THE IMPACT OF AN IBR MLV ON ESTROUS CYCLE PARAMETERS, ANTI-MÜLLERIAN HORMONE CONCENTRATIONS AND THE INFLAMMATORY PROFILES OF NULLIPAROUS HEIFERS

by

CAITLIN LARK WIDENER

(Under the Direction of Jillian F. Bohlen)

ABSTRACT

Twenty-four heifers in were synchronized for estrus using a 7-day CIDR protocol with 2 injections of $PGF_{2\alpha}$, one at CIDR removal and one 16 hours later. All animals were calf-hood vaccinated with an available BRDC vaccine with a MLV IBR component. At Heat 2, heifers were vaccinated with either the same MLV IBR or a BRDC vaccine with a Killed IBR component. There was no significant effect of vaccine on P4 concentrations, E2 concentrations, estrous cycle lengths or first service conception rates ($P>0.05$). There was a decrease ($P<0.05$) in circulating AMH concentrations from Cycle 1 to 2 and 1 to 3, but there was no difference ($P>0.05$) between vaccine types. There was no vaccine difference ($P>0.05$) between post-vaccination titers, circulating white blood cell concentrations or neutrophil activity.

INDEX WORDS: AMH, IBR MLV, Neutrophil ROS, Cyclicity, Dairy Heifers, BRDC

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DEDICATION

To My Family and Friends

Thank you for being my strength and support thorough this crazy process called Graduate School.

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First, I would like to thank Dr. Jillian Bohlen for the opportunity she provided and the chance she took on me. From our time together at Clemson until now, you have been a supportive (and tough!) mentor and friend, and I truly appreciate the opportunities you have provided me. Your friendship is priceless. Thank you Dr. Graves for being a guiding hand throughout this process, your opinions and experience have been invaluable. Thank you Dr. Hurley for both your guidance and making those mornings in the lab brighter. Thank you to the UGA Teaching Dairy farm crew for all your help, from moving to breeding heifers. Thank you to the labs across campus and beyond that gave me the opportunity to work with them and complete this project.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

An Evaluation of Cyclicity and Endocrinology in Nulliparous Heifers

Cattle are a polyestrous species that maintain an average 21-day estrous cycle. Unlike some other ruminant species, cattle are not confined to a single breeding season. This cycle is controlled by two major ovarian structures; the ovulatory follicle and the corpus luteum (CL) and their associated hormones, estrogen (E2) and progesterone (P4) respectively (Forde et al., 2011). The presence of these structures allows the cycle to be easily split into two phases, the follicular and luteal, and four stages: estrus, metestrus, diestrus and proestrus (Figure 1.1).

The follicular phase accounts for approximately 20% of the bovine estrous cycle and is defined as the timeframe from CL regression to ovulation of the dominant follicle. The follicular phase can be further broken down into two of the stages, proestrus and estrus. Proestrus begins on approximately day 17 of the cycle and during this time there is a rapidly growing pre-ovulatory follicle and regression of the CL (Hansel and Convey, 1983). Estrus is defined as the timeframe during which a female exhibits secondary sex characteristics that indicate her sexually receptivity to the male (Ireland, 1987). Estrus is commonly used to designate the first day of the cycle (d 0) because it is observable via secondary sexual characteristics. Estrus lasts 6 to 24 hours with an average

durations of 15 hours, and is directly correlated with ovulation in the bovine, which occurs 24 to 32 hours after the onset of standing estrus (Diskin, 2008).

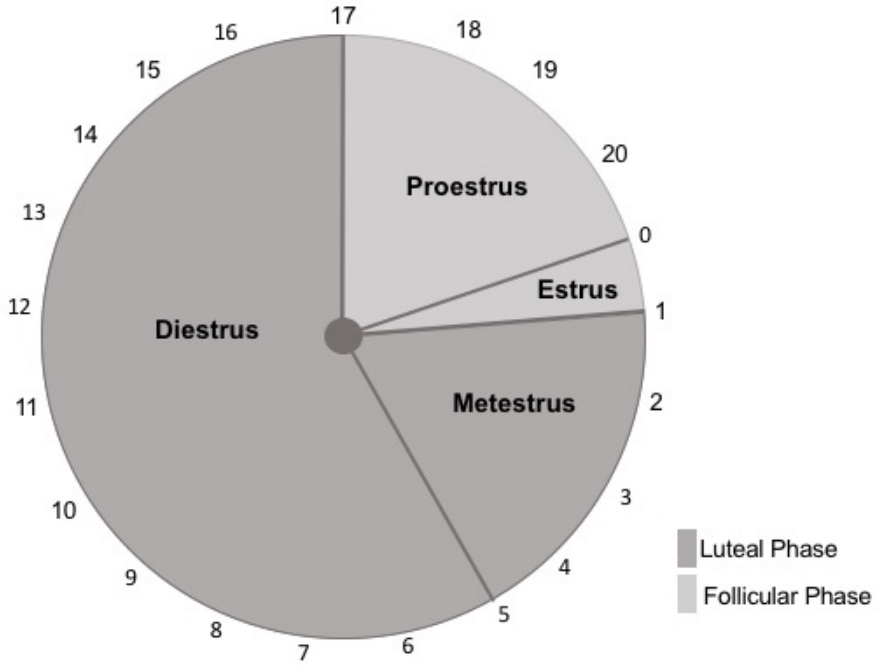

Figure 1.1: Graphic representation of the phases and stages of the bovine estrous cycle. Numbers surrounding the graph correspond to the day of the estrous cycle. The stages of the cycle are labeled within their corresponding phase.

The luteal phase represents the remainder of the cycle (80%), from ovulation to CL regression, during which time the CL is the dominant structure on the ovary. The early part of this phase, until approximately day 5 of the cycle, is defined as the stage metestrus (Forde et al., 2011). During this time, the recently ruptured follicle is being transformed into a functional CL. Finally, the longest stage of the estrous cycle is diestrus, which lasts approximately day 5 to day 16 of the cycle. During this stage, the CL reaches maximal size and progesterone

production. The hormones produced by the follicle and CL communicate with the brain through a complex system known as the hypothalamic-pituitary-gonadal axis (Senger, 2012).

Hypothalamic-Pituitary-Gonadal Axis

The hypothalamic-pituitary-gonadal (HPG) axis is the primary control mechanism of cyclicity in vertebrate species. This unique system utilizes both the nervous system and the endocrine system to regulate and coordinate all of the processes necessary for reproductive function (Figure 1.2). In brief, the hypothalamus is the control center, releasing gonadotropin releasing hormone (GnRH). The hypothalamus is connected to the anterior pituitary via a portal blood system. This prevents GnRH from having to enter the circulatory system to reach its target tissue, allowing small changes in concentrations to have a meaningful impact on secretions of the anterior pituitary (Carmel et al., 1976). These secretions (LH and FSH) are delivered to the gonads through a capillary network, where they influence the development and maintenance of different ovarian structures. These ovarian structures (follicle and CL) create and release their specific hormones into circulation to communicate back with the hypothalamus, in either a negative or positive feedback (Bearden et al., 2004 and Senger, 2012).

Within the hypothalamus there are two functional areas related to the release of GnRH, the tonic and surge centers. The tonic center is more sensitive to negative feedback. It is the negative feedback signaling of progesterone (P4), produced by the CL, to this center that maintains a small level of GnRH, and thus

small pulses of LH and FSH, preventing the formation of a pre-ovulatory follicle. The surge center, which is sensitive to positive feedback, is responsible for the larger surges of GnRH that lead to ovulation of the dominant follicle (Roche, 1996). As progesterone is removed through luteolysis, the dominant follicle is allowed to undergo exponential growth, producing more and more estrogen (E2). Once this estrogen production reaches a threshold level, it can begin to influence the surge center of GnRH. This large release of GnRH leads to the spike in LH necessary for ovulation of that dominant follicle.

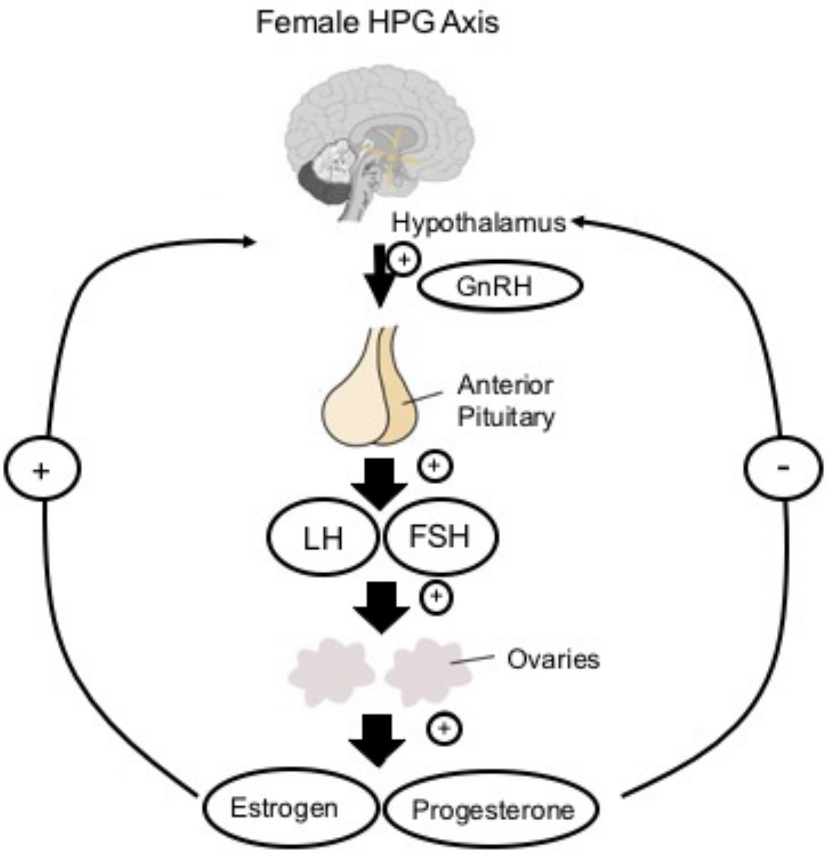

Figure 1.2: Depiction of the Hypothalamic-Pituitary-Gonadal axis of the female, including the primary hormonal secretions from each portion.

CL Formation

As the luteal phase begins, the cells remaining from the erupted follicle will be transformed into the large and small luteal cells of the CL through the process of lutenization. The granulosa cells go on to form the large luteal cells and the theca interna form the small luteal cells in response to pulsatile releases of LH (Figure 1.3). Inhibition of these LH pulses will reduce both the function and the structure of the forming CL (Quintal-Franco et. al., 1999). Both cells types are capable of producing progesterone, but large luteal cells have the additional capability of producing granules containing oxytocin, which is important for cycle regulation (Alila et. al., 1988).

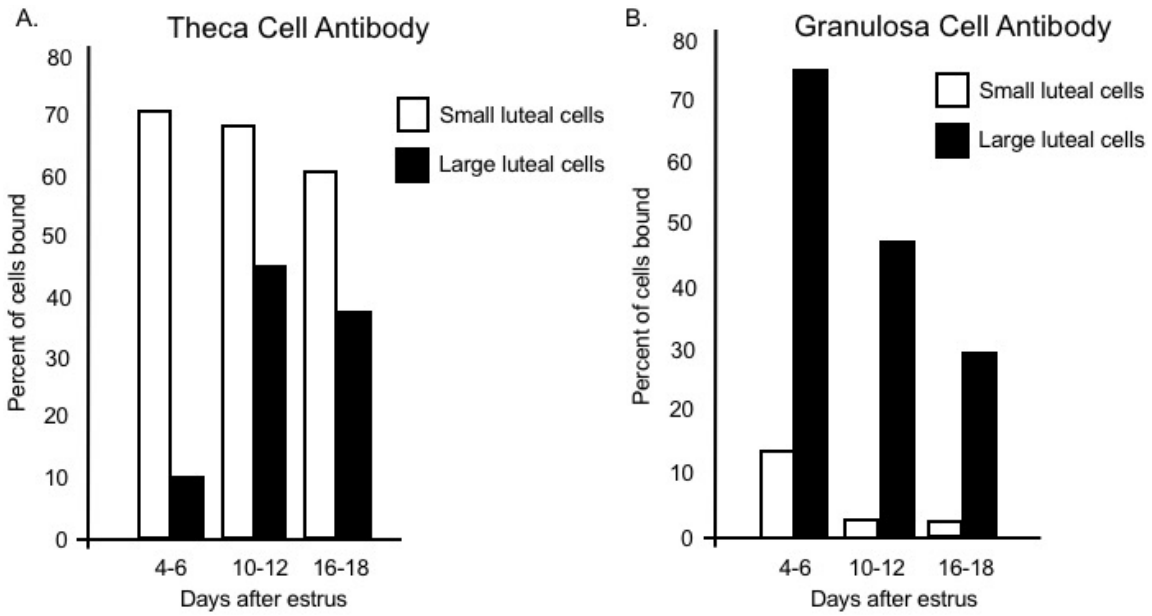

Figure 1.3: Binding of theca cell (A) and granulosa cell (B) monoclonal antibody to luteal cell types as an indicator of cell derivation. (Adapted from Alila and Hansel, 1984).

Progesterone plays an important role in the regulation of the estrous cycle. Primarily, it maintains a small, pulsatile release of GnRH from the hypothalamus. The CL will continue to increase in mass until approximately day 10-11 and will then plateau until approximately day 16 (Siqueira et al., 2009).

Follicular Waves

Throughout the luteal phase there are most commonly 2 follicular waves, in the cow or 3 follicular waves, in the nulliparous heifer (Figure 1.4). With this, in heifers waves emerge on approximately days 0, 9, and 16 while in cows, waves emerge on approximately days 0 and 10 (Bearden et al., 2004).

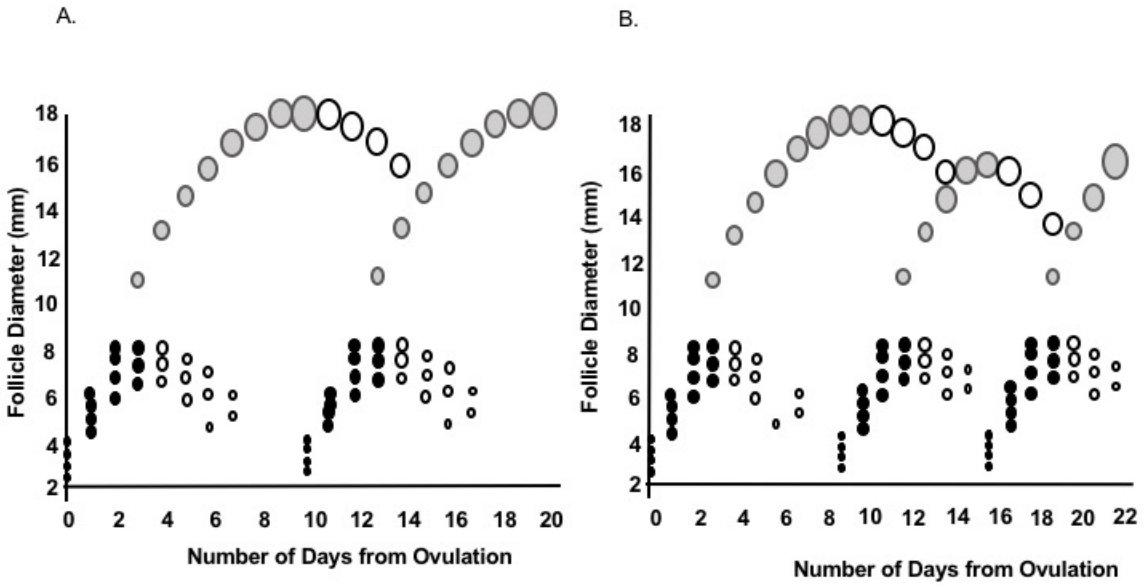

Figure 1.4: Schematic representation of 2-wave (A) and 3-wave (B) follicular growth during the estrous cycle of primiparous and multiparous cows and nulliparous heifers. Black circles represent growing follicles, the grey circles represent the dominant follicle and the white circles represent atretic follicles. (Adapted from Fricke, 2001).

For a 2-wave cycle, each wave lasts approximately 10 to 11 days. For a 3-wave cycle, the second and third waves are much shorter, with a shortened interval from emergence to ovulation in the third wave (Noseir, 2003).

The time immediately following ovulation is a peculiar time point for the ovary as there is not an endocranially active structure present for nearly 5 days. During this time, there is a rise in circulating FSH, leading to the recruitment of the 3mm gonadotropin sensitive follicles and the first follicular wave (Hendriksen et al., 2000). In cows treated with exogenous FSH beginning on Day 2 of the estrous cycle there was the development of as many as 19 estrogen active follicles by day 5 (Fricke et al., 1994). These follicles initiate the first follicular wave.

