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Changes in Whole Blood Parameters in Beef Heifers May Contribute to Delayed Pubertal Attainment

Jessica A. Keane
University of Nebraska-Lincoln, jessica.keane@huskers.unl.edu

CHANGES IN WHOLE BLOOD PARAMETERS IN BEEF HEIFERS MAY CONTRIBUTE TO DELAYED PUBERTAL ATTAINMENT

Jessica A. Keane

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CHANGES IN WHOLE BLOOD PARAMETERS IN BEEF HEIFERS MAY CONTRIBUTE TO DELAYED PUBERTAL ATTAINMENT

Jessica A. Keane, M.S.

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Advisor: Andrea S. Cupp

Pubertal development in beef heifers is a transient period that has many contributing factors. Previously in our lab, progesterone (P4) concentrations collected from heifers at weaning (Oct) to breeding (May) were used to identify four distinct pubertal groups: Typical, Early, Start-Stop, and Non-Cycling. There is limited research on heifers and the impacts of whole blood cell populations, particularly WBCs, during pubertal development; however, girls with differing puberty onset displayed altered concentrations of hematocrit, white blood cells (WBC), and hemoglobin concentrations. Thus, our hypothesis was that heifer pubertal groups with delayed or precocious puberty may have increased white blood cells or altered whole blood parameters contributing to abnormal pubertal development. Weekly blood samples during the pre and peripubertal period were collected to obtain progesterone concentrations from previously identified pubertal classifications. Monthly blood samples were also analyzed with a HemaTrue ® Veterinary Hematology Analyzer to determine whole blood parameters. Additionally, blood smears were used to count specific WBC populations. All blood parameters were found to change during the collection period suggesting that weight and maturity directly impacts hematological and immunological factors. Early heifers tended to have reduced granulocyte cells ($p=0.07$) when compared to Typical (controls) but was not different from Start-Stop and Non-Cycling. Additionally, red cell distribution width to platelet ratio tended to be greater in Early compared to Typical

and Non-Cycling heifers. Early and Start-Stop heifers that initiate puberty earlier had increased hematocrit ($p=0.04$) when compared to heifers with delayed puberty (Non-Cycling). Finally, Non-Cycling, heifers had reduced hemoglobin levels ($p=0.03$) compared to all other pubertal groups. Thus, hematological factors such as reduced granulocytes, increased hematocrit, and increased red blood cell width per platelet number may promote increased growth and development resulting in the probability that heifers will initiate puberty earlier. Furthermore, reductions in hemoglobin concentrations or functionality may delay pubertal attainment.

DEDICATIONS

To my grandma Hargrave and grandpa Keane. While you were unable to watch me finish my degree, I am confident you would be proud in my academic growth and endeavors. Thank you for your constant support and encouragement throughout my life.

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CHAPTER 1: REVIEW OF THE LITERATURE

Introduction

Beef production accounts for a large portion of agriculture receipts in the state of Nebraska and is therefore a large economic resource for beef cattle producers. Replacement heifers in cow-calf operations is one of the largest costs associated with cow-calf operations. To enhance producer profitability, selection of heifers with increased reproductive longevity is necessary. Heifers that achieve puberty earlier have longer reproductive longevity allowing producers to recover their development costs after 3-5 calves (Perry, 2012). Our laboratory has evaluated how heifers achieve puberty and identified 4 different pubertal classifications: Early, Typical, Start-Stop and Non-Cycling (Nafziger et al., 2021). Therefore, the next step was to determine factors that may affect pubertal attainment to cause these differences in pubertal attainment. Many investigators have evaluated environment, plane of nutrition, male exposure, and health status (Izard and Vandenbergh, 1982; Nelsen et al., 1982; Hansen et al., 1983; Schillo et al., 1983; Roberson et al., 1991; Martin et al., 1992). However, little information is available on how hematological and immunological factors impact puberty in beef heifers. It has been shown that these parameters shift during the peripubertal period in girls and boys and may contribute to alterations in timing of attainment of puberty (Timmons et al., 2006; Pluncevic Gligoroska et al., 2019). This is largely due to hormonal changes that establish molecular, physiological, and anatomical differences altering the immune response (Lamason et al., 2006; Pluncevic Gligoroska et al., 2019). Currently, very little information is present in the literature in regard to how the immune system interacts with the reproductive system and impact on pubertal development. Therefore, this thesis provides

the first step in characterizing immunological and hematological factors during pubertal development in beef heifers.

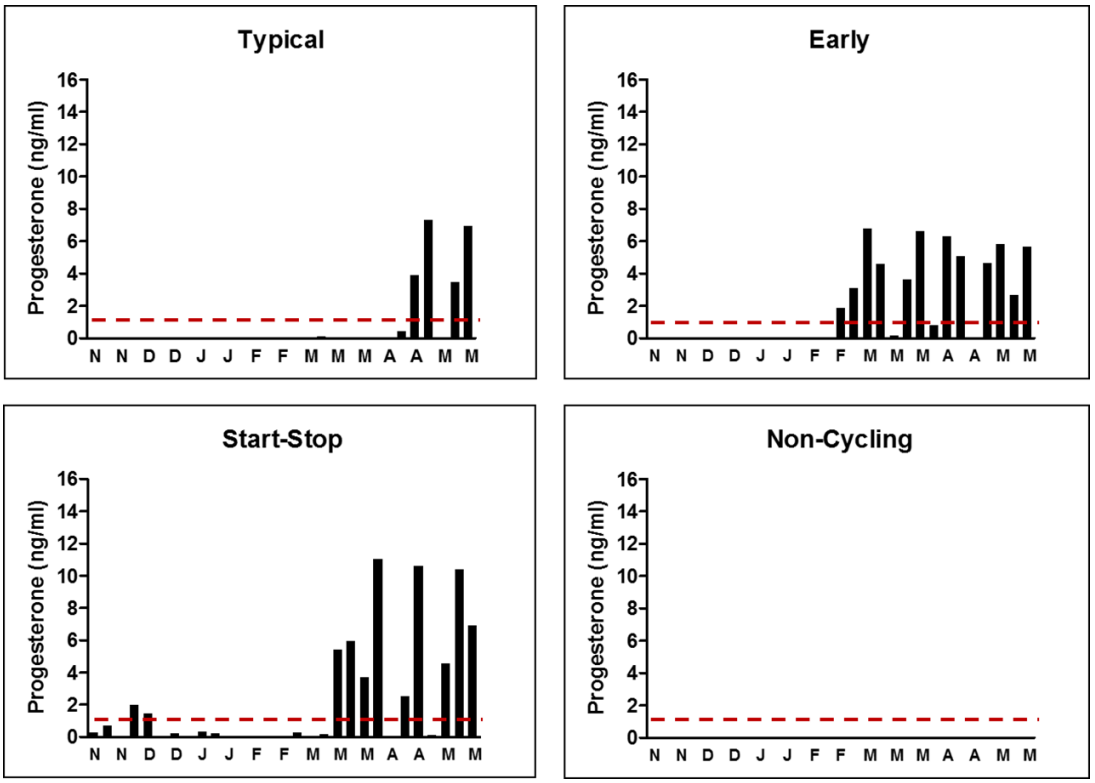

Figure 1-1. Representative progesterone profiles of Typical, Early, Start-Stop, and Non-Cycling heifers from weaning (October) to breeding (May). Since beginning the study in 2012, 44% of heifers have been classified as Typiccal, 25% Early, 17% Start-Stop, and 14% Non-Cycling. From Nafziger et al., 2021 DOI: 10.1093/biolre/ioab044.

Hypothalamic-Pituitary-Gonadal Axis

The hypothalamic-pituitary-gonadal (HPG) axis is the major endocrine signaling pathway associated with the reproductive system (Sarkar et al., 1976). The pathway is comprised of the hypothalamus, anterior pituitary, and gonads (specifically ovaries in the female). During the peripubertal process, the connections between the hypothalamus, pituitary, and ovary are established and estrous cyclicity is initiated (Atkins et al., 2013). If appropriate connections are not established, delayed or irregular cyclicity may occur

resulting in abnormal pubertal attainment (Cooper et al., 2000; Stoker et al., 2000). In Nafziger et al., a pubertal classification Start-Stop was identified that has an initial rise in progesterone of greater than 1 ng/ml; however, cyclicity is not maintained and 50% of the females resume cyclicity later during the pubertal process while another 50% do not (Nafziger et al., 2021). Also, a Non-Cycling pubertal group was identified that did not initiate cyclicity prior to breeding. We do not know why these females did not achieve regular cyclicity, but it appears that communication between the hypothalamus, pituitary, and ovary was not established appropriately in these females or was delayed in getting established.

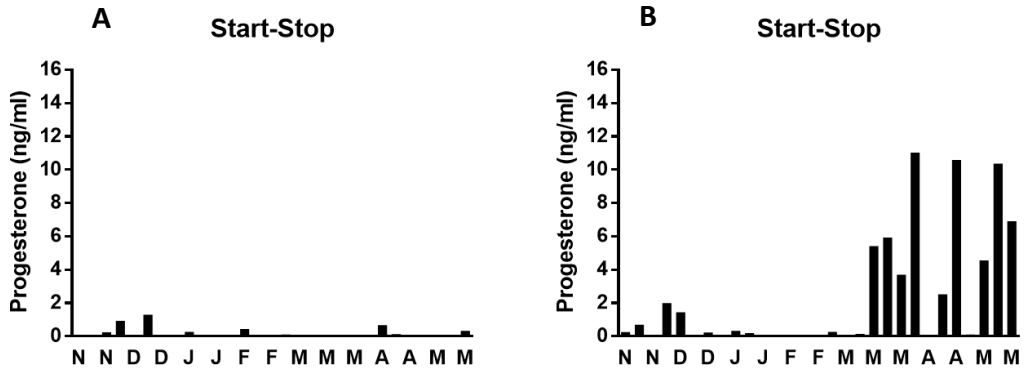

Figure 1-2. Representative progesterone profiles of Start-Stop heifers from weaning (Oct) to breeding (May). Graph A represents a heifer that initiated cyclicity during November and December, but failed to continue cycling. Graph B represents a heifer that also initiated cyclicity during November and December, but resumed cyclicity in March and continued to cycle through the collection period. From Nafziger et al., 2021 DOI: 10.1093/biolre/ioab044.

The hypothalamus originates in the diencephalon of the brain that is functionally important for regulating body temperature, metabolic processes, autonomic functions, and the reproductive system (Maffucci and Gore, 2009; Sanchez Jimenez and De Jesus, 2021). The hypothalamus synthesizes and secretes gonadotropin releasing hormone. Gonadotropin releasing hormone (GnRH) is a decapeptide that has several isoforms in mammalian species (Metallinou et al., 2007). The most common isoform is comprised of

the 10 amino acid sequence pGlu-His- Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (Wang et al., 2012). In mature mammalian females, GnRH is secreted from two distinct regions of the hypothalamus: the tonic and surge centers. The tonic center provides pulsatile basal secretion of GnRH while the surge center releases high frequency and amplitude of GnRH (Karsch, 1987). The tonic and surge centers are controlled by neurons located in the arcuate (ARC) and preoptic area (POA), respectively (Marshall et al., 2001; Sanchez Jimenez and De Jesus, 2021). The surge center is unique to females and undergoes development during the pre and peripubertal period. The high frequency and amplitude of GnRH results in the release of luteinizing hormone (LH) in a surge like fashion that will result in ovulation (Vasantha and Kona, 2016). Once GnRH is released, it will be transported via the hypothalamic-hypophyseal portal system to bind to its G-protein-coupled receptor on pituitary gonadotrophs to stimulate the release of gonadotropins luteinizing hormone (LH) and follicle stimulating hormone (FSH) (Rispoli and Nett, 2005). Inappropriate maturation of the tonic and surge centers of the hypothalamus can result in puberty not being established in females (Marques et al., 2000; Crowe A. and Mullen P., 2013). The regulation of GnRH secretion is tightly controlled by other interconnected neurons in the hypothalamus. One of these GnRH regulators is the neuropeptide kisspeptin (Marques et al., 2000).

Kisspeptin is encoded by the *Kiss1* gene that stimulates GnRH secretion through binding of kisspeptin receptor (Kiss1R), previously referred to as G protein-coupled receptor 54 (GPR54), located on GnRH neuron bodies (Seminara et al., 2003; Oakley et al., 2009; Pinilla et al., 2012). Kisspeptin protein is encoded in two distinct regions of the hypothalamus, the preoptic area (POA) and arcuate nucleus (ARC) (Lehman et al., 2013).

Kisspeptin's role in regulating GnRH secretion is largely due to estrogen receptor (ER)-alpha, progesterone receptor, and androgen receptors that are co-localized with kisspeptin neurons (Lehman et al., 2010). Additionally, it has also been noted that kisspeptin neurons in ARC also have receptors for prolactin and insulin as a subset of KNDy neurons (Li et al., 2011; Lehman et al., 2013).

Kisspeptin is unique in that its synthesis comes from multiple signals allowing informed input when stimulating the reproductive system through GnRH stimulation. As described, kisspeptin plays an intricate role in GnRH stimulation and secretion. This kisspeptin regulation begins during the pre and peripubertal developmental periods. While this process is not entirely understood, *Kiss1* mRNA levels in the ARC are elevated and increase in signaling during puberty (Terasawa et al., 2013). Kiss1R levels also increase in the POA and ARC areas of the hypothalamus during puberty (Takase et al., 2009). It has been shown that as heifers approach puberty, fewer ER receptors are present in the hypothalamus and this potentially allows for desensitization to estrogen (Day et al., 1987; Atkins et al., 2013). These factors contribute to an overall increased secretion of kisspeptin allowing for additional stimulation of GnRH neurons (Roa et al., 2008). While the exact mechanisms contributing to GnRH release are not fully understood, increases in kisspeptin and desensitization of centers in the hypothalamus to estrogen enable GnRH to be released to stimulate gonadotropin secretions from the anterior pituitary (Terasawa et al., 2013).

Upon stimulation of the anterior pituitary, gonadotropins LH and FSH are secreted and will bind to their receptors on the ovary. LH and FSH are glycoproteins that consist of the same noncovalently associated α-subunits (Hagen and McNeilly, 1975; Maghuin-Rogister et al., 1975) and distinct β subunits (Mullen P. et al., 2013; Nedresky and Singh,

2020) . While both hormones are made of the same α subunit, the variation in the β subunit of the second polypeptide chain and attached carbohydrates in the amino acid sequence distinguish function of both hormones and each β subunit comes from different β subunit genes (Palermo, 2007). The β subunit of LH is comprised of 120 amino acids whereas the β subunit of FSH is comprised of 111 amino acids (Mullen P. et al., 2013; Nedresky and Singh, 2020). These differences give the glycoproteins a high degree of specificity for their respective receptors.

It is important to note that the pulsatile secretion rate of LH and FSH are influenced by the number of oligosaccharides attached to the β subunit (Mullen P. et al., 2013). These differences in attached carbohydrates dictate the circulating half-life of the respective hormone. For example, the half-lives of GnRH, LH, and FSH are approximately 2-4 mins, 20 mins, and 3-4 hours respectively (Kumar and Sharma, 2014). With GnRH being a small decapeptide and few carbohydrates attached to the β subunit of LH, these hormones are cleared from circulation much more quickly compared to FSH. These differences influence physiological impacts on receptive tissues. In response to FSH and LH's action on the ovary, steroids are produced within the theca and granulosa cells which may diffuse into general circulation. Depending on age and reproductive cyclicity, these hormones positively or negatively stimulate the secretion of GnRH and the gonadotrophs completing the HPG axis.

The process of pubertal attainment is defined when ovulation is accompanied with visual signs of estrus followed by normal luteal function (Perry, 2016). The first ovulation ensues once the hypothalamic-pituitary axis loses the inhibitory effect of estradiol (Day et al., 1987). This results in progressive secretion of LH to stimulate additional androgen

production to generate estradiol from growing follicles. Eventually, an LH surge is induced resulting in ovulation of an antral follicle (Moran et al., 1989).

Steroidogenesis

Ovarian steroidogenesis can be defined by the two-cell, two-gonadotropin theory (Fortune, 1986). Follicles develop through primordial to antral stages as somatic cells differentiate into theca and granulosa cells (Nikitin and Vorob'eva, 1988; Hsueh et al., 2000; McGee and Hsueh, 2000; Binelli and Murphy, 2010). Theca and granulosa cells are the two somatic cell types involved in producing sex steroid hormones.

Steroidogenesis in the ovary begins with cholesterol as the precursor for androgen and estrogen synthesis. Cholesterol is a 27-carbon steroid alcohol that has four fused rings and hydroxyl polar head. It is obtained through dietary means and synthesis by the liver. In the body, cholesterol is important for eukaryotic cell membranes in which it helps modulate fluidity and permeability and is the metabolic precursor for steroid hormones and bile acids (Gimpl and Gehrig-Burger, 2007). To produce steroid hormones, cholesterol is transported into the cytoplasm of the cell. Steroidogenic acute regulatory protein (StAR) regulates the transport of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane (Miller, 2007). StAR is a necessary protein involved in steroidogenesis (Clark et al., 1995; Manna et al., 2003; Manna et al., 2009).

Once cholesterol is transferred into the mitochondria, the enzyme cytochrome P450 side-chain cleavage (P450scc or CYP11A1) converts cholesterol to pregnenolone. This conversion removes the side chain at carbon 17 of the D ring (Black et al., 1994). The P450 enzyme family that chemically modifies cholesterol to generate the steroid hormones is commonly used throughout steroidogenesis. These enzymes are classified as hydroxylases

and typically alter the side groups on the cholesterol rings as demonstrated with P450scc. These subtle alterations in cleavage of the side groups allows distinct binding of the steroid hormone to the specific steroid receptor. In addition to the P450 enzyme family, hydroxysteroid dehydrogenases are also primary enzymes involved in steroidogenesis (Miller, 1988). Pregnenolone is the precursor hormone that has the capacity to be converted to any steroid hormone through two alternate pathways: Δ4 and Δ5. These are the two most predominant pathways found in mammals with humans, bovine, sheep, and primates favoring the Δ5 pathway (Conley and Bird, 1997).

Theca interna cells are located along the exterior of the basement membrane of the follicle and are responsible for androgen synthesis (Fortune and Armstrong, 1977). Once cholesterol is in the cell, expression and activity of the enzymes responsible for converting cholesterol to an androgen are activated with LH binding to its extracellular receptor, luteinizing hormone receptor (LHR) (Fortune, 1986). Binding of LH causes the activation of membrane bound G-proteins. This G-protein complex is broken into two components where the α subunit binds to the activated GTP (Baird et al., 1981). This component then binds and activates adenylyl cyclase to catalyze the formation of cyclic adenosine monophosphate (cAMP), a secondary messenger, from ATP. cAMP then activates protein kinase A (PKA) which is responsible for activating enzymes to convert the substrates into products. In these steps of steroidogenesis for theca interna cells, cholesterol is the substrate with the product being a species dependent androgen. For example, the major androgen for humans is testosterone whereas in cattle it is androstenedione (Fortune, 1986). After the androgen is produced, it diffuses through the theca interna cell to enter the granulosa cells. Granulosa cells are the second cell type in the two-cell model of steroidogenesis (Fortune,

1986). Follicle Stimulating Hormone (FSH) binds to its receptor, follicle stimulating hormone receptor (FSHR), located on the extracellular membrane of the granulosa cell to cause activation of cyclic adenosine monophosphate (cAMP). Then, cAMP activates PKA which increases the aromatization of the androgen to estradiol through the induction of CYP19A1 which converts androgens into estrogens (Palermo, 2007). Theca interna cells are unable to produce estradiol due to the lack of the enzyme CYP19A1. This results in the necessity of two cells being involved in the steroid production. Upon generation of the steroid hormones, freely circulating sex hormone binding globulin (SHBG) will bind to steroid hormones, testosterone and estradiol, to allow circulation of these hormone throughout the body. SHBG is a glycoprotein that is required to facilitate this transportation because of the polar, steroid profile of these hormones that do not allow them to be soluble in blood and circulate unless they our bound to a binding protein (Selby, 1990).

