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Attainment and Maintenance of Pubertal Cyclicity May Predict High A4 Cows with Reduced Fertility

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ATTAINMENT AND MAINTENANCE OF PUBERTAL CYCLICITY MAY PREDICT HIGH A4 COWS WITH REDUCED FERTILITY

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ATTAINMENT AND MAINTENANCE OF PUBERTAL CYCLICITY MAY PREDICT HIGH A4 COWS WITH REDUCED FERTILITY

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Puberty attainment was investigated in heifers. Blood samples were collected from weaning until breeding during 6 years from heifers born in 2012-2017. Plasma progesterone concentrations ≥ 1 ng/ml were used to indicate cyclicity, and heifers were categorized into one of four puberty groups using SAS: 1) Early heifers reached puberty before March 12 and cycled continuously during the sampling period, 2) Typical heifers reached puberty on or after March 12 and cycled continuously, 3) Start-Stop heifers had at least one occurrence of progesterone ≥ 1 ng/ml but discontinued cycling during the sampling period, and 4) Non-Cycling heifers had no occurrence of progesterone ≥ 1 ng/ml during the sampling period.

Early and Typical Heifers had increased average daily gain during the preweaning period, resulting in higher adjusted yearling weights heifers compared to Start-Stop and Non-Cycling heifers. At pre-breeding ultrasound, Early heifers had the highest percentage reproductive tract score 5, followed by Typical, Start-Stop, and Non-Cycling heifers. At breeding, a greater percentage of Typical and Early heifers displayed estrus in response to prostaglandin and were artificially inseminated, followed by Start-Stop heifers and Non-Cycling heifers. All heifers were exposed to bulls, and overall pregnancy rate did not differ between puberty groups. Fewer Non-Cycling heifers calved in the first

21 days of the calving season. Approximately 10 heifers from each puberty group were intensively studied in years 2014-2017 during the peripubertal period. Non-Cycling heifers had reduced progesterone concentrations and increased mean FSH, and Start-Stop and Non-Cycling heifers appeared to have reduced SHBG compared to Typical and Early heifers. At the conclusion of the intensive study, heifers were stimulated with FSH and ovariectomized. Interestingly, Non-Cycling ovarian cortex secreted more androstenedione in culture media compared to Typical and Early cortex. Ovarian cortex from cows previously classified as Start-Stop or Non-Cycling also secreted increased androstenedione in culture media compared to cortex from Typical cows. Since ovarian cortex from High A4 cows secretes increased androstenedione in culture media, these findings together with endocrine and fertility parameters indicate that Start-Stop and Non-Cycling heifers have similar High A4 cow traits which may result in their reduced fertility.

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Chapter 1 : Review of the Literature

INTRODUCTION

Puberty is a dynamic and critical timepoint in the life of a beef heifer. It is at this time that a heifer is able to conceive and reproduce, allowing her to return a profit to the farmer after the cost and time invested in her development. On average, Bos taurus beef heifers in North America reach puberty at 10-14 months, but herd averages for age at puberty range from 9.6 to 22 months (R. L. Larson, White, & Laflin, 2016). In the United States beef industry, it is crucial for heifers to reach puberty early enough to become pregnant and deliver a calf at two years of age (Ferrell, 1982; Perry, 2016). Heifers that reach puberty and deliver a calf within this time window have increased fertility over their lifespan and last a longer time in the herd, returning more dollars of weaned calf back to the producer for the investment cost required for their development (D. M. Larson & Funston, 2009; Perry & Cushman, 2013). In order for a heifer to give birth at two years old, she must become pregnant by 15 months of age. Because previous studies have suggested that a heifer's first few estrous cycles after puberty may be less fertile, it is therefore ideal for her to be pubertal by 12-13 months of age in order to have a high conception rate at 15 months (Byerley, Staigmiller, Berardinelli, & Short, 1987).

In heifers, pubertal attainment is marked by behavioral estrus (showing receptivity to the male) and is followed by ovulation and formation of a functional corpus luteum on the ovary (Atkins, Pohler, & Smith, 2013; R. L. Larson et al., 2016; Perry, 2012; Tena-Sempere, 2013). Therefore, the time leading up to puberty must be characterized by development of both the reproductive tract organs and signaling centers in the brain, as well as establishment of the communication between the brain and

reproductive tract through the hypothalamic-pituitary-gonadal axis (Perry, 2016; Tena-Sempere, 2013).

PHYSIOLOGY OF PUBERTY

Development of the Female Reproductive Tract

In order for puberty to occur, a heifer's reproductive tract must be substantially mature. Sex differentiation occurs in the bovine fetus, and development of the reproductive tract continues from birth until after puberty (Atkins et al., 2013; Desjardins & Hafs, 1969).

The uterus has a biphasic growth pattern with the first rapid increase in uterine diameter occurring from 2-10 weeks of age and the second period of growth from 8-15 months of age (Atkins et al., 2013; Honaramooz et al., 2004). During the peripubertal and pubertal periods, as antral follicle diameter increases, more estradiol is produced, and uterine growth progresses (Atkins et al., 2013; Desjardins & Hafs, 1969; Honaramooz et al., 2004). This uterine growth occurs up to and even after puberty (Atkins et al., 2013).

The ovary has a biphasic growth pattern similar to the uterus, with rapid growth from 2-14 weeks of age and 30-60 weeks of age (Atkins et al., 2013; Desjardins & Hafs, 1969; Honaramooz et al., 2004). The ovaries are the location where oocytes are produced and released, and they are also the primary site of steroid production in the mammalian female. The ovary is made of up of two zones, the cortex and medulla. The medulla is the inner region that is vascularized. The cortex is the outer shell of the ovary where primordial and developing follicles are located.

Ovarian Follicle Development

A heifer is born with all the oocytes she will ever have in her life (B. H. Erickson, 1966). This pool of oocytes, called the ovarian reserve, is in the form of primordial follicles (Fortune, Cushman, Wahl, & Kito, 2000; Fortune, Yang, Allen, & Herrick, 2013). Follicles are the unit in which oocytes are found in the ovary. Each follicle contains an oocyte surrounded by layer(s) of cells. Primordial follicles, which make up the ovarian reserve, contain oocytes arrested in meiosis surrounded by one layer of squamous pre-granulosa cells (Fortune et al., 2000; Hummitzsch et al., 2013; Rüsse, 1983; Young & McNeilly, 2010). Even before birth, groups of primordial follicles are activated each day, and activated primordial follicles can develop into primary, secondary, and small antral follicles (Fortune et al., 2013).