These recruited follicles experience 3 days of similar growth rates and achieve a size of approximately 6 to 8mm by day 3 of the cycle (estrus=0) (Ginther et al., 1996). At this point, a decline in FSH and increase in LH coincide with a change in the gonadotropin responsiveness of some of these follicles, leading one follicle to diverge from the pool (Ginther et al., 1997 and Beg and Ginther, 2006). Throughout this common growth phase, no single follicle exerts control or suppression, although in 60% of cattle the largest of these follicles will coincidentally move on to dominance (Ginther et al., 1996). In cattle, as the growing pool reaches 5mm, they obtain the ability to suppress FSH. Effective suppression is related to the number of 5mm follicles on the ovary and their production of inhibin and E2 (Gibbons et al., 1999, Ginther et al., 1996). This suppression may lower circulating FSH concentration below the level required by

subordinate follicles, but still allow the newly deviated follicle to grow (Ginther et al., 1999).

This selected follicle will grow at an expedited rate compared to its cohorts, and begin to suppress them through the production of E2 and inhibin (Beg and Ginther, 2006 and Ginther et al. 1996). Inhibin acts directly on the anterior pituitary to suppress FSH production but does not alter LH production (Carroll et al., 1989). Successful deviation of this follicle is completed when its continued growth is accompanied by the cessation or reduction in growth of the subordinate follicles (Ginther, 2000). During the 6 day growing phase, the follicle continues to increase E2 production in direct relation to follicular size. This now dominant follicle can continue to grow in the face of decreased circulating FSH because it shifts to a dependence on LH. There is a consistent release of LH from the anterior pituitary throughout the luteal phase, and when this basal release is suppressed by a GnRH agonist, the largest follicle will not grow beyond 7-9mm (Gong at al., 1995). Thus, this basal release of LH is vital for the development of the dominant follicle.

This growing phase is followed by a static phase, with a drop in circulating E2 (Noseir, 2003). This drop in E2 production is temporally related to a rise in circulating FSH, which in turn initiates the subsequent follicular wave (Adams et al., 1993). In a nonovulatory wave the largest follicle will undergo atresia, as the negative feedback of luteal P4 prevents the E2 rise and stimulation of the hypothalamic surge center and subsequent LH necessary for ovulation (Senger, 2012).

Luteolysis

In the normally cycling and non-pregnant female, there is a need for self-regulation of the reproductive cycle. On approximately day 15 the CL begins the process of degradation or luteolysis. This is primarily controlled by the production of Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) by the uterus and oxytocin (OT) by the CL, which work in a positive feedback loop. Throughout the luteal phase, the high levels of P4 increase the accumulation of the necessary precursors and enzymes required for the production of $PGF_{2\alpha}$ (Silvia et al., 1991). Additionally, prolonged exposure to P4, around 12 days, will increase the concentration of the oxytocin receptor (OTR) within the uterine endometrium, increasing uterine response to OT secretion as indicated by $PGF_{2\alpha}$ secretion (Vallet et al., 1990). During this time, the large luteal cells are producing small levels of oxytocin, housed in intracellular granules (Schams and Berisha, 2004).

By approximately day 15, the uterus has collected enough $PGF_{2\alpha}$ precursors and there is enough expression of the OTR to begin small, pulsatile releases of $PGF_{2\alpha}$. This binds to the low-frequency prostaglandin receptor (LFPR) of the CL, releasing OT (McCracken et al., 1995). It is at this point that oxytocin binding to the uterine OTR is able to produce more $PGF_{2\alpha}$, which enters the uterine vein and then diffuses into the ovarian artery beginning the process of luteolysis (McCracken et al., 1999). In cows that have undergone hysterectomy of the uterine horn ipsilateral to the CL, there is a prolonged functional lifespan of the CL because the circulating $PGF_{2\alpha}$ from the contralateral horn is not sufficient to induce normal lutelolysis. However, if the uterine vein or ovarian artery

ipsilateral to the CL in that animal is connected to the contralateral horn, the CL will maintain a more normal life-span; highlighting the importance of both the uterus and the venoarterial connection between the uterus and the ovary (Mapletoft et al., 1976).

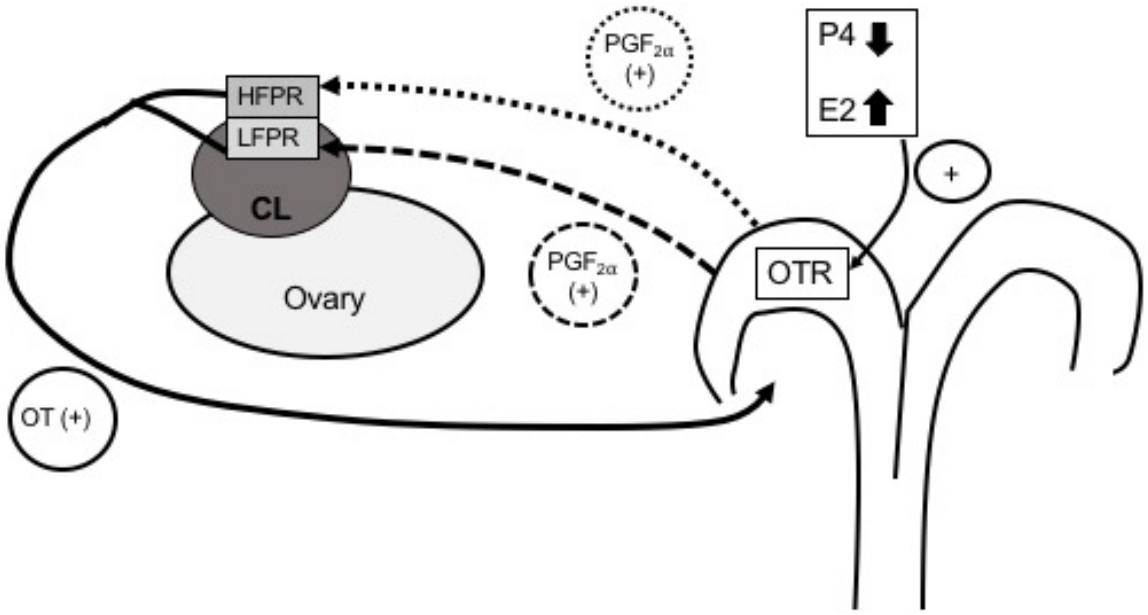

Figure 1.5: A representation of the positive feedback loop associated with the process of luteolysis. (Adapted from McCracken et al., 1995).

Coinciding with this pulsatile release, there is a decrease in P4 and a rise in E2 from the developing follicle. E2 enhances the positive feedback loop between the CL and uterus by increasing the transcription and sensitivity of the uterine OTR (Gimpl and Fahrenholz, 2001). As the cascade builds, higher releases of $PGF_{2\alpha}$ bind to the high frequency prostaglandin receptor (HFPR), enhancing the positive feedback mechanism, summarized in Figure 1.4. The role of follicular E2 in the process of luteolysis in vivo was confirmed by Araujo et al. (2009). By removing all follicles >4mm on cycle days 9-21, researchers were

able to delay both the rise and peak E2 and delay the onset of luteolysis to day 18 (Araujo et al., 2009). Within hours of the onset of this process the CL has lost its functional role, the ability to produce P4 at adequate levels to maintain suppression of the dominant follicle. The structural portion of the CL will remain for approximately 12 hours or more (Schams and Berisha, 2004). This small, nonfunctional structure is referred to as the corpus albicans. This point represents the transition from the luteal to the follicular phase of the estrous cycle.

Ovulation and Estrus

During the final follicular wave of the estrous cycle, a follicle will deviate from the wave of follicles just as in the preceding waves. However, the deviation and subsequent growth of this particular follicle coincides with the degradation of the CL and the beginning of the follicular phase and the stage of proestrus. A follicle achieves ovulatory capacity at approximately 10mm, and this size is associated with the development of LH receptors (LHr) in the granulosa cells of this follicle (Sartori et al., 2001 and Beg et al., 2001). This corresponds to about one day after the initiation of follicular deviation. In their 2001 experiment, Beg et al. found that in comparing the largest and second largest follicle, when the largest follicle attained a diameter of approximately 8.5mm, there was both an increase in E2 concentration of the follicular fluid and an increase LHr mRNA expression by the granulosa cells. This increase in LHr occurred 1 diameter range before diameter deviation between the two follicles, implicating the role of LHr in follicular deviation (Beg et al., 2001).

The binding of LH, in addition to basal binding of FSH, increases the aromatase activity of the granulosa cells of the pre-ovulatory follicle (Ginther et al, 1996). Aromatase is the enzyme responsible for the conversion testosterone, sourced from the theca interna cells, into E2 (McNatty et al., 1984). As the CL undergoes luteolysis at the beginning of proestrus, the inhibitory actions of P4 are removed. This allows for a 12 to 18-fold rise in E2 expression by the dominant, pre-ovulatory follicle and a subsequent increase in uterine motility, the expression of estrus, and ultimately timely ovulation (Bridges and Fortune, 2003). GnRH secretion increases in both frequency and amplitude, as a response to rising estrogen, and this in turn increases the secretion of LH and FSH, feeding the growing follicle. As the follicle continues to grow, it produces and increases the circulating concentrations of E2 directly related to its size (Vasconcelos et al., 2001). Once a sufficient concentration of E2 is achieved, the final stage of the cycle begins.

The final stage of the estrous cycle is estrus. Estrous behaviors and signs common in cattle include: increased restlessness, increased vocalization, increased vaginal mucus production and homosexual behavior including chin resting, riding herd mates, and standing to be ridden by herd mates (Bearden et al., 2004). These behaviors are expressed in response to the actions of E2 on the hypothalamus in the absence of P4 (Blache et al., 1991). Estrous behaviors can even be induced by administration of exogenous E2 to ovariectomized heifers and cows (Allrich, 1994). The onset of standing estrus, most often used to determine timing of AI, is preceded by a 19-fold increase in circulating LH,

followed by ovulation 32 hours after this peak (Swanson and Hafs, 1971). This LH surge is associated with the cascade of events responsible for ovulation of the dominant follicle.

Pre-ovulatory follicular fluid analyzed for E2, P4 and testosterone concentrations relative to the LH surge showed changing characteristics (Table 1.1).

Table 1.1: Concentrations of estrogen, progesterone, and testosterone in bovine follicular fluid relative to the LH peak. (Adapted from Dieleman et al., 1983).

Hours relative to LH Surge	Estrogen Concentration (μmol/l)	Progesterone Concentration (μmol/l)	Testosterone Concentration (μmol/l)
Onset of estrus	6.05	0.387	0.165
0-6 hours post	constant	0.727	0.241
6-20 hours post	dropping	0.387	0.031
20+ hours post	0.461	1.51	low

The increase of P4 concentrations 20+ hours after the LH surge was associated with degradation of the follicular basement membrane and the invasion of phagocytic cells of the innate immune system (Dieleman et al., 1983). In animals administered a progesterone inhibitor, ovulation will be blocked. (Espey and Lipner, 1994). This follicular P4 binds to an induced P4 receptor on the granulosa cells of the pre-ovulatory follicle and induces the production of the proteases necessary to degrade wall of the follicle. Transgenic mice that lack the granulosa P4 receptors do not have adequate production of these proteases, and thus suffer from anovulation of the pre-ovulatory follicle (Robker et al., 2000). The signaling of and subsequent appearance of phagocytes within the pre-ovulatory

follicle is not well understood, but these cells do appear to contribute to the degradation of the basement membrane (Norman and Brannstrom, 1994). In addition to these changes in steroid concentrations, there is a considerable rise in the concentrations of $PGF_{2\alpha}$ and PGE_2. The rise of these two inflammatory prostaglandins (PGs), particularly $PGF_{2\alpha}$, is imperative for successful ovulation of the follicle (Algire et al., 1992).

These PGs act locally to induce contraction of the ovarian smooth muscle, allowing the female gamete to erupt and enter the reproductive tract (Espey, 1978). With this action, the follicular phase has come to an end and the luteal phase begins again, completing one estrous cycle. The size of the subsequent CL and its progesterone production, formed from the remaining tissue of the ovulatory follicle, is related to the size of the ovulated follicle (Vasconcelos et al., 2001). Thus, the ovulatory follicle has a direct impact on CL size and function in the subsequent estrous cycle.

Deviations from Normal Cyclicity

The nulliparous heifer is considered the most fertile animal on any cattle operation. Unlike her older herd mates, she does not have the metabolic stress of lactation or any trauma from a previous calving that can alter her reproductive competence. However, this does not mean that virgin heifers cannot deviate from normal. There are a variety of management considerations, hormonal imbalances, and diseases that can cause abnormal cyclcity or negatively impact heifer fertility.

Nutritional Causes of Abnormal Cyclicity

The nutritional status of an animal has a lasting impact on all physiological processes, including reproduction. When considering virgin heifers, the earliest point nutrition influences reproductive cyclicity is in the achievement of puberty. Pre-pubertal heifers will utilize the nutrients provided in their diets to first meet their maintenance and then growth requirements. Once she achieves an adequate size, or an adequate plain of nutrition, a young female will begin to store any excess energy as fat (Patterson et al., 1992). These fat cells will begin to produce the hormone leptin, which has receptors at the blood-brain barrier. It is through this connection with the blood-brain-barrier that adequate leptin concentrations are able to stimulate the production of GnRH. Leptin production is directly correlated with total body fat. Once an adequate level of leptin is achieved, indicating an excess of energy, the body will initiate puberty as it now has the ability to support the growth of offspring (Garcia et al., 2002). Puberty may be delayed in heifers that do not receive adequate nutrition.

Heifers must then maintain an adequate plain of nutrition to remain cycling and achieve pregnancy. Nutrient restriction in growing heifers can alter LH and FSH secretion, reduce follicular diameter, reduce circulating estrogen and cause anovulation (Mackey et al., 1999 and Bossis et al., 1999). Even short-term (18-19 day) dietary restriction of heifers will reduce the concentrations of estrogen within follicular fluid and reduce the expression of the FSH receptor on granulosa cells in both ovulatory and anovulatory follicles. Furthermore, the anovulatory follicle in restricted heifers displayed reduce transcription of the genes regulating

the ability of theca interna cells to respond to LH (Walsh et al., 2012). It appears that these large, anovulatory follicles undergo atresia and a new wave initiates, again without ovulation, leading to functionally anestrous animals. When heifers are returned to an appropriate plain of nutrition, they will return to cyclcity but the first estrous cycle may be shortened to as few as 10 days. The subsequent cycle will achieve a normal duration (Bossis et al., 2000).

Heat Stress and Abnormal Cyclicity

All cycling animals on a dairy farm experience reduced reproductive capabilities as a response to heat stress and virgin heifers are not excluded from this group. Thankfully, they do not have to battle the added metabolic heat that lactating animals do, but they can still be impacted by prolonged exposure to high ambient temperatures. Ambient temperatures are best classified by the temperature-humidity index (THI), which accounts for both temperature and humidity. An elevated THI, especially when prolonged as during the summer months, has lasting impact on all aspects of fertility including a reduction in circulating estrogen and compromised follicular growth.

The dominant follicle of an animal experiencing heat stress is generally smaller with less fluid than a non-stressed control. Interestingly, the subordinate follicles in a non-shaded group were larger than their shaded counterparts, indicating a compromised ability of the heat stressed dominant follicle to suppress other follicles (Badinga et al., 1993). In heat stressed cows, there are higher and extended surges of circulating FSH, even noted during the pre-ovulatory period when FSH should be at a basal level. This high circulation has

been associated with a marked decrease in circulating inhibin when comparing a heat stressed animal to her cooled counterparts (Roth, 1998; Wolfenson et al., 1995). The dominant follicles of heat stressed cattle also experience compromised aromatase activity, reducing circulating estrogen (Wolfenson et al., 1995). The theca cells of these follicles have a reduced capacity to produce the necessary precursor, androstenedione, for E2 production by the granulosa cells (Wolfenson et al., 2000).

This ineffective suppression by the dominant follicle may also cause failure of proper angiogenesis, reducing the availability of energy and cellular components necessary for oocyte development and maturity. Additionally, reduced estrogen will cause a decreased duration and intensity of estrus, contributing to the overall reduction in fertility seen in heat stressed animals (Bearden et al., 2004). A final consequence of heat stress is the possibility for delayed ovulation as a consequence of both low E2, and thus a delayed LH spike and delayed luteolysis (Gilad et al., 1993; Wilson, 1998). Not only is she not capable of proper reproductive cyclicity and oocyte maturity, but she may be missed by human breeding technicians or bred at an inappropriate time relative to estrus.

Hormonal Imbalances and Abnormal Cyclicity

Although the body utilizes a highly regulated mechanism to control the reproductive cycle, never allowing a single hormone to be completely responsible for an action through the HPG axis, there are instances where a hormonal imbalance occurs. These can be caused by exogenous issues, like feeding

plants high in phytoestrogens, or endogenous disruptions in the normal endocrine control of reproduction, such as the presence of a follicular cyst. Phytoestrogens in feedstuffs may disrupt normal cyclcity by binding the the estrogen receptors of the HPG axis, blocking the ability of endogenous estrogen to bind (Mathieson and Kitts, 1980). These chemicals can lead to prolonged estrus, irregular cycles, and formation of cystic ovaries. Follicular cysts and other endogenous endocrine disruptors lead to prolonged ovarian structures that exert control over the estrous cycle (Gaverick, 1997). These structures essentially remove the ability of the estrous cycle to self-regulate by exerting dominating control over the HPG axis through abnormal steroid hormone production.