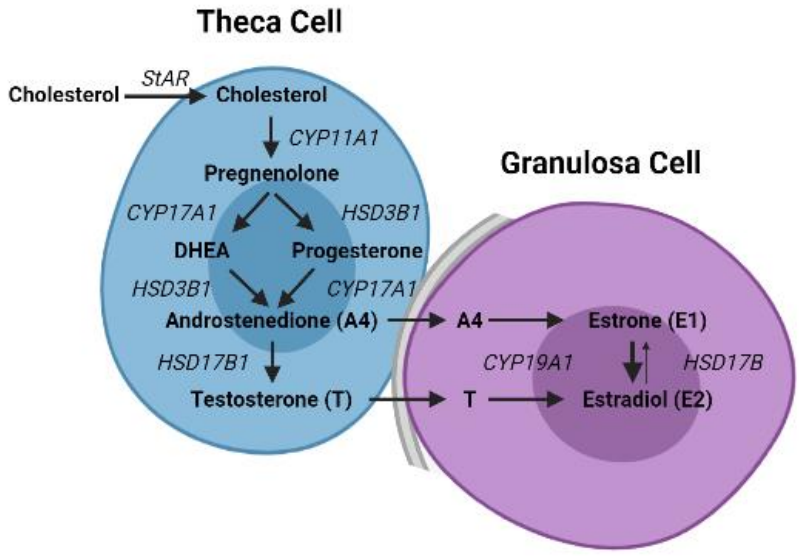

Figure 1-3*. Illustration of the steroidogenesis pathway emphasizing the roles of the theca and granulosa cells producing estradiol in the two-cell, two-gonadotropin theory. Cholesterol is the biological precursor for producing the sex steroid hormones.*

Follicular Dynamics

In the ovary, developing follicles containing oocytes will grow and regress in response to gonadotropins. The initial stage of follicular development are primordial follicles which are composed of oocytes surrounded by a single layer of flattened squamous cells known as pre-granulosa cells (Fortune, 2003). Oocytes are present in the indifferent gonad prior to development of an ovary. They migrate from the yolk sack to hindgut and then genital ridge of the indifferent gonad. Granulosa cells arise from coelomic epithelium of the genital ridge and migrate into the gonad prior to follicle formation with the oocytes form oogonial clusters (Liu et al., 2015; Ariza et al., 2016; Strauss and Williams, 2019). Primordial follicles develop into primary follicles due to the differentiation of the granulosa cell morphology from squamous into cuboidal cells. Early primary follicles have cuboidal

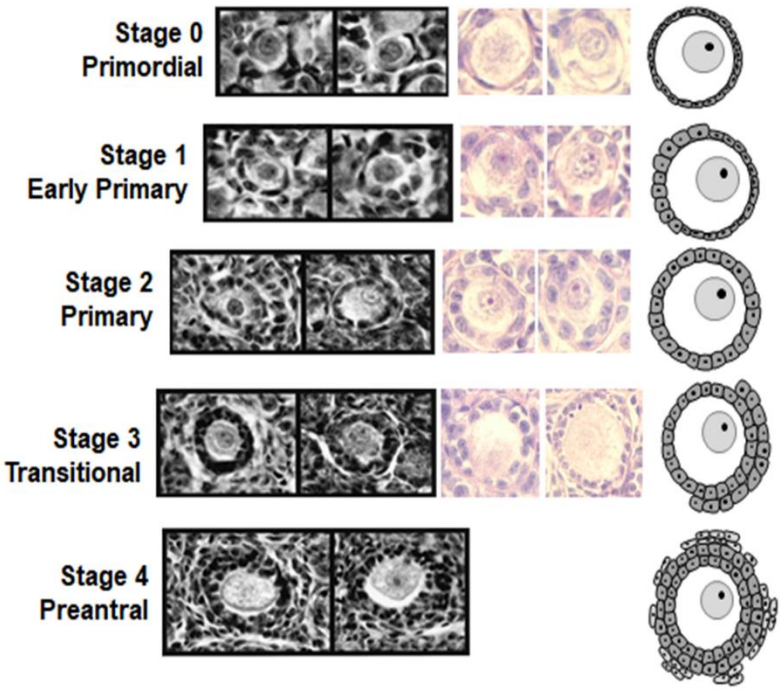

Figure 1-4*. Follicle staging of primordial, primary, and antral follicles. Primordial follicles are identifiable with a single layer of squamous cells. Developed squamous cells to cuboidal are identifiable in primary follicles. Secondary follicles begin having a secondary layer of cells.*

and squamous cells surrounding the oocyte (Eppig, 2001). Primary follicles transition to secondary follicles which contain two or more layers of cuboidal granulosa cells surrounding the oocyte and have two distinct cell layers comprised of granulosa and theca cells (Young and McNeilly, 2010). The theca cells are thought to differentiate from fibroblast-like mesenchymal cells in the stromal compartment and they migrate to the basement membrane surrounding the granulosa layer (Liu et al., 2015; Ariza et al., 2016; Strauss and Williams, 2019).

These secondary follicles continue development into antral follicles or go through atresia. Secondary follicles begin accumulating follicular fluid and have three distinctive cell layers made of theca externa, theca interna, and granulosa cells (Eppig, 2001). Secondary follicles will be selected to continue to develop into antral follicles. Antral follicles are characterized by containing an antrum, or fluid filled cavity (Richards, 1994). In monovulatory species, typically one antral follicle will become a large dominant follicle that will ovulate under the correct endocrine milieu. This will release the oocyte for potential fertilization. During any of these transition growth periods, the follicles can enter atresia, also known as apoptosis or cell death, rather than continuing to grow into the next phase (Richards, 1994; Eppig, 2001). Once a follicle ovulates, some of the granulosa and theca cells will remain a part of the ovary and will develop into a corpus luteum (Smith et al., 1994).

The corpus luteum (CL) forms from somatic cells, theca and granulosa, that remain after the oocyte is ovulated from the follicle. The CL produces several hormones including progesterone, oxytocin, relaxin, inhibin, and activin which are essential for reproductive regulation and communication to other tissues in the body (Messinis et al., 2009). The

presence of the CL will inhibit estrus and ovulation. The process of growing, regressing, and ovulating follicles is described in the bovine estrous cycle.

Bovine Estrous Cycle

Pubertal cattle undergo dynamic estrous cyclicity with a displayed estrous and ovulation of a dominant ovarian follicle. The estrous cycle can be divided into two major phases: follicular and luteal phases. Estradiol is the predominant hormone produced in the follicular phase while progesterone is the predominant hormone in the luteal phase.

The estrous cycle begins with the ovulation of a dominant follicle. During the process of ovulation, mural granulosa and theca cells remain a part of the ovary and undergo the process of luteinization to form large and small luteal cells, respectively (Hoyer and Niswender, 1985; Farin et al., 1989; Smith et al., 1994). This process is characterized by a morphological and functional change to enable progesterone to be the predominant hormone produced as estrogen declines. The process of luteinization develops the corpus luteum (CL) that begins emerging after 2-3 days (Dieleman and Blankenstein, 1985). The luteal phase begins with the emergence of a corpus luteum on the ovary. This phase will last approximately from days 1 to 17 of the estrous cycle (Rajakoski, 1960; Choudary et al., 1968; Sirois and Fortune, 1988; Taylor and Rajamahendran, 1991). The HPG axis is suppressed by the presence of progesterone inhibiting GnRH secretion. Progesterone remains elevated if pregnancy recognition signaled by the conceptus occurs. Interferon-tau is the specific pregnancy recognition hormone in ruminants (Forde and Lonergan, 2017). If pregnancy recognition does not occur in time, the sustained progesterone from the corpus luteum will result in the secretion of prostaglandin F2α effectively regressing the corpus luteum (Taylor and Rajamahendran, 1991).

Occurring approximately days 18 to 21, the follicular phase will produce substantial estradiol from growing antral follicles. This group of antral follicles is characterized as one follicular wave that will result in typically one dominant antral follicle ovulating (Sirois and Fortune, 1988; Bao and Garverick, 1998). During the duration of an estrous cycle, 2 to 3 follicular waves usually occur (Sirois and Fortune, 1988). Due to the elevated progesterone during the luteal phase, the leading 1 to 2 follicular waves result in the dominant follicle becoming atretic and regressing. However, the removal of the inhibitory progesterone enables the last follicular wave of the estrous cycle to develop an ovulatory follicle (Stock and Fortune, 1993). This follicle will usually reach 10-15 mm in diameter in cattle (Perry et al., 2007). The increasing estradiol production from the dominant follicle will eventually induce GnRH pulses and therefore LH surge to ovulate the follicle. This results in the generation of a new corpus luteum and estrous cycle to begin.

Factors Impacting Pubertal Attainment

Puberty is a transient period of development that results in the display of estrus and ovulation with the capability to become pregnant for females (Patterson et al., 1992). There are numerous factors that contribute to an animal becoming pubertal including but not limited to nutrition, environmental factors, presence of male, and genetics. These factors ultimately culminate to pubertal development that can have long-lasting production impacts for the producer.

The nutritional plane and overall weight of prepubertal beef heifers has a positive correlation to age at pubertal development (Nelsen et al., 1982). It has been illustrated that puberty is initiated in heifers when they typically reach 55-65% of their mature body weight (Freetly et al., 2011). It is crucial that heifers are allowed sufficient time to grow as

it has been shown that heifers born earlier in the calving season are heavier at breeding and are therefore likely to have higher pregnancy rates (Wiltbank et al., 1985). By feeding heifers at a higher nutritional plane, age at puberty is decreased and allows for breeding and pregnancy to occur sooner (Short and Bellows, 1971). Nutrition and whole-body reserves are critical factors in initiating puberty.

One method of indicating whole-body reserves is through monitoring levels of the hormone leptin. Leptin is predominantly produced and released by white adipose tissue and regulated through nutritional and metabolic status (Chilliard et al., 2005). This hormone is crucial for the initiation of puberty in cattle and other mammals. Without the production of leptin from the *ob* gene in adipocytes, individuals will be hypogonadal and infertile (Strobel et al., 1998). However, when leptin is supplemented in *ob/ob* mice deficient in leptin, puberty can be triggered (Chehab et al., 1997; Mounzih et al., 1997). It has been indicated that leptin is able to affect the central reproductive axis through its own receptors and neuropeptide Y (NPY) to indicate the overall metabolic state of the individual (Zieba et al., 2005). These metabolic factors are important signals to indicate if pregnancy can be supported and therefore for pubertal development to progress.

Heifer's age at puberty is impacted by varying environments. Season of birth for cattle can contribute to pubertal onset (Schillo et al., 1983). This is likely the result of variations in photoperiod, temperature, and/or hormonal balance contributing to metabolic status and gonadal hormones (Hansen et al., 1983; Schillo et al., 1983; Garcia et al., 2002). It has also been observed that cattle maintained in cold stress environments had an increase in dry matter intake while animals maintained in heat stress environments had a decreased intake of dry matter. These differences in environment and feed intake contribute to the

overall metabolic status and weight of the animal and negatively impacts pubertal attainment (Fox and Tylutki, 1998). Regardless of season of birth for heifers, it appears that birth of offspring occur more often in spring or summer (Kinder et al., 1987).

Interaction and contact of the male, commonly referred to as bull exposure for cattle, helps to hasten the onset of puberty in beef heifers (Izard and Vandenbergh, 1982; Roberson et al., 1991). The main hypothesized mechanisms in which bull exposure advances the onset of puberty is through exposure of pheromones. In a study conducted with goats, it was shown that exposure to the male pheromones increased pulsatile release of GnRH (Hamada et al., 1996).

There is significant genetic variation in onset of puberty between beef cattle breeds (Martin et al., 1992). Genetic selection from producers has been the driving factor for genetic diversity among breeds. Selection for weight, specifically percentage of mature body weight, and age is the predominant factor contributing to age at puberty. Heifers from larger mature breeds typically achieve puberty at a later age and at a heavier weight compared to smaller mature breeds (Martin et al., 1992). Crossbreeds also typically reach puberty earlier than pedigree animals (Ferrell, 1982).

Immune System

The immune system in multicellular organisms is critical for protecting the body from pathogens and regulating inflammation. Through exposure to pathogens and acquisition of antibodies, the immune system develops and changes to respond to future foreign material. In addition to responding to foreign material, the peripheral immune system has been shown to develop during the pre and peripubertal period. This is largely impart due to hormonal changes that establish molecular, physiological, and anatomical

differences altering the immune response (Lamason et al., 2006; Pluncevic Gligoroska et al., 2019). Sex steroid hormones, such as estradiol and testosterone, have the capacity to influence lymphocyte and macrophage function (Shames, 2002). For example, after pubertal attainment, women have been shown to have stronger innate and adaptive immune responses when compared to men (Klein and Flanagan, 2016; Laffont et al., 2017). This can lead to higher risk for autoimmune diseases that are more sex-specific (Brenhouse and Schwarz, 2016). Unfortunately, there is little information available with less than 10% of immunology articles accounting for sex in models and even less information available focusing on the pubertal development period (Beery and Zucker, 2011). Nevertheless, the immune and reproductive systems are intricately intertwined and should be the subject for future investigations.

Innate and Adaptive Immunity

Multicellular organisms have developed a complex and highly specific immune system to respond to foreign pathogens within the body. The immune system has two primary components of immune response that are innate and adaptive immunity.

The innate immune response is commonly known for having a quickly reacting and broad-spectrum defense to non-self antigen detection. This ability allows the body to respond to foreign pathogens within hours of microorganism exposure (Hoffmann et al., 1999). Neutrophils, eosinophils, basophils, and monocytes are the primary leukocytes involved in the innate immune response. The adaptive immune response is intrinsically slower to responding to a foreign pathogen invasion, but unlike the innate immune response it acts in a highly specific and targeted attack. The adaptive immune response is highly efficient because clonal expansion of immune cells enables rapid production of antibodies

targeted to a specific antigen (Dempsey et al., 2003). The adaptive immune system primarily uses lymphocytes and its ability to differentiate naive B, T, and natural killer cells into mature effector cells in its immune response.

Before dissecting the inflammatory response and antibody production, it is important to characterize the cells involved in the body's immune system. Leukocytes, or white blood cells, are cells that respond to irritants or antigen to begin the inflammatory process. All leukocytes originate from the pluripotent hematopoietic stem cells in bone marrow and differentiate in the thymus, bone marrow, or blood circulation (Seita and Weissman, 2010; Woods et al., 2011; Zlotoff and Bhandoola, 2011). Leukocytes are divided into two main classifications: granulocytes and agranulocytes. Granulocytes are white blood cells that contain secretory granules in the cytoplasm such as, neutrophils, eosinophils, and basophils. Agranulocytes, the second leukocyte classification, are white blood cells that lack granules in the cytoplasm, these include lymphocytes and monocytes.

Neutrophils

Neutrophils are the first responders and most readily available immune cell. These cells are produced in the bone marrow from differentiated committed granulocyte progenitor cells. CCAAT/*e*nhancer binding *p*rotein α and PU.1 are transcription factors critical for progenitor cells to become committed to the generation of granulocytes, granulocytopoiesis (Iwasaki et al, 2005). Growth factor independent-1 is essential for the specific differentiation of neutrophils (Karsunky et al., 2002). Neutrophils enter endothelial tissues to become activated. Once activated, neutrophils phagocytose pathogens and release enzymes, such as peroxide, from their granules (Teng et al., 2017). With the production of granulocyte-colony stimulating factor (G-CSF), cytokines are generated and

released into the peripheral tissue (Borregaard, 2010). With the secretion of chemokines and cytokines in circulation, acute inflammation will begin to recruit additional leukocytes to the activation site.

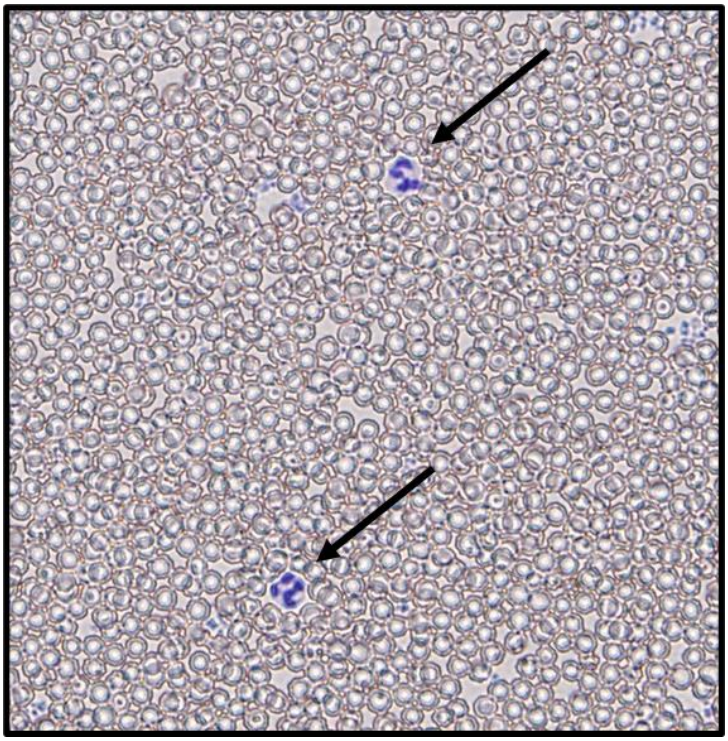

Figure 1-5*. Image of bovine neutrophils. Neutrophils are morphologically discerned by the presence of dark granules surrounded by a clear cytoplasm. These cells have a smooth, round shape.*

Eosinophils

Eosinophils have several roles in the immune system including responding to allergens, fighting viral and parasitic infections, and removing fibrin from tissues (Riddle and Barnhart, 1965; Kay et al., 1997; Humbles et al., 2004). Differentiation of eosinophils is dependent on transcription factor interferon consensus sequence binding protein (ICSBP) and GATA-binding factor 1 (GATA-1). GATA-1 has been identified on the X chromosome of humans and mice that associates with a double GATA sequence commonly related to numerous eosinophil dependent genes including granule protein genes, CC-chemokine receptor 3, interleukin 5 receptor alpha chain, and GATA-1 (Zimmermann et

al., 2000; Yu et al., 2002). Eosinophils produce reactive oxygen species (superoxide and hydrogen peroxide) through the NADPH oxidase, or NOX, pathway, which allows for the degradation of pathogens (Brown and Griendling, 2009; Ravin and Loy, 2015). In addition to the reactive oxygen species, several proteins are released including: major basic protein, eosinophil cationic protein, and eosinophil-derived neurotoxin are dispersed at sites of possible infection to assist in the recruitment of additional leukocytes and combat any pathogen present (Haman et al., 1991; Blanchard and Rothenberg, 2009). The above-mentioned factors also assist in the breakdown of fibrin from eosinophil chemotactic factors and collagenases which are present in the granules. These proteins degrade type I and III collagen that cause fibrosis in the tissue (Scott and Miller, pg. 273, 2011). Eosinophils synthesize several cytokines, chemokines, and lipid mediators that are associated with the proinflammatory process such as IL-2 and 10, RANTES, leukotriene C4, and GM-CSF (Blanchard and Rothenberg, 2009).