Primary follicles have a single layer of cuboidal granulosa cells around the oocyte (Braw-Tal & Yossefi, 1997; Shimizu, 2016). Secondary follicles have two or more layers of granulosa cells (Braw-Tal & Yossefi, 1997; Lussier, Matton, & Dufour, 1987; Young & McNeilly, 2010). As secondary follicles continue to develop into antral follicles, which contain a large antrum or fluid-filled cavity, theca cells begin to differentiate outside the layers of granulosa cell (Magoffin & Weitsman, 1994). At this point in development, follicles become responsive to the gonadotropins secreted from the anterior pituitary gland, follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Adams, 1999; Magoffin & Weitsman, 1994; Shimizu, 2016).

Small groups of antral follicles are targeted by FSH from the anterior pituitary, which causes them to increase in size and secrete estradiol (Adams, Matteri, Kastelic, Ko, & Ginther, 1992). This is known as recruitment. Following increased growth and

secretion of estradiol, a few of these antral follicles are selected for future growth, while others become atretic (Adams, Jaiswal, Singh, & Malhi, 2008; Savio, Keenan, Boland, & Roche, 1988). One follicle then becomes dominant, where it grows in size and produces estradiol as other follicles (known as subordinate follicles) regress (Savio et al., 1988). This process of recruitment, selection, growth, and dominance of ovarian follicles occurs repeatedly in the ovary, and is known as a follicular wave. Follicular waves occur in young heifers from the time they are 2 weeks old (A. C. Evans, Adams, & Rawlings, 1994).

Prior to puberty and the maturation of the HPG axis, antral follicles are exposed to very low concentrations of LH and FSH (Kinder, Bergfeld, Wehrman, Peters, & Kojima, 1995). As a result, they are smaller in size, produce less estradiol, and prepubertal dominant follicles will not ovulate but will simply become atretic, leading to a new follicle wave after the next rise in FSH (Atkins et al., 2013; Day, Imakawa, Wolfe, Kittok, & Kinder, 1987). As heifers get closer to puberty attainment, dominant follicles increase in diameter and secrete more estradiol (Bergfeld et al., 1994; Day et al., 1987).

After puberty and the establishment of a regular estrous cycle, there will be 2-3 follicular waves per estrous cycle, and a dominant follicle arising from the second or third follicular wave will be stimulated to ovulate, meaning the oocyte will be released from the follicle (Adams, 1999; Ginther, 2016). The resulting site forms a temporary corpus hemorrhagicum which becomes highly vascularized and after several days becomes a corpus luteum where theca and granulosa cells become luteinized and produce progesterone (M. F. Smith, 1986).

Theca and Granulosa Cells

Two primary cell types support maturation of the oocyte in each developing follicle: theca and granulosa cells. In response to LH and FSH, these two cell types work together to produce estradiol; this is referred to as the two-cell, two-gonadotropin theory (Fortune & Hansel, 1979; Hatzirodos, Hummitzsch, Irving-Rodgers, & Rodgers, 2015; Voss & Fortune, 1993). The basis of the two-cell, two-gonadotropin theory is that LH targets theca cells to convert cholesterol into androgens, and FSH targets granulosa cells to convert androgens produced in the theca cells into estrogens.

Theca cells are located outside the basal lamina of the ovarian follicle (G. F. Erickson, Magoffin, Dyer, & Hofeditz, 1985). There are two populations of theca cells, the theca interna and theca externa (Hatzirodos et al., 2015; Magoffin, 2005). These layers of theca cells provide structural support and vascularization for the follicle (O'Shea, Hay, & Cran, 1978). Theca cells have LH receptors and contain steroidogenic enzymes to produce androgens in response to LH (G. F. Erickson et al., 1985).

Granulosa cells directly surround the antrum of the follicle and nurture the developing oocytes (Hummitzsch et al., 2013). They also contact the surface of the theca cells. Androgens produced by the theca cells are aromatized to estrogens by granulosa cells in response to FSH (Hansel & Convey, 1983). As follicles progress in development under the stimulation of FSH on granulosa cells and produce more estradiol, granulosa cells also attain LH receptors which allows the follicle to respond to an LH surge (G. F. Erickson, Wang, & Hsueh, 1979).

Hypothalamic-Pituitary-Gonadal Axis

In addition to the development of the parts of the reproductive tract, puberty attainment requires the maturation of the hypothalamic-pituitary-gonadal (HPG) axis (Day & Anderson, 1998). This axis contains three key organs: the hypothalamus, anterior pituitary, and ovaries. Each of these organs produces one or more of the major hormones crucial to puberty attainment and cyclicity in the bovine female.

The key signaling molecule produced by the hypothalamus is gonadotropin releasing hormone (GnRH), a decapeptide hormone (Dierschke, Bhattacharya, Atkinson, & Knobil, 1970). Secretion of GnRH is triggered by signals from other neurons in the hypothalamus that innervate GnRH neurons, and it can be secreted from either the surge center or the tonic center, two groups of GnRH-secreting hypothalamic nuclei (Karsch, 1987). The GnRH neurons in the tonic center of the hypothalamus produce a constant and low rate of GnRH pulses. In the surge center of the hypothalamus, which is unique to females, neurons also produce low basal rates of GnRH similar to the tonic center prior to puberty. After puberty, however, the surge center can be stimulated by high concentrations of estradiol in the absence of progesterone to release a large quantity of GnRH (the "GnRH surge") once per estrous cycle (Clarke, 2002).

After its release is triggered from neurons in the hypothalamus, GnRH travels through the hypophyseal portal system and binds to seven transmembrane G protein coupled receptors (7TMGPCR) on gonadotrope cells in the anterior pituitary, causing an increase in the concentration of available intracellular calcium in the gonadotropes and promoting secretion of protein hormones, LH and FSH, from the anterior pituitary (Day & Anderson, 1998; Day et al., 1987; Millar, 2005; Neves, Ram, & Iyengar, 2002). After

its release into the bloodstream, FSH binds to its 7TMGPCR, the FSHR, on the granulosa cells of the ovary and triggers Gs to activate adenylate cyclase and upregulate cAMP, resulting in increased activation of steroidogenic enzymes in the granulosa cells (Neves et al., 2002). Meanwhile, LH produced by the anterior pituitary also travels through the bloodstream to the ovary, where it binds to its 7TMGPCR, the LHCGR, on theca cells and later-stage granulosa cells and activates Gs and Gq (Baird, Swanston, & McNeilly, 1981). There, the same signaling cascades cause an increase in steroidogenic enzymes in the theca cells (Baird et al., 1981; Bremer, 2010; Voutilainen, Tapanainen, Chung, Matteson, & Miller, 1986).