There are two primary types of cysts that can form on the ovary, follicular and luteal. Follicular cysts result from a large, dominant follicle that does not successfully ovulate. These retained follicles produce high levels of both estrogen and androgens, leading to the primary outward sign of a follicular cyst: nymphomania (Garverick, 1997). Animals with a follicular cyst will seem to be in constant heat, with an inter estrus interval of 3 to 10 days. If the condition is allowed to persist, the androgens produced by the cyst may lead to the masculinization of a female (Bearden et al., 2004). On some occasions, the walls of a follicular cyst will thicken and begin to produce progesterone, transforming it into a luteal cyst. Progesterone production is a response to the spontaneous lutenization of the theca, granulosa or both cells of the cystic follicle as a response to prolonged LH exposure (Garverick, 1997). Experimentally, cysts can be induced through a variety of methods including: exogenous

estrogen and progesterone in amounts similar to the end of gestation (Erb et al., 1973), exogenous adrenocortitropic hormone (Liptrap and McNally, 1976), or an LH antagonist (Nadaraja and Hansel, 1976). Each of these treatments alter the normal cascade of events that lead to ovulation, indicating the wide variety of causes of follicular and luteal cysts.

A less problematic ovarian abnormality is the cystic CL. This is simply a developing CL that captured an abnormal amount of follicular fluid during lutenization. Some capture of follicular fluid is normal during CL development, 7 to 8 mm in diameter when measured via ultrasound (Bearden et al., 2004). In their 1988 study, Okuda et al., examined the morphology of 706 CLs. A central cavity was found in 42.1% of developing CLs, 33.7% of fully developed CLs, and 11.1% of regressing CLs. Cavities may disappear as the CL continues its increase in mass or may exhibit a cobweb appearance on ultrasound, a sign the body is attempting to luteinize the fluid (Bearden et al., 2004). The presence of excessive fluid inhibits the ability of the remaining follicular cells to effectively transform into luteal cells, diminishing the luteal tissue area of the CL. As progesterone production is highly correlated with luteal tissue area and luteal tissue heterogeneity, the presence of a persistent cavity would impair progesterone production and could alter estrous cycle length (Siqueira et al., 2009 and Mann, 2009).

Health and Fertility: The Bovine Respiratory Disease Complex

The bovine respiratory disease complex (BRDC) is a combination of pathogens that impact cattle production all over the world. The greatest

economic impact on the dairy industry is the abortive nature of these viral pathogens (Houe, 2003). Regardless of cause, the average cost of a lost pregnancy in a lactating Holstein dairy herd is estimated at $555, and this cost increases as the day of gestation increases (De Vries, 2006). The best way to manage for these diseases in the implementation of an effective vaccination program with a wide variety of commercially available vaccines, including both killed viral particles and modified live viruses.

BRDC is caused by both viral and bacterial pathogens. The common viral components which are vaccinated against are: bovine respiratory syncytial virus (BRSV), parainfluenza 3 (PI3), bovine viral diarrhea virus (BVDV) and infectious bovine rhinotracheitis (IBR). Some of the bacterial pathogens that are involved with BRDC *Mannheimia haemolytica*, *Pastuerella multicoda*, and *Mycoplasma bovis*. A true BRDC infection results from infection by two or more of these pathogens, presenting as an acute respiratory infection, most commonly in young calves or recently shipped animals (Tyler and Ensminger, 2006).

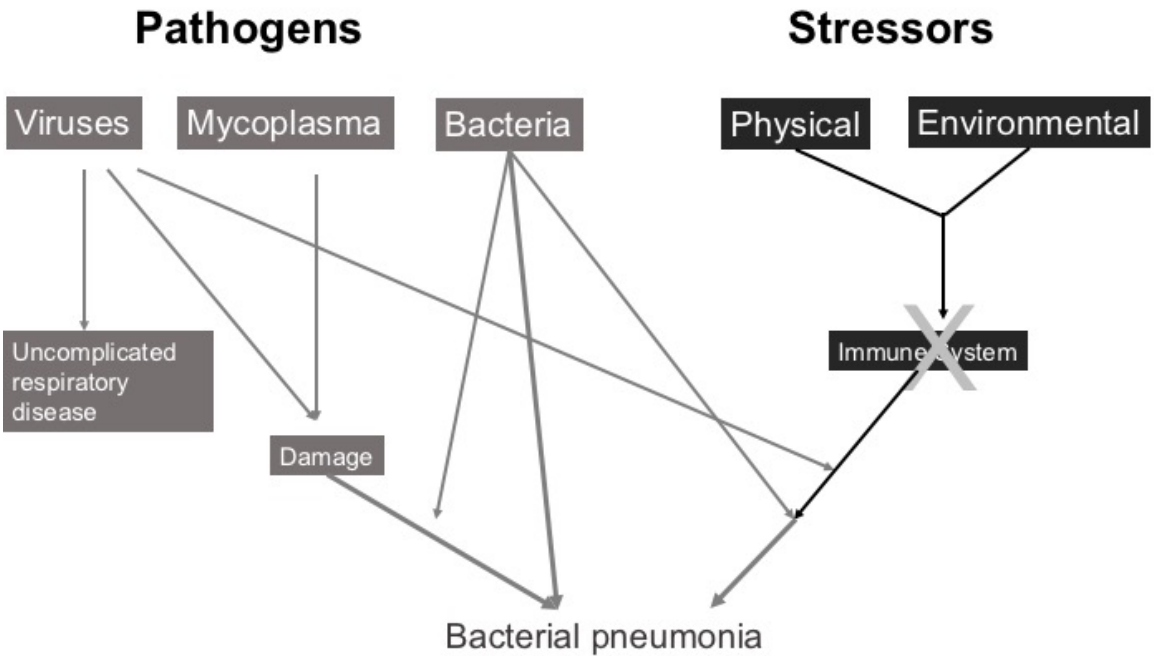

Figure 1.6: Representation of the complex interactions that lead to bovine respiratory disease. The viruses alone can cause uncomplicated respiratory disease just as the bacteria can induce pneumonia.

However, it is when these pathogens interact with each-other to cause tissue damage or with an immunosuppressed animal, as a result of stress, that a true BRDC infection takes hold (Figure 1.6).

These viral pathogens have been linked with abortion of both dairy and beef herds across the United States (Table 1.2). Experimental infection with BVDV on day 100 of gestation has induced abortion rates as high as 40%, although field observations are lower, as observed in Table 1.3 (Done et al., 1980). IBR is best recognized as a cause of “abortion storms” when susceptible, unvaccinated animals are exposed, with a 25-60% abortion rate (Kelling, 2007). Historically, those abortive materials submitted for analysis by a lab implicate IBR

as the most prevalent BRDC component cause of infectious abortion, when compared to other BRDC pathogens (Anderson, 2007).

Table 1.2: Infectious causes of bovine abortion during mid- to late-gestation. (Adapted from Anderson, 2007)

Location	North East	Midwest	California	Midwest	California
Survey period	1960-1970	1968-1972	1985-1989	1980-1989	1998-2003
Production	Dairy	Beef	Dairy	Mixed	Mixed
Total cases	3812	2544	468	8692	2296
Total etiologic diagnoses (%)	888 (23.3%)	899 (35.3%)	213 (45.5%)	2942 (32.8%)	1019 (44.4%)

Table 1.3: Categories of etiologic diagnoses with percentage of diagnosed abortions. Viral components of the BRDC have been shaded (Adapted from Anderson, 2007)

Location	North East	Midwest	California	Midwest	California
Survey period	1960-1970	1968-1972	1985-1989	1980-1989	1998-2003
Sporadic bacteria	33.9%	48.9%	25.3%		24.7%
Brucellosis	<1%	1.6%	0%		0%
Listeriosis	2%	1.7%	<1%	<1%	<1%
Salmonellosis	<1%	0%	3.3%	4.1%	1.6%
Leptospirosis	10.7%	0%	4.2%	<1%	5.9%
Campylobacter	10.6%	8.5%	1.8%	2.7%	3.7%
Epizootic bovine abortion	0%	2.4%	3.7%	1.8%	6.5%
Mycotic	21.9%	10%	2.3%	0%	3%
IBR	12.6%	44.9%	8.4%	16.2%	3%
PI3			<1%	<1%	
BVDV	2%	<1%	3.3%	13.8%	4%
Neospora	0%	0%	41.8%	0%	50.9%

IBR is most often associated with infection by bovine herpesvirus 1 (BoHV-1), an alphaherpesvirus. The *Alphaherpesvirinae* subfamily contains viruses characterized by: a large host range, a short replication cycle and the ability to induce latent infection, primarily in neurons (Muylkens et al., 2007). IBR abortions can occur at any gestational age, but most often between 4 and 8 months of gestation. There is an incubation period between primary infection and observed abortion of 15-64 days and abortion is not always preceded by clinical respiratory disease. This is complicated by the ability of the virus to lay dormant, especially within the sensory neurons of the peripheral nervous system and become re-activated by stress (Enquist et al., 1998). Taking advantage of systemic infection through viremia, the virus is able to encounter and cross the placenta, inducing a lethal infection of the fetus (Chow et al., 1964 and Owen et al., 1964). Although the exact mechanism of fetal access is not understood, it most likely arises from the ability of BoHV-1 to infect and replicate within macrophages, thus gaining access to the fetus through the placental crossover of these white blood cells (Owen at al., 1964; Forman et al., 1982). The placenta is also degraded, but this is most likely secondary to the death of the fetus (Molello et al., 1966). In addition to fetal lesion and infection, IBR virus has been isolated from ovarian structures (Bielanski et al., 1993).

In a 1985 study Van der Maaten and Miller infected 12 nulliparous angus heifers with one of two IBR viral isolates the day after breeding by an IBR negative bull. Heifers were exposed to the virus by one of three mechanisms: aerosol, intramuscular injection or intravenous injection. Heifers were evaluated

for clinical signs of an acute respiratory infection and were harvested 12-15 days post inoculation for samples of the reproductive tract. Virus was isolated from ovarian tissue of 7 heifers, primarily from luteal tissue. Two of the heifers had luteal cavities surrounded by hemorrhagic necrosis and with only a small ring of functional luteal tissue on the CL. Two additional heifers' CLs where overtaken by necrosis. Virus was isolated from follicular structures of 3 heifers exhibiting varying degrees of inflammation and abnormal structure. One heifer also exhibited necrosis and inflammation of the ovarian stroma. The aerosol inoculated heifers (4) developed severe respiratory lesions but no ovarian lesions. The virus remained localized to the respiratory tract of these animals and lacked the systemic viremia of the other inoculation routes (Van der Maaten and Miller, 1985). Similar lesions and structural alterations were observed on the ovaries of heifers inoculated with early IBR MLVs (Van der Maaten et al., 1985 and Smith et al., 1990). These alterations in CL development, follicular development, and generalized ovarian necrosis could have long lasting impacts on a heifer's fertility.

Oophoritis, or inflammation of the ovary can be a devastating pathological condition. The ovary itself it a highly vascular tissue, especially during the short time period preceding ovulation. It is through this highly vascular network that pathogens may come into contact with the ovary and any associated structures (Grooms et al., 1998). This inflammation is mediated through the non-specific innate immune system. Macrophages and several cytokines and chemokines play an important role in normal ovarian functions, and any disruption or change

in these concentrations as a result to pathogenic insult could alter the normal ovarian dynamics (Tabibzadeh, 1994). Macrophages in particular are present in both healthy and atretic follicles. When these cells are removed from either CLs or follicles, there was a marked increase in overall follicular atresia and hemorrhage within 16 hours. Following treatment, there was structural damage and necrosis throughout the ovary. Macrophages play an important role in the maintenance of ovarian vasculature (Turner et al., 2011).

Cytokines are a large family of secreted proteins that regulate and coordinate the cells of the innate and adaptive immune systems. Chemokines, a specific subset of cytokines, primarily regulate immune cell recruitment and migration to sites of immunological compromise (Abbas et al., 2015). Some of these mediators play a role in the process of luteolysis, thereby impacting the development and timing of the pre-ovulatory follicle. In a 1999 study, Penny et al. collected and staged CLs from an abattoir as stage I (1-5 days), stage II (6-12 days), stage III (13-18 days) and stage IV (19-21 days) and around natural luteolysis (14-20 days). Both stage IV and natural luteolysis (day 16+) CLs experienced increases numbers of T lymphocytes, although the recruitment mechanism is not understood.

In vitro studies have specifically shown that the inflammatory mediators tumor necrosis factor α (TNF-α) and interferon gamma (IFN-γ) are cytotoxic to bovine luteal cells suggesting they may play a role in in vivo luteolysis (Petroff et al., 2001). These cytokines, along with others, are known to be produced by lymphocytes, indicating their role in functional luteolysis. Additionally,

macrophages, a stationary and circulating phagocytic cell of the innate immune system, were isolated from both stage I and stage IV CLs. Their presence implicates the innate immune system in both early CL development and luteolysis. Most interesting, a significant number of eosinophils, another cell of the innate immune system, were present in stage I CLs, although their function there is not well understood (Penny et al., 1999).

Additionally, the granulosa cells of dominant follicles may play a role in immune function at the ovarian level. These cells express Toll-like receptors (TLR) 2 and 4, which bind bacterial pathogen-associated molecular patterns (PAMPs). When the pathways associated with these receptors are activated, the endocrine function of the granulosa cells is interrupted and inflammatory responses are initiated (Price et al., 2013). This inflammatory signaling would lead to a disruption of cytokine production, recruit cells of the innate immune system, and cascade back to increased inflammation (Abas et al., 2015). Inflammation is an early reaction to immunological challenge. Inflammation can be a protective and effective method of fighting infection, but can also cause long lasting damage to host tissues. If live viruses or viral particles of IBR are encountered within the ovary, it may be this inflammatory reaction to these invaders that not only induces oophritis but could disrupt normal ovarian function.

IBR MLV Implications

In 2004, labels for IBR MLV vaccines were revised to include use in pregnant animals. Prior to these revisions, only killed component vaccines were recommended for use in pregnant animals. In order to ensure the safety of

pregnancy, the new instructions require that an animal have been exposed to the MLV within 12 months prior to this vaccination. If not, there is an increased risk of abortion as a reaction to the vaccine, with fetal material exhibiting lesions similar to an IBR induced abortion. However, a recent trial by the Wyoming State Veterinary Laboratory indicates that this pre-exposure may not be protective against vaccine induced abortion. Fifty-five angus crossbred heifers were vaccinated three times pre-breeding with a BRD complex vaccine that included MLV components. Then, at approximately 7 months of gestation, heifers were re-vaccinated with the same MLV. 8 of these heifers had observed abortions, six of which had diagnostic work that confirmed the presence of IBR in the fetal tissue. Of the remaining 47, 41 calved. This leaves another 6 heifers with a loss of pregnancy (Figure 1.7)

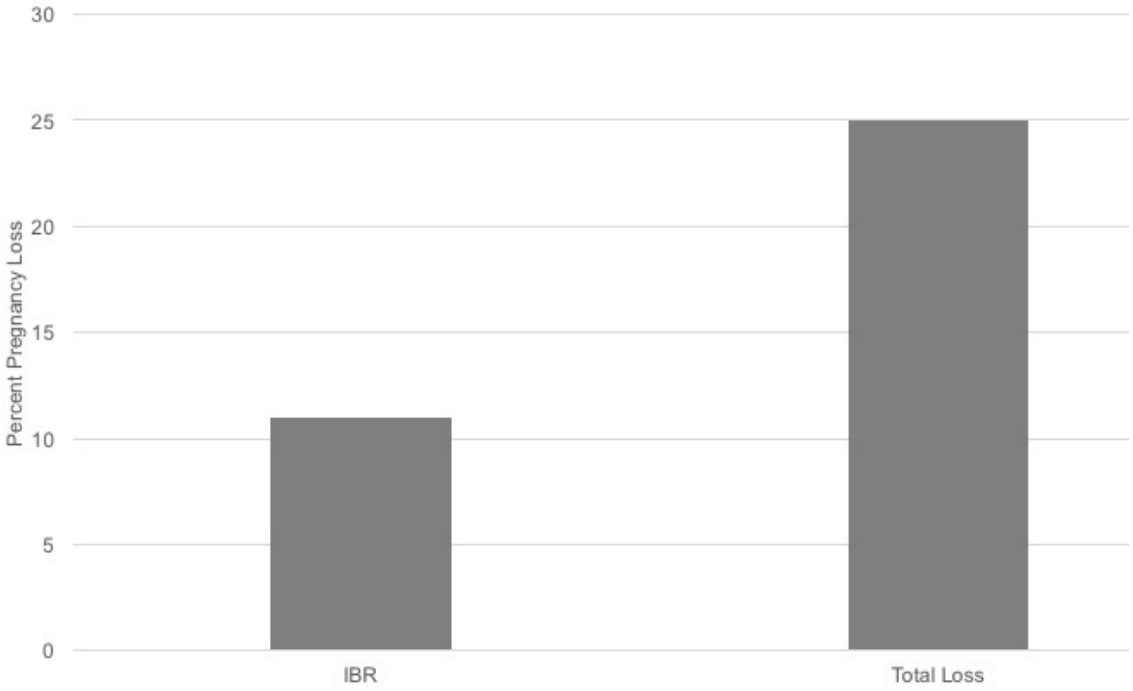

Figure 1.7: Percent pregnancy loss attributed to IBR and the total pregnancy loss for 55 IBR MLV vaccinated beef heifers. (Adapted from O'Toole et al., 2012).