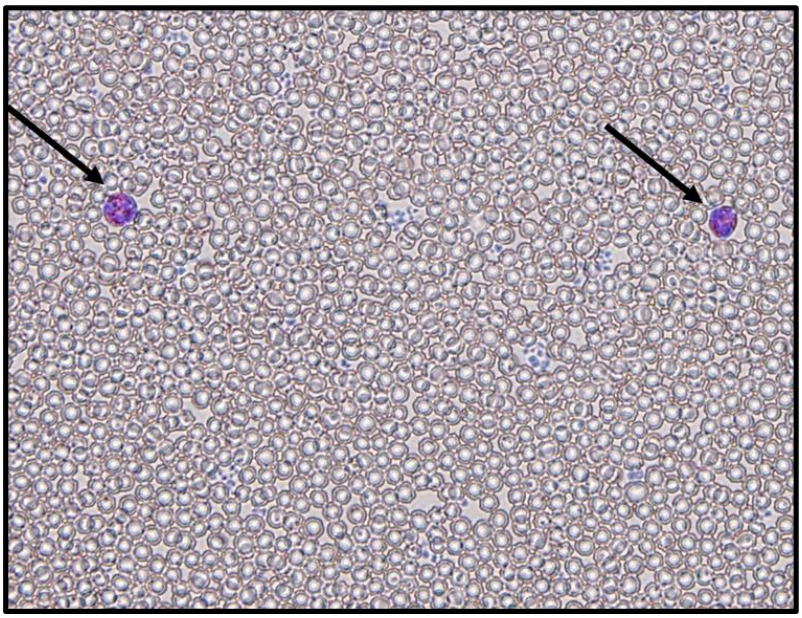

Figure 1-6*. Image of bovine eosinophils. Eosinophils are most easily identified by a pink, acidic dye binding to the cytoplasm. Additionally, granules can be identified within the cell.*

Basophils

Basophils are the primary responders to allergens and the major cause for the inflammatory reaction. Basophils are produced from the hematopoietic stem cells and mature within the bone marrow. Through the expression of over 30 surface receptors, including high-affinity IgE receptors, FcεRI, cytokine receptors interleukin (IL)-3R, IL-5R, and GM-CSFR, and chemokine receptors CCR2 and CCR3, basophils become activated to release histamines comprised of proteoglycans (Stone et al., 2010). Additionally, leukotriene 4, leukotriene D4, and leukotriene E4 are produced from the basophils to increase vascular permeability and bronchoconstrictors. These factors cause the contraction of smooth muscle and dilation of capillaries promoting blow flood to the allergen site to recruit additional leukocytes (Siracusa et al., 2013).

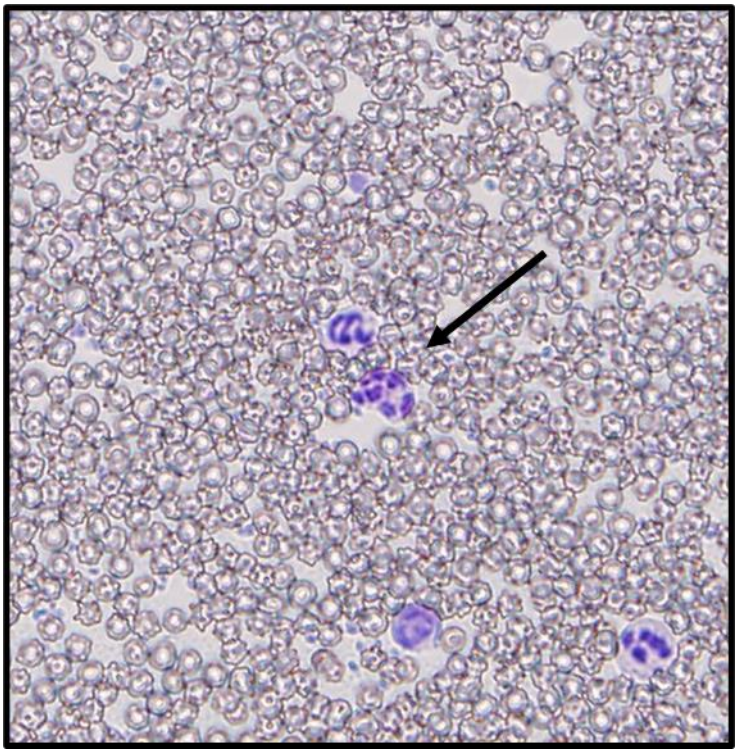

Figure 1-7*. Image of bovine basophil. The distinguishing factor in identifying basophils compared to other granulocytes is that the granules are present on the exterior of the cell. Additionally, purple, basic dye adheres more to basophils to generate a purple/pink appearance to the cytoplasm.*

Lymphocytes

Lymphocytes are the primary leukocyte in the adaptive immune response of the body. The cells originate from common lymphoid progenitor cells in the bone marrow and thymus and are found in circulation throughout the body and peripheral tissues. When activated, these cells differentiate into T and B lymphocytes and natural killer cells (Bonilla and Oettgen, 2010). B lymphocytes originate in the bone marrow and are committed to maturation through expression of signal-transduction receptors CD19 and CD34 (Blom and Spits, 2006). T lymphocytes originate in the thymus. Under experimental conditions, multiple types of progenitors can generate T cells. These cells are selected when gene expression for DN2 and DN3 phenotypic stages (c-Kit^{+} CD44^{+} CD25^{+} and c-Kit^{low} CD44^{-} CD25^{+} respectively) occur to eliminate alternative hematopoietic fates (Carpenter and Bosselut, 2010; Rothenberg, 2011). In their development, gene segments are spliced to create a diverse population of receptors to identify pathogens. When pathogens are identified, long-live memory T and B cells are created to establish a faster immune response with subsequent encounters of the pathogen.

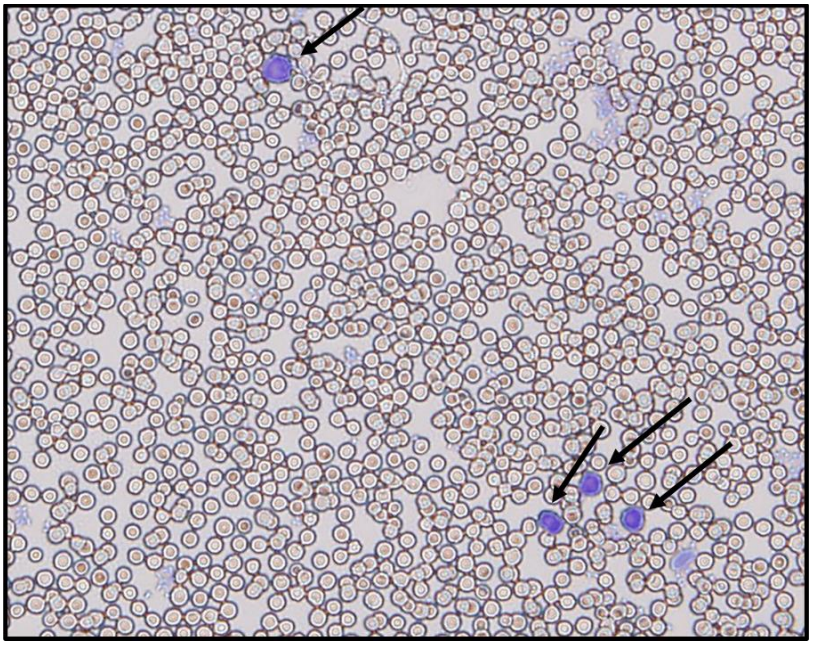

Figure 1-8*. Image of bovine lymphocytes. Lymphocytes are small and circular with a smooth exterior wall. Additionally, two cytoplasmic membranes give the distinguishing characteristic of lymphocytes.*

Monocytes

Monocytes are primarily associated with the innate immune system but play functional roles in adaptive immunity. Monocytes develop within the bone marrow and differentiate from macrophage-DC precursor cells. After entering circulation, environmental factors dictate monocyte differentiation into either macrophage 1 (M1) or macrophage 2 (M2) cells. M1 cells are proinflammatory and recruit additional white blood cells to increase cytokines produced at the pathogen site. M2 cells are anti-inflammatory and promote cell proliferation and repair through collagen synthesis and fibrosis (Italiani and Boraschi, 2014). Both cells are phagocytic and engulf pathogenic microorganisms (Weiss et al., 2009). Monocytes differentiate into macrophages based on specific subpopulations and genetic markers (Muñoz et al., 2020). For example, $CCR2^{hi}Ly6C^{+}$ subpopulation monocytes have preferential differentiation to M1 macrophages whereas $CCR^{low}Ly6C^{-}$ subpopulation monocytes are attributed to M2 macrophages (Yang et al., 2014). The literature suggests that, due to the high plasticity

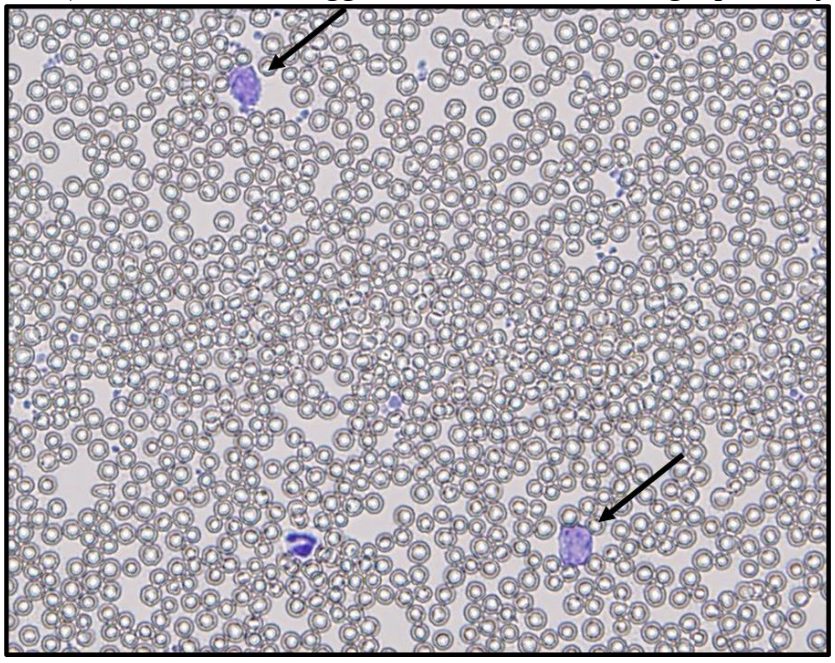

Figure 1-9*. Image of bovine monocytes. Monocytes are the largest of the agranulocyte and granulocyte white blood cells. These cells have abnormally shaped cell walls with no distinction of granules or secondary cell wall.*

nature of monocytes, either the upregulation or downregulation of Ly6C can result in monocyte differentiate to either M1 or M2 (Yang et al., 2014).

Ovarian Inflammation

The process of ovulation is an inflammatory response. Cytokines and chemokines are essential for ovulation to occur in the normal estrous cycle (Stassi et al., 2019). Activated T lymphocytes, macrophages, monocytes, and neutrophils will infiltrate the theca layer during the follicular phase and be present in higher concentrations of preovulatory follicles (Duffy et al., 2019). The follicular phase is the normal progression of primordial follicles developing to antral follicles for ovulation. These infiltrated leukocytes will secrete numerous chemokines and cytokines to recruit additional leukocytes to the ovary (Stassi et al., 2019). Some of these chemokines and cytokines present are granulocyte-macrophage-colony stimulating factor (GM-CSF), monocyte chemoattractant protein 1 (MCP-1), interleukin (IL) 8 and 6 proteins, chemokine ligand 20 (CCL20), regulated upon activation of normal T cell expressed and secreted (RANTES), interferon alpha (INFα), and IFNβ are just a few signaling factors normally expressed in preovulatory follicles that recruit additional leukocytes (Stassi et al., 2017; Snider and Wood, 2019).

Interestingly, GM-CSF and IL-8 also regulate ovarian cell function (Stassi et al., 2017). In the steroidogenesis pathway discussed earlier, GM-CSF decreases granulosa cell proliferation and synthesis of 17β-estradiol and progesterone (Zhang and An, 2007) which will result in the accumulation of androgens. IL-8 also inhibits estradiol synthesis in granulosa cells but increases progesterone synthesis in both theca and granulosa cells

(Shimizu et al., 2012). These cytokines are essential for healthy ovulation to release the oocyte for fertilization.

Summary

Initiation and maintaining estrous cyclicity is essential for pubertal development. Failure to establish regular cyclicity can negatively impact profitability of beef producers. There are numerous factors that contribute to pubertal development including nutrition, weight, environmental factors, presence of male, and genetics. These components regulate the hypothalamic-pituitary-gonadal axis that helps to determine a physiological set-point where energy can be deviated to the reproductive system.

While it has been well established that the previously described factors are essential for the initiation of puberty, one area of emerging research is how the interplay of hematological and immunological factors can impact pubertal attainment. Gonadal hormones can impact development and function of white and red blood cell development. Estradiol receptors are present on several lymphoid tissues, lymphocytes, macrophages, and dendritic cells (Klein and Flanagan, 2016). Progesterone has a broad anti-inflammatory effect on macrophages and dendritic cells by inhibiting IL-1β and TNF secretion (Butts et al., 2007; Jones et al., 2010). Additionally, P4 can also antagonize the TLR and NF-κB pathways that will inhibit the inflammatory process (Butts et al., 2007). Androgens also have a broad anti-inflammatory effect where inhibition can be caused by TNFα, inducible nitric oxide synthase, and nitric oxide pathways by macrophages (D'Agostino et al., 1999; Lechner et al., 2005). Androgens are also able to increase IL-10 and TGFβ that will promote anti-inflammatory responses (D'Agostino et al., 1999; Liva and Voskuhl, 2001). These studies have established that gonadal hormones influence hematological and

immunological factors. However, very little information is present on potential roles of different immunological factors on puberty. Therefore, the current study is the first step in determining if alterations in hematological and immunological factors effect different pubertal classifications in beef heifers during the pre and peripubertal period which might contribute to altered pubertal attainment.

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CHAPTER II: CHANGES IN WHOLE BLOOD PARAMETERS IN BEEF HEIFERS MAY CONTRIBUTE TO DELAYED PUBERAL ATTAINMENT

ABSTRACT

Pubertal development in beef heifers can be affected by many factors. Previously in our lab, we identified four distinct pubertal groups: Typical, Early, Start-Stop, and Non-Cycling through progesterone profiling during the peripubertal period. Our next step was to determine factors contributing to these pubertal classifications. While limited research is available in cattle, whole blood parameters such as hematocrit, white blood cells (WBC) and hemoglobin concentrations do change in girls during pubertal onset. Thus, our hypothesis was that heifers with altered pubertal attainment would have correlated differences in whole blood cell parameters that may contribute to their precocious or delayed puberty. Weekly blood samples were collected during the pre and peripubertal period on 2019 born heifers to obtain progesterone profiles for pubertal classifications. From collections, 62 heifers were classified as Typical, 17 as Early, 11 as Start-Stop, and 22 as Non-Cycling. Monthly blood samples were analyzed with a HemaTrue ® Veterinary Hematology Analyzer to determine whole blood parameters and blood smears were used to count specific WBC populations. Early heifers tended to have reduced granulocytes ($p=0.07$) compared to Typical (controls). Early and Start-Stop heifers that initiate puberty earlier had greater hematocrit ($p=0.04$) compared to Non-Cycling heifers that have delayed pubertal attainment. Hemoglobin concentrations ($p=0.03$) were greater for Early and Start-Stop heifers compared to Non-Cycling heifers. Additionally, red blood cell (RBC) distribution width to platelet ratio tended to be greater in Early compared to Typical and

Non-Cycling heifers (p=0.07). However, blood smears revealed no differences for numbers of granulocytes or agranulocytes among pubertal groups. All parameters changed over the collection as age increased in all groups suggesting that maturity had effects on these hematological parameters. Hematological factors such as reduced granulocytes, increased hematocrit, and red blood cell width per platelet number may promote increased growth and development to allow for the initiation of puberty earlier. Furthermore, reductions in hemoglobin concentrations or functionality may delay pubertal attainment as we observed in the Non-Cycling heifers. Further investigation into these hematological factors will provide linkages between reduced granulocytes, RBC production/destruction, altered hemoglobin production, and impacts on timing of attainment of puberty in heifers.

INTRODUCTION

Over the past few decades, sexually dimorphic differences between innate and adaptive immune responses have been investigated in human and animal models (Pluncevic Gligoroska et al., 2019). Generally, women have been shown to have stronger innate and adaptive immune responses when compared to men (Klein and Flanagan, 2016; Laffont et al., 2017). This factor is crucial in understanding how diseases and infections are combatted differently between sexes (McDonald et al., 2016) and potentially if different puberty events (estrogen-centric vs androgen-centric) may cause these altered response. Hematological and immunological parameters are widely used as an evaluative tool to assist in diagnosis and treatment of diseases (Pérez-de-Heredia et al., 2015; Kone et al., 2017; Herman et al., 2018). Several of these parameters include white blood cell (WBC) counts of granulocyte and agranulocytes, red blood cell (RBC) counts and associated factors such as hemoglobin (HGB) concentrations, hematocrit (HCT) levels, and analysis of platelet (PLT) counts and concentrations. It has been previously noted that a shift in these hematological and immunological parameters occur during the peripubertal period in girls and boys and may contribute to alterations in timing of attainment of puberty (Timmons et al., 2006; Pluncevic Gligoroska et al., 2019).

Puberty is a critical developmental period that establishes the capacity for females to conceive and produce offspring. Initiation of regular cyclicity is essential for reproductive success (Perry, 2016). Hormonal changes occur during puberty to establish molecular, physiological, and anatomical differences particularly in regards to the immune response, but it is unclear if differences in immunological parameters can contribute to timing of pubertal attainment (Lamason et al., 2006; Pluncevic Gligoroska et al., 2019).

While some of this information on immunological parameter has been illustrated in humans, less than 10% of immunology articles account for sex in experimental models and even less information is available on cattle during the period of pubertal development (Beery and Zucker, 2011). After an evaluation of the current literature, there is a large discrepancy in information investigating hematological and immunological parameters during the peripubertal period in beef heifers (Klein and Flanagan, 2016). From previous research, we have determined that heifers in our herd achieve puberty differently and have developed pubertal classifications to reflect these differences: Typical, Early, Start-Stop and Non-Cycling. Our next step was to determine what factors may cause these changes in timing of puberty and if whole blood parameters collected during the pubertal period contributed to differences in pubertal classification. Thus, in the current study, we collected immunological and hematological cell populations in whole blood of heifers from weaning to breeding to determine if any parameters correlated with pubertal classification that may impact pubertal attainment which in turn would impact reproductive success and longevity.

MATERIALS AND METHODS

Ethics

The University of Nebraska-Lincoln Institutional Animal Care and Use Committee approved all procedures and facilities used in this experiment.

Animals

The physiology herd at the University of Nebraska-Lincoln are maintained at the Eastern Nebraska Research and Extension Center (ENREC) in Mead, NE. 112 beef heifers born around March of 2019 were utilized for this study. These heifers are a composite breed comprised of ¾ Red Angus and ¼ Simmental. Heifers are maintained with dams

until weaning in mid-October at approximately 6-7 months of age where they are separated to additional pasture space at ENREC.

Blood Sample Collection

Weekly blood samples were collected 1 week before weaning in October (pre-weaning sample) to the week before breeding in May (Figure 2-1). Samples were obtained using coccygeal venipuncture into glass vacutainer blood collection tubes (Becton, Dickinson and Company, Franklin Lakes, NJ) containing 12 mg EDTA and placed on ice for transportation. Blood samples were centrifuged at 700 g for 30 minutes at 4° C within 6 hours of collection (Nafziger et al., 2021). Plasma samples were aliquoted and stored in 1.5 ml tubes (Reference: MCT-150-L-C, Corning Incorporated, Corning, NY) at -20° C until hormonal analysis.

Progesterone Radioimmunoassay

Plasma samples were processed utilizing a radioimmunoassay (RIA) to detect progesterone (P4) concentration (Nafziger et al., 2021). Each sample was run in duplicate and averaged to determine P4 concentration for that sample. Samples with a coefficient of variation (CV) greater than 15% were re-analyzed. Progesterone concentrations were determined using the ImmuChem^{TM} Coated Tube Progesterone ^{125}I RIA kit (ICN Pharmaceuticals, Inc., Costa Mesa, CA; intra-assay CV= 5.64%, inter-assay CV=7.43%)

Puberty Group Classification

Progesterone concentrations were utilized to classify pubertal attainment in heifers as published (Nafziger et al., 2021). Briefly, heifers are considered pubertal when P4 ≥ 1 ng/ml and there is continuous cyclicity. Continuous cyclicity was determined to be when P4 samples ≥ 1 ng/ml are greater 50% of the sampling period on and after the initial

pubertal date. In contrast, heifers that have samples less than 50% of samples where P4 samples ≥ 1 ng/ml during the sampling period after the puberty date are considered to have discontinuous cyclicity. The four pubertal classifications (Nafziger et al., 2021) identified were: 1) Early – initial P4 ≥ 1 ng/ml before March 12 with continuous cyclicity; 2) Typical – initial P4 ≥ 1 ng/ml after March 12 with continuous cyclicity. March 12th was used to distinguish Early and Typical pubertal groups because it marked the 25th percentile for puberty date in historical UNL physiology herd data; 3) Start-Stop – had an initial P4 ≥ 1 ng/ml but discontinuous cyclicity; 4) Non-Cycling – had no P4 ≥ 1 ng/ml during the sampling period. A SAS program developed to identify pubertal classification (Nafziger et al., 2021) was used with the criteria above.

