treatment, with no differences from FULL treatment. Combined LV followed the same pattern of response, but differences were detected later, at d 4 post-treatment. Decrease in LV is a result of

vast tissue remodeling (Miyamoto et al., 2009). Therefore, morphological effects of such process take longer to be detected with ultrasonography.

Differences on combined LV and LBF reduction between CLO doses were associated with parity. No differences on combined LV or LBF were observed between CLO dose strategy in 1st and 2nd parity cows. Third-plus parity cows were affected differently by different doses of CLO. At d 4 post-treatment 3rd+ parity cows had lower combined LV when treated DOUBLE in comparison FULL. Combined LBF was lower in 3rd+ parity cows that received DOUBLE in comparison to FULL at d 2 and d 4 after treatment.

Lower combined LV or LBF did not translate into greater PR/AI in cows treated with DOUBLE. Instead, synchronized cows treated with FULL had greater PR/AI at d 24 post-AI. Those were cows that had overall greater combined LBF at d 2, and greater combined LV at d 4 post-treatment. Amount of combined LBF measured at d 2 or 4 post-AI was not a predictor of pregnancy at d 24 post-AI. However, combined LBF measured at d 2 and 4 was as a predictor of pregnancy at d 34 post-AI. Probability of pregnancy increased as combined LBF decreased. Souza et al. (2007) found a similar association with circulating levels of P_4 and probability of pregnancy 58 to 64 d post-AI. Circulating P_4 levels > 0.5 ng/mL 2 d after $PGF_{2\alpha}$ treatment decreased PR/AI in about 50%.

Logistic regression analysis of combined LBF post-treatment provided evidence that greater amount of LBF decreases the probability of pregnancy. Data presented in Chapter 2 indicates that heifers with complete luteal regression had disappearance of LBF 4 d following treatment. However, treatment with different doses of CLO in lactating dairy cows only resulted in a small proportion of cows with complete LBF disappearance (LBF = 0). Thus, different approaches were utilized to determine a cutoff based on combined LBF and verify its relationship

with PR/AI outcomes. Median individual LBF measured 4 d post-treatment was utilized as an indicator of complete luteal regression. Only 263 out of 607 lactating dairy cows met these criteria (43.4%). There was no difference in complete luteal regression between CLO dose strategies. Cows that regressed both d 7 and d 14 LBF below their median cutoff had greater PR/AI at d 24 post-AI. Cows with LBF below the median also had increased levels of PSPSB at d 24 post-AI. Nonetheless, cows that maintained LBF above the median still averaged overall 52.6% PR/AI at d 24 post-AI. This indicates that high LBF around TAI does not interfere with fertility at the same proportion as high P_4 around TAI does.

Accurate cutoffs for luteal regression can be determined when P_4 levels are measured in highly sensitive assays. Previous studies utilized a highly accurate RIA that is no longer available. Even slight increases in P_4 levels detected in the low end of this assay resulted in drastic decrease in PR/AI (Brusveen et al., 2009; Martins et al., 2011a). Utilizing color Doppler was not sensitive enough to capture a similar relationship between LBF and PR/AI. Utilizing the median LBF cutoff only captured a group of cows with markedly low LBF 4 d post-AI. It was not possible to find a precise amount of LBF where pregnancies decreased significantly or stopped happening. It seems that intermediate/high LBF still could be present in regressing CL without negatively impacting chances of pregnancy. In the regressing CL, bFGF is involved with selective cellular death. It allows for large luteal microvessels to remain in the CL and steroidogenic cells to involute (Reynolds et al., 2000). Intermediate LBF detected around the regressing CL could be present in response to vasoactive substances produced in adjacent ovarian structures (i.e. follicles and newly formed CL). It is possible that color Doppler signals detected around regressing CL were from these preserved large microvessels that still were subject to hemodynamic changes induced via vasoactive substances.

In summary, there is no other study published at this time that compared FULL vs. DOUBLE doses of CLO. But there are now at least n = 4 studies (Carvalho et al., 2015; Wiltbank et al., 2015; Heidari et al., 2017; Borchardt et al., 2018) in the literature that compared FULL vs. TWO/24 and reported significant improvements in fertility of lactating dairy cows with an additional dose of CLO or DIN. And another study that indicated TWO/24 enhanced luteolysis compared to FULL (Brusveen et al., 2009). But, in the current study, there were no evidence of differences on overall PR/AI between CLO dose strategies. However, cows treated a FULL (0.5 mg of CLO) experienced greater early pregnancy loss from d 24 to 34 post-AI. The greater proportion of losses was detected within older cows (3^{rd}+ parity) treated with FULL. Third-plus parity cows also had the greatest amount of LV at d 4, and greatest LBF at d 2 and 4 after treatment with FULL which could explain the differences. Multiparous cows (2^{nd} and 3^{rd}+ parity) did not have greater PR/AI when treated TWO/24 or DOUBLE. Surprisingly, synchronized cows that received FULL had greater PR/AI at 24 d, but not at 34 and 62 d post-AI. Interestingly, PR/AI in cows that received DOUBLE did not differ between the two synchronization statuses. Synchronized cows treated with DOUBLE had similar PR/AI in comparison to non-synchronized cows at d 24, 34 and 62 post-AI. High ovulation rates following GnRH treatments of DO and GGPG were the main factor to modulate PR/AI outcomes in this dataset. Amount of LBF present at d 2 and 4 post-treatment was a predictor of pregnancy 34 d post-AI. There was a decrease in PR/AI when cows had LBF above the median cutoff at d 4 post-treatment. However, LBF measured with color Doppler at a d 4 was not sensitive enough to detect complete luteal regression in lactating dairy cows.

We recommend that multiparous cows should be treated with double doses of CLO in order to avoid lower fertility in non-synchronized cows and greater pregnancy losses. Treatment with

double dose of CLO (1.0 mg) will have a dramatic impact on the amount of labor needed for an additional dose a day later. Thus, double dosing CLO will have a positive impact on dairy profit.

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