There are implications for pre-breeding vaccinations of virgin heifers as well. Historic and recent research indicates the use of a MLV IBR may have negative impacts on breeding success in these animals. Early versions of the IBR MLV exhibited negative fertility impacts on cycling, virgin heifers (Chiang et al., 1990; Smith et al. 1990; Van der Maaten et al., 1985). In a 1990 study Chiang et al. administered a commercially available IBR MLV intramuscularly to 10 nulliparous Hereford heifers at synchronized estrus. 9 synchronized heifers were not vaccinated as controls. The heifers were then placed with proven bulls for 35 days to allow for 2 estrous cycle for breeding. The heifers did not exhibit any clinical signs of disease and there was no virus isolated from nasal or vaginal swabs. Sixty days after the bulls were removed, rectal palpation indicated a 100% conception rate for the control heifers and a 70% conception rate for the vaccinated group.

Although not statistically different, these result were confirmed by a 100% calving rate for the control group and a 60% calving rate for the treatment heifers. One treatment heifer experienced an apparent abortion after pregnancy confirmation (Chiang et al., 1990). A major implication of this study was that naïve heifers may be negatively impacted by IBR MLVs, especially when administered at estrus. This finding by Chiang and others suggests that heifers be vaccinated with the IBR MLV as young calves, prior to breeding and even prior to puberty as an attempt to bypass this disruption in fertility.

Increases in the understanding of viral replication, infection and interactions with the immune system have allowed for new developments in the

MLV arena. In a more recent study, Perry et al. (2013) utilized 59 crossbred beef heifers to study the impacts of a modern IBR MLV on naïve heifers. Heifers were split into the following groups:

Group 1: initial vaccination 36 days prior to AI with a commercially available inactivated (2-Killed) combination vaccine and re-vaccinated 8 days prior to AI, both subcutaneously

Group 2: vaccinated 8 days prior to AI with the same commercially available inactivated (1-Killed) combination vaccine

Group 3: vaccinated 8 days prior to AI with a commercially available MLV combination vaccine

Group 4: initial vaccination 36 days prior to AI with a sterile diluent (sham) and re-vaccinated 8 days prior to breeding, both subcutaneously

Heifers were estrous synchronized and AI bred at two time points (60 and 72 hours after CIDR removal). They were then exposed to healthy virgin 18-month old bulls for two weeks to complete the breeding season. The percent of abnormal estrous cycle following vaccination can be observed in Figure 1.8.

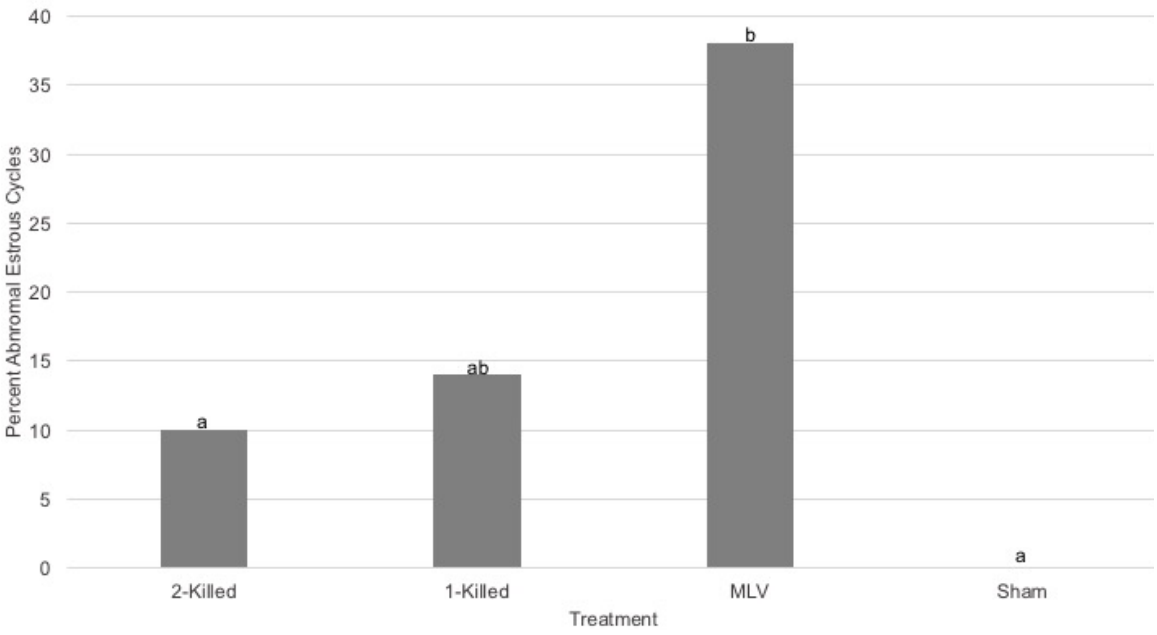

Figure 1.8: Percent abnormal estrous cycles following vaccination. Different letters represent statistically different abnormal estrous cycles. Note that 2-Killed, 1-Killed, and Sham are not different. However, MLV is different from both 2-Killed and Sham, but not 1-Killed. (Adapted from Perry et al., 2013).

MLV vaccinated heifers also experienced a decreased pregnancy rate (48%) when compared to the other groups of heifers (90%) at the completion of the breeding season. This study reaffirmed the need for both previous exposures to the IBR MLV in virgin heifers and that these vaccines should not be administered on top of or too close to anticipated breeding.

The use of BRD complex vaccines that contain modified virus, along with other modified and attenuated viruses, provide animals with a longer lasting and stronger response (Ellis, 1992). The use of IBR MLVs as part of a multivalent vaccine provide rapid protection against viral challenge, in as few as 72 to 96

hours (Fairbanks et al., 2004). This elevated response should provide the animal with more protective immunity, which is an important consideration for producers of all levels. This would mean that neonatal calves could receive increased protection from colostral transfer of these antibodies and any traveling animals would be better protected off-farm. However, questions still remain as to the impact these vaccines can have on heifer fertility.

Objectives of the Study

The objectives of the upcoming study were to examine the impact of a BRDC vaccine with an IBR MLV component on fertility, estrous cycle parameters, AMH concentrations, and the innate inflammatory profile of nulliparous heifers. The first part of the study focuses on the fertility and cyclicity of these heifers. In an attempt to gain a complete picture, heifers were examined for ovarian structures, circulating steroid hormones, vaccine titers and circulating concentrations of AMH. The second piece of the study was used to investigate any differences in the innate inflammatory reaction between the two vaccine types over time and until breeding. This intensive pilot study aimed to scrutinize any differences between ovarian function or fertility of vaccinated dairy heifers.

CHAPTER 2

INFLUENCE OF A BRDC VACCINE WITH A MLV OF KV IBR COMPONENT ON ESTROUS CYCLE PARAMTERS AND ANTI-MÜLLERIAN HORMONE CONCENTRATION IN NULLIPAROUS HEIFERS

Abstract

The objective of this study was to examine the impact of a BRDC vaccine with a MLV IBR component on estrous cycle parameters and the follicular pool. Twenty-four Holstein heifers (mean±SD; 12.4±0.5 months) in two replicates (Spring n=10 and Fall n=14) were synchronized for estrus using a 7-day CIDR protocol with 2 injections of $PGF_{2\alpha}$, one at CIDR removal and a follow-up injection 16 hours later. Heifers were calf-hood vaccinated with an IBR MLV. Heifers were observed for one complete estrous cycle to establish normal cyclicity. At Heat 2, heifers were vaccinated with either the calf-hood MLV (MLV; n=12) or a BRDC vaccine with a killed (K; n=12) IBR component. Heifers were blocked into treatment groups according to pre-vaccination BVDV serum neutralizing (SN) titers. Heifers were then tracked for two complete estrous cycles. Serum samples for E2 and progesterone P4 and ultrasound of ovarian structures were collected to track cyclicity every-other-day. Serum samples for Anti-Müllerian Hormone (AMH) were collected at estrus and mid-cycle to evaluate the follicular pool. Data was normalized with ovulation as day 0. Data were analyzed with the PROC MIXED procedure of SAS with cycle number, season, and vaccine as fixed

effects. The model for P4 analysis added day of cycle as a fixed effect. There was no difference (P>0.05) in post-vaccination titers. Vaccination had no impact on P4 concentrations, P4 area under the curve, luteal tissue area, peak E2 production, or estrous cycle lengths (P>0.05). Overall variables that affected AMH concentrations were: season (Spring= 138.92 ± 43.1 pg/mL, P=0.0043), vaccine type (MLV= -92.4 ± 42.9 pg/mL, P=0.0435), and cycle number (P<0.0001). AMH concentration decreased between cycles 1 and 2 and cycles 1 and 3 for MLV vaccinated heifers (P<0.0003). AMH concentrations of cycle 2 were numerically lower between vaccine types (K=308.22±33.3 pg/mL, MLV=181.13 ± 32.9 pg/mL; P=0.0953) though not statistically different. This may be due to low animal numbers, the variability between animals, or the differences observed in the Fall killed vaccine (-70.93 ± 21.1 pg/mL, P=0.0145) from cycle 1 to 2, but not in the Spring killed vaccine (3.40 ± 45.1 pg/mL, P=0.9969). AMH was weakly correlated with small follicle count (r^2=0.15, P<0.0001). Although no differences were seen in overall cycle parameters, these differences in AMH concentrations may indicate a reduction of the follicular pool as a result of vaccination with an IBR MLV.

Introduction

Maintaining an adequate and appropriate vaccination program against the bovine respiratory disease complex (BRDC) is vital to the reproductive health of a dairy farm. Not only can these pathogens induce systematic respiratory infection, but they are also associated with mid- to late-gestation abortion in pregnant animals (Anderson, 2007). Until 2004, only BRDC vaccines with Killed

components were recommended for use in pregnant animals, as previous versions of the MLV components were implicated as a cause of abortion, with particular focus on the IBR MLVs (Kirkbride at al., 1973). These modern IBR MLVs have been associated with decreased fertility, inducing both abnormal estrous cycle lengths and reduced pregnancy rates when compared to Killed (K) and sham vaccinated heifers (Perry et al., 2013). Based on these findings, recommendations on vaccine packaging have changed again to advise a pre-breeding vaccination of at least 60 days (Zoetis, 2015).

Both the IBR virus itself and the IBR MLV have a longstanding history as a causative agent of abnormal cyclicity in cattle. Both live virus IBR MLVs have been associated with necrotizing oophritis of the ovarian stroma, hemorrhagic necrosis of the CL, and inflammation of antral follicles (Van der Maaten and Miller, 1985). These alterations in the normal structure associated with inflammation may have implications for the function of these ovarian structures. With this inflammation there is the possibility and probability for recruitment of cells of the innate immune system to the ovary and associated structures. These cells can be damaging to surrounding host tissues when they engage and engulf cells identified as non-self; particularly neutrophils when they release an oxidative burst (Nathan, 2006). Additionally, macrophages, a circulating white blood cell, are important in the maintenance of ovarian vasculature. Disrupting this population of immune cells induces a marked increase in follicular atresia and hemorrhage (Turner at al., 2011). Vaccination on top of breeding has shown

delayed and altered luteinization during the first week post estrus (Spire et al., 1996).

Though not well studied, there may be implications for the long-term development of follicles as well. In a study of 59 synchronized beef heifers, 38% of the MLV vaccinated heifers experienced an abnormal estrous cycle following vaccination. Although these heifers returned to normal, at the conclusion of the breeding season they still experienced a significantly lower pregnancy rate (48% versus 90%), indicating that there may have been damage or alterations to the follicular pool (Perry et al., 2013). One novel tool in the study of the gonadotropin sensitive follicular pool is Anti-Müllerian Hormone (AMH). This glycoprotein is a part of the transforming growth factor (TGF)-β family, the same as another follicular product, inhibin (Cate et al., 1986). Granulosa cells of all stages have the capability to produce AMH, but its production is most often associated with the granulosa cells of primary follicles (Weene at al., 2004). In heifers, circulating AMH is associated with these small follicles, which be available for deviation in approximately 60 days (Ireland et al., 2008; Lussier et al., 1987). Historically AMH has been best characterized as a predictor for super ovulation response (Ireland et al., 2008; Rico et al., 2009). Thus, changes in the circulating concentrations of AMH may be indicative of a reduction in the viability of the follicular pool.

The objectives of this study were to 1) determine the impact of vaccination with a MLV or K IBR component vaccine on basic cycle parameters and pregnancy, 2) to establish if there was a change in the steroid production and

size of ovarian structures (CL and follicle) after vaccination, and 3) to investigate the effect of vaccination on the gonadotropin sensitive follicular pool through changes in circulating AMH. It was hypothesized that the MLV vaccinated heifers would exhibit more abnormal estrous cycle post-vaccination, with decreased P4 and E2 production, and have lower circulating AMH, indicating an alteration to the follicular pool.

Materials and Methods

Animal Management

Twenty-four virgin Holstein heifers 12.4 ± 0.5 months of age and in two replicates (Spring n=10, Fall n=16) were selected for enrollment in the trial. The heifers were born and raised on the University of Georgia Teaching Dairy in Athens, Georgia. The heifers were housed on pasture and fed a heifer TMR designed to meet their maintenance, growth, and reproductive needs once daily. All heifers were calf-hood (5 to 6 months of age) vaccinated with Cattlemaster Gold 5 LP5.

Estrous synchronization

Heifers where synchronized in the following manner using a standard CIDR-synch protocol. On D 0 each heifer received an intravaginal CIDR (EAZI-BREED CIDR, Zoetis, Florham Park, NJ) insert designed to release progesterone for the synchronization of the estrous cycle. On D 7, CIDRS were removed and examined for the presences of purulent discharge or foul odor, indicators of possible reproductive tract irritations or infections, with any noted. At CIDR removal, each heifer was administered 25mg of $PGF_{2\alpha}$ (Luatlyse, Zoetis,

Florham Park, NJ) and a heat mount detector (Estrotect, Hermitage, TN) was applied. Approximately 16 hours after the initial injection, a second dose of $PGF_{2\alpha}$ was administered in an attempt to remove variability in the response time. Heifers were then observed for estrus (Heat 1).

Vaccination

At approximately the second observed estrus post CIDR removal (Heat 2), heifers were sub-cutaneously vaccinated with either a commercially available IBR MLV (Cattlemaster Gold 5 LP5) or Killed (Triangle 10; K) vaccine. These vaccines both contained parainfluenza$_3$, bovine respiratory syncytial virus, bovine viral diarrhea virus types 1 and 2, IBR, and 5 *Leptospira* species. The Killed IBR in Traingle 10 was the same strain and the MLV IBR in the Cattlemaster. Figure 2.1 provides a basic timeline of the experimental protocol.

Estrus and breeding determination

Heat mount detectors were applied to each heifer to aid in the detection of estrus. Animals were observed at least three times daily during heats 1-3 for 20 minutes. Detectors were recorded as “No,” “1/4,” “1/2,” “3/4,” or “All” rubbed at each heat observation. A heifer was recorded as in estrus when there was both an observation of the animal standing to be ridden by a herd mate and the presence of a heat mount detector with $\geq$75% of the silver coating removed.

For the breeding heat (Heat 4) animals were observed at specific time points; 6am, 2pm and 6pm. Animals were bred according to the timeframes outline in table 2.1.

Table 2.1: The approximate breeding time for each heat observation during Heat 4, the breeding heat.

Time observed standing	Approximate time bred
6 am (suspect standing overnight)	9:30 am on the same day
6 am – 2 pm	6 pm on the same day
2 pm – 6 pm	7 am the next day

All animals were mated bred via artificial insemination by experienced inseminators employed by the UGA Teaching Dairy.

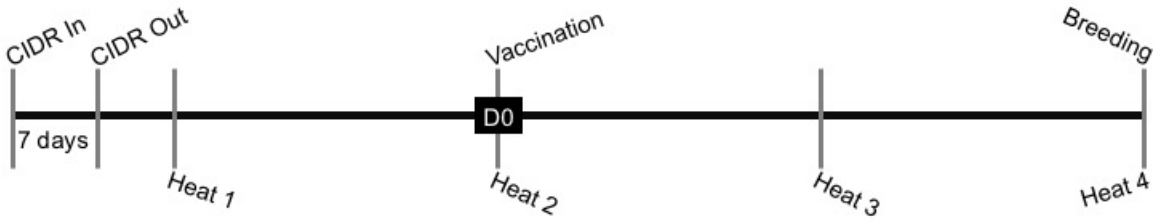

Figure 2.1: Experimental timeline of events.