White Blood Cell and Whole Blood Analysis

HemaTrue

Whole blood parameters were calculated using a HemaTrue® Veterinary Hematology Analyzer and corresponding reagents (Heska, Loveland, Colorado) (Zinicola et al., 2019). Approximately 20 µl was taken from each weekly blood sample to run the analyses prior to centrifugation to obtain plasma for P4 evaluation. Parameters analyzed include total white blood cell count (WBC), lymphocyte concentration in absolute number and percentage (LYM), monocyte concentration in absolute number and percentage (MONO), granulocyte concentration in absolute number and percentage (GRAN), hematocrit (HCT), Mean Cell Volume of RBCs (MCV), Red Cell Distribution Width in absolute number and percentage (RDW), RBC size distribution histogram Hemoglobin Concentration (HGB), Mean Cell Hemoglobin Concentration (MCHC), Mean Cell Hemoglobin (MCH), Total Red Blood Cell Count (RBC), Total Platelet Count (PLT), Mean Platelet Volume (MPV),

and PLT size distribution histogram. Additionally, PLT to LYM and RDW to PLT ratios were compared to better understand what is occurring within and between the pubertal groups.

Analysis of whole blood with the HemaTrue® occurred monthly from weaning (October) to breeding (May) with an additional collection taken two weeks after weaning (Figure 2-1). This sample was taken in case the weaning caused stress. It has been shown that stress can cause progesterone secretion from the adrenal gland in women, rats, and cattle (Bova et al., 2014; Herrera et al., 2016; Fernandez-Novo et al., 2020).

Blood Smears

To better examine and identify white blood cell populations during the pubertal development period, periodical timepoints were selected to evaluate blood smears and stain to allow for identification of different white blood cells through morphology in each sample. Four of the monthly HemaTrue® collections (October, December, February, and April) were selected to conduct a blood smear analysis. Seven µl of whole blood were placed on microscope slides (Catalog No: 12-550-01, Fisher Scientific Company LLC, Atlanta, GA) prior to centrifugation (Figure 2-1). Using a second slide, blood was smeared to thinly coat the slide. This process was repeated with each sample for a total of two slides per animal/collection. Slides were then allowed at least 24 hours to dry. Once fully dried, slides were subjected to a Differential Quick Stain (Catalog No: 26096-25, Electron Microscopy Sciences, Hatfield, PA) following an established procedure accompanying the stain. After adding stain, the slides dried for an additional 24 hours. Once dried, all samples were counted for white blood cell populations by a minimum of two technicians. Each individual technician averaged their counts between the two slides collected for an

individual animal for each collection. Neutrophils are identified by dark granules surrounded by a clear cytoplasm. Eosinophils are discerned by a pink, acidic dye that binds to the cytoplasm with the addition of granules present. Basophils are distinguished by granules present on the exterior of the cell. Additionally, purple, basic dye adheres to the cytoplasm to generate a purple/pink appearance. The presence of two cytoplasmic membranes is the distinguishing feature for lymphocytes. Monocytes are the largest of the agranulocyte and granulocyte white blood cells. They have a distinct abnormal shaped cell with no granules or secondary cell wall present.

Statistical Analysis

Data analyses were conducted using SAS v9.4. Mean ± standard error of the mean (SEM) are utilized for all data presented. All analyses illustrated significance when $P \leq 0.05$ and a tendency when $P \leq 0.10$ and > 0.05. Blood smears and HemaTrue® parameters were analyzed with a repeated measure using the GLIMMIX procedure in SAS. Parameters were compared by pubertal group, date collection, and date collection*pubertal group (Figures 3-16 A, C, and D). The correlation of weight on the respective HemaTrue® variable was compared to linear, quadratic, and cubic polynomials to best illustrate the relationship between these parameters. If a polynomial was not significant ($P \leq 0.05$), it was removed from the final code (Figures 3-16 B).

RESULTS

HemaTrue®

Total White Blood Cell Count

Total white blood cell count was measured to indicate inflammation or infection in particular pubertal groups. However, there were no differences in total while blood cell

count in pubertal classifications (Figure 2-3A). Repeated measures analysis of white blood cell count over the collection period did demonstrate that total WBC count declined from weaning to breeding over all heifers (Figure 2-3C). Changes in weight of heifers had no effect on this parameter (Figure 2-3B).

Lymphocyte, Monocyte, and Granulocyte

Lymphocyte, monocyte, and granulocyte counts can be indicative of the overall health, reproductive and metabolic status, and adaptability to environmental stress of the animal (Latimer, 2011; Pandey et al., 2017; Noya et al., 2019). Lymphocyte and monocyte populations were not different between pubertal groups (Figure 2-4A and 2-5A), and both types of cells were decreased over the collection period (Figure 2-4C and 2-5C). Granulocyte populations tended to be different between pubertal groups with Early having reduced amounts compared to Typical heifers (Figure 2-6A). Interestingly, even though granulocyte populations were different over the collection period, there was not a distinct trend over the collection time (Figure 2-6C). For Lymphocyte, monocyte, and granulocyte cell numbers, changes in weight of heifers did not affect cell numbers (Figures 2-4B, 2-5B, and 2-6B).

Mean Cell Volume of RBC and Red Cell Distribution Width

Elevated mean cell volume of red blood cell (MCV) has been associated with anemia, liver disease, alcohol use, hematologic disease, hypothyroidism, and malignancy in adult humans (Solak et al., 2013). There was no difference in MCV between puberty groups (Figure 2-7A). However, mean cell volume of RBC was different with a distinct increase over time (Figure 2-7C). Additionally, weight tended to have a positive, linear

relationship with MCV (Figure 2-7B) indicating that as heifers increased in weight there was an increase in MCV.

Red cell distribution width (RDW) is the measurement of heterogeneity of red blood cells and has been positively correlated to cardiovascular disease, neurovascular complications, sepsis, and hepatitis as well as inflammation in adults (Yilmaz et al., 2014). In the current study, the RDW was not different between pubertal groups or influenced by weight during the pubertal period (Figure 2-8A and 2-8B). However, the repeated measures analysis over all heifers demonstrated an overall increase from weaning to breeding (Figure 2-8C).

Hematocrit and Total Red Blood Cell Count

Hematocrit is an evaluation of the volume of red blood cells compared to the total blood volume and can be used to evaluate anemia in patients (Billett, 1990). Hematocrit was different between groups with Early and Start-Stop heifers greater than Non-Cycling heifers (Figure 2-9A). Additionally, hematocrit over all heifers significantly declined over the collection period (Figure 2-9C). Weight had a linear interaction with hematocrit. As weight increased, hematocrit increased in value (Figure 2-9B). Total RBC count was also not different between pubertal groups nor had an interaction with heifer weight (Figure 2-10A and 2-10B). Total RBC count decreased over the collection period (Figure 2-10C).

Hemoglobin Concentration and Mean Cell Hemoglobin Concentration

Hemoglobin concentration was significantly different between pubertal groups with Typical, Early, and Start-Stop heifers having greater concentrations compared to Non-Cycling heifers (Figure 2-11A). Weight also had a linear relationship where, as weight increased, hemoglobin concentrations increased (Figure 2-11B). Additionally, hemoglobin

concentration decreased over the collection period (Figure 2-11C). When pubertal groups were compared to collection period there were no differences (Figure 2-11D).

Mean cell hemoglobin concentration was not different between pubertal groups and increasing weight for the heifers did not affect this parameter (Figure 2-12A and 2-12B). Over time hemoglobin concentration was different (Figure 2-12C).

Total Platelet Count and Mean Platelet Volume

Total platelet count and mean platelet volume have been used as tools to diagnose vascular diseases and infectious and inflammatory processes in adult humans (Biljak et al., 2011; Wang et al., 2011). Total platelet count was not different between pubertal groups (Figure 2-13A) but decreased over the collection period (Figure 2-13C). Additionally, heifer weight tended to have a positive, linear interaction with total platelet count (Figure 2-13B). Mean platelet volume was also not different between pubertal groups (Figure 2-14A). Heifer weight had no effect on mean platelet volume (Figure 2-14B), which declined over the collection period (2-14C).

Platelet to Lymphocyte Ratio and Red Cell Distribution Width to Platelet Ratio (RDW)

Two additional parameters were compared and analyzed to better understand what is occurring between our puberty groups. Total platelet count was compared to lymphocyte populations to generate a platelet to lymphocyte ratio (PLR). PLR has become a novel inflammatory marker that has helped predict several human diseases primarily, cardiovascular disease. Elevated PLR is associated more frequently with disease detection (Ye et al., 2019). From this analysis, there were no differences detected between pubertal groups or an interaction with heifer weight (Figure 2-15A and 2-15B). However, there was a decrease over the collection period (Figure 2-15C).

RDW was compared to total platelet count to create RDW to platelet ratio. In previous studies, RDW to platelet ratio has been found to predict elevated fibrosis levels in adult patients with chronic hepatitis (Chen et al., 2013). RDW to platelet ratio tended to be different with Early heifers having greater RDWa:PLT ratio compared to Typical and Non-Cycling heifers (Figure 2-16A), but there was no impacting factor of heifer weight (Figure 2-16B). Additionally, RDW increased over the collection period (Figure 2-16C).

Blood Smears

Granulocyte

There were no differences in amounts of neutrophils, basophils, or eosinophils when analyzed among pubertal groups (Figures 2-17A, D, and G). When analyzing all heifers irrespective of pubertal classification there was a difference in neutrophils, basophils, and eosinophils over time of the collection with neutrophils and eosinophils increasing with basophils declining overall (Figures 2-17B, E, and H).

Agranulocyte

Both lymphocytes and monocytes were not different between pubertal groups (Figures 2-18A and D). However, monocyte counts for all heifers declined over the collection period (Figure 2-18E). Indicating that as heifers became more sexually mature, the amount of cells for both of these WBCs were reduced.

DISCUSSION

The current study provides novel information related to whole blood parameters during pubertal development and how these parameters may contribute to alterations in pubertal attainment in beef heifers. Since only one year of data was analyzed, we are reporting differences in heifers born in 2019 born. Interestingly, heifers initiating puberty

earlier demonstrated tendencies for reductions in granulocytes (Early vs Typical), increases in RDWa:PLT ratio (Early vs Typical and Non-Cycling), and increases in hematocrit (Early and Start-Stop vs Non-Cycling). This suggests that reductions in certain WBCs and increases in RBC per volume of plasma may allow for earlier reproductive maturation and initiation of puberty. These parameters may also be key to enhanced growth and weight gain detected in Early heifers (Nafziger et al., 2021). In contrast, Non-Cycling heifers have delayed pubertal onset and reduced hemoglobin concentrations compared to all other groups suggesting that factors affecting hemoglobin production and function may contribute to delayed puberty.

White blood cells play a crucial role in the innate and adaptive immune response in controlling pathogen invasion in vertebrate species. Different leukocyte populations are indicative of several physiological factors (Latimer, 2011; Pandey et al., 2017; Noya et al., 2019). In humans, varying leukocyte populations has been expressed during the peripubertal period in girls and boys (Timmons et al., 2006). White blood cells are essential for communicating pro- and anti-inflammatory cytokines and chemokines to regulate acute and chronic inflammation (Cronkite and Strutt, 2018; Yahfoufi et al., 2020). Elevated chronic inflammation during the peripubertal period can result in alterations in the hypothalamic-pituitary-gonadal axis and delay or arrest puberty (Grob and Zacharin, 2020). From this literature, we sought to evaluate if differences in leukocyte populations could be used as a potential evaluative tool to identify alterations and delays in pubertal attainment in beef heifers.

In the current study, granulocyte populations tended to be reduced in Early heifers compared to Typical (Figure 2-6A). This can indicate that Early heifers are less sensitive

or do not have as much inflammation as Typical heifers. Neutrophils comprise the majority of the granulocyte population (Kobayashi et al., 2005). Neutrophils secrete chemokines and cytokines into circulation causing acute inflammation that will recruit additional leukocytes to this tissue.

No differences seen between the other leukocyte populations (Figures 2-3A, 2-4A, and 2-5A) that explain potential delays in pubertal attainment. Reduced granulocyte numbers in Early heifers compared to Typical heifers may provide reduced inflammation allowing advanced pubertal development.

Hematocrit is an evaluation of the volume of red blood cells compared to the total blood volume and is key for evaluating anemia in patients (Billett, 1990). Reduced hematocrit levels are common in menstruating girls and as a result boys usually have higher levels (Pluncevic Gligoroska et al., 2019). Reduced hematocrit levels have been associated with reduced CO2 removal from the lungs, internal parasitic infection, genetic disorders, deficiencies in metabolic enzymes, and destruction of RBC's through oxidative stress (Craig, 1988; Volk et al., 2019; Kells et al., 2020; May et al., 2020). In contrast, elevated hematocrit levels are commonly caused by severe clinical dehydration, but can also be a determinant of thrombotic risk in polycythemia and erythrocytosis in adult humans (Gordeuk et al., 2019).

The majority of oxygen consumed by tissues is due to mitochondrial respiration during the process of oxidative phosphorylation in the electron transport chain (Pittman, 2011). In previous studies, adult cattle have been shown to have hematocrit levels at

~37.8% whereas calves are at ~33.7% (Lin et al., 2020). As heifer calves age, elevated hematocrit levels are necessary to maintain additional mitochondria.

In our study, hematocrit values were greatest in heifers that initiated cyclicity early, Early and Start-Stop heifers, compared to Non-Cycling heifers (Figure 4A) and increased as weight increased (Figure 4B). Additionally, Early heifers have been shown to be heavier in weight during development than Non-Cycling (Nafziger et al., 2021). In our current study hematocrit levels have been shown to be increased as heifers increase in body weight. Body weight or percentage of mature weight in heifers is directly positively correlated with timing of pubertal onset (Martin et al., 2008). Start-Stop heifers did not have increased body weight previously and still appear to be the lightest as yearlings; however, they are the category of heifers that initiate cyclicity at the earliest time period. We speculate that stress may be a factor in their rise in progesterone at this early time period, but; it is puzzling that they would also have increased hematocrit similar to Early heifers.

Weight has been shown to increase as hematocrit levels increase illustrating that lighter weight animals are less likely to have adequate or excess hematocrit levels (Figure 2-9B). While there were no weight differences in our 2019 heifers (Figures 2-2 A-F), historically it has been shown that our Non-Cycling heifers have lower body weight compared to the other pubertal classifications (Nafziger et al., 2021). Including data from 2012-2019, we observe that Non-Cycling heifers are lighter at weaning than Typical or Early but are not different at yearling. Thus, it is possible that Non-Cycling heifers may be slightly anemic. Furthermore, lower hematocrit levels have been associated with reduced weight and growth characteristics illustrating that Non-Cycling heifers may be less efficient for growth during the pre-pubertal period.

It can also be speculated that reduced oxygen binding would result in increased CO_2 concentrations in the blood. Previously, it has been shown that as hematocrit levels decrease, CO_2 amounts released per mol of hemoglobin increased while the intracellular bound CO_2 concentration decreased (Takiwaki et al., 1983). This resulted in higher pH levels in association with lowered hematocrit levels. pH levels are tightly regulated to maintain cell viability. Unregulated levels can result in numerous cell metabolism and function failures involving membrane lipid fluidity, ionic status of cell metabolites, signal transduction within and between cells, ATP production, protein synthesis, and apoptosis and posttranslational modification of proteins (Chesler, 2003; Obara et al., 2008). Therefore, reduced hematocrit levels shown in Non-Cycling heifers may be an indication of altered physiological changes that contribute to delayed ovulation.

Hemoglobin concentration and mean cell hemoglobin concentration have been evaluated during the peripubertal period in humans. In girls, hemoglobin levels will increase and eventually plateau while in boys it will continue to increase (Garvin, 2004). Hemoglobin is tightly correlated to testosterone levels in boys with severely reduced testosterone resulting in reduced hemoglobin levels (Krabbe et al., 1978; Thomsen et al., 1986). Alterations in androgen production in males may therefore contribute to abnormalities in hemoglobin levels. Hemoglobin is important for maximal oxygen consumption. Relatively small decreases in hemoglobin can subsequently reduce oxygen transport to working muscles (Hunter et al., 2001; Andreacci et al., 2004). Alterations in hemoglobin may therefore contribute to altered or delayed pubertal attainment as less oxygen and subsequently more carbon dioxide is present in the body and with the reproductive organs.

Hemoglobin concentration was greater in Typical, Early, and Start-Stop heifers compared to Non-Cycling heifers. There were no differences seen between pubertal groups for mean cell hemoglobin concentration. For both parameters, they declined over the collection period while as in girls it typically increases. Similarly to hematocrit levels, hemoglobin concentrations also increased as weight increased indicating that animals with lighter weight would have reduced hemoglobin concentrations (Figure 2-11B). Additionally, Non-Cycling heifers have reduced reproductive development and maturity compared to the other pubertal classifications when analyzing reproductive tract score, uterine horn diameter, and their estrous response to synchronization with PGF2α (Nafziger et al., 2021). Therefore, reduced hemoglobin and hematocrit levels may indicate hindered sexual maturity of the reproductive system as a result of lowered weight during pubertal development.

Altered levels of mean cell volume of red blood cell (MCV), red cell distribution width (RDW), and total red blood cell count have been used as evaluative tools for numerous human diseases including anemia, sepsis, hepatitis, hematologic disease, and cardiovascular diseases (Solak et al., 2013; Yilmaz et al., 2014). Elevated RDW levels have particularly been associated to be positively correlated to inflammation. In humans, differences in these hematological variables emerges during the peripubertal period (Pluncevic Gligoroska et al., 2019). In the study, we found no differences between these parameters and our pubertal groups (Figures 2-7A, 2-8A, and 2-10A). However, MCV and RDW increased over the collection period while total red blood cell count declined (Figures 2-7C, 2-8C, and 2-10C). This is likely the result of the heifers growing and gaining weight over the trial period. The calculation for MCV best accounts for this as it takes the average

of the red blood cells in accordance with hematocrit (volume of red blood cells). During the study, MCV tended to increase when weight increased indicating that lighter animals were more likely to have reduced MCV (Figure 2-7B). By accounting for the average red blood cells over the total volume, MCV directly illustrates that more red blood cells are present as the animals mature.

Similarly to RDW, elevated platelet counts have been shown in response to inflammatory markers and cytokines such as IL-1 and IL-6 (Unsal, 2005; Zareifar et al., 2014). Total platelet count and mean platelet volume did not vary between the pubertal groups. Both parameters had a steady decline over the collection period. While platelet levels can indicate inflammation in adults, without analyzing inflammatory markers over the collection, we cannot conclude that inflammation is present or absent within our study. Additionally, it is important to note that some potential error with platelet values can occur if inadequate anticoagulation happens within the samples (Billett, 1990). While this may have occurred to a certain degree within our study, sufficient number of heifers present would dilute any error that occurred.

Total platelet count was compared to lymphocyte populations to generate a platelet to lymphocyte ratio (PLR). PLR has become a novel inflammatory marker to detect low-grade inflammation (Ye et al., 2019). In unexplained infertility (UI) in vitro studies, elevated PLR resulted in fewer implantations among patients (Tola, 2018; Kowsar et al., 2021). PLR has been found to be significantly correlated to elevated 17-OH progesterone which can be an indication of polycystic ovary syndrome (PCOS) (Pergialiotis et al., 2018). Overall, reproductive failure has been associated with this parameter. In our study, PLR was not different between pubertal groups, but gradually declined over the collection

period. This would indicate that it is not contributing to potential inflammation that would alter pubertal attainment.