Activation of the LH receptor causes an increase in levels of steroidogenic acute regulatory protein (StAR), the rate-limiting enzyme for the initiation of steroidogenesis that imports cholesterol from the cytoplasm into the mitochondria of granulosa and theca cells (Lavoie & King, 2009; Manna, Dyson, & Stocco, 2009; Rosenfield & Ehrmann, 2016; Stocco & Clark, 1996). (Manna et al., 2009). Cholesterol is the precursor for steroid hormones. Once cholesterol is in the mitochondria, cytochrome P450 side-chain cleavage (P450scc), an enzyme encoded by CYP11A, converts cholesterol into pregnenolone (Halkerston, Eichhorn, & Hechter, 1961; Stone & Hecter, 1955. 3B-hydroxysteroid dehydrogenase type 2 (3BHSD2) in the cytoplasm converts pregnenolone to progesterone (Lachance et al., 1990; Lorence, Murry, Trant, & Mason, 1990; Thomas, Myers, & Strickler, 1989). Cytochrome P450c17, an enzyme encoded by CYP17A1, is present only in the theca cells. Cytochrome P450c17 converts pregnenolone into 17-hydroxypregnenolone and then into dehydroepiandrosterone (DHEA) which can be converted to androstenedione; it also converts progesterone into 17-hydroxyprogesterone

and then into androstenedione (Auchus, Lee, & Miller, 1998; Conley & Bird, 1997; Fan et al., 1992; Miller & Auchus, 2011). In the granulosa cells, DHEA or androstenedione can be aromatized into estrone and then modified into estradiol (Miller & Auchus, 2011; Simpson et al., 1994).

Progesterone

Progesterone is the primary product of ovarian steroidogenesis during the luteal phase (Miller & Auchus, 2011). It is produced from the luteal cells within the corpus luteum formed after ovulation. After release in the theca cells, progesterone is modified into other steroidogenic products or released into the bloodstream.

Progesterone release occurs in significant biological levels after puberty when the ovary is capable of forming a corpus luteum. Circulating progesterone concentrations are often used as a biomarker of puberty attainment. Previous studies have used variations of 1-3 repeated occurrences of progesterone within ranges of 1-2 ng/ml as a threshold level to suggest pubertal attainment. Several studies consider puberty the first time heifers have circulating progesterone concentrations > 1 ng/ml (Honaramooz, Chandolia, Beard, & Rawlings, 1999; Piccolo et al., 2018). In a study by Taylor et al. (2017), puberty was considered to be the first time heifers had 2 consecutive weekly samples where progesterone ≥ 1 ng/ml, and a study by Cardoso et al. (2014) considered puberty in heifers the first time 3 consecutive samples had progesterone ≥ 1 ng/ml. Moriel et al. (2014) considered puberty the first occurrence of progesterone > 1.5 ng/ml. One other example of progesterone concentrations used to indicate puberty attainment was a 2015 study where puberty was considered to be 7 days before the date of the first sample that

contained either 2 ng/ml P4 or the first of 2 consecutive samples where P4 > 1 ng/ml (Gunn, Schoonmaker, Lemenager, & Bridges, 2015).

Estradiol

Estradiol is the primary product of ovarian steroidogenesis during the follicular phase (Miller & Auchus, 2011). After estradiol is produced by the aromatization of androgens in the ovarian granulosa cells, it can diffuse out of the ovary and into the bloodstream. Estradiol elicits its effects on target tissues by acting through both a plasma membrane receptor, GPR30, as well as nuclear receptors (Funakoshi, Yanai, Shinoda, Kawano, & Mizukami, 2006). The most well-known mechanism of action for estradiol is through nuclear receptors. Because of its lipophilic structure, estradiol can diffuse through the plasma membrane into target cells and bind to intracellular nuclear receptors. There are two nuclear estrogen receptors: ERα and ERβ (H. R. Lee, Kim, & Choi, 2012). These intracellular estradiol receptors are located in the hypothalamus of heifers as early as 1-2 weeks of age (Armstrong & Villee, 1977).

Prior to puberty, estradiol provides negative feedback to the hypothalamus and anterior pituitary and prevents a GnRH and LH surge. This negative feedback declines as puberty approaches and is a possible mechanism for puberty attainment (Day et al., 1984; Schillo, Dierschke, & Hauser, 1982). As puberty approaches, estradiol goes from providing extremely negative feedback on GnRH when low levels of estradiol are present to less negative feedback as more estradiol is produced and finally to providing positive feedback on GnRH after sustained high levels of estradiol. One possible explanation for this change in the negative feedback of estradiol that allows or regulates the initiation of

puberty is a decline in the number of estradiol receptors in the hypothalamus and anterior pituitary (Day et al., 1987).

Sex Hormone Binding Globulin

Estrogens and androgens are both steroid hormones, and due to their lipophilic structure they cannot travel freely through the bloodstream. These sex hormones have a specialized carrier protein called sex hormone binding globulin (SHBG), first identified as sex steroid binding protein (SBP) (De Moor, Heyns, & Bouillon, 1972; Lermite & Terqui, 1991). SHBG is a glycoprotein produced primarily by liver hepatocytes and released into the bloodstream to bind free estrogens and androgens. SHBG is also found in many other organs, including the ovaries, uterus, and brain (Rosner, Hryb, Kahn, Nakhla, & Romas, 2010). Sex hormones bound by SHBG are not available for biological use in the body, so SHBG can control the amount of biologically active estrogens and androgens available (Selby, 1990).

In girls, SHBG is greater before puberty and declines around the time of puberty (Pinkney et al., 2014). This would allow for an increase in bioavailable sex steroids. Decreased SHBG in girls was predictive of earlier puberty (Pinkney et al., 2014). In addition, an increase in adiposity and decrease in circulating leptin were correlated with a prepubertal decrease in SHBG in girls (Pinkney et al., 2014).

Reduced circulating SHBG concentrations are seen in metabolic disorders such as obesity, type II diabetes, insulin resistance, and non-alcoholic fatty liver disease (Aydın & Winters, 2016; Deswal, Yadav, & Dang, 2018). Reductions in SHBG is also seen in Polycystic Ovary Syndrome (PCOS) and is partly responsible for excess androgens due

to the increased amount of biological androgens in the presence of lower SHBG (Deswal et al., 2018).

Changes in the HPG Axis During Puberty

The HPG axis is in place before puberty occurs. In prepubertal heifers, GnRH release occurs in relatively low quantities and at relatively low frequencies by both the surge center and the tonic center (Day & Anderson, 1998). This results in reduced release of LH and FSH by the anterior pituitary, and low concentrations of steroid hormones (estrogens and progesterones) produced by the ovary (Day & Anderson, 1998). These small quantities of steroid hormones (primarily estrogen) provide strong negative feedback to GnRH neurons in the hypothalamus and inhibit GnRH release (Day & Anderson, 1998). One explanation for the strong negative feedback of estradiol prior to puberty is that the greatest number of estradiol receptors are present in the hypothalamus during the prepubertal period, allowing estradiol to have its strongest inhibitory effect on GnRH (Day et al., 1987). Estradiol also inhibits LH release through negative feedback at the anterior pituitary (Perry, 2012). Due to the negative feedback of estradiol, there is reduced GnRH, LH and FSH, resulting in less estradiol produced by the HPG axis before puberty.