Serum Neutralizing Titers

Utilizing vaccination as D0, samples for BoHV-1 and BVDV-1 titers were collected both pre and post vaccination. Samples were collected D-7 for Spring as heifers were randomly assigned a vaccine treatment. However, in an attempt to balance the study for pre-vaccination titers to BVDV-1, samples were collected on D-11 for Fall heifers (n=16) to allow the lab to return results prior to vaccination. Post vaccination titers were collected on D+22 in order to capture the peak antibody production in response to this booster vaccination. Blood was collected via coccygeal venipuncture into vacutainer tubes containing no additives. Samples were then transported on ice back to the lab and allowed to

clot in a 4ºC refrigerator overnight. The following morning, the tubes were shaken to release the clot and the samples were centrifuged at 3500 rpm for 15 minutes. Approximately 1mL of serum was pipetted into a clean and labeled microfuge tube for each animal. Samples were then heat inactivated in a 56ºC water bath for 30 minutes. After heat inactivation, samples were allowed to return to room temperature and then frozen at -20ºC for future analysis by the Athens Veterinary Diagnostic Lab.

Because IBR tends to induce a weak antibody response and is not sensitive to boostering, BVDV-1 titers were used as a monitor of vaccine response. BVDV-1 is very sensitive to boostering, controlled almost entirely by antibody production and the vaccines themselves are made to induce a high level of response.

Progesterone, Estrogen, Ultrasound, and AMH

Beginning 2 days after CIDR removal, heifers were evaluated for ovarian structures via trans rectal ultrasound every-other-day (Figure 2.2). During each scan, ovarian maps were created for both the left and right ovaries to track the development of ovarian structures over time. All follicles 5mm in diameter and larger were measured and mapped and any follicles under 5mm were counted and recorded as a total number, not individually placed. Additionally, the corpus luteum was measured, both total size and the cavity, if present. Each structure was measured twice to ensure accuracy. Maps were stored for future evaluation. Commensurate with each ultrasound, blood was collected via coccygeal

venipuncture into vacutainer tubes containing no additives for progesterone and estradiol analysis. Serum was collected for AMH analysis every fifth day.

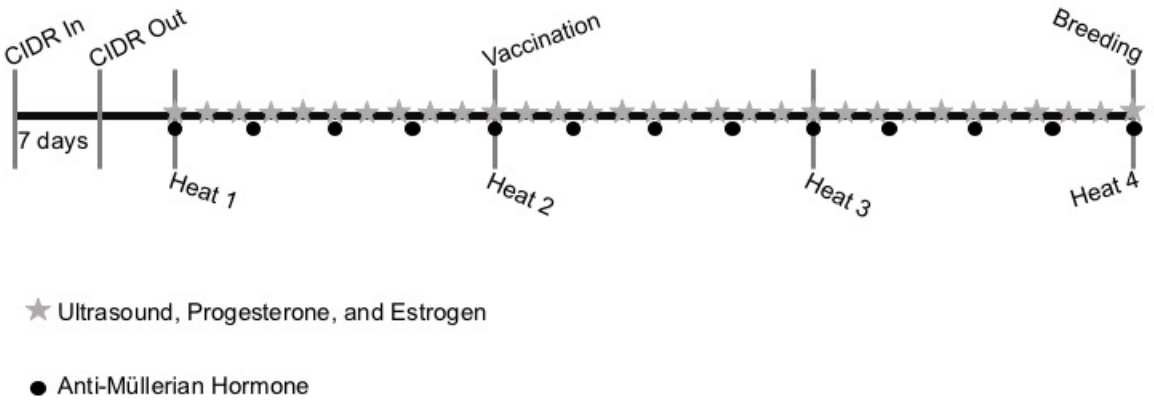

Figure 2.2: The approximate sampling timeline for reproductive parameters. The stars represent every-other-day ultrasound and sample collection for P4 and E2. The black circles represent every fifth day sample collection for AMH.

Blood Processing and Storage for Hormone Analysis

Blood samples were kept cool and transported to the lab on ice. They were then placed in a 4ºC overnight to clot. The next morning, blood was centrifuged at 3500 rpm for 15 minutes at 4ºC. Serum was transferred to a storage tube labeled with the heifer number and sample date and frozen at -20°C for future analysis.

Hormone Analysis

Radioimmunoassay for P4 and E2 concentrations in serum samples were performed by an outside laboratory that used the kits produced by MP Biomedicals (Solon, OH). The P4 was performed according to kit instructions.

The E2 analysis did involve an ether extraction step to increase the reliability of the assay. For the ether extraction, 1mL of serum was pipetted into a borosilicate glass tube. Then, 2mL of ether, anhydrous baker analyzed A.C.S. reagent was added and the tube was capped and shaken for 10 minutes. Tubes were then frozen to -20°C for 2-3 hours, until the serum was solidly frozen. The ether supernatant was poured into a 12x75mm culture tube and placed into a Thermolyne Dr-Bath set to 45°C to evaporate the ether.

Frozen serum was shipped to and analyzed for AMH by MOFA Global (Mount Horeb, WI) utilizing their developed enzyme-linked immunosorbent assay for AMH analysis.

Statistical Analysis

Data was normalized with day ovulation as day 0. Data were analyzed with the PROC MIXED procedure of SAS (Cary, NC) as a repeated measure with the following fixed effects: cycle number, season, vaccine type and day-of-cycle.

Results

SN Vaccine Titers

There was no difference in pre-vaccination or post-vaccination titers for IBR or BVDV for the MLV or K vaccine, as observed in Table 2.2 ($P>0.05$).

Table 2.2: Pre- and post-vaccination SN Titers for MLV and K vaccinated heifers.

	MLV (mean ± SE)	K (mean ± SE)	P Value
IBR Pre-Vac	7.14 ± 2.10	1.75 ± 2.15	0.0871
IBR Post-Vac	99.43 ± 19.87	80.80 ± 20.36	0.5194
BVDV Pre-Vac	87.52 ± 44.00	56.20 ± 45.09	0.6240
BVDV Post-Vac	432 ± 38.68	378 ± 39.63	0.3401

There were differences in pre-vaccination titers for IBR and post-vaccination titers for BVDV by season, show in Table 2.3 (P<0.01)

Table 2.3: Pre- and post-vaccination SN Titers for Spring 2015 and Fall 2015 replicates.

	Spring (mean ± SE)	Fall (mean ± SE)	P Value
IBR Pre-Vac	0 ± 2.29	8.89 ± 1.96	0.0073
IBR Post-Vac	60.8 ± 21.63	119.43 ± 18.48	0.0513
BVDV Pre-Vac	9.86 ± 47.89	133.86 ± 40.94	0.0618
BVDV Post-Vac	298.00 ± 42.10	512.00 ± 35.98	0.0008

Estrous Cycle Length and Pregnancy

Because of a high level of variability observed in the amount and duration of estrous activity in the heifers, ovulation was used as D0 for all reproductive cycle parameters. There were no differences between estrous cycle lengths by vaccine, by replicate, within vaccine or within replicate (Table 2.4, P>0.1). There was no difference in first service conception rate between vaccines, although the MLV vaccinated heifers were numerically higher at 66.67% when compared to 41.67% for the K group (Figure 2.3, P>0.1). There was no difference in first service conception rates between seasons, but the Fall replicate was numerically lower at 42.86% than the Spring replicate at 70.00% (Figure 2.4, P>0.1)

Table 2.4: Estrous cycle lengths, in days, by vaccine and replicate.

	Spring (mean ± SE)	Fall (mean ± SE)	P Value	MLV (mean ± SE)	K(mean ± SE)	P Value
Cycle						
1	20.80 ± 0.66	19.50 ± 0.56	0.1488	20.39 ± 0.61	19.91 ± 0.61	0.5921
2	21.90 ± 0.58	20.57 ± 0.49	0.0964	21.40 ± 0.54	21.07 ± 0.54	0.6707
3	21.40 ± 0.65	20.42 ± 0.55	0.2666	21.16 ± 0.60	20.67 ± 0.60	0.5741
	P > 0.1	P > 0.1		P > 0.1	P > 0.1	

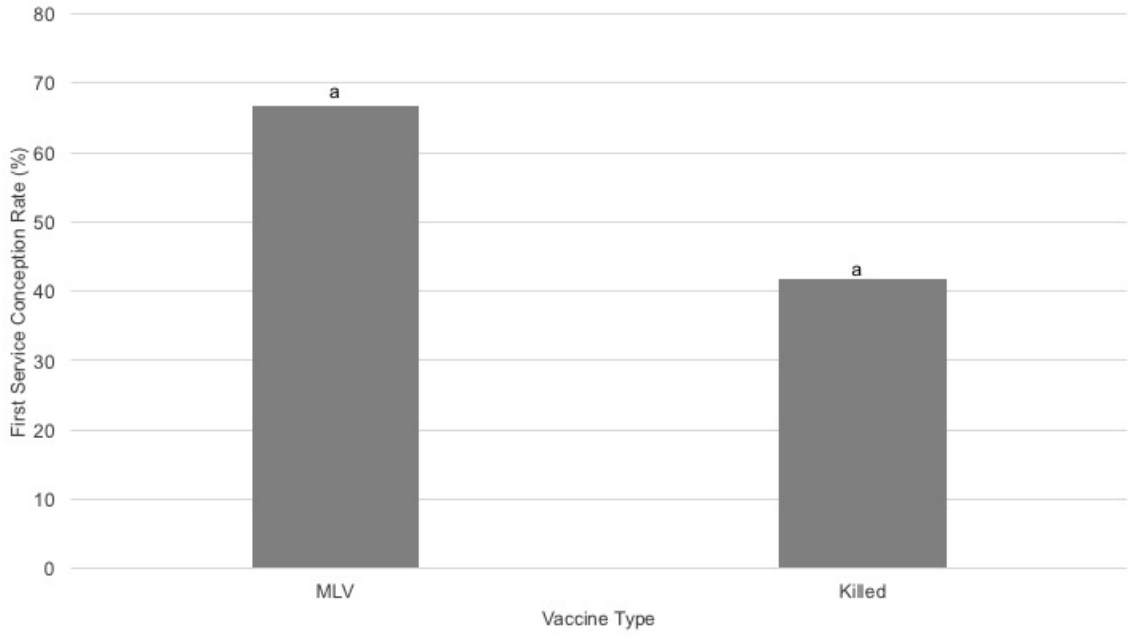

Figure 2.3: First service conception rates by vaccine type (P=0.2191).

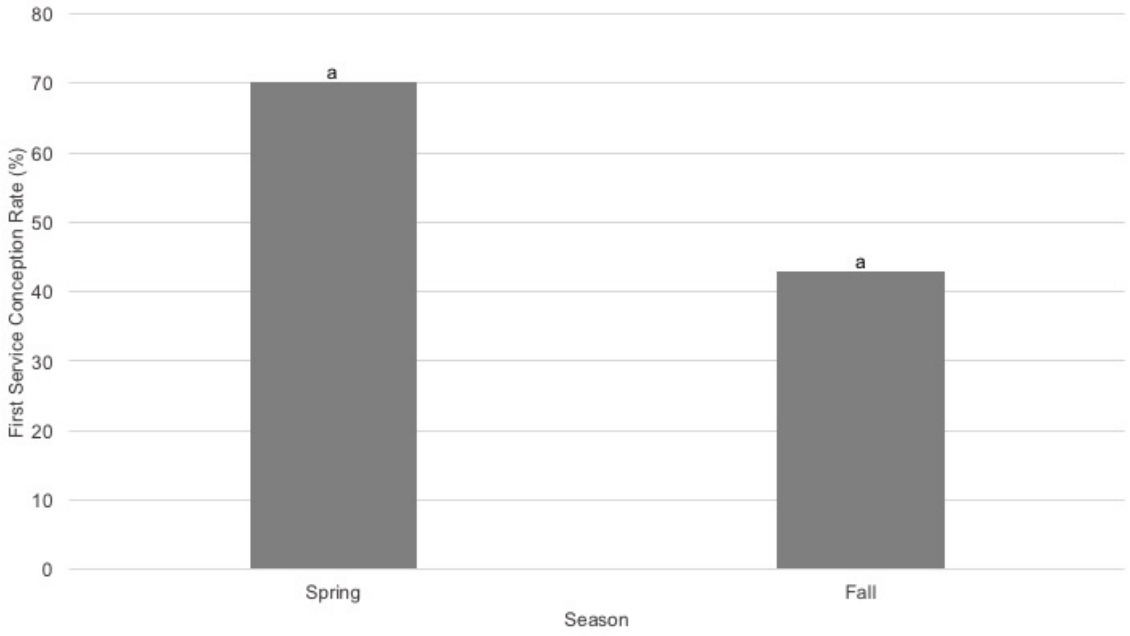

Figure 2.4: First service conception rates by season (P=0.1883).

Luteal Tissue Are and Progesterone Production

There was no difference in luteal tissue area between vaccines or within vaccines, both by cycle (Figure 2.5, P>0.1). Progesterone concentrations were analyzed as an area under the curve. There was no difference in progesterone under the curve between vaccines or between cycle within vaccine, as observed in Table 2.5 (P>0.1). The Fall replicate, regardless of vaccine type, had a significantly lower area under the curve in Cycle 1 than the Spring, as seen in Table 2.6 (P=0.0346). However, there were no differences in subsequent cycles or between the cycles within each season (P>01).

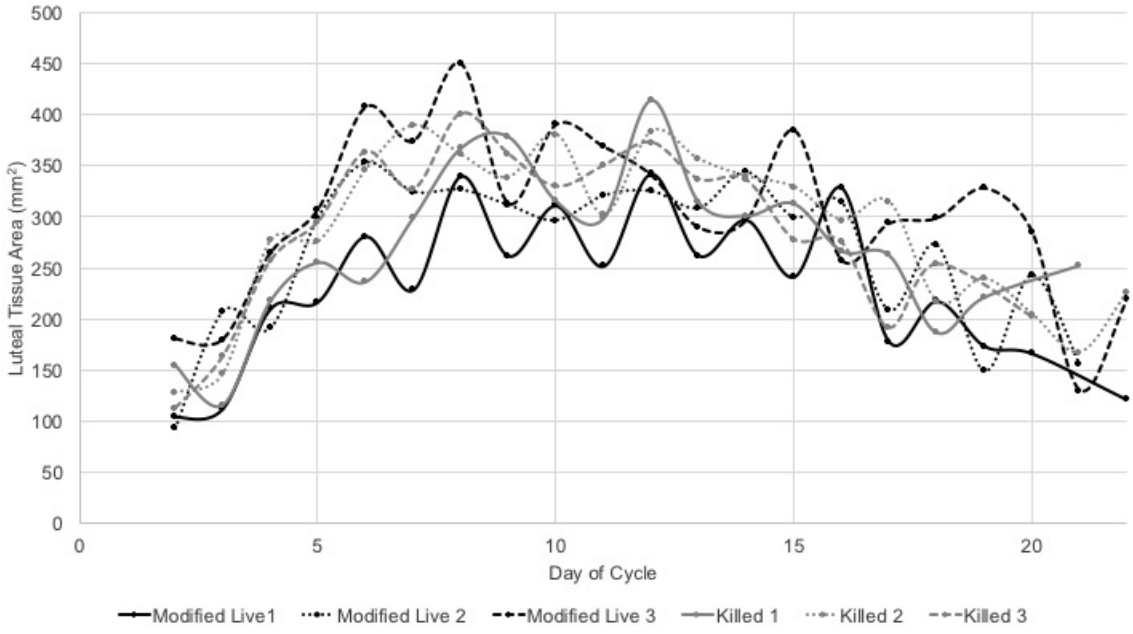

Figure 2.5: Luteal tissue are by vaccine and cycle (P>0.1).

Table 2.5: Progesterone under the curve for MLV and K vaccinated heifers, by cycle.

	Modified Live (mean ± SE)	Killed (mean ± SE)	Difference (mean ± SE)	P Value
Cycle				
1	71.96 ± 7.20	66.14 ± 7.65	5.82 ± 10.51	0.9934
2	69.72 ± 7.20	74.80 ± 7.65	-5.08 ± 10.51	0.9965
3	79.01 ± 7.20	71.47 ± 7.65	7.54 ± 10.51	0.9786
	P > 0.1	P > 0.1		

Table 2.6: Progesterone under the curve for the Spring and Fall replicates.

	Fall (mean ± SE)	Spring (mean ± SE)	Difference (mean ± SE)	P Value
Cycle				
1	52.54 ± 6.82	85.56 ± 7.99	-33.02 ± 10.51	0.0346
2	65.28 ± 6.82	79.24 ± 7.99	-13.97 ± 10.51	0.7672
3	75.07 ± 6.82	75.41 ± 7.99	-0.34 ± 10.51	1.000
	P>0.1	P>0.1		

Follicular Size and Estrogen Production

There was no difference in peak estrogen production or the size of the pre-ovulatory follicle between vaccine types (P>0.05).