RDW was compared to total platelet count to create RDW to platelet ratio. Increases in this parameter is used to detect elevated fibrosis levels in human adult patients with chronic hepatitis (Chen et al., 2013). In our study, Early heifers had elevated RDW to platelet ratio compared to Typical and Non-Cycling heifers with overall levels continually increasing over the collection period. This parameter has been used to indicate fibrosis in humans (Chen et al., 2013). Elevated steroid production can result increases in fibrosis levels. Early heifers have been illustrated to have greater progesterone concentrations over the developmental period as a result of obtaining puberty sooner (Nafziger et al., 2021). Therefore, this may indicate heifers who begin estrous cyclicity sooner and have elevated steroid production. Since Early heifers initiate cyclicity sooner in the study and ovulation is an inflammatory event the increase in this ratio may indicate changes that reflect a greater amount of ovulations within Early heifers. To support this theory, estrous cycles would need to be correlated with weekly samples and parameters obtained at closer intervals than we have conducted in our study.

CONCLUSION

Heifers that attain puberty early may have altered hematological factors that contribute to their increased weight and advanced sexual maturity. A tendency for reduction in granulocytes may indicate reduced inflammation in Early heifers allowing for normal processes to occur without any impediments to growth and development. Increased hematocrit and RDWa:PLT may also allow for increased efficiency of growth and utilization of nutrients contributing to the increased progesterone concentrations measured

during the pre and peripubertal period (Nafziger et al., 2021). Conversely, Non-Cycling heifers that have delayed pubertal onset displayed reduced hemoglobin levels which may contribute to their impaired growth, sexual maturity, and infertility in their first reproductive cycle (Nafziger et al., 2021). Overall, the hematological parameters evaluated in the current manuscript may be due to genetic, prenatal, or altered metabolic capacity in these different heifer pubertal groups. Further investigation will provide linkages between altered RBC parameters, inflammation or lack of inflammation, and delayed puberty in heifers.

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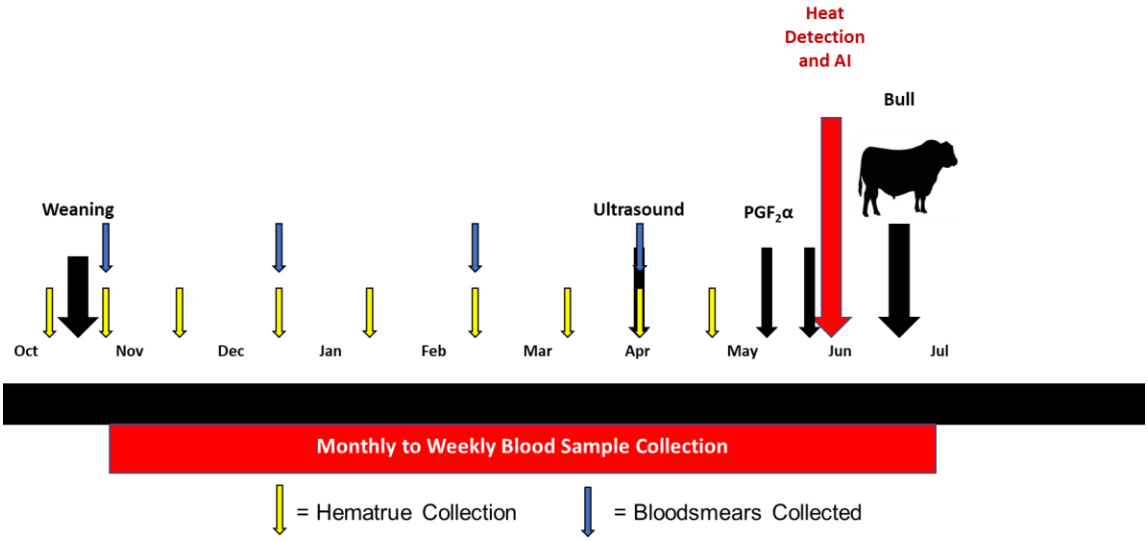

Figure 2-1. Experimental Timeline of Hematrue and Bloodsmear Collections. Weekly plasma collections for pubertal classification begins prior to weaning in October and continues until breeding in May. During this time, monthly Hematrue analysis of whole blood samples occur indicated by the yellow arrow. Additional analysis of white blood cell populations by blood smears occur every other month and are represented by the blue arrows. In addition to analyzing whole blood, ultrasonography for reproductive maturity occurs in April. To prepare for breeding, all heifers are synchronized by two shots of prostaglandin F2α two weeks apart. After synchronization, heifers are bred by artificial insemination (AI) from estrus heat detection. The remaining heifers that are not bred by AI are naturally serviced by the bull.

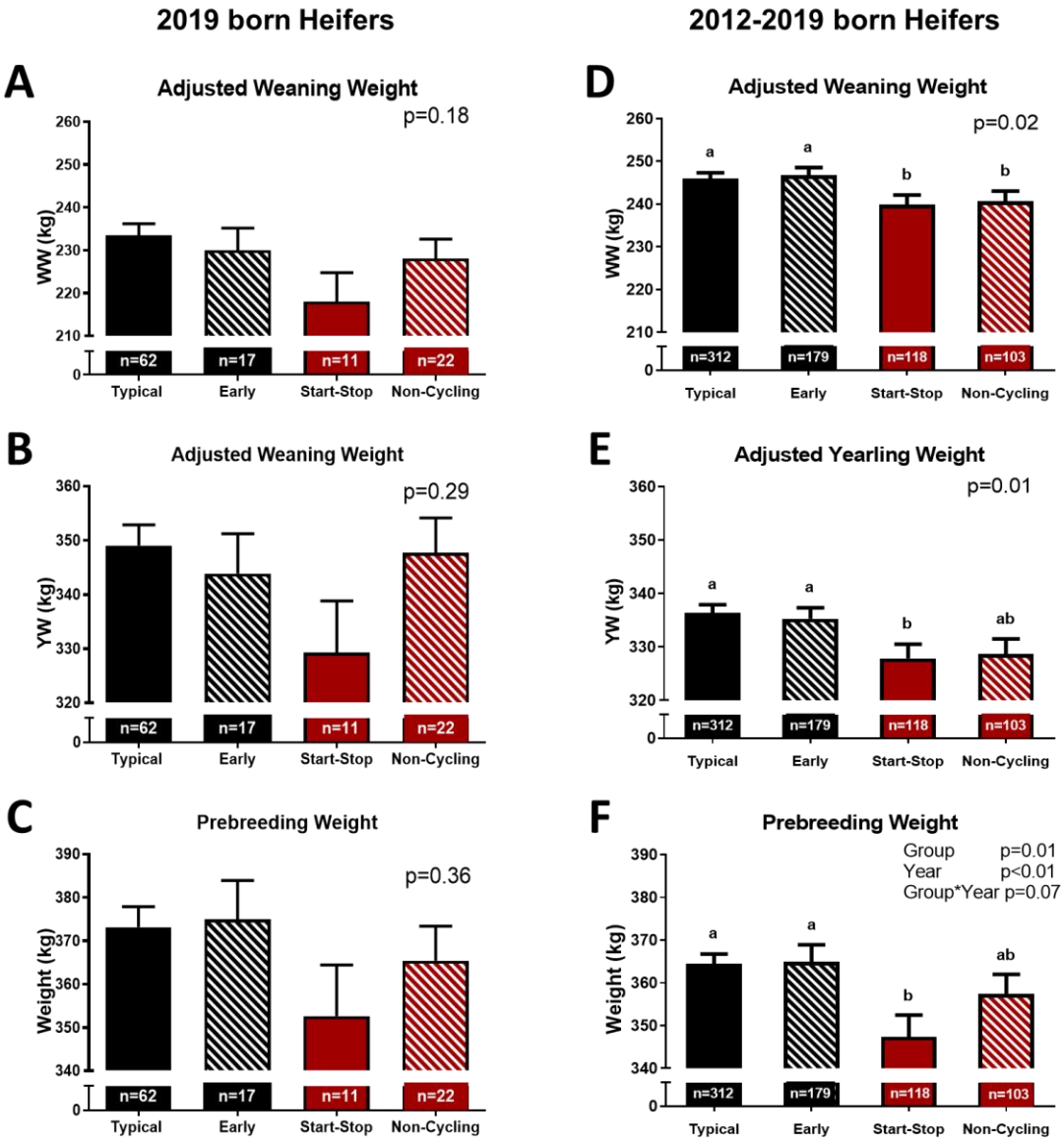

Figure 2-2. Assessment of weight for 2019 (left;A-C) vs 2012-2019 (right;D-F) born heifers. While no differences in weight were seen in just the 2019 born heifers, Start-Stop and Non-Cycling heifers are shown to be lighter adjusted weaning weight (WW). Nafziger et al., 2021 DOI: 10.1093/biolre/ioab044.

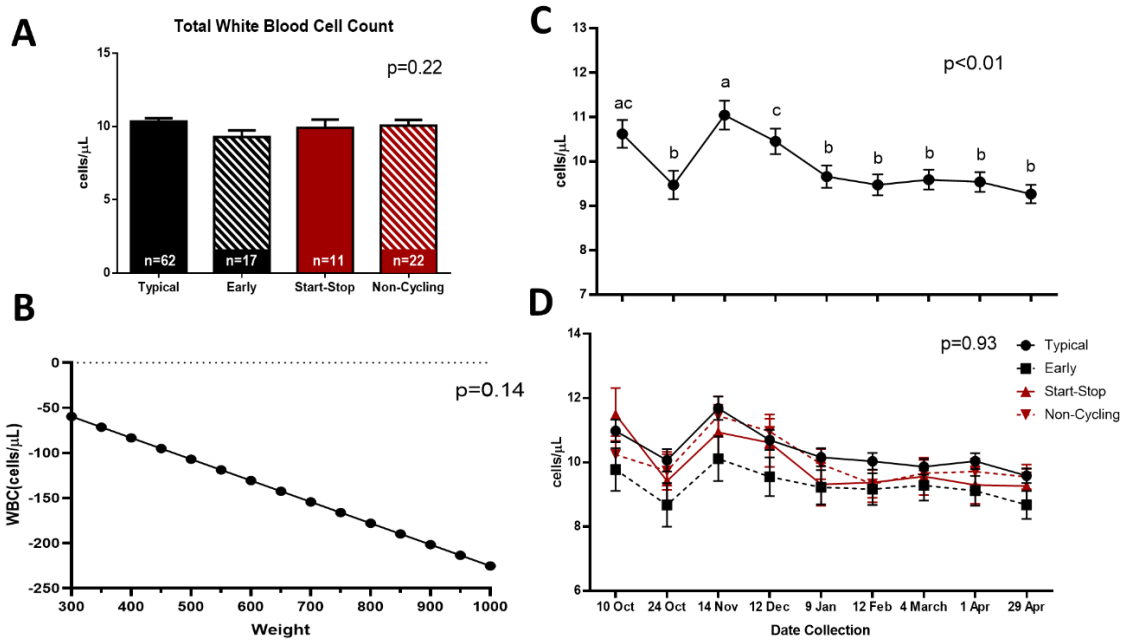

Figure 2-3. Pubertal Group (A), Weight Correlation (B), Collection over time (C), and Collections*Group for Total White Blood Cell (WBC) Count (D). No differences were seen between pubertal groups and weight did not affect total WBC count. A decline over the collection period was observed.

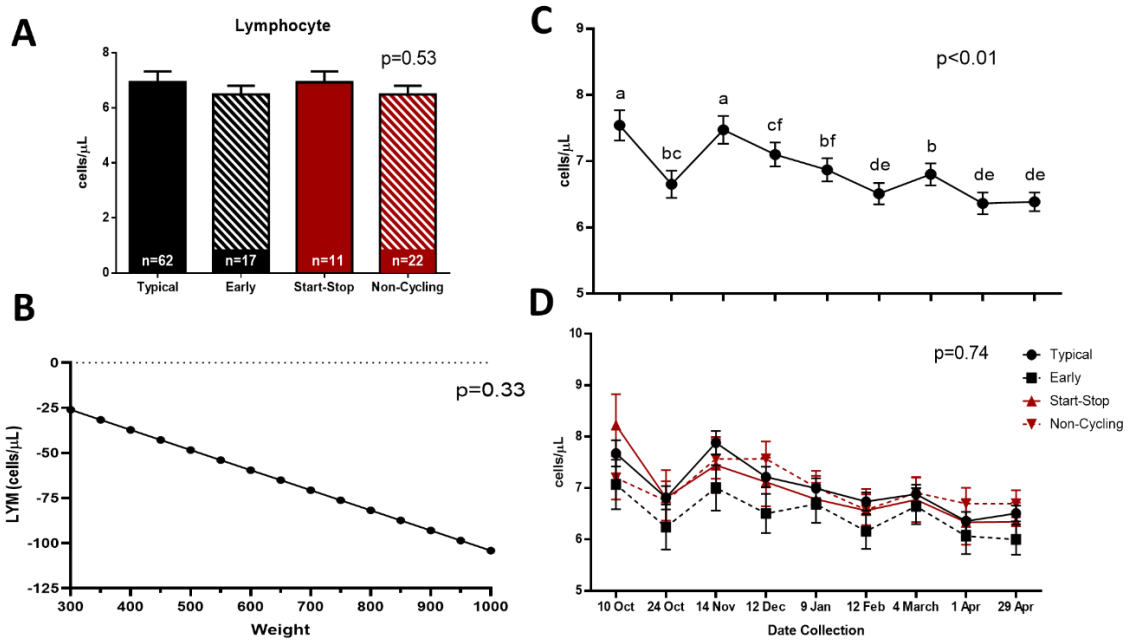

Figure 2-4. Pubertal Group (A), Weight Correlation (B), Collection over time (C), and Collections*Group for lymphocyte (D). No differences were seen between pubertal groups and weight did not affect lymphocyte populations. A decline in lymphocytes was observed over the collection period.

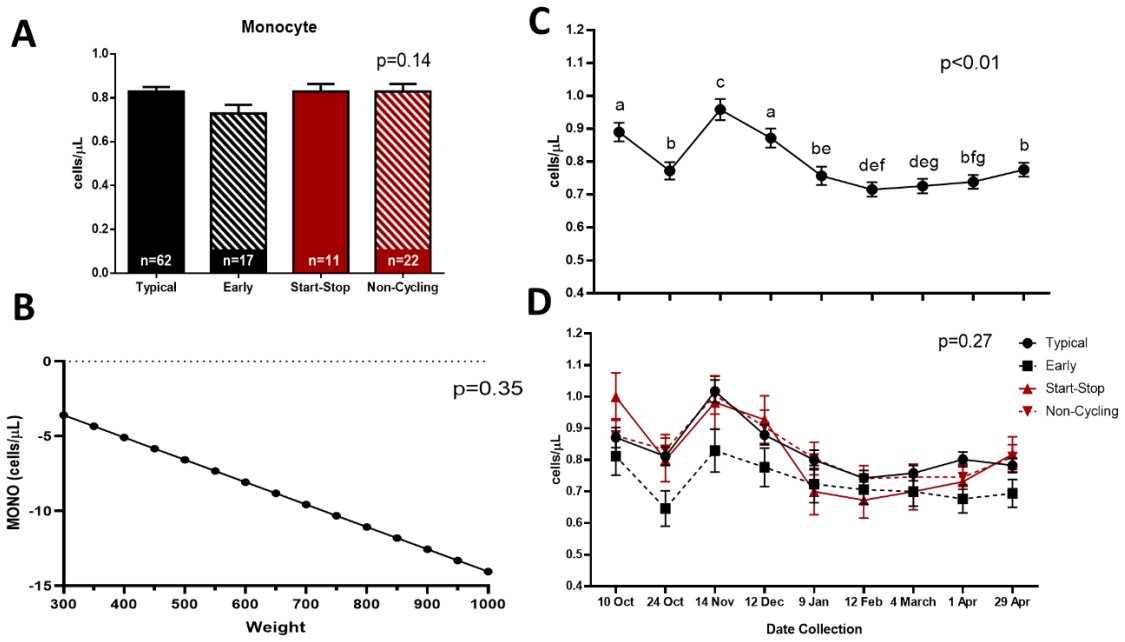

Figure 2-5. Pubertal Group (A), Weight Correlation (B), Collection over time (C), and Collections*Group for Monocyte (D). Monocyte populations were not different between pubertal groups or impacted by weight. A decline in monocytes was observed over the collection period.

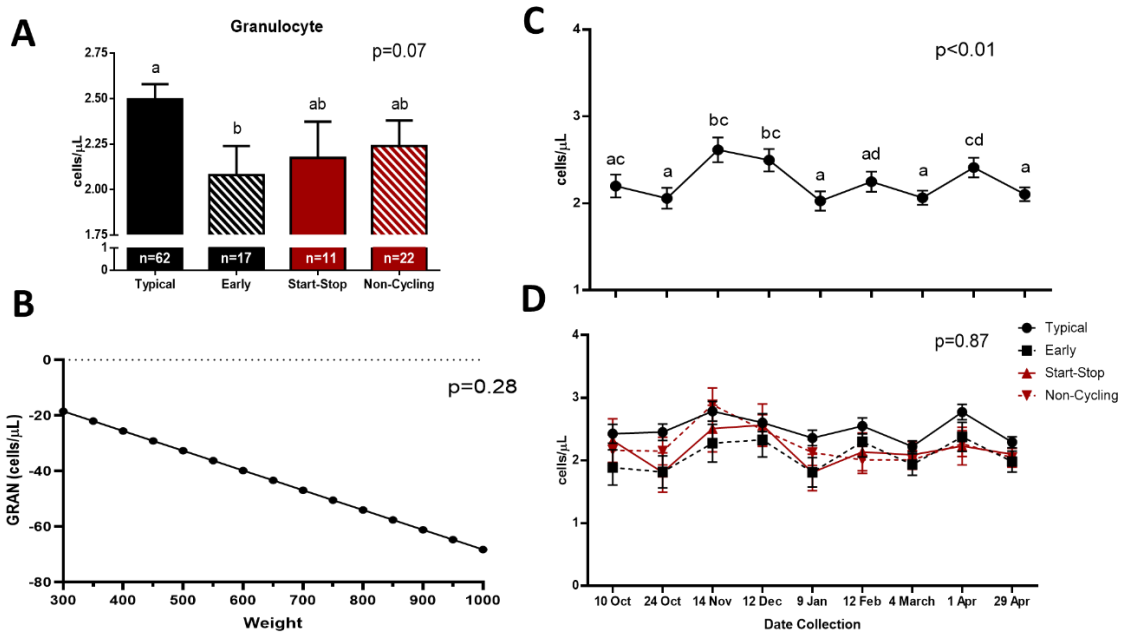

Figure 2-6. Pubertal Group (A), Weight Correlation (B), Collection over time (C), and Collections*Group for Granulocyte (D). Granulocytes tended to be different between Typical and Early heifers. Weight was not an impacting factor on granulocyte numbers. Granulocytes varied over the collection period.

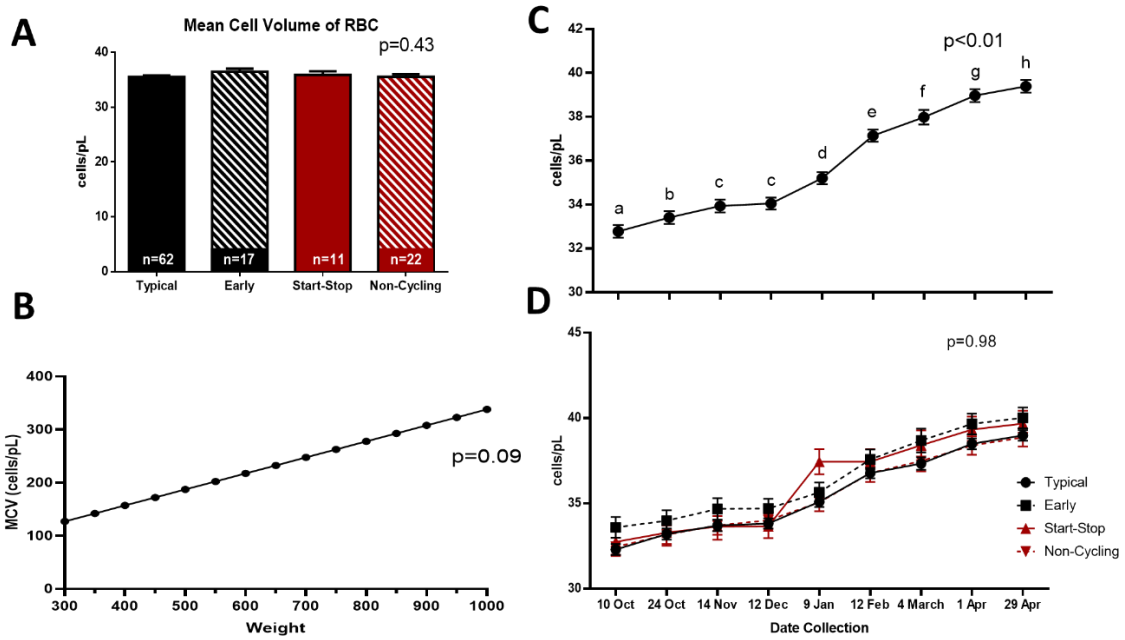

Figure 2-7. Pubertal Group, Collection (A), Weight Correlation (B), Collection over time (C), and Collections*Group for Mean Cell Volume (MCV) of RBC (D). Pubertal class was not different for MCV. As weight increased, MCV tended to increase in value. Over the collection period, MCV increased.