As heifers enter the peripubertal period (period of about 50 days just prior to puberty), the negative feedback of estradiol on GnRH and LH begins to decline (Day & Anderson, 1998). Decreased numbers of estradiol receptors are present in the hypothalamus, allowing less of an inhibitory effect of estradiol on GnRH neurons. As a result, increased GnRH secretion is permitted, which allows for increased secretion of LH and FSH (Kinder et al., 1995). LH and FSH promote dominant follicles on the ovary to

have increased diameter and secrete more estradiol (Day & Anderson, 1998). This decline in the negative feedback of estradiol promotes continually increasing secretion of GnRH from the hypothalamus, increasing LH and FSH from the anterior pituitary, and increasing estradiol from the ovarian follicles (Bergfelt, Smith, Adams, & Ginther, 1997). The increase in estradiol secreted from growing dominant follicles on the ovaries appears to hasten the decline in the negative feedback of estradiol, which allows even more GnRH to be released.

At the time of puberty, fewer estradiol receptors are present in crucial areas of the hypothalamus (Day et al., 1987). Ovarian follicles are increased in size, and the large dominant follicles produce high quantities of estrogen. As the amount of estradiol released from the ovaries increases, the negative feedback of estradiol on GnRH neurons becomes positive, and the GnRH surge center is stimulated by high concentrations of estradiol in the absence of progesterone to release a large quantity of GnRH. This GnRH surge results in high frequencies of LH released from the anterior pituitary (Alves et al., 2015; Rahe, Owens, Fleeger, Newton, & Harms, 1980). This is called the LH surge and leads to ovulation of the dominant follicle on the ovary, making the female able to conceive (Alves et al., 2015; Rahe et al., 1980). After ovulation, a corpus luteum forms on the ovary at the site of ovulation, and the luteal cells begin producing progesterone.

Estrous Cycle

Puberty marks the beginning of estrous cycles, which will continue from a heifer's pubertal estrus during her entire reproductive lifespan except for during pregnancy or periods of anestrus due to illness, injury, or infertility. The bovine estrous cycle lasts 20-22 days, with an average of 20 days for 2 follicular waves and an average

of 22 days for 3 follicular waves (Adams, 1999; Adams et al., 2008; Rajakoski, 1960). The estrous cycle is comprised of two phases, the luteal phase and the follicular phase. The luteal phase is dominated by production of progesterone, while the follicular phase is dominated by production of estradiol.

Ovulation is considered to be day 0 of the estrous cycle. After ovulation, estradiol drops rapidly and a corpus luteum forms from the ovulatory site 2-3 days later, which produces progesterone (Dieleman & Blankenstein, 1985). The presence of a corpus luteum on the ovary marks the luteal phase, which lasts approximately from days 1 to 17 of the estrous cycle (C. Taylor & Rajamahendran, 1991). During the luteal phase, progesterone and estradiol suppress GnRH (Boer et al., 2011; N. P. Evans, Dahl, Mauger, & Karsch, 1995; Goodman, Gibson, Skinner, & Lehman, 2002). If pregnancy is not achieved, the production of progesterone eventually initiates secretion of prostaglandin which causes regression of the corpus luteum (C. Taylor & Rajamahendran, 1991).

The follicular phase lasts from days 18-21 of the cycle and is dominated by estradiol secretion. During each estrous cycle, 2-3 follicular waves occur, each initiated by an increase in FSH released from the anterior pituitary (Bergfelt et al., 1997; Boer et al., 2011). During the first or first two follicular waves, which occur during the luteal phase, the dominant follicle regresses because there is not an appropriate environment for ovulation. During the last follicular wave, negligible progesterone from the regressed corpus luteum fails to inhibit LH, which allows for peak production of estradiol and behavioral estrus followed by a GnRH surge and ovulation of the dominant follicle. After ovulation, a new corpus luteum forms and the estrous cycle begins again.

FACTORS REGULATING PUBERTY ATTAINMENT

Physiological Importance of Regulating Puberty Attainment

During puberty, energy begins to be channeled away from critical body systems such as homeostasis of basic life functions and growth and is funneled toward a non-essential body system: reproduction. Because puberty permits energy divergence from these critical body systems, it is tightly regulated. There are multiple factors that initiate and/or regulate signaling pathways of pubertal development. Some of the most crucial factors that regulate puberty in beef heifers are body weight and plane of nutrition/rate of gain (Perry, 2012, 2016). These factors are communicated to the reproductive axis through metabolic signals that affect GnRH release and puberty attainment. Some of these metabolic signals include hormones such as leptin and neurohormones such as kisspeptins, agouti-related peptide (AgRP), and neuropeptide Y (NPY). Other factors that are important regulators of puberty attainment are environment/season, bull exposure, and genetics (Perry, 2012, 2016).

Body Weight

The time at which heifers reach puberty is highly variable; according to a recent review, 19-100% of heifers reach puberty before the breeding season (Perry, 2016). Rather than achieving puberty at a consistent chronological age, heifers reach puberty at a consistent physiological age (R. N. Funston & Deutscher, 2004). Most heifers reach puberty at a certain weight and body condition according to their breed; several studies have concluded that heifers are 55-65% of mature body weight when puberty is reached and suggest a target weight of 55-65% at breeding (R. N. Funston & Deutscher, 2004; Patterson et al., 1992; Perry, 2016).

Mature weight for beef cattle is usually measured at 4-7 years of age. Because an individual heifer's mature body weight is not able to be measured until later in life, but producers need to select and feed heifers to reach their target body weight at breeding, target body weight can be measured as a percent of herd or breed average (Stockton, Wilson, Feuz, Stalker, & Funston, 2013). Percent herd average weight (PHAW) is often used to estimate PMBW and is calculated using the heifer's body weight as a percentage of the herd average weight (Stockton et al., 2013).

Plane of Nutrition

Nutritional intake (short-term and long-term) and rate of gain also influence age at puberty. Preweaning body weight gain has a larger impact on reducing age at puberty than postweaning body weight gain (Buskirk, Faulkner, & Ireland, 1995; Patterson et al., 1992; Perry, 2016). Feeding high concentrate diet for only 10 weeks preweaning increased LH pulse frequency, decreased negative feedback of estradiol, and significantly decreased age at puberty (Gasser, Behlke, Grum, & Day, 2006; Gasser, Bridges, et al., 2006; Gasser, Burke, et al., 2006; Gasser, Grum, et al., 2006). Interestingly, heifers with higher rates of gain reached puberty at younger ages than heifers with decreased rates of gain, but there was no difference due to whether the high gain was achieved through a high forage or a high concentrate diet (Allen et al., 2017). The period of time that seems to allow rate of gain to have the largest impact on reducing age at puberty is from 4-8 months of age; this coincides with the time period when leptin, a signal of adiposity, has its largest effect on age at puberty (Allen et al., 2017; Cardoso et al., 2014). Different feeding strategies can be used to encourage puberty development in heifers. For example,

stair-step feeding using feed restriction followed by ad libitum feed resulted in compensatory gain and initiation of puberty (Allen et al., 2017; Cardoso et al., 2014).