Table 2.7: Type 3 tests for fixed effect for estrogen

Season	Vaccine	Follicle Size	Cycle
0.1458	0.0870	0.4044	0.9256

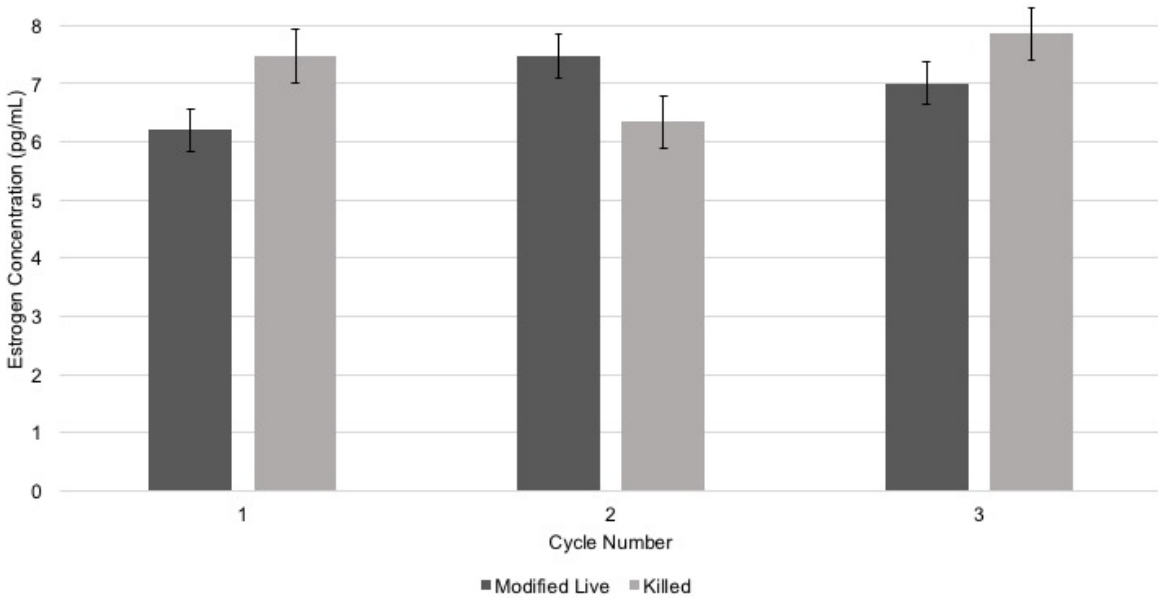

Figure 2.6: Average estrogen concentrations between vaccine types, by cycle (P>0.1).

Anti-Müllerian Hormone

Every-fifth-day samples for AMH collected during the observational cycle were evaluated for any apparent variability within the cycle (Figure 2.7). Noticing a peak concentration at approximately estrus and a lower amount at

approximately mid cycle, the samples closest to these points were chosen for Cycles 2 and 3 for analysis. There was no difference between the Estrus and Mid-cycle AMH concentrations (Figure 2.8, P > 0.1).

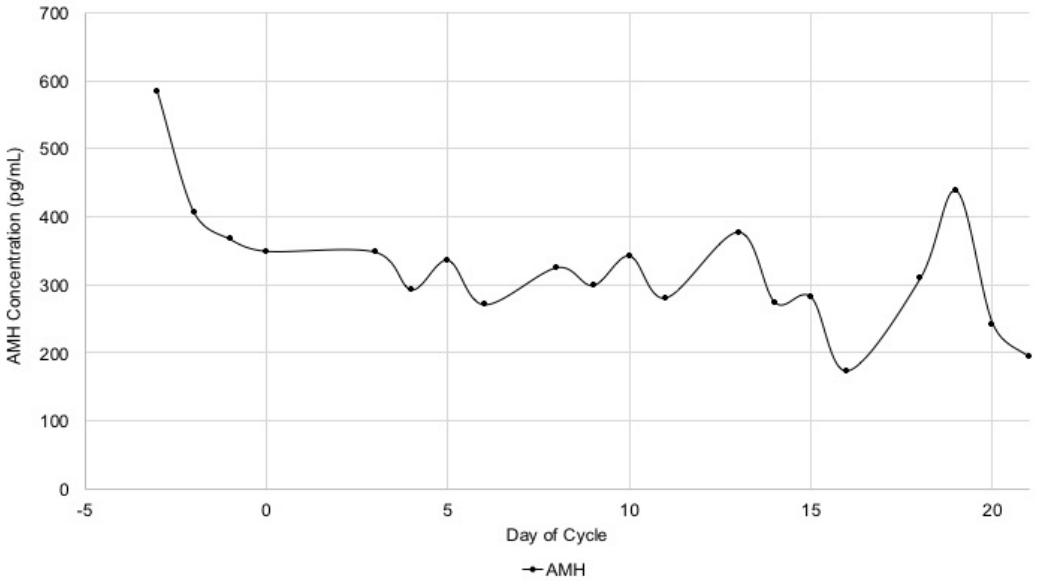

Figure 2.7: AMH concentration for the observational (first) cycle by day of cycle with ovulation as Day 0.

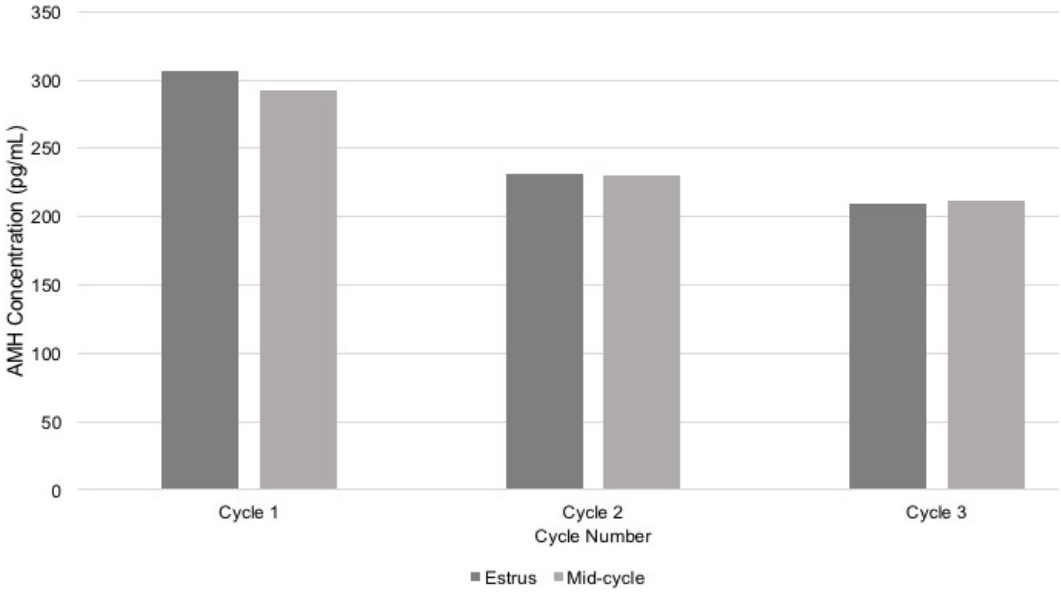

Figure 2.8: Circulating AMH values at Estrus and Mid-cycle for each cycle (P>0.1).

With no differences within cycle, these value (Estrus and Mid-cycle) were averaged for analysis. There were no differences between Cycle 1 AMH concentrations (P>0.1). Visualized in Figure 2.9, the Spring MLV, Fall MLV, and Fall K groups the AMH concentrations for both Cycles 2 and 3 were significantly lower that for Cycle 1 (P<0.02). For the same groups, Cycles 2 and 3 were not different (P>0.1). There were no differences between any of the cycles for the Spring K group (P>0.1).

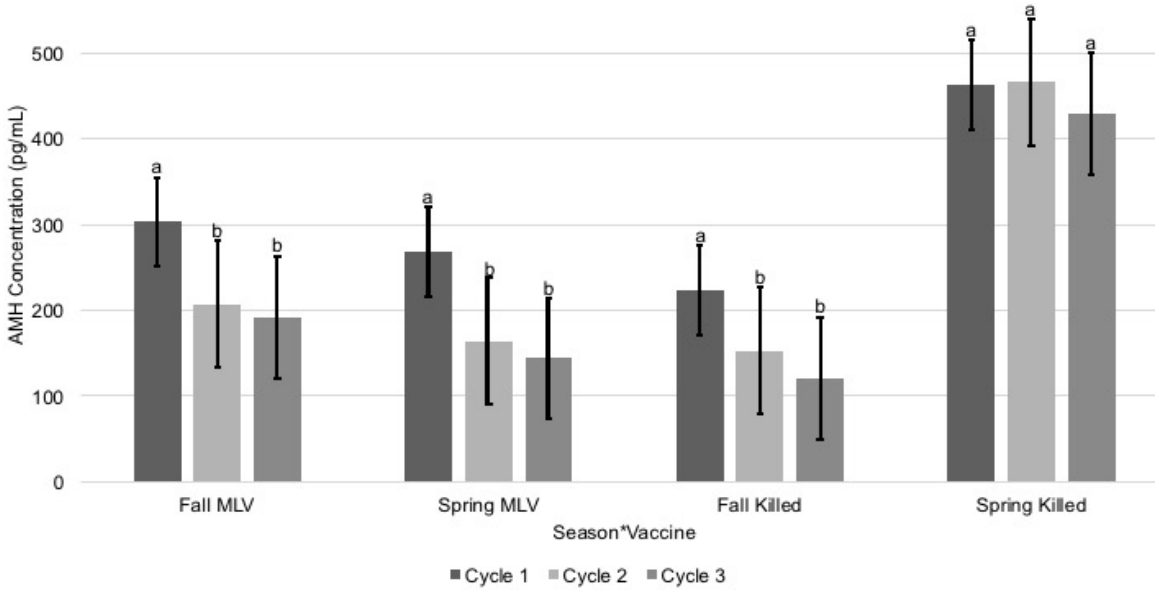

Figure 2.9: AMH concentrations for each cycle by season and vaccine. There were no differences between Cycle 1 concentrations.

Discussion

These findings are inconsistent with previous work (Chiang et al., 1990; Perry et al., 2013). In the Perry study, 38% of the MLV vaccinated heifers experienced an abnormal estrous cycle (less than 15 days) during the first cycle

post-vaccination. In the current study, there were no alterations in peak E2 production by the pre-ovulatory follicle or P4 production by the CL, and thus there were no vast alterations in cyclcity. Finally, there were also no difference in first service conception rates between vaccine types, although this could be attributed to low animal numbers and the accuracy of the heat detection and breeding program. A major difference between these studies is that all of the animals in the current study were calf-hood vaccinated with a BRDC vaccine with a MLV IBR component. Most previous work has used samples of naïve heifers. Since this study used heifers with adequate previous exposure, it stands to reason that the effects of vaccination would be different than those seen in a naïve group.

In a previous work, seropositive heifers were vaccinated with an IBR MLV and no lesions were found on the ovarian tissue of animals. However, there was evidence for delayed or altered CL development post-vaccination, as blood samples obtained after estrus (6-7 d) where below 2 ng/mL (Spire et al., 1996). This proposed delay in P4 production was not observed in the present study, as all animals had at least 2 ng/mL of circulating P4 by d 6-7 in both treatment cycles. However, in the cycle immediately following vaccination (Cycle 2) there was an observed increase in the number of CLs with a cavity, or lumen. In fact, 2 heifers did not fully complete luteinization by the subsequent estrus. In addition to the overall higher rate of lumens, any heifers exhibited lumens with an odd, almost lobular shape. Although these lumens did not significantly alter P4 production in this study, it is still important to note that CL heterogeneity and P4 production are highly correlated (Siqueira et al., 2009 and Mann, 2009).

It takes approximately 3 estrous cycles for a bovine follicle to grow from 0.13 mm to deviation (Lussier et al., 1987). Although the granulosa cells of all stages of follicular development can produce AMH (Vigier et al., 1984). AMH expression increases until the follicle reaches 4mm in diameter, and then begins to taper off (Weenen et al., 2004). In the current study, AMH concentrations decreased after vaccination with a MLV for both replicates and a K for the Fall replicate. This decrease in AMH concentration was not seen for the Spring K group, and they appeared to be an overall higher AMH producing group. This elevated AMH production is because of the presence of 2 heifers in this group that were extraordinary in both the number of small follicles present on their ovaries during ultrasound and circulating AMH concentrations. By the conclusion of the study, approximately 40 days after vaccination, AMH levels had not returned to pre-vaccination levels. This sustained decrease in circulating AMH may indicate a reduction in the viability of the gonadotropin sensitive follicular pool as a result of vaccination.

CHAPTER 3

IMPACT OF A BRDC VACCINE WITH A MLV OR KV IBR COMPONENT ON THE INNATE INFLAMMATORY PROFILE OF NULLIPAROUS HEIFERS

Abstract

To investigate the difference in the inflammatory response between bovine respiratory disease complex (BRDC) vaccines containing either a modified live vaccine (MLV) or a killed component for infectious bovine rhinotracheitis (IBR), 28 Holstein heifers (mean ± SD; 12.4 ± 0.5 months) in two replicates (Spring n=12 and Fall n=16) were synchronized for estrus using a 7-day CIDR protocol. This protocol included 2 injections of $PGF_{2\alpha}$, one at CIDR removal and a follow-up injection 16 hours later. All animals were calf-hood vaccinated with an available BRDC vaccine with a modified live IBR component. At approximately Heat 2, heifers were vaccinated with either the calf-hood MLV (n=14) or a BRDC vaccine with a killed (n=14) IBR component. Heifers were vaccination blocked according to pre-vaccination bovine viral diarrhea virus (BVDV) serum neutralizing (SN) titers. On day -7, relative to vaccination, a complete blood count (CBC) and an assay to measure neutrophil activity, as indicated by the relative presence of reactive oxygen species (ROS) were performed to establish a baseline immune profile. Two heifers were removed from the trial for pre-existing immunological challenge. These assays were repeated on day 1 post-vaccination, day 3 post-vaccination and then weekly until the heifer was bred (Heat 4). Data were

analyzed with the PROC MIXED procedure of SAS. The fixed effects for the model were: season, vaccine type and week relative to vaccination. There was no difference (P>0.05) in post-vaccination SN titers. Vaccine type had no significant effect (P>0.05) on any of the cell types measured by the CBC. Season did have a significant effect (P=0.0008) on the circulating lymphocytes, with the Fall heifers exhibiting higher average lymphocytes on day 1 and weeks 2, 3, 4, 5, and 6 post-vaccination. The ROS response ratio was not impacted by vaccine (P>0.05) but was influenced (P<0.0001) by both season and the season*week interaction. When comparing seasons, Spring heifers maintained a higher average response ratio when compared to the Fall heifers on Day 3 and weeks 1, 2, 3, 5, and 6 post-vaccination, which is substantiated by their higher average circulating granulocytes (P<0.05). These seasonal differences may be a consequence the severe immunological challenge experienced by the Fall group shortly after vaccination which may have obscured their true vaccine response.

Introduction

All vaccination programs have a singular goal: to stimulate a sufficient and long-term memory immunity to a pathogen. To accomplish these goals there are two types of vaccines, modified live vaccines (MLV) and killed (K). MLVs are exactly as they sound, they represent a live version of the pathogen of interest that has been altered to prevent the virus from inducing symptoms of the disease. Conversely, a K virus is a non-replicating whole virus and a mixture of cellular components from culture. K vaccines are usually combined with an

adjuvant which helps stimulate the immune response as the antigens used in K vaccines tend to be poorly immunogenic (Coffman et al., 2010).

For either vaccine to be successful, they must stimulate signs of danger in the vaccinated animal. For a MLV, this is accomplished through the low-level infection itself. These vaccines cause local damage to the injection site and then begin to replicate within host cells, producing the necessary antigen for presentation to the adaptive immune system. For a K vaccine, the necessary antigens are trapped in a matrix to be released over time while the adjuvant induces local damage to cause signs of danger. However, since there is no opportunity for replication or building of the virus within the host, it is this limited period of damage and danger that limits the response to K vaccines.

There are advantages and disadvantages to both MLV and K vaccination. The primary advantage of MLV is the rapid and long-lived immunological memory induced by these vaccines. In as little as 72 to 96 hours, an IBR MLV, as part of a multivalent vaccine provided rapid protection against an IBR challenge in calves (Fairbanks et al., 2004). However, there is always the risk, though small, that the MLV will mutate and regain its full infectious capabilities (Meeusen et al., 2007). They are generally mild, and reproduce similar immune protection as recovering from the disease itself. The primary advantage of K vaccines is that there are some pathogens that a MLV has simply not been successfully developed for or they are not practical. Additionally, K vaccines are much more stable to store and do not require mixing of wet and freeze-dried parts to make the complete vaccine (Meeusen et al., 2007). But, they do lack the extended

protection from disease often observed in MLV counterparts and effectiveness can be highly dependent on strain (Patel, 2005). Both of these vaccine types work by stimulating both the non-specific innate and the highly specific adaptive immune systems.

Although the two types of immunity are often thought of as separate entities, there is a large amount of communication between them. When antigen is discovered, the innate immune system reacts first, recognizing a pathogen as non-self and making an attempt to clear it. The actions of this system often have the purposeful side effect of inflammation. Inflammation increases blood flow to the area of attack, allowing for the infiltration of more cells. However, this inflammation can also cause damage to host tissues (Abbas et al., 2015). In addition to clearing the antigen, these innate immune cells also activate and communicate with the adaptive immune system. These cells include monocytes and granulocytes, particularly neutrophils.