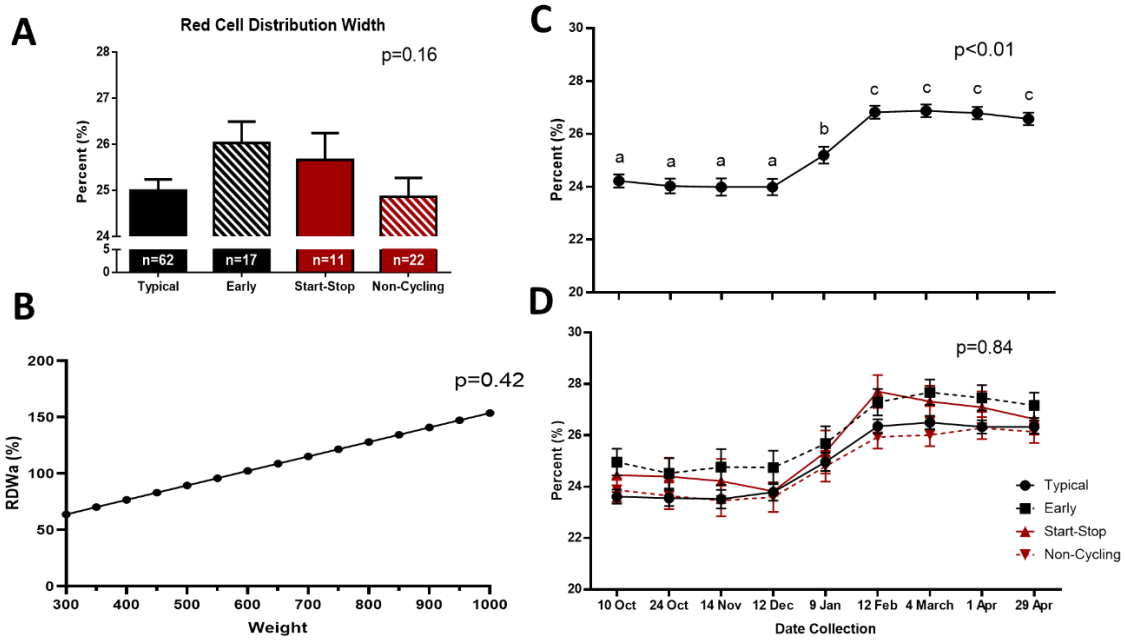

Figure 2-8. Pubertal Group, Collection (A), Weight Correlation (B), Collection over time (C), and Collections*Group for Red Cell Distribution Width (RDW) (D). RDW was not different between pubertal groups or impacted by weight. Over the collection period, RDW increased before plateauing.

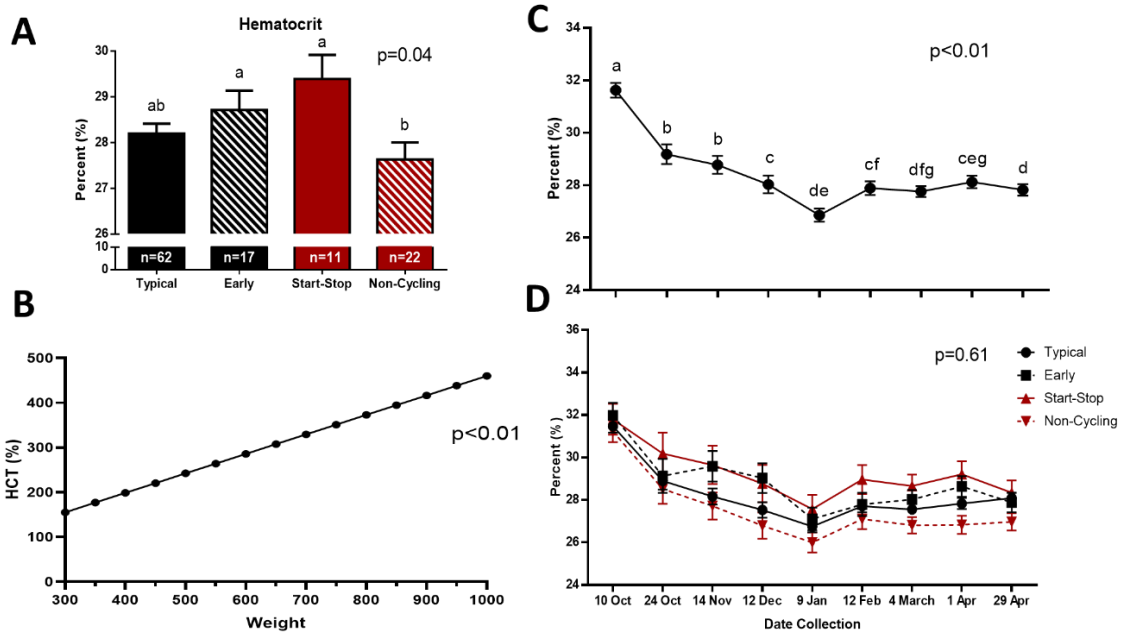

Figure 2-9. Pubertal Group, Collection (A), Weight Correlation (B), Collection over time (C), and Collections*Group for Hematocrit (D). Early and Start-Stop heifers had the greatest hematocrit percentage compared to Non-Cycling heifers. Hematocrit also increased as weight increased. During the collection period, hematocrit declined.

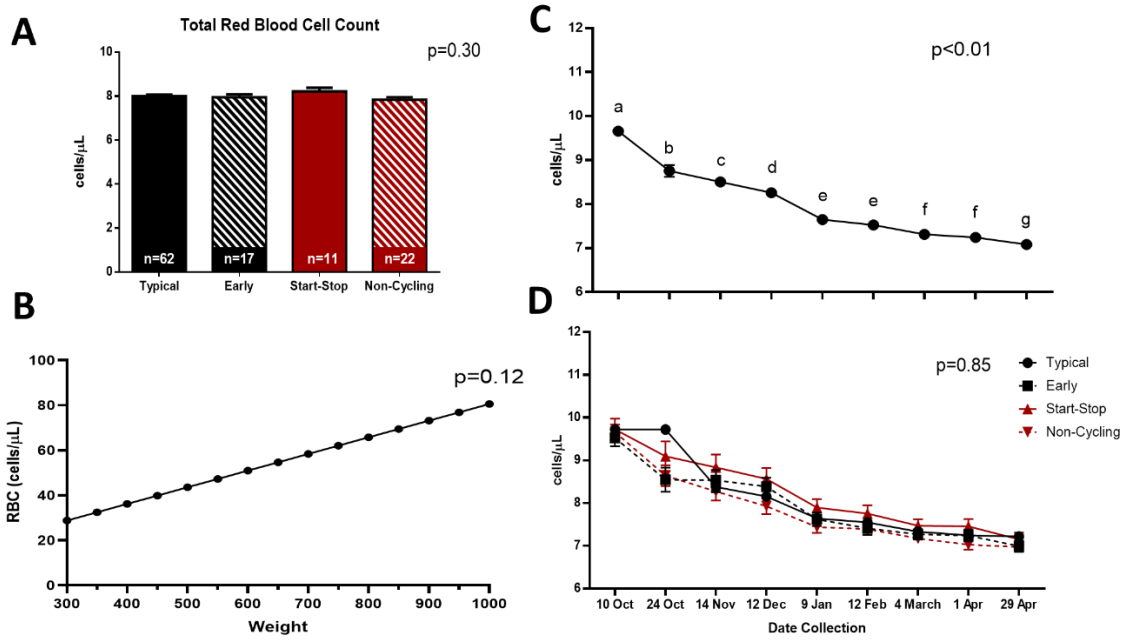

Figure 2-10. Pubertal Group, Collection (A), Weight Correlation (B), Collection over time (C), and Collections*Group for Total Red Blood Cell (RBC) Count (D). Puberty classification and weight were not different for RBC. Additionally, RBC declined over the collection period.

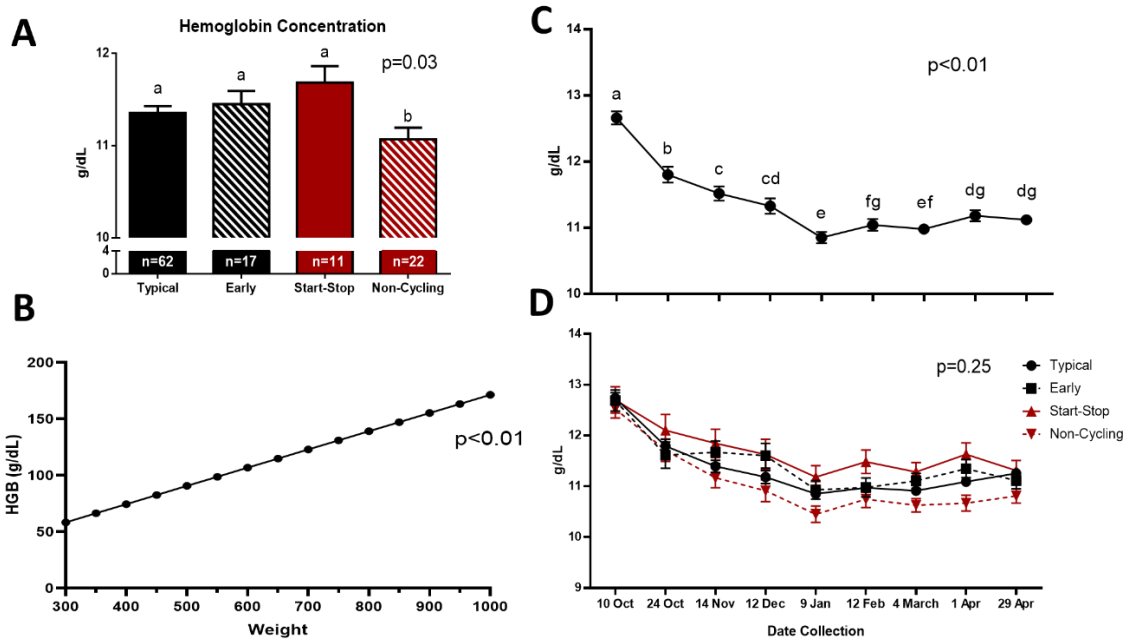

Figure 2-11. Pubertal Group, Collection (A), Weight Correlation (B), Collection over time (C), and Collections*Group for Hemoglobin Concentration (D). Hemoglobin was greatest in Typical, Early, and Start-Stop heifers. Hemoglobin also increased as weight increased. Additionally, hematocrit declined over the collection period.

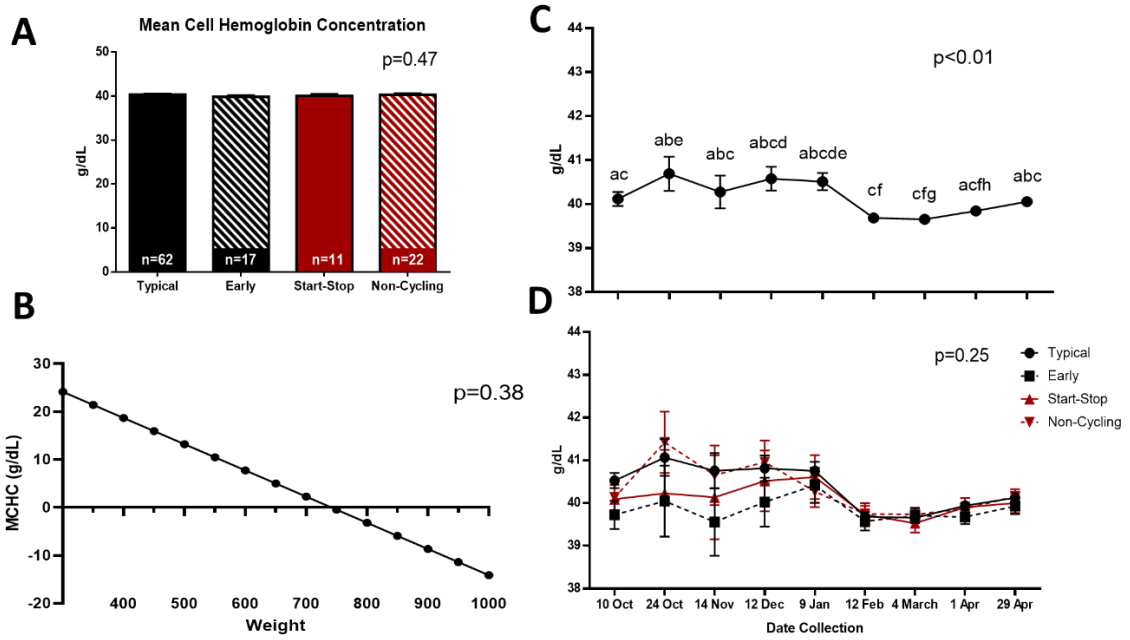

Figure 2-12. Pubertal Group, Collection (A), Weight Correlation (B), Collection over time (C), and Collections*Group for Mean Cell Hemoglobin Concentration (MCHC) (D). Puberty classification and weight were not impacted by MCHC. Additionally, MCHC was different over the collection period.

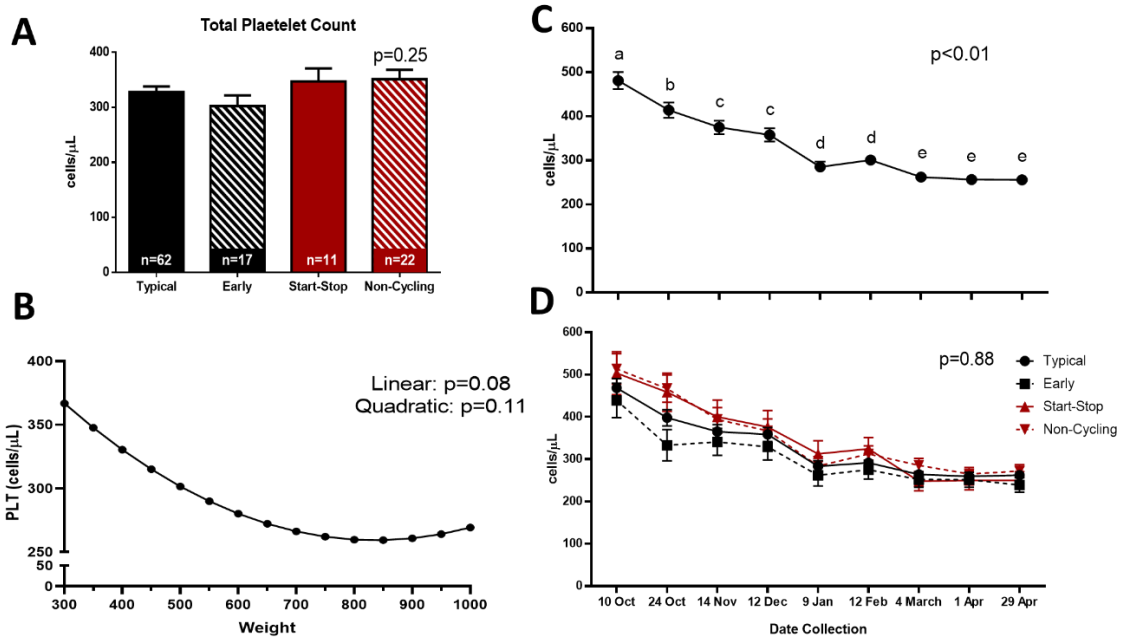

Figure 2-13. Pubertal Group, Collection (A), Weight Correlation (B), Collection over time (C), and Collections*Group for Total Platelet Count (D). Platelet count was not different between pubertal groups. However, platelet declined over the collection period and as weight increased.

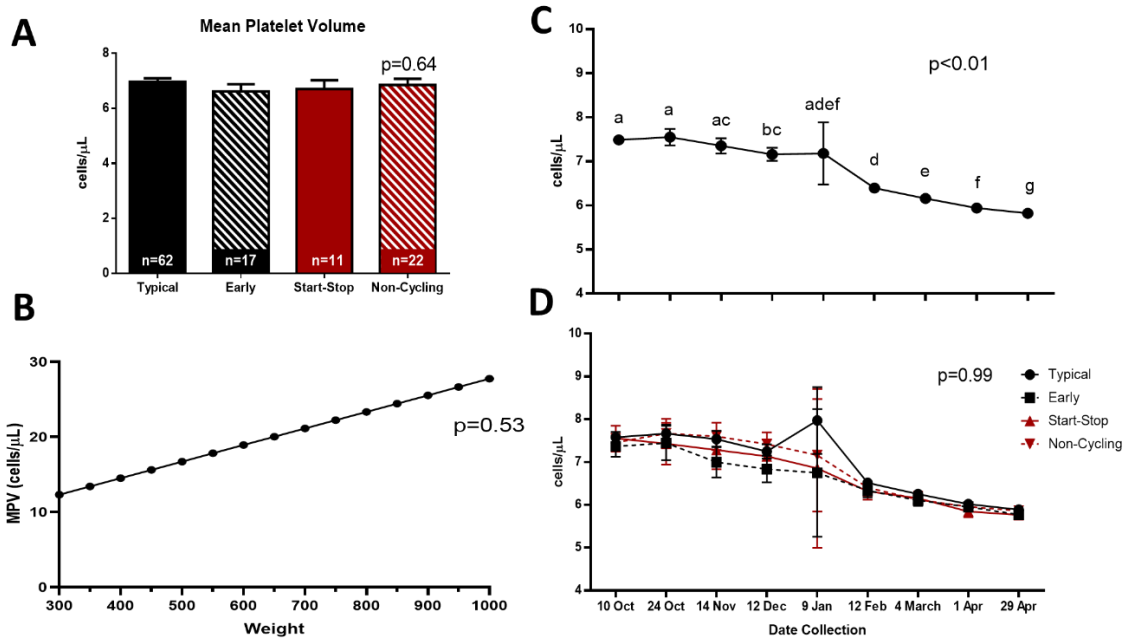

Figure 2-14. Pubertal Group, Collection (A), Weight Correlation (B), Collection over time (C), and Collections*Group for Mean Platelet Volume (D). Mean platelet volume was not different between pubertal groups or influenced by weight. Mean platelet volume declined over the collection period.

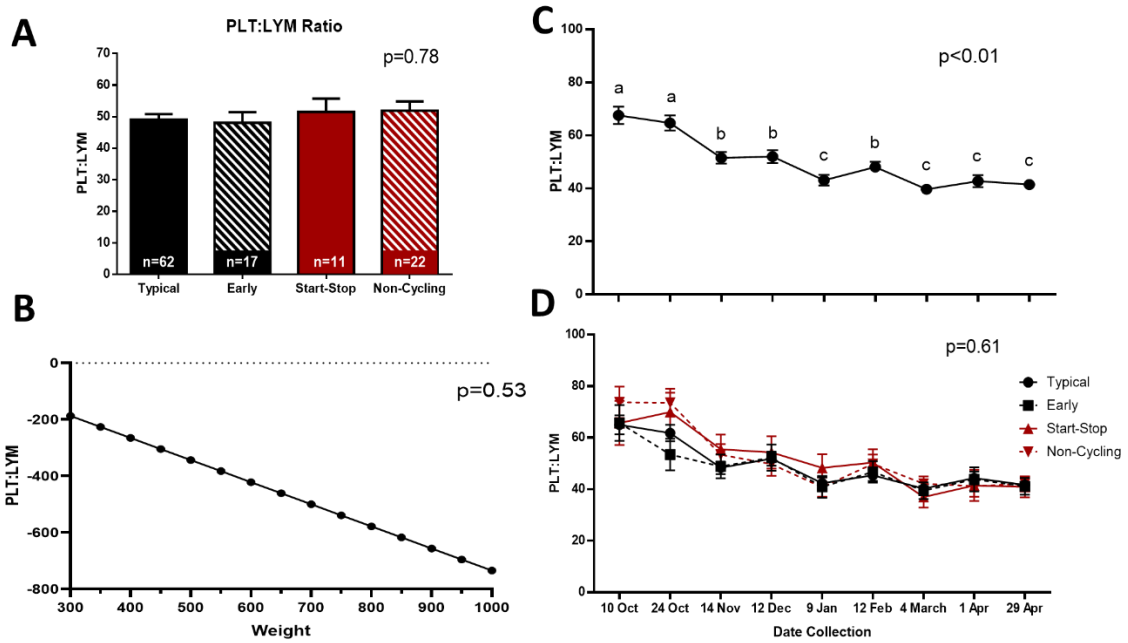

Figure 2-15. Pubertal Group, Collection (A), Weight Correlation (B), Collection over time (C), and Collections*Group for Platelet to Lymphocyte Ratio (PLR) (D). Puberty classification was not different for PLR and was not influenced by weight. Over the collection period, PLR declined.