Metabolic Signaling

Sufficient nutrient status is a key regulator of pubertal attainment in heifers. This energy state is communicated to the reproductive axis through energy signaling molecules that act on neurons in the hypothalamus and regulate feed intake as well as reproductive function. GnRH neurons are innervated by other neurons, which when stimulated cause or prevent GnRH release from the anterior pituitary. The increase in GnRH release from hypothalamic neurons at puberty is thought to be due to changes in afferent inputs to GnRH neurons (Perera & Plant, 1997). Hormones such as leptin and neuropeptides such as POMC, α-MSH, kisspeptins, and NPY, are all signals of metabolic state that also influence the release of GnRH and regulate puberty attainment.

In the arcuate nucleus (ARC) of the hypothalamus are two neuronal centers that regulate food intake and control energy balance: the orexigenic center and the anorexigenic center (Wen, Wang, Gong, & Zhou, 2018). The orexigenic center induces appetite and stimulates food intake, whereas the anorexigenic center sends satiety signals that block food intake (Wen et al., 2018). In addition to regulating food intake, the factors produced by these centers also regulate endocrine control of the hypothalamic-pituitary-gonadal (HPG) axis.

POMC

Proopiomelanocortin (POMC) and Cocaine-and amphetamine-regulated transcript (CART) are neuropeptides produced by neurons in the anorexigenic center of the ARC. These anorexigenic signaling molecules act on other hypothalamic centers to signal

satiety and reduce food intake. Since satiety is associated with adequate energy balance, these peptides also signal to the reproductive axis that adequate energy is available for reproduction, increasing GnRH release and thus promoting puberty attainment. Diets high in energy resulted in increased POMC in the ARC (Allen et al., 2017).

POMC is the precursor for several other hormones when cleaved by enzymes located in different parts of the hypothalamus. One product of POMC is α-Melanocortin Stimulating Hormone (α-MSH). α-MSH is a signal of high energy, and it signals through the anorexigenic center to decrease food intake. As a signal of high energy, α-MSH is also correlated with a rise in GnRH release. In heifers fed high-energy diets, post-translational processing of POMC produced increased α-MSH (Cardoso, Alves, Sharpton, Williams, & Amstalden, 2015).

POMC neurons directly innervate GnRH neurons in rats, and POMC fibers also synapse with kisspeptin neurons in sheep, so it is unknown whether POMC neurons in heifers directly stimulate GnRH neurons or indirectly act on GnRH neurons through α-MSH and kisspeptin signaling (Cardoso et al., 2015). In heifers approaching puberty with increased body mass and energy available, POMC likely acts through secretion of α-MSH to indirectly stimulate GnRH neurons (Cardoso et al., 2015). POMC neurons also have leptin receptors, so POMC signaling may be stimulated by increased body fat and increased circulating leptin (Cardoso et al., 2015).

NPY

Neurons in the orexigenic center of the ARC produce Neuropeptide Y (NPY) and Agouti-related peptide (AgRP); these peptides act on other centers in the hypothalamus to

stimulate food intake and, as a signal of energy deficit, inhibit the reproductive endocrine axis and decrease GnRH release.

NPY is a neuropeptide that signals for low energy and decreases during increased body weight gain (Alves et al., 2015). In rats, NPY administered to the hypothalamus inhibits puberty attainment, whereas administering an NPY antagonist has been shown to cause progression of puberty (Pierroz, Gruaz, d'Alièves, & Aubert, 1995; Pralong, Voirol, Giacomini, Gaillard, & Grouzmann, 2000). As puberty progressed, there was reduced expression of NPY in the ARC; reduced NPY in the ARC was also observed in heifers on a high gain diet compared to a low gain diet (Alves et al., 2015). Diets high in energy have been demonstrated to result in decreased number of NPY projections to GnRH neurons (Alves et al., 2015). NPY is known to inhibit GnRH secretion, and this inhibition must be decreased during puberty to allow an increase in GnRH secretion (Gazal et al., 1998). It is unknown if NPY acts directly on GnRH neurons, or if NPY acts indirectly through kisspeptins or other neurons to cause changes in GnRH release. Metabolic changes have been shown to impact NPY nerve projections into GnRH or kisspeptin neurons, which supports the hypothesis that metabolic state of the animal may determine synapses between NYP, GnRH, kisspeptin, and other neurons, allowing changes in innervation of GnRH neurons and determining GnRH release in response to stimuli (Alves et al., 2015).

Establishment of NPY projections in certain areas of the brain is also regulated by leptin (Bouret, Draper, & Simerly, 2004; Pinto et al., 2004). Leptin inhibits NPY expression and possibly is responsible for programming NPY innervation of GnRH neurons (Alves et al., 2015; Stephens et al., 1995). Therefore, leptin may be part of the

mechanism responsible for reducing NPY expression and innervation of GnRH neurons at puberty which allows for decreased NPY inhibition of GnRH and increased GnRH release (Alves et al., 2015).

Leptin

Leptin is a peptide hormone produced by white adipose tissue (Zhang et al., 1994). The more fat there is in the body, the greater amount of circulating leptin that is present in the body (Fernandez-Fernandez et al., 2006). Likewise, the higher the current energy intake, the more leptin that is produced (Ahima, Saper, Flier, & Elmquist, 2000). In this way leptin production is regulated by both current plane of nutrition as well as long-term nutrition (Perry, 2012). Leptin is a signaling molecule that communicates to the brain the fatness of the body in order to maintain homeostasis in energy storage and food intake (Ahima et al., 2000; Zhang et al., 1994).

Once produced by adipocytes, leptin travels through the blood either free or bound by a plasma protein and binds to its receptors on plasma membranes of cells in the arcuate nucleus (ARC) of the hypothalamus (Morrison, Morton, Niswender, Gelling, & Schwartz, 2005). In the ARC, leptin binding initiates a signaling cascade that inhibits NPY/AgRP neurons (Morrison et al., 2005). Simultaneously, leptin stimulates the production of POMC (Barrios-Correa, Estrada, & Contreras, 2018). In these ways leptin promotes appetite suppression.