Monocytes are an encompassing term for a variety of mononuclear phagocytes of the innate immune system (Wiktor-Jedrzejczak and Gordon, 1996). Of important note for vaccination are the macrophages and dendritic cells (DC). These cells function to present antigen to antigen-specific T-lymphocytes of the adaptive immune system assisting with the development of memory to a novel pathogen or the activation of memory against a previously seen pathogen (Geissmann et al., 2003). Thus, adequate stimulation of the innate immune system is a key step in the ability of a vaccine to provide immunity. Additionally, macrophages have important function as surveyors of the body, they are present

in nearly every tissue and play important roles in both the onset of inflammation and the return to homeostasis (Abbas et al., 2015).

Neutrophils are an abundant circulating white blood cell. They play a significant role in host defense, particularly in defense against bacterial and fungal infections (Nathan, 2006). These granulocytes also maintain a functional position in the development of the inflammatory response, but have long been considered to be an unsophisticated cell (Mocsai, 2013). Their primary weapon is the release of an oxidative burst, in conjunction with phagocytosis of the pathogen (Leto and Geiszt, 2006). Historically considered an effector cell with little influence on the adaptive immune system, recent works have found that activated neutrophils play a major role as a source of B-cell stimulating substances and may even mediate T-cell independent antibody response (Puga et al., 2011). Thus, these cells may play a key role in the stimulation of both the antibody producing and memory B-cells.

The objective of this study was to investigate the differences in innate inflammatory and immune responses between a commercially available BRDC vaccine with either a MLV or K IBR component. In order to evaluate the following parameters were examined 1) the total circulating white blood cells and their relative concentrations, 2) the activity level of circulating neutrophils as indicated by the presence of reactive oxygen species (ROS) and 3) utilizing serum neutralizing (SN) vaccine titers to indicate the adaptive immune response. It was hypothesized that the MLV vaccinated heifers would experience an elevated and sustained inflammatory response when compared to the K vaccinated heifers.

Materials and Methods

Animal Management

26 Holstein heifers 12.4 ± 0.5 months of age and in two replicates (Spring n=10, Fall n=16) were selected for enrollment in the trial. The heifers were born and raised on the University of Georgia Teaching Dairy in Athens Georgia. The heifers were housed on pasture and fed a heifer TMR designed to meet their maintenance, growth, and reproductive needs once daily. All heifers were calf-hood (5 to 6 months of age) vaccinated with Cattlemaster Gold 5 LP5. Animals were synchronized for estrus using a 7-day CIDR protocol with 2 injections of $PGF_{2\alpha}$, one at CIDR removal and a follow-up injection 16 hours later.

Experimental Design

At approximately 12.4 months of age, animals were synchronized for estrus using a 7-day CIDR protocol with 2 injections of $PGF_{2\alpha}$, one at CIDR removal and a follow-up injection 16 hours later. At CIDR removal, a heat mount detector was applied to each heifer and they were observed for estrus (Heat 1). At approximately the second estrus after CIDR removal (Heat 2), heifers were vaccinated with either Cattlemaster 5 LP5 or a commercially available BRDC vaccine with a Killed (K) IBR component (Figure 3.1). Treatment groups were blocked based on pre-vaccination BVDV titers. The heifers' immunological profile was then tracked until the week of breeding.

Vaccination

At approximately Heat 2, heifers were sub-cutaneously vaccinated with either a commercially available IBR MLV (Cattlemaster Gold 5 LP5) or Killed (Triangle 10) vaccine. These vaccines both contained parainfluenza$_3$, bovine respiratory syncytial virus, bovine viral diarrhea virus types 1 and 2, IBR, and 5 *Leptospira* species. The Killed IBR in Traingle 10 was the same strain and the MLV IBR in the Cattlemaster. Figure 3.1 provides a basic timeline of the experimental protocol.

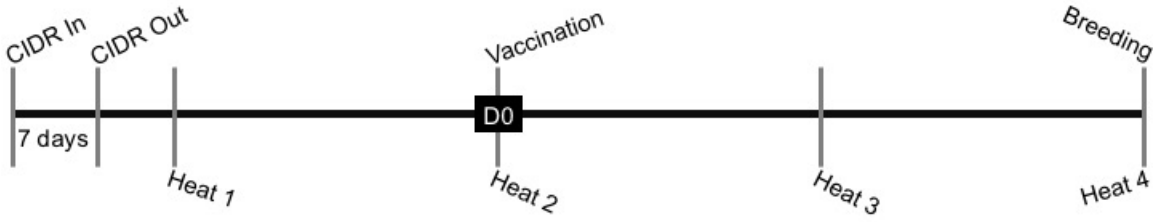

Figure 3.1: Experimental timeline of events.

Serum Neutralizing Titers

Samples for BoHV-1 and BVDV-1 titers were collected both pre and post vaccination. Samples were collected D-7 for group one as heifer were randomly assigned a vaccine treatment. However, in an attempt to balance the study for pre-vaccination titers to BVDV-1, samples were collected on D-11 for group 2 to allow the lab to return results prior to vaccination. Post vaccination titers were collected on D+22 in order to capture the peak antibody production in response to this booster vaccination. Blood was collected via coccygeal venipuncture into

vacutainer tubes containing no additives. Samples were then transported on ice back to the lab and allowed to clot in a 4ºC refrigerator overnight. The following morning, the tubes were shaken to release the clot and the samples were centrifuged at 3500 rpm for 15 minutes. Approximately 1mL of serum was pipetted into a clean and labeled microfuge tube for each animal. Samples were then heat inactivated in a 56ºC water bath for 30 minutes. After heat inactivation, samples were allowed to return to room temperature and then frozen at -20ºC for future analysis by the Athens Veterinary Diagnostic Lab.

Because IBR tends to induce a weak antibody response and is not sensitive to boostering, BVDV-1 titers were used as a monitor of vaccine response. BVDV-1 is very sensitive to boostering, controlled almost entirely by antibody production and the vaccines themselves are made to induce a high level of response.

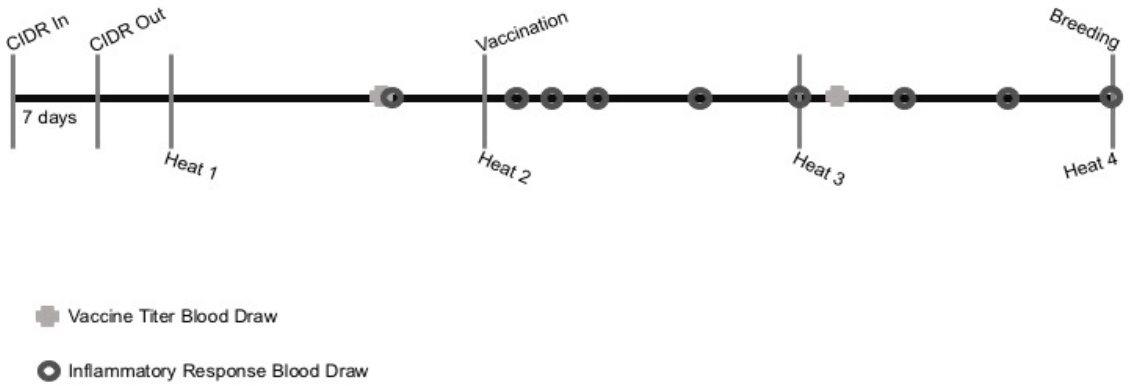

Figure 3.2: A representation of the timeline for sample collection for inflammatory response measurements. The crosses represent sample collections for vaccine titers and the open circles represent sample collections for inflammatory response.

Blood collection

7mL of whole blood was collected into 7mL vacutainer tubes containing EDTA weekly for each animal from the week prior to vaccination (D-7), the first day after vaccination (D1), the third day post vaccination (D3) and then weekly through the week of breeding. Samples were collected via coccygeal venipuncture and the tube was inverted 10 times to ensure the EDTA was well mixed into the collected blood. The tube was then placed in a warmed cooler and, after all samples had been collected, transported back to the lab for immediate analysis. Once arriving in the lab, tubes were inverted an additional 10 times to prevent the formation of clots and the sample was then partitioned for both reactive oxygen species (ROS) activity analysis (3mL) and a complete blood count (1mL).

ROS

3mL of whole blood was transferred into a 16x100 mm sterile tube. 6mL of Tris-amonium chloride, previously prepared, was then added to each tube to induce red blood cell lysis. Tube were inverted 10 times to mix and incubated at 37ºC for 30 minutes. While samples were incubating, the other reagents were prepared. First, from the frozen stock solution ($1x10^{-3}$M) PMA a serial dilution in 900µL RPMI-1640 (without Phenol red) containing 10% heat-inactivated fetal bovine serum and 2mM L-glutamine (RPMI) was performed to achieve the working stock concentration of $1x10^{-6}$M PMA. Next, DHR-123, a fluorescent dye, was prepared from frozen stock solution by adding 30mL of the stock to 3mL of RPMI. The RPMI was previously prepared and in stock and was used as the

media for the assay. Once incubation was complete, the tubes containing each heifer sample were centrifuged at 1200 rpm for 10 minutes. Upon completion, each sample was examined for the presence of a small, white pellet of collected cells at the bottom of the tube. The supernatant was gently poured off and cells were washed. For the wash, 5mL of 1X PBS was added to each tube, tubes were vortexed for approximately 5 seconds to suspend cells, and tubes were then centrifuged at 1200 rpm for 5 minutes. Upon completion of the wash, supernatant was gently poured off. This wash step was repeated twice. After the final wash, supernatant was removed and cells were suspended in 1mL of 1XPBS. 50µl of this suspension were placed into a 1.5mL microfuge tube contains 450µl of trypan blue. The microfuge tube was mixed by vortexing and then 10µl of stained sample were loaded into a cell counting chip. At least 100 cells were counted, and using the following equation, the volume of RPMI necessary to achieve a concentration of $4x10^6$ cells per mL was performed:

*#/mL=viable cells/#SQ * dilution factor (10) * volume factor (10^4)*

After determination of the necessary volume of RMPI-1640 (without Phenol red), cells were mixed with media to the appropriate concentration and plated (Table 3.1)

Table 3.1: A representation of the 96-well plate for the ROS assay.

	1	2	3	4	5	6	…	11	12
A	Media	Media + Dye	H1	H1 + PMA	H2	H2+ PMA	…	H5	H5 + PMA
B	Media	Media + Dye	H1	H1 + PMA	H2	H2 + PMA	…	H5	H5 + PMA
C	Media	Media + Dye	H1	H1 + PMA	H2	H2 + PMA	…	H5	H5 + PMA
D	Media	Media + Dye	H1	H1 + PMA	H2	H2 + PMA	…	H5	H5 + PMA
E	H6	H6 + PMA	H7	H7 + PMA	H8	H8 + PMA	…	H11	H11 + PMA
F	H6	H6 + PMA	H7	H7 + PMA	H8	H8 + PMA	…	H11	H11 + PMA
G	H6	H6 + PMA	H7	H7 + PMA	H8	H8 + PMA	…	H11	H11 + PMA
H	H6	H6 + PMA	H7	H7 + PMA	H8	H8 + PMA	…	H11	H11 + PMA

To A-D1 add only 100µl of medium; To A-D2 100µl of medium and 10µl of DH-R123
To all other wells, add 100µl of cells from the heifer indicated 1 to n to the even wells add 100µl of $1x10^{-6}$M PMA.
To all wells except A-D 1, add 10µl of DH-R123.

Plate(s) were then incubated at 37ºC for 2 hours. Upon the completion of incubation, plates were read for the fluorescent signal using the Thermo-plate reader (with Ascent software) with the 485-538 program. Results were recorded in an Excel spread sheet for further analysis.

CBC

Upon arrival in the lab, and after inversion of the samples to prevent clotting, 1mL of whole blood was placed into a labeled 1.5mL microfuge tube. A Heska analyzer was used for complete blood count (CBC) analysis. Samples were run on the cow programs for analysis of: white blood cells, proportion and number of lymphocytes, proportion and number of monocytes, proportion and number of granulocytes, the granulocyte-to-lymphocyte ratio, and the total number of platelets. Analysis were saved for future evaluation.

Statistical Analysis

Inflammatory data were evaluated using the PROC MIXED procedure of SAS (Cary, N.C.). The fixed effects for the repeated measures model where: season, vaccine type and week relative to vaccination. Additionally, the pre-vaccination measurement was factored into the model as a fixed effect. Vaccine titers were examined using the PROC GLM procedure; the model included season and vaccine type as fixed effects.

Results

SN Titers

There was no difference in pre-vaccination or post-vaccination titers for IBR or BVDV for the MLV or K vaccine, as observed in Table 2.1 ($P>0.05$).

Table 3.2: Pre- and post-vaccination SN Titers for MLV and K vaccinated heifers.

	Modified Live (mean ± SE)	Killed (mean ± SE)	P Value
IBR Pre-Vac	7.14 ± 2.10	1.75 ± 2.15	0.0871
IBR Post-Vac	99.43 ± 19.87	80.80 ± 20.36	0.5194
BVDV Pre-Vac	87.52 ± 44.00	56.20 ± 45.09	0.6240
BVDV Post-Vac	432 ± 38.68	378 ± 39.63	0.3401

There were differences in pre-vaccination titers for IBR and post-vaccination titers for BVDV by season, show in table 2.2 ($P<0.01$)

Table 3.3: Pre- and post-vaccination SN Titers for Spring 2015 and Fall 2015 replicates.

	Spring (mean ± SE)	Fall (mean ± SE)	P Value
IBR Pre-Vac	0 ± 2.29	8.89 ± 1.96	0.0073
IBR Post-Vac	60.8 ± 21.63	119.43 ± 18.48	0.0513
BVDV Pre-Vac	9.86 ± 47.89	133.86 ± 40.94	0.0618
BVDV Post-Vac	298.00 ± 42.10	512.00 ± 35.98	0.0008

Complete Blood Count

There was no difference by season or vaccine in total circulating white blood cells or monocytes throughout the trial (P>0.1). As presented in Figure 3.3, there was no difference in circulating lymphocytes at any point after vaccination between the MLV and K vaccinated heifers (P.0.1). Additionally, there was no difference in post-vaccination circulation of granulocytes, where neutrophils are housed in the CBC, by vaccine type (Figure 3.4, P>0.1).

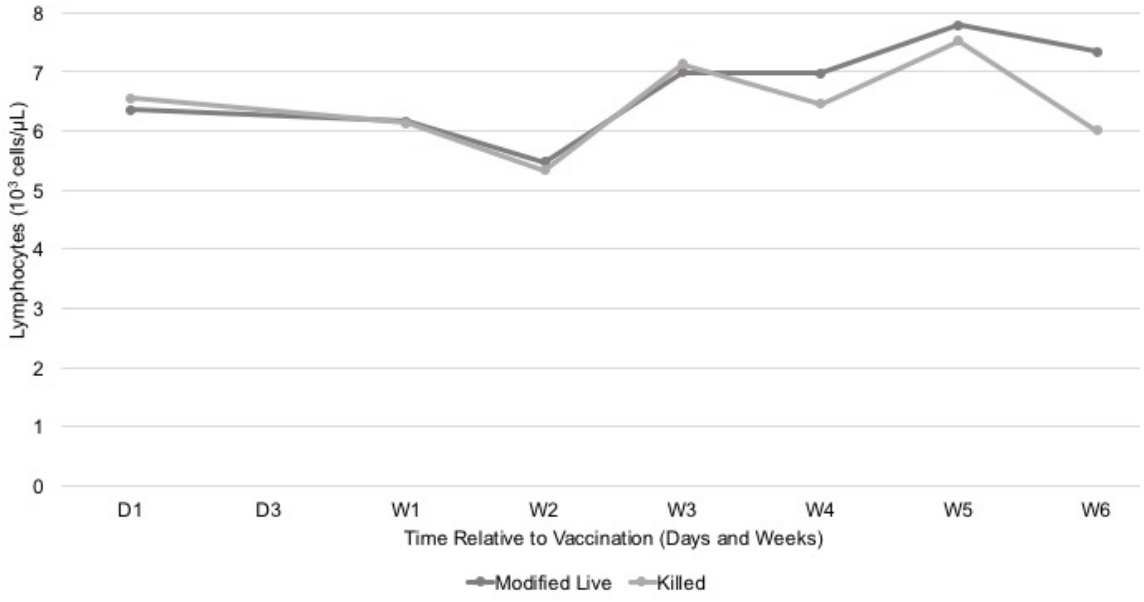

Figure 3.3: Circulating lymphocytes post-vaccination by vaccine type (P>0.1).