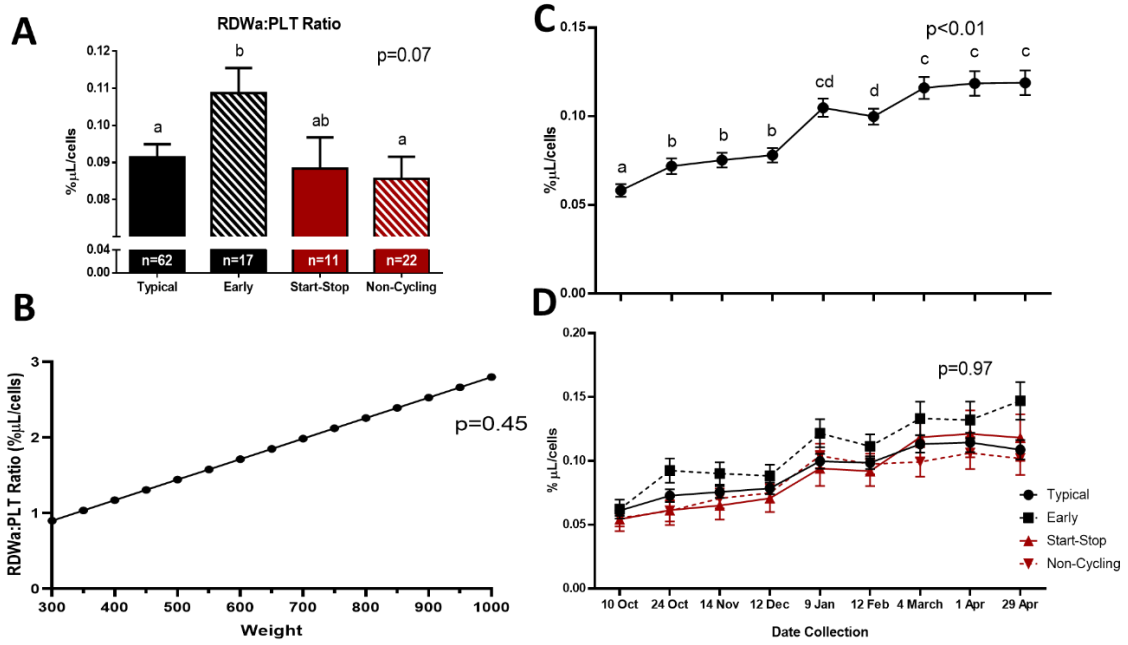

Figure 2-16. Pubertal Group, Collection (A), Weight Correlation (B), Collection over time (C), and Collections*Group for Red Cell Distribution Width (RDW) to Platelet Ratio (D). Early heifers tended to have greater RDW to platelet ratio compared to Non-Cycling heifers. Weight did not influence RDW to platelet ratio, but increased over the collection period.

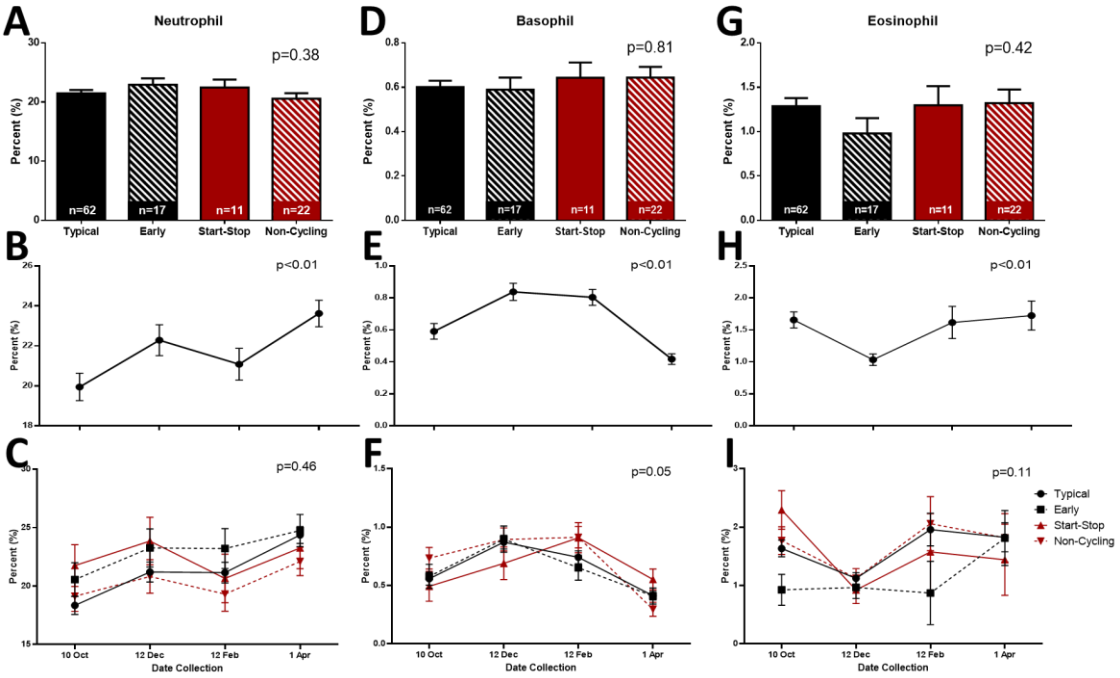

Figure 2-17. Pubertal Group, Collection over time, and Collections*Group for Neutrophil (A-C), Basophil (D-F), and Eosinophil (G-I). Neutrophil, basophil, and eosinophil were not different between pubertal groups. However, neutrophil and eosinophil increased over the collection period while basophil declined overall.

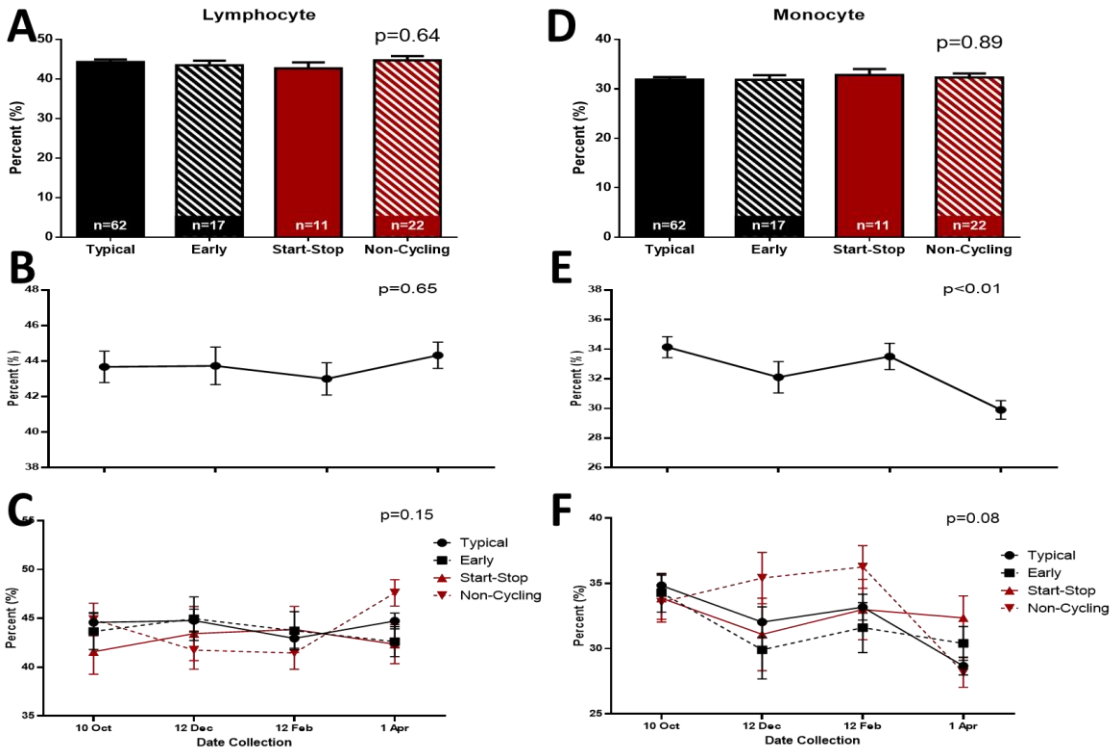

Figure 2-18. Pubertal Group, Collection over time, and Collections*Group for Lymphocyte (A-C) and Monocyte (D-F). Lymphocyte and monocyte were not different between pubertal groups. Lymphocyte did not vary over the collection period, while monocyte declined over time.

CHAPTER 1 APPENDIX

ABSTRACT

Over the past decade, our lab has developed and optimized a method to categorize pubertal attainment in beef heifers. Estrus cyclicity and initiation of puberty is determined by plasma progesterone (P4) concentrations ≥ 1 ng/ml analyzed over the course of weaning (Oct) to breeding (May). This has identified four distinct pubertal groups: Typical, Early, Start-Stop, and Non-Cycling. Initial methodology of defining puberty utilized monthly to bi-monthly plasma sampling over the 2012-2013 years. However, as our study has advanced, weekly plasma sampling is taken from weaning to breeding beginning in February of 2014 continuing to the present. Since our frequency of collections has changed, our aim was to confirm that results derived with earlier year less frequent sampling (2012-2014) were similar to yearly only with frequent (weekly) sampling. So we compared 2012-2016 data to 2015-2018-9 born heifer parameters in the current study. All production factors published in Nafziger et al., 2021 were evaluated to categorize production value and reproductive development and success. Of the growth production value factors, there were no changes detected amongst adjusted birth weight (BW), adjusted weaning weight (WW), and weaning to yearling average daily gain (ADG). Minor changes were seen for both adjusted yearling weight birth to weaning ADG where Typical and Early heifers were heavier compared to Start-Stop and Non-Cycling in 2015-current. In evaluating birth to yearling weight per day of age (WDA), a large changed occurred where initially Start-Stop heifers were lighter compared to the other pubertal groups. However, in 2015-current, there are no differences between the pubertal groups. Observation of reproductive development

and success factors found no differences for antral follicle count, uterine horn diameter, estrous response to prostaglandin F2α, conception to artificial insemination (AI) to those bred via AI, or overall pregnancy differences. Minor differences were seen in heifers categorized as a reproductive tract score of 5 where Typical heifers are no longer different to Start-Stop. Prebreeding weight also found that Early heifers are heaviest followed by Typical and then Start-Stop and Non-Cycling in 2015-current. Additionally, calving within the first 21 days showed that Typical heifers are more likely to when compared to Start-Stop. While minor differences are observed, these results conclude that frequency differences in plasma progesterone sampling did not vastly alter observed production and reproductive factors analyzed between our pubertal groups.

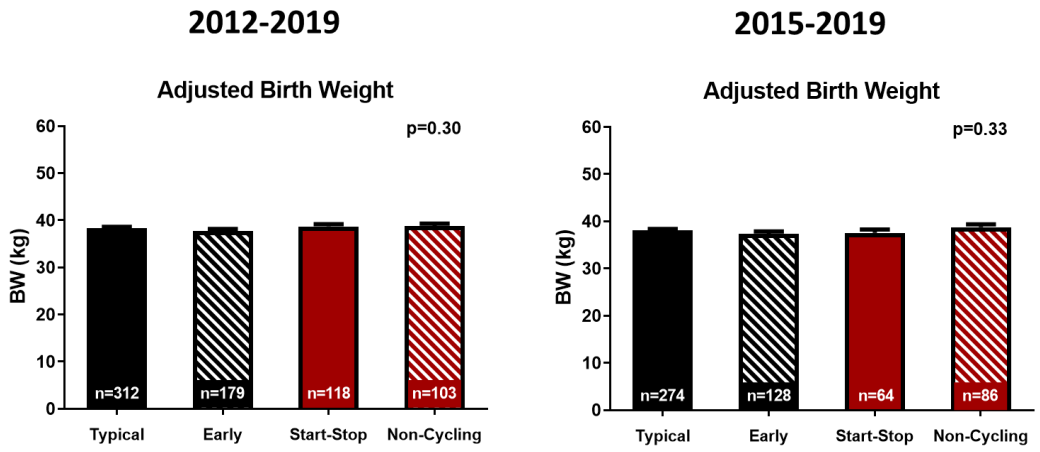

Figure A-1. Comparison of Adjusted Birth Weight for 2012-2019 and 2015-2019 heifers to determine effects of frequency of blood collection in heifer pubertal classifications. No differences were seen between progesterone sampling frequency.

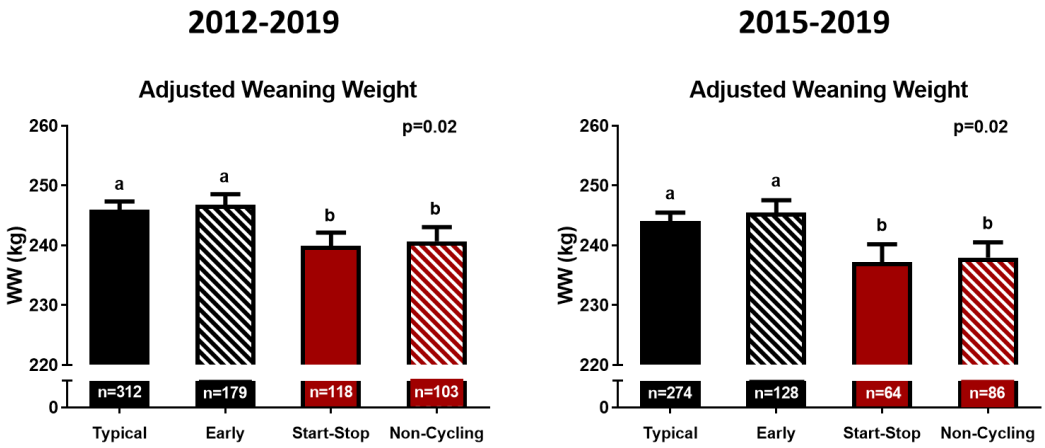

Figure A-2. Comparison of Adjusted Weaning Weight for 2012-2019 and 2015-2019 heifers to determine effects of frequency of blood collection in heifer pubertal classifications. No differences were seen between progesterone sampling frequency.

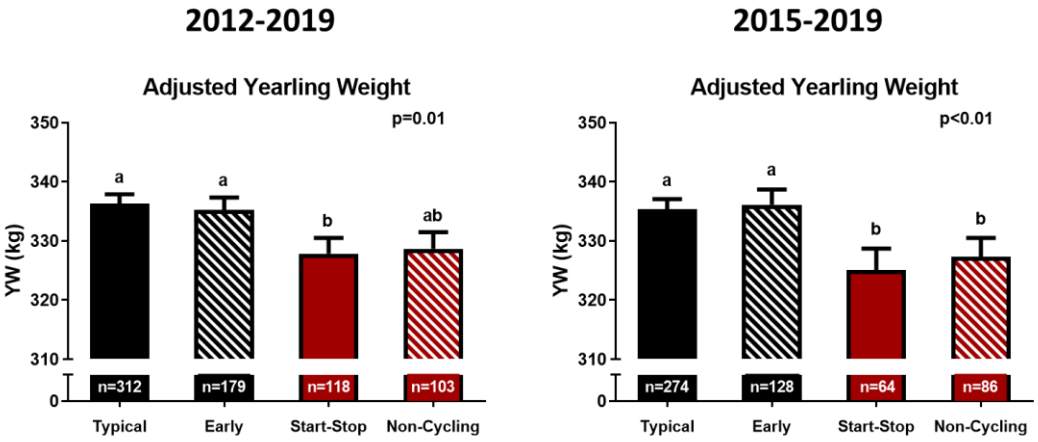

Figure A-3. Comparison of Adjusted Yearling Weight for 2012-2019 and 2015-2019 heifers to determine effects of frequency of blood collection in heifer pubertal classifications. With increased progesterone sampling, Start-Stop now have lowered adjusted yearling weight compared to Typical and Early heifers.

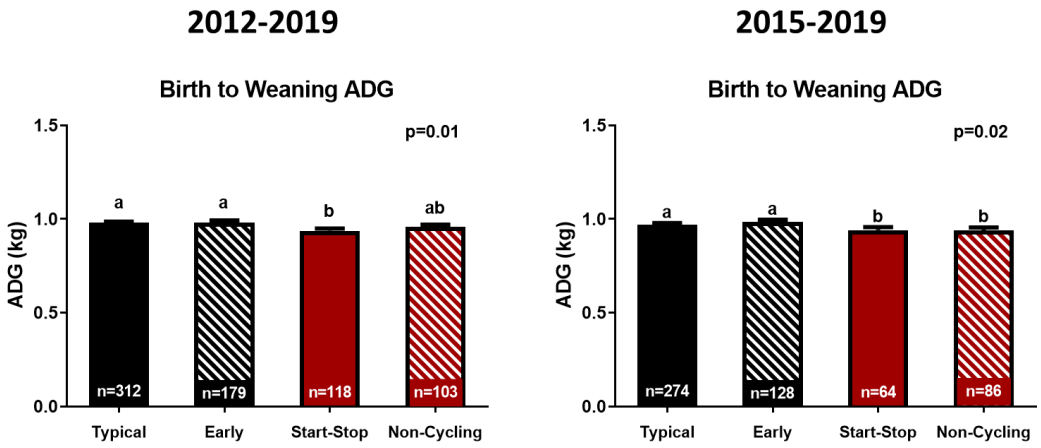

Figure A-4. Comparison of Birth to Weaning Average Daily Gain for 2012-2019 and 2015-2019 heifers to determine effects of frequency of blood collection in heifer pubertal classifications. Increased progesterone sampling resulted in lower birth to weaning ADG for Non-Cycling heifers compared to Typical and Early heifers.

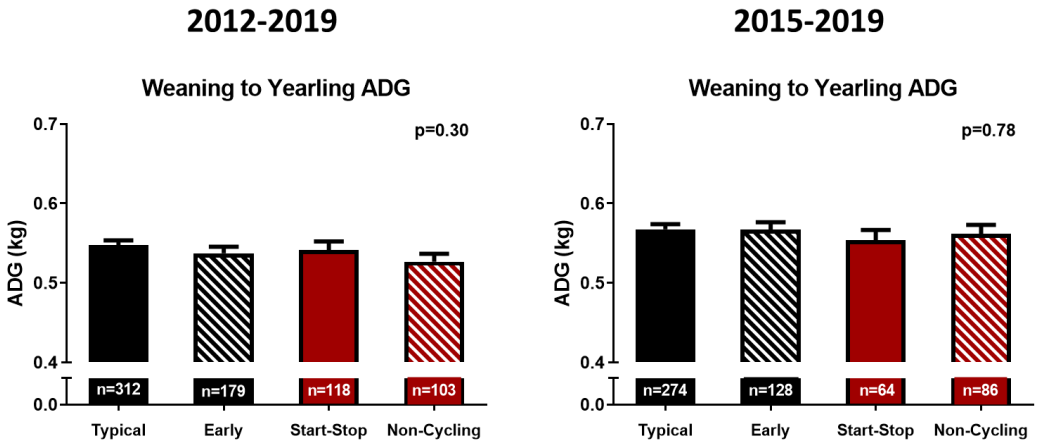

Figure A-5. Comparison of Weaning to Yearling Average Daily Gain for 2012-2019 and 2015-2019 heifers to determine effects of frequency of blood collection in heifer pubertal classifications. No differences were seen between progesterone sampling frequency.