As heifers mature and accumulate increased body fat, their circulating leptin concentrations rise (Ahrén, Larsson, Wilhelmsson, Näsman, & Olsson, 1997; Yu & Kim, 2012). This keeps appetite in check and maintains homeostasis in body mass. In addition to its role in regulating metabolism, leptin also is crucial for the initiation of puberty. It is

known that leptin concentrations regulate the onset of puberty; under low leptin concentrations puberty does not occur, and at high leptin concentrations puberty takes place (Ahima, Dushay, Flier, Prabakaran, & Flier, 1997; El-Eshmawy, Abdel Aal, & El Hawary, 2010; Farooqi, 2002). Increases in leptin and *Lep*, the gene that encodes leptin, occur during puberty (Garcia et al., 2002).

Previous studies have suggested that leptin acts at the level of the hypothalamus to regulate GnRH secretion; however, GnRH neurons do not have leptin receptors, so it appears that leptin regulation of GnRH occurs indirectly through intermediate signaling molecules (Cunningham, Clifton, & Steiner, 1999; Finn et al., 1998; Hâkansson, Brown, Ghilardi, Skoda, & Meister, 1998; Williams et al., 2002). Leptin-deficient mice have age-appropriate gonad and gonadotrope development but decreased GnRH at time of expected puberty, which indicates that leptin is crucial for GnRH release but not reproductive organ or somatic maturation (Batt et al., 1982; Elias & Purohit, 2013). One current hypothesis is that leptin acts on ARC neurons to promote kisspeptin release. There is some evidence that leptin acts through kisspeptin receptors and kisspeptin signaling to activate GnRH neurons (see following section: Kisspeptin) (Cortés, Carrera, Rioseco, Pablo del Río, & Vigil, 2015). However, there is also evidence that leptin signaling regulates reproduction by acting in areas of the hypothalamus that have no kisspeptin receptors (Louis et al., 2011), so there may be a mechanism other than kisspeptin that leptin acts through.

There are six known leptin receptors, all of which are encoded by the *Lepr* gene (Myers, 2004). Of these six isoforms, only LepRb is currently known to be physiologically active (Bjørbaek, Uotani, da Silva, & Flier, 1997; Myers, 2004). The

highest affinity of leptin binding to LepR has been seen in the ARC, where LepRb is expressed in POMC neurons, NPY/AgRP, and Kiss1 neurons (Banks, Kastin, Huang, Jaspan, & Maness, 1996; Baskin, Breininger, & Schwartz, 1999; Cheung, Clifton, & Steiner, 1997; J. T. Smith, Acohido, Clifton, & Steiner, 2006). LepRb when bound activates the JAK/STAT signaling pathway, which causes phosphorylation of STAT3 (Myers, 2004). STAT3 then translocates to the cell nucleus and acts as a transcription factor (Myers, 2004). However, it seems that STAT3 is not the only signaling pathway activated when leptin binds to LepRb (Elias & Purohit, 2013). Studies have shown that leptin also activates a phosphoinositide 3-kinase (PI3K) pathway which may have an impact on reproduction (Myers, 2004). Research has demonstrated that blocking PI3K blocks leptin signaling through the POMC and AgRP neurons in the ARC; therefore, it is likely that leptin signals through PI3K to activate POMC and AgRP neurons (Al-Qassab et al., 2009). However, it is unknown if this PI3K pathway has an impact on the effect of leptin signaling on puberty attainment. Mammalian target of rapamycin (mTOR) signaling pathways are also possible pathways used by leptin to cause metabolic and reproductive/pubertal signaling (Sanchez-Garrido & Tena-Sempere, 2013).

In conclusion, there appears to be a minimum amount of body fat required for heifers to reach puberty. A heifer's amount of body fat is communicated to her brain by a corresponding concentration of circulating leptin, which indicates to the brain that the body has enough energy for puberty through the activation of GnRH neurons (Ahrén et al., 1997; Williams et al., 2002). Circulating leptin increases as pubertal development progresses (Williams et al., 2002). Although leptin is highly likely a key regulator in the

attainment of puberty, it is unknown the exact method by which this hormone promotes release of GnRH.

Kisspeptins

Kisspeptins are a family of neuropeptides involved with the regulation of metabolism, reproduction, and puberty attainment (J. H. Lee et al., 1996). In humans, kisspeptins are encoded by the *Kiss1* gene and are made in the preoptic area (POA) and ARC of the hypothalamus (Lehman, Merkley, Coolen, & Goodman, 2010). After their release, kisspeptins bind to their G-protein coupled receptor Gpr54, activating G-protein activated phospholipase C and increasing intracellular calcium ion concentrations through second messengers IP_3 and DAG to cause specific effects on the target cells (Constantin, Caligioni, Stojilkovic, & Wray, 2009; Messager et al., 2005; Oakley, Clifton, & Steiner, 2009). Kisspeptins are involved in hypothalamic signaling of reproduction and metabolism, although their exact mechanisms of action are mostly hypotheses at this point (Cortés et al., 2015; Sanchez-Garrido & Tena-Sempere, 2013).

It is known that kisspeptin Gpr54 receptors are found on GnRH neurons and stimulate GnRH neurons to secrete GnRH (Messager et al., 2005). An increase in *Kiss1*, the gene encoding kisspeptins, has been documented during puberty in rats and monkeys (Navarro et al., 2004; Shahab et al., 2005). In mice, puberty is accompanied by an increased sensitivity to kisspeptin that is shown by an increase in LH and GnRH production during kisspeptin stimulation (Han et al., 2005). In addition, Gpr54 knockout mice do not reach puberty, indicating the kisspeptin signaling through its receptor is essential for puberty attainment in mice (Holmes, 2014).

While it is unknown how kisspeptins promote puberty, there is a pubertal increase in number of Gpr54 found in GnRH neurons and an increase in signaling efficiency through Gpr54 during puberty (Han et al., 2005; Herbison, de Tassigny, Doran, & Colledge, 2010). Normally, increased kisspeptin signaling would lead to desensitization of Gpr54, but during puberty in rats, there is less desensitization occurring during increased kisspeptin signaling which allows more activation of GnRH neurons without negative feedback at the level of the kisspeptin receptor (Roa et al., 2008). The combination of these mechanisms possibly promotes the activation of the HPG axis.

Kisspeptins also seem to play a role in metabolism. Research has suggested that a rise in leptin leads to increased kisspeptin secretion, inducing binding to Gpr54 and activation of GnRH neurons (Teles, Silveira, Tusset, & Latronico, 2011). Although it has been suggested that leptin directly activates kisspeptin neurons to upregulate GnRH secretion and influence the HPG axis, it seems that there are more complex systems at play that promote the idea of more indirect pathways with yet unknown intermediate signaling steps (Sanchez-Garrido & Tena-Sempere, 2013).