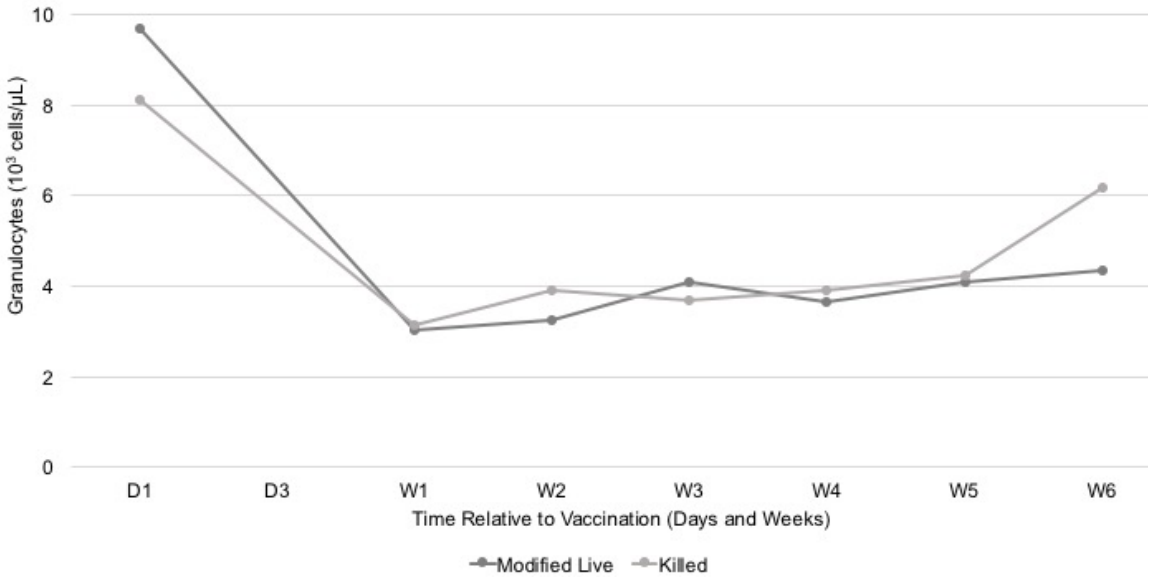

Figure 3.4: Circulating granulocytes post-vaccination by vaccine type (P>0.1).

The Fall replicate did exhibit overall higher numbers of circulating lymphocytes post-vaccination, with significantly higher numbers on D1, W3, W4, W5, and W6 (Figure 3.5, P<0.05). Conversely, there was no difference in circulating granulocytes between seasons, although the Spring replicate remained numerically higher post-vaccination (Figure 3.6, P>0.1).

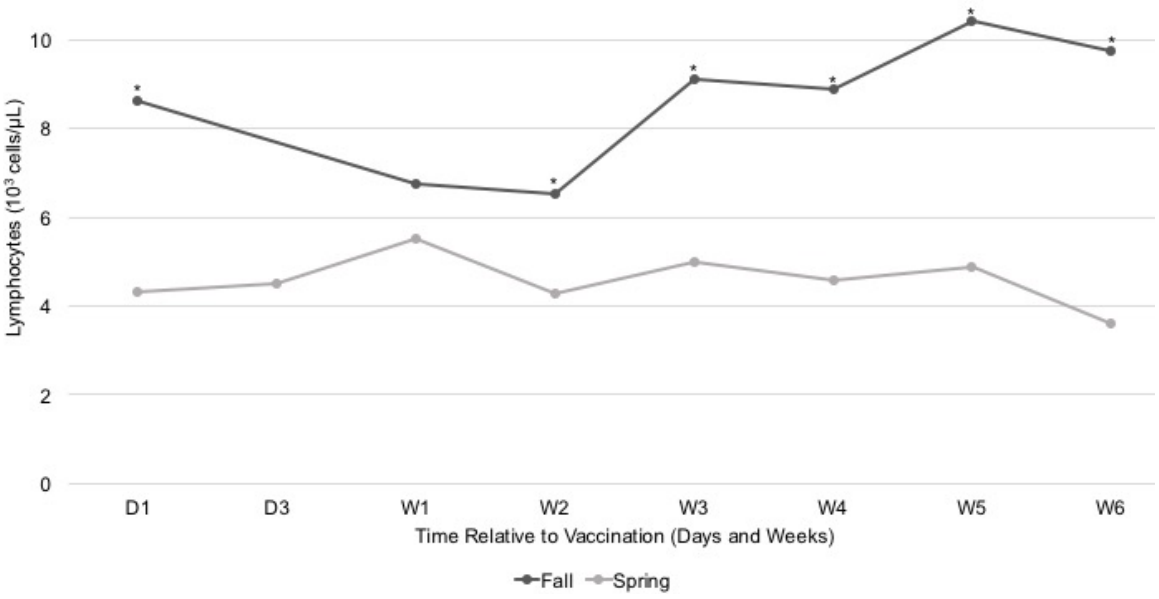

Figure 3.5: Circulating lymphocytes post-vaccination by season. A * denotes a P<0.5.

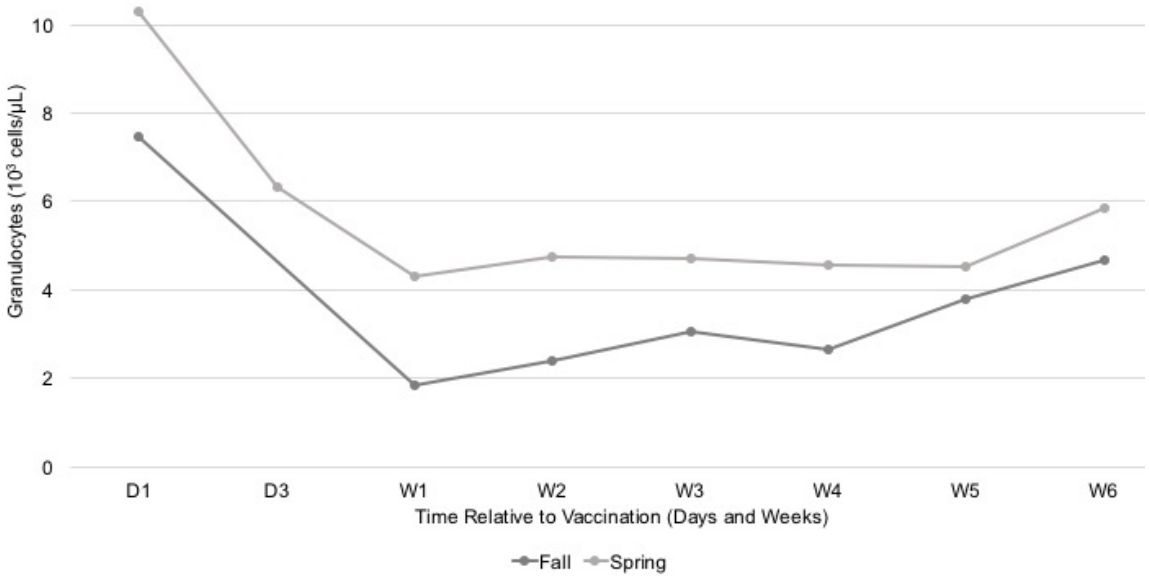

Figure 3.6: Circulating granulocytes post-vaccination by season.

Neutrophil Activity

As seen in Figure 3.7, there was no difference in the ROS response post-vaccination between the MLV or K vaccinated heifer (P>0.1). However, the Spring replicate exhibited a significantly higher ROS response ratio on D3, W1, W2, W3, W5, and W6 post vaccination, as observed in Figure 3.8 (P<0.05).

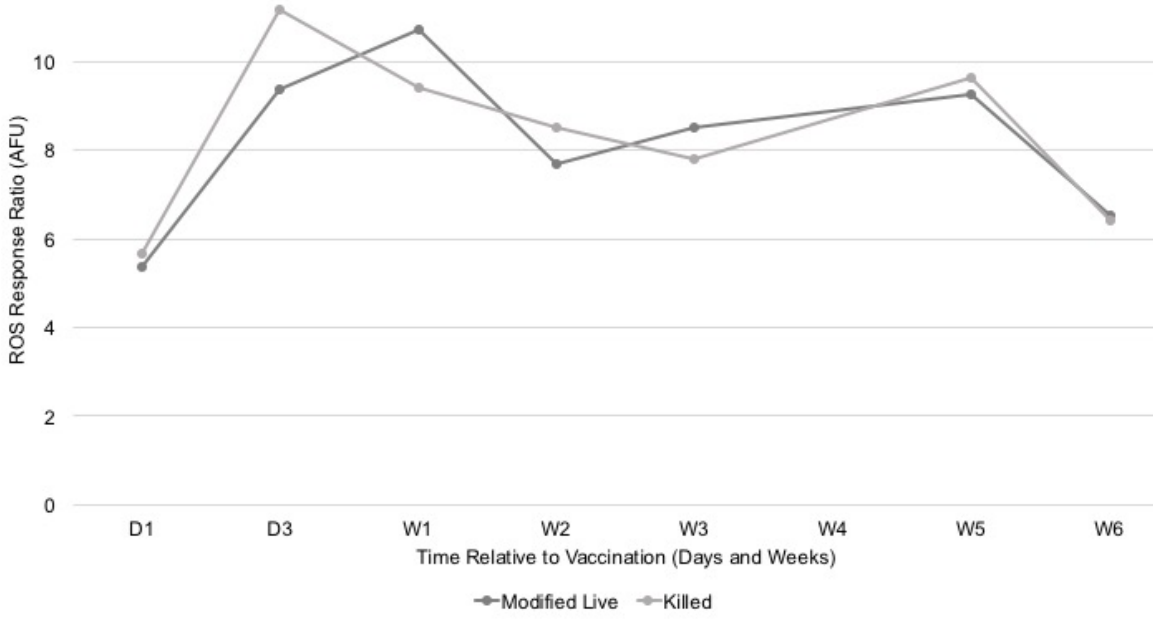

Figure 3.7: ROS response ratio between vaccine types (P>0.1). Note the numerically faster and large onset of response by the K vaccinated group and the more delated response activation of the MLV vaccinated group.

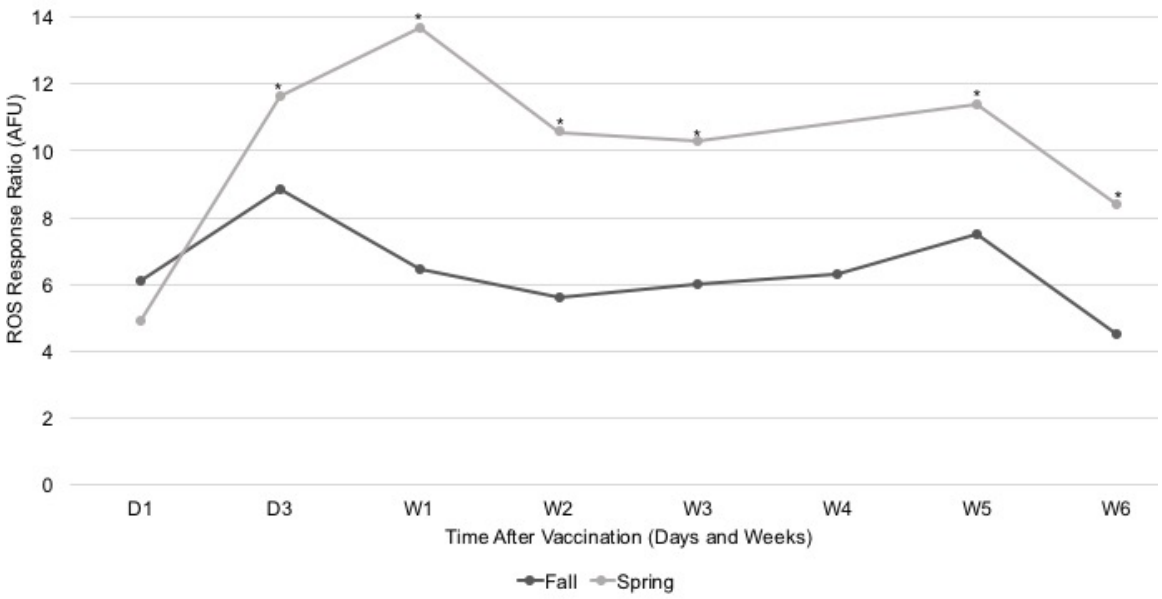

Figure 3.8: ROS response ratio between the replicates. A * denotes a P<0.05.

Discussion

When evaluating the immunological data for this experiment, it is important to note that the Fall replicate experienced a severe immunological disturbance. This disturbance presented approximately 7 days after vaccination as scours that lasted around one week. Thus, when the heifers were vaccinated they were probably in the process of incubating this pathogen and the early stages of reacting to it. Although the heifers in this study were not tested, and thus the pathogen or combination of pathogens cannot be positively identified, scouring calves born in the neighboring field tested positive for rotavirus, cryptosporidia, and salmonella. It is important to note that the post-vaccination titers for the Fall replicate were higher than the Spring. Now, this is in contradiction with the idea that you should not vaccinate sick animals because there will be a lack of protection induced by the vaccine. However, it may be that

because these heifers were vaccinated prior to the onset of clinical symptoms they were still able to mount an immune response to the vaccine and the pathogen.

The observed seasonal differences can be explained. The overall higher circulating lymphocytes of the Fall replicate may be indicative of the adaptive immune response mounted by the heifers. Lymphocytes house T and B cells of the adaptive immune system, and in particular B cells are responsible for the production of antibodies against a pathogen, both newly and previously encountered agents (Abbas et al., 2015). Combined with the lack of difference in circulating granulocytes between seasons, and it would appear that the Fall heifers were undergoing contraction of the innate response and expansion of the adaptive response.

In preparation of the ROS assay for neutrophil activity, problems were encountered with the neutrophils of the Fall replicate. When viewed under the microscope for counting, the exterior portions of the neutrophils were tattered for many heifers, and especially on D3, W1, and W2 there were more than 15% dead cells. One heifer on the W1 collection did not even have 100 total cells to be counted for the assay. It appeared that the neutrophils were not holding up to the lysis procedure with Tris-ammonium chloride, as there would be extremely large pellets of intracellular material present after the initial wash. On these days in particular, the data collected from many Fall replicate heifers simply could not be used because of a lack of cell viability. Interestingly, some of the Fall

replicate exhibited basophilia at this time, indicating a possible parasitic infection in addition to the disruptive diarrheal pathogen.

There were no differences in the number or cells or their responses between vaccine types. However, the ROS assay showed the classic response difference between a K and a MLV vaccine. There was a faster and larger onset of ROS activity for the K vaccinated heifers, as the presence of the adjuvant in the vaccine signaled danger to the innate immune system, of which the neutrophil is a primary responder. The MLV vaccinated heifers, on the other hand, exhibited a delayed response to vaccination as the MLV components of the combination vaccine needed time to replicate and expand within the host to induce a response in the innate immune system.

Although it does appear that the two vaccine types induced a similar reaction pattern on the circulating white blood cell populations it may be that some of the true differences in vaccine response was masked by the disease of the Fall replicate.

CHAPTER 4

IMPLICATIONS

The advantages of a BRDC vaccine with a MLV IBR component have led to their widespread use, especially in calves. These types of vaccines have been shown to provide rapid and long lasting protection when administered according to label directions. However, concerns remain about their use in pregnant animals, even with previous exposure, and breeding age heifers. The IBR MLV is most often identified as the cause of a vaccine-related fertility complication, such as abortion or an abnormal estrous cycle.

In order to better grasp the widespread impact of the inflammatory response activated by vaccination with an IBR MLV, the innate inflammatory information was gathered. The hope was that a higher rate or more extended inflammatory response related to vaccination would coincide with differences in estrous cycle parameters and ovarian function observed in MLV vaccinated heifers. However, in disagreements with previous work, there were no alterations in cyclicity between the MLV vaccinated animals and the K; nor between the observational (Cycle 1) and 2 treatment cycles. Since the current study utilized heifers that had received adequate calf-hood vaccination, with 3 heifers receiving an additional calf-hood vaccination, it would appear that this may have protected these animals from the adverse impacts MLV vaccination can have on fertility. Thus, it may be important for producers to be consistent in

their vaccination protocol and brand choice. Making sure that calves are properly vaccinated so that when they are re-vaccinated prior to breeding, those heifers can achieve pregnancy in a timely manner and enter the lactating or production herd as scheduled.

However, neither vaccine type was completely free of adverse impacts. It appears that vaccination, and more consistently with an IBR MLV, that there is an impact on the follicular pool. There was a decrease in circulating AMH post-vaccination that had not returned to pre-vaccination levels by the conclusion of the trial (approximately 40 days). This decrease in AMH may be connected to a decrease in the size and/or viability of the follicular pool. Since a return to normal circulating AMH was not observed, it may behoove producers to administer their pre-breeding vaccine at least 60 days prior to timed AI or the onset of the breeding season.

Finally, there are some recommendations for future study. First and foremost, a less intensive sampling schedule, particularly reproductive sampling, would allow for a larger sample size. A larger sample size might increase the power of the study, helping to elucidate some of the numerical, but not significant, difference observed in this study. Additionally, the leukocytes collected for the ROS assay could be plated on MDBK (bovine kidney) cells. After two days, these cells could be examined for a cytopathic effect, indicating the presence of circulating and replicating IBR as a result of vaccination. Overall, there is still a need for investigation of the use of IBR MLVs in breeding age

heifers, to both develop an appropriate and accurate timeline and to further understand the impacts on ovarian function.

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