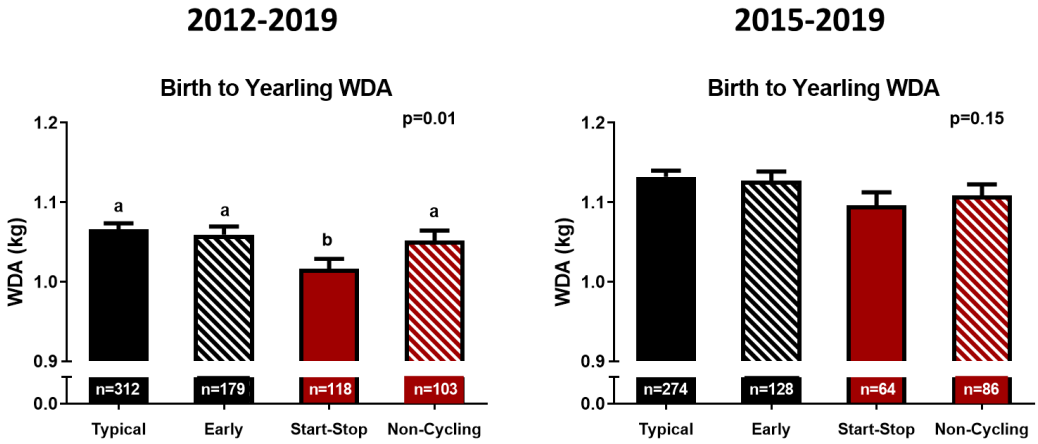

Figure A-6. Comparison of Birth to Weaning Weight per Day of Age for 2012-2019 and 2015-201 heifers to determine effects of frequency of blood collection in heifer pubertal classifications. Increased progesterone sampling resulted in no differences for birth to yearling WDA.

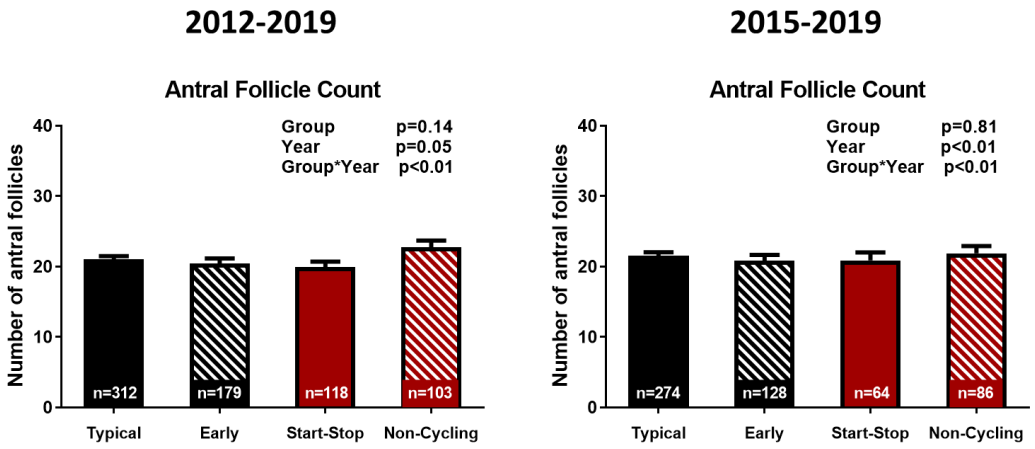

Figure A-7. Comparison of Antral Follicle Count for 2012-2019 and 2015-2019 heifers to determine effects of frequency of blood collection in heifer pubertal classifications. No differences were seen when progesterone sampling frequency increased.

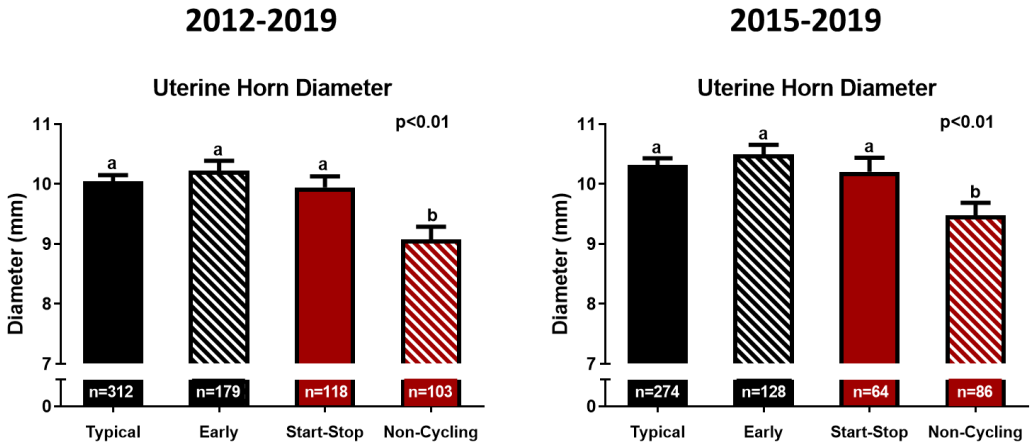

Figure A-8. Comparison of Uterine Horn Diameter for 2012-2019 and 2015-2019 heifers to determine effects of frequency of blood collection in heifer pubertal classifications. No changes were observed when progesterone sampling frequency increased.

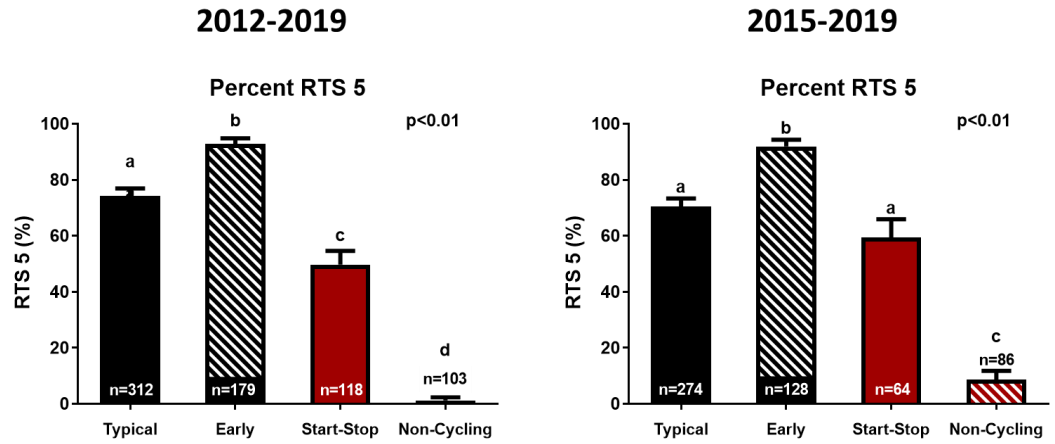

Figure A-9. Comparison of Percent of Reproductive Tract Score of 5 for 2012-2019 and 2015-201 heifers to determine effects of frequency of blood collection in heifer pubertal classifications. Increased progesterone sampling resulted in Start-Stop heifers no longer being different when compared to Typical heifers.

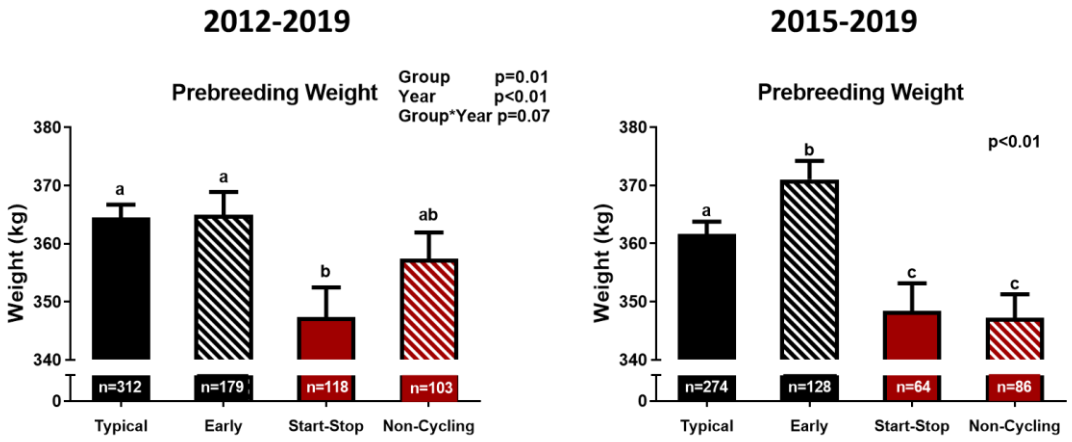

Figure A-10. Comparison of Prebreeding Weight for 2012-2019 and 2015-2019 heifers to determine effects of frequency of blood collection in heifer pubertal classifications. After increasing progesterone sampling frequency, Early heifers had the greatest prebreeding weight followed by Typical and then Start-Stop and Non-Cycling heifers.

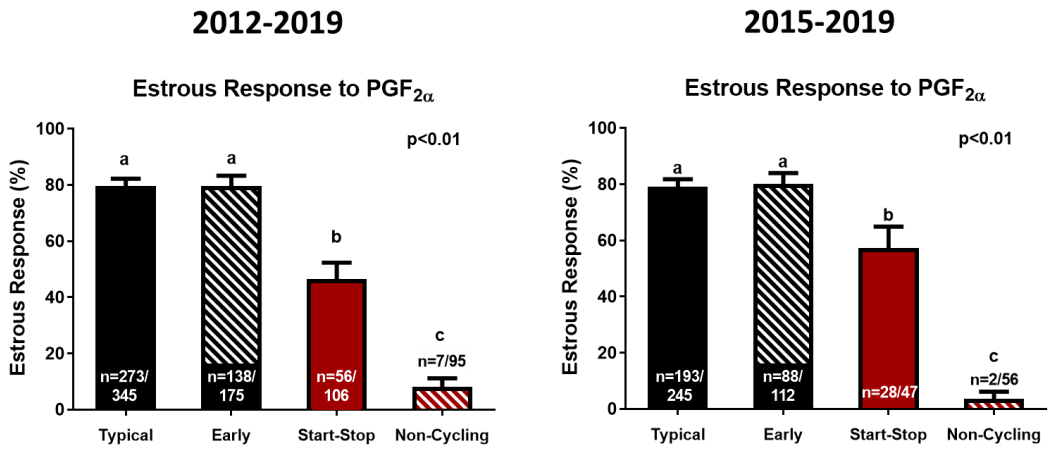

Figure A-11. Comparison of Estrous Response to Prostaglandin F2α for 2012-2019 and 2015-2019 heifers to determine effects of frequency of blood collection in heifer pubertal classifications. No differences were seen with increased progesterone sampling frequency.

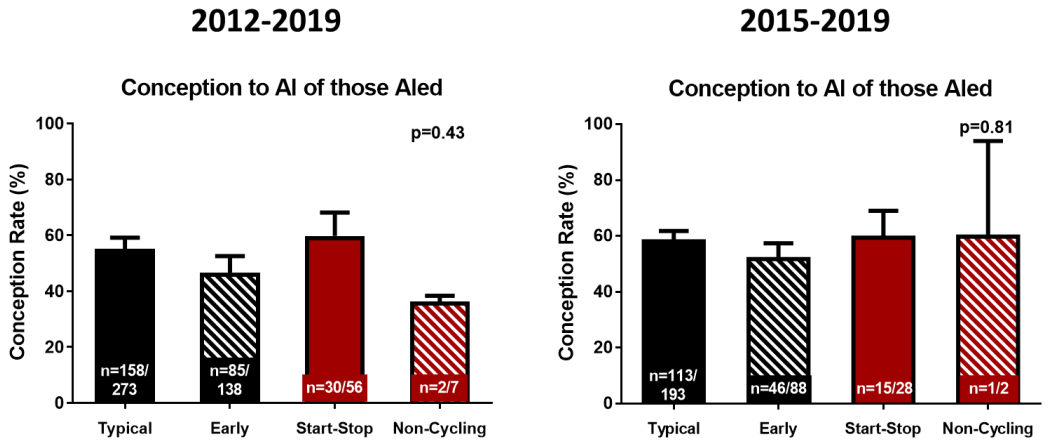

Figure A-12. Comparison of Conception to Artificial Insemination (AI) for those bred to AI for 2012-2019 and 2015-2019 heifers to determine effects of frequency of blood collection in heifer pubertal classifications. No differences were seen with increased progesterone sampling frequency.

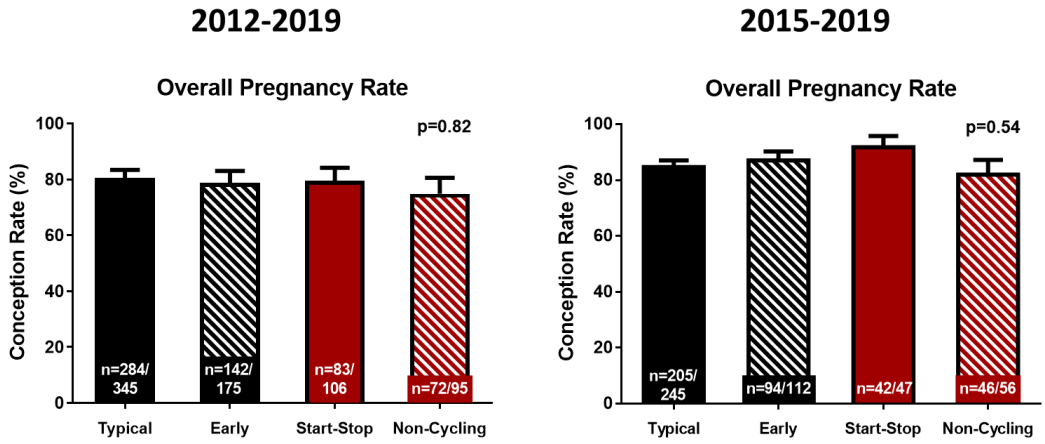

Figure A-13. Comparison of Overall Pregnancy Rate for 2012-2019 and 2015-2019 heifers to determine effects of frequency of blood collection in heifer pubertal classifications. No differences were seen with increased progesterone sampling frequency.

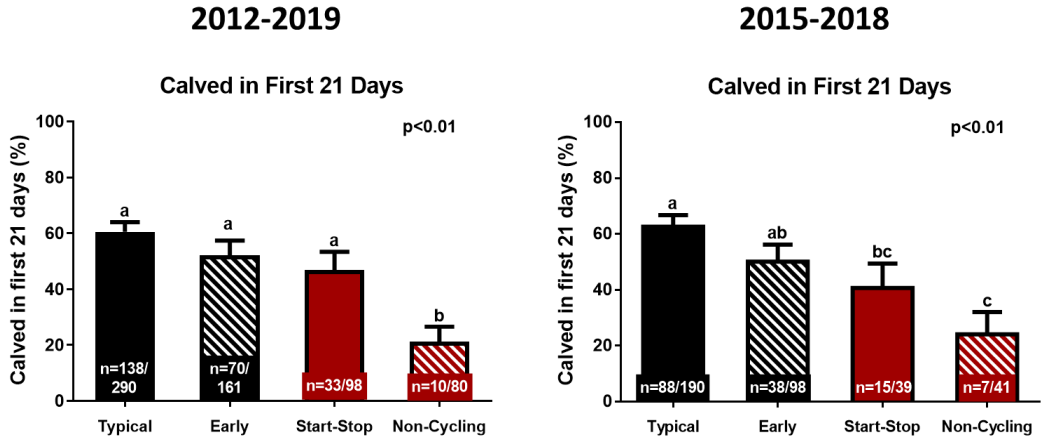

Figure A-14. Comparison of Calving within the 1st 21 Days for 2012-2019 and 2015-2019. Comparison of Calving within the 1st 21 Days for 2012-2019 and 2015-2019 heifers to determine effects of frequency of blood collection in heifer pubertal classifications. After increasing progesterone sampling, Start-Stop was now different from Early and Typical and similar to Non-Cycling had reduced calves born in the first 21 days of calving.

CHAPTER 2 APPENDIX

SUMMARY

A standard operating protocol was established to maintain consistency in operating the HemaTrue® Veterinary Hematology Analyze. This protocol describes the procedures for initial setup, running a whole blood EDTA tube, replacement of reagents, admission into standby mode, and common troubleshooting instructions.

Hematrue Operating Protocol

Setup Procedure

1. For initial setup and activation, tap the screen and select exit standby mode.
    a. The system will do a complete run through and will take approximately a minute to complete.
2. The system will finish standby mode and automatically bring up the daily startup menu.
    a. Perform a background check to make sure there are no residual readings from previous runs.
        i. It is critical that the values are within the error range, preferably 0. If the machine fails the first background check, rerun an additional background. It should work, but if not contact Eileen, Rachel, or Jessica for troubleshooting.

Running a Sample

1. Once the background checks have been completed, a sample is ready to run.
2. Invert the sample multiple times to ensure it is well mixed.
    a. The machine can mix it for you while it is setting up/processing a sample. Place the sample in the front turning wheel for the sample to be mixed.
    b. It is important to make sure there are minimal/no clots present in the sample. Clots can easily clog the tubing within the machine. If there is a clot, use a wooden stick to gently swirl the blood.
3. Once the sample is mixed, select new sample on the screen.
4. To change species (default is sheep), press previous until species is bovine
5. Add in animal ID with number keypad
6. Insert the sample probe to the sample and press the start plate. This will initiate the analysis.
    a. Make sure the tube is removed swiftly from the probe to allow it to be cleaned.
    b. It will take ~1 min for the sample to be processed
7. The results for the sample will be viewable on the sample screen.
    a. It is recommended to take a picture of the processed sample and be ready to add the next sample. Results can then be recorded while the next sample is being processed. This will ensure efficiency in processing all heifer samples.

Note: It is important to do background checks throughout the processing of the samples. Normally after ~14 samples, do 2 background checks to ensure there is minimal interference for the upcoming samples.

Changing an Empty, Low, or Expired Reagent Container

1. While the Hematrue is not processing a sample, press menu to access the main menu screen.
2. Press Reagent Setup and then Enter New Reagent to change a reagent container
3. Use the handheld scanner to scan barcodes 1 and 2 on the reagent container.

Note: Barcodes 1 and 2 to be scanned when replacing a reagent bottle. The handheld scanner is found on the right backside of the machine.

4. Remove the used reagent bottle and insert the new reagent bottle with the appropriate transfer tubing.

Entering Standby Mode

1. Once all samples are processed, run background check(s) to clear the Hematrue system. Usually 2 backgrounds checks are necessary but do as many as necessary until there are no residual readings and/or the values are within the error range (preferably 0).
2. After completing the background checks, select menu to pull up the main menu screen.
3. Select Enter Standby mode to prepare the machine for the next run.

a. DO NOT PRESS SHUTDOWN. The machine needs to continually run and be primed before it can process samples. Entering shutdown will result in the machine needing to be reprimed before it can run any new samples.

Common Troubleshooting Tips

While the Hematrue generally works with little error, it can be a fickle machine.

No Values for White Blood Cells

- This error commonly occurs if a reagent runs out or the Hematrue was allowed to "run dry"
- To fix this, make sure there is sufficient reagents according to the machine.
    - Select menu to pull up the main menu. Select New Reagent as if to add a new reagent. It should say at the bottom of the screen how many runs are left for each specific reagent. The diluent will need to be changed/added the most frequently and is the most likely thing to run out of. If there is "0 Runs Left", replace with the respective reagent.
    - Likely, the system will need to be primed before another sample can be rerun even with the new reagents. From the main menu screen, select prime system. Once this is completed, the samples should be processed smoothly.

No Values for Platelets

- This error does not occur frequently, but in some cases there will be no platelet value for the sample. If this occurs, run the necessary amount of background checks to clear the system. Once the system is cleared, rerun the sample. This does not always work, but it has worked in the past. If after running the sample twice and no platelet value is obtained, make a note and continue to process the remaining samples.