Environmental Factors

The United States beef industry utilizes both fall and spring calving seasons. Season of birth as well as season of development can affect puberty attainment in heifers (Garcia et al., 2002; Schillo, Hansen, Kamwanja, Dierschke, & Hauser, 1983; Zain El Abdein & Makkawi, 2006). Season of birth has also been shown to cause differences in patterns of LH secretion around time of puberty (Honaramooz et al., 1999). These effects may be due to photoperiod, temperature, rate of gain, or a combination of mechanisms (Schillo et al., 1983).

Longer day length has been correlated to decreased age at puberty in beef heifers (Schillo et al., 1983). Photoperiod mechanisms that influence puberty attainment in heifers may signal through metabolites such as leptin. Circulating leptin levels have been shown to fluctuate seasonally in mature cows, increasing from winter to summer with no accompanying change in body weight (Garcia et al., 2002). This mechanism could also be a contributing factor in stimulating puberty attainment.

Bull Exposure

Bull exposure is also a factor that can influence puberty attainment in heifers. There seems to be an interaction between growth rate and bull exposure that hastens puberty attainment in beef heifers (Roberson et al., 1991). Heifers at a certain weight range and between 12 to 14 months are most influenced by bull exposure prior to puberty (Rekwot, Ogwu, Oyedipe, & Sekoni, 2001; Roberson et al., 1991). Heifers exposed to sterile bulls or androgenized steers reached puberty earlier than heifers not exposed to sterile bulls (Fiol, Quintans, & Ungerfeld, 2010; Roberson et al., 1991).

The mechanism by which bull exposure hastens puberty attainment is hypothesized to be pheromone-dependent. Exposure to bull urine hastens puberty attainment in pre-pubertal heifers (Roberson et al., 1991). In female mice, a similar phenomenon of accelerated puberty after exposure to male mice or their urine is due to an androgen-dependent pheromone; in cattle, the mechanism is suggested to be through pheromones but is still unproven (Rekwot et al., 2001; Roberson et al., 1991).

Genetics

Age at puberty is largely determined by weight and age within breed (Martin, Brinks, Bourdon, & Cundiff, 1992). Smaller breeds usually reach puberty earlier than

breeds with a larger mature size (Martin et al., 1992). In addition, breeds with increased milk production reach puberty earlier than those with lower milk production (Martin et al., 1992). Heterosis achieved through cross-breeding also decreases age at puberty (Martin et al., 1992).

Age at puberty in beef cattle is estimated to have a heritability of 0.41 (Laster, Smith, Cundiff, & Gregory, 1979). However, age at puberty is a quantitative trait impacted by many different genes (Fortes et al., 2016). Genome-wide association studies have identified many genes that influence puberty and the interactions of these genes, many of which are the target of future and current research studies (Fortes et al., 2016). These genes may interact with nutritional status of heifers or their rate of gain prior to puberty. One study found that elevated levels of body weight gain in heifers during their development caused changes in DNA methylation in the ARC (Alves et al., 2017). Since many metabolic signaling pathways that interact with the HPG axis occur in the ARC of the hypothalamus, these changes in methylation correlated with increased rate of gain may be one mechanism that genes program age at puberty in heifers (Alves et al., 2017).

TOOLS TO EVALUATE/PREDICT PUBERTAL STATUS

Reproductive Tract Score

A five-point reproductive tract scoring (RTS) system can be used to determine if heifers have reached puberty prior to breeding. This system utilizes rectal palpation or ultrasound of heifers when they are 10-12 months old (Holm, Thompson, & Irons, 2009). Each heifer is scored on her sexual maturity based on the diameter of her uterine horns, the size of her ovaries, and the presence of large follicles or corpora lutea (Anderson,

LeFever, Brinks, & Odde, 1991). A heifer with a score of 1 is immature, and a heifer with a score of 5 is cycling (Atkins et al., 2013).

Prepubertal heifers receive a RTS of 1 or 2. A heifer with a RTS of 1 has a uterine horn diameter less than 20 mm, small ovarian size, and no palpable follicles on either ovary. A heifer with a RTS of 2 has a slightly larger uterine horn diameter and slightly larger ovarian size, with 8 mm follicles present. Peripubertal heifers receive a RTS of 3. These heifers have a uterine horn diameter of 20-25 mm, medium ovarian size, and 8-10 mm follicles. Finally, heifers presumed to be pubertal receive a RTS of 4 or 5. Heifers with a RTS of 4 have thick, toned uterine horns, large ovarian size, and follicles greater than 10 mm. In order to receive a score of 5, there must be a corpus luteum present on at least one ovary. This guarantees that the heifer has reached puberty and is cycling. Heifers with RTS of 4 or 5 had greater A.I conception rates, greater overall pregnancy rates, an earlier calving day and calves with increased weaning weights (Holm et al., 2009). Many producers choose not to have a veterinarian perform a pre-breeding reproductive tract examination because they believe that choosing the oldest and heaviest heifers will insure that they are pubertal. There is some evidence, however, that the larger uterus associated with a greater RTS may contribute to the improved fertility in these heifers much more than whether she had a corpus luteum at palpation or not.

Antral Follicle Count

Antral follicle count (AFC), or the number of antral follicles present on the ovaries of a heifer at one time, varies from heifer to heifer but remains consistent between repeated measurements on the same individuals (Burns, Jimenez-Krassel, Ireland, Knight, & Ireland, 2005; Ireland et al., 2008). Antral follicle count is positively correlated

to the size of the ovarian reserve, which is correlated to reproductive longevity (Ireland et al., 2008; Monniaux et al., 2014). In addition, antral follicle count is positively correlated to increased growth and development, greater progesterone concentrations, and a decreased age at puberty (Santa Cruz, Cushman, & Viñoles, 2018).

Antral follicle count is correlated to ovarian size and uterine development and can be used to indicate readiness for breeding (R. A. Cushman et al., 2009; Rawlings, Evans, Honaramooz, & Bartlewski, 2003). Because AFC is not influenced by stage of the estrous cycle, prebreeding AFC can be a useful strategy in selecting replacement heifers (R. A. Cushman et al., 2009). This may be a connection between antral follicle count and fertility, because heifers with diminished numbers of antral follicles had smaller uteri and decreased uterine protein production (McNeel et al., 2017).

Studies have shown that heifers with increased antral follicle counts at prebreeding had an increased heifer pregnancy rate (R. A. Cushman et al., 2009) and calve earlier as first-calf heifers (R.A. Cushman, McNeel, & Freetly, 2014; McNeel & Cushman, 2015). However, it is interesting to note that two studies have shown increased AFC in prepubertal heifers has antagonistic genomic correlations to heifer pregnancy rate (Júnior et al., 2017; Snelling et al., 2012).

A Note on Precocious Puberty

Caution must be used in selecting heavily for decreased age at puberty. Although puberty attainment by 12 months of age is desirable, selecting for early puberty can lead to increased occurrence of precocious puberty in beef heifers (Perry & Cushman, 2013). Heifers reaching puberty before weaning are at risk for exposure to fertile bulls pastured with their dams, and may become pregnant early (Wehrman, Kojima, Sanchez, Mariscal,

& Kinder, 1996). Pregnancy in heifers before achieving a target body size or body weight can lead to increased dystocia and increased calf death loss (Wehrman et al., 1996). In addition, precocious heifers that become pregnant before entering the feedlot will have reduced feed efficiency and growth as well as increased labor costs (Wehrman et al., 1996).

Interestingly, a study by Wehrman et al. (1996) recorded weekly circulating progesterone values from a herd of beef heifers and found that heifers reaching precocious puberty showed evidence of temporary luteal function at an early age, resumed anestrus, and then resumed cyclicity again at a normal age for achieving puberty. This study hypothesized that precocious puberty occurs when the inhibitory effects of estradiol are delayed (Wehrman et al., 1996). In this case, estradiol from follicles on the ovary could induce an LH surge, leading to ovulation and cyclity without the negative feedback of estradiol. The paper suggested that when estradiol began to have an inhibitory effect on the HPG axis, this would cease cyclicity leading to temporary anestrus until at a normal weight and age signaling would be appropriate for cyclicity to resume (Wehrman et al., 1996).

In conclusion, selection for age at puberty must focus on achieving an optimal range of age at puberty rather than strictly targeting a decreased age at puberty.

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Chapter 2 : Attainment and Maintenance of Pubertal Cyclicity May Predict Reproductive Longevity

ABSTRACT

Puberty attainment was investigated in heifers born in 2012-2017 by collecting blood samples, weights and phenotypic data from weaning to breeding. The threshold for puberty and cyclicity was indicated by progesterone concentrations ≥ 1 ng/ml. A SAS program was developed to categorize heifers into one of four puberty groups based on puberty date and whether or not cyclicity continued: 1) Early heifers reached puberty before March 12 and cycled continuously during the sampling period, 2) Typical heifers reached puberty on or after March 12 and cycled continuously during the sampling period, 3) Start-Stop heifers had at least one occurrence of progesterone ≥ 1 ng/ml but discontinued cycling during the sampling period, and 4) Non-Cycling heifers had no occurrence of progesterone ≥ 1 ng/ml during the sampling period.

Early and Typical heifers had greater adjusted weaning weights than Start-Stop heifers, and greater adjusted yearling weights compared to Start-Stop and Non-Cycling heifers. At prebreeding ultrasound, Early heifers had the highest percentage reproductive tract scores of 5, followed by Typical, Start-Stop, and Non-Cycling heifers. At breeding, heifers were given 2 injections of prostaglandin $F_{2\alpha}$ 14-days apart, and all heifers that showed estrus were artificially inseminated. Typical and Early heifers displayed the greatest percentage estrus in response to prostaglandin, and a greater percentage of Start-Stop heifers displayed estrus and were artificially inseminated compared to Non-Cycling heifers. After this, all heifers were exposed to bulls, and overall pregnancy rate did not

differ between puberty groups. A greater percentage of Typical, Early, and Start-Stop heifers calved in the first 21 days of the calving season compared to Non-Cycling heifers.

Start-Stop heifers were characterized as either one of two subgroups in order to investigate their puberty attainment more closely: 1) Start-Stop-Start (SSS) heifers showed evidence of continuous cyclicity in May prior to breeding, 2) Start-Stop-Discontinuous (SSD) heifers had no evidence of continuous cyclicity in May prior to breeding. Between these two subgroups, SSS heifers tended to have heavier adjusted weaning weights and prebreeding weights. Significantly more SSS heifers had a reproductive tract score of 5 at prebreeding, responded to prostaglandin by showing estrus and were artificially inseminated, and calved within the first 21 days of the calving period.

INTRODUCTION

We have identified a population of High Androstenedione (A4) cows in the UNL physiology herd that are often anovulatory resulting in irregular estrous cycles, altered metabolism and a 17 percent reduction in calving rate (Summers et al., 2014). These High A4 cows are characterized by excess androstenedione (30 fold greater) in the follicular fluid of their dominant follicle (Summers et al., 2014). When pieces of ovarian cortex from High A4 cows were cultured, they secreted excess androstenedione into culture media (Abedal-Majed, unpublished). On average, High A4 cows reached puberty 45 days earlier than control cows (P=0.17) (Summers et al., 2014).

Previous studies have used different threshold levels of progesterone to identify puberty in beef heifers. Ranges of 1-2 ng/ml circulating progesterone (P4) are a common

threshold level of progesterone to suggest luteal function and cyclicity. One study collected samples 2-3 times per week and considered puberty the first occurrence of P4 > 1 ng/ml (Honaramooz et al., 1999). In a study by Piccolo et al. (2018), samples were collected every 9-10 days, and puberty was considered to be the first occurrence of P4 > 1 ng/ml. In another study, plasma samples were collected weekly, and puberty was considered to be reached after 2 consecutive weeks of P4 ≥ 1 ng/ml (E. G. Taylor et al., 2017). Cardoso et al. (2014) collected samples every 3 to 4 days, and puberty was considered the first time 3 consecutive samples had P4 ≥ 1 ng/ml. In a study by Moriel et al. (2014), samples were collected every 10 days, and puberty was considered to be reached after 2 consecutive samples where P4 > 1.5 ng/ml. One other example of progesterone concentrations used to indicate puberty attainment was a 2015 study where samples were collected every 7 days, and puberty was considered to be 7 days before the date of the first sample that contained either 2 ng/ml P4 or the first of 2 consecutive samples where P4 > 1 ng/ml (Gunn et al., 2015).

In the following experiment, 1 ng/ml P4 was used as a threshold to indicate puberty and cyclicity. Our hypothesis that the manner heifers achieve puberty may indicate if they are predisposed to become the High A4 population of cows with reduced fertility. We also hypothesized that attainment of puberty may be a predictor of reproductive longevity in heifers. Heifers with discontinued cyclicity during puberty attainment may have irregular reproductive cycles, anovulation, and infertility throughout their reproductive lifespan.

MATERIALS AND METHODS

Animals

A total of 611 beef heifers across six years born in 2012-2017 were used in this experiment. See Table 2-1 for the number of heifers in the experiment born each year. All heifers were from the physiology herd at the University of Nebraska-Lincoln and were kept at the Eastern Nebraska Research and Extension Center (ENREC). The physiology herd is made up of approximately 250 Red Angus composite cows. About 50% of cows were AI sired by Red Angus bulls, and about 50% were sired by Red Angus x Simmental composite herd bulls. During the earlier years of this project (2012-2014), up to 10% of calves were sired by MARC III bulls, a composite breed of ¼ Red Angus, ¼ Hereford, ¼ Pinzgauer, and ¼ Red Poll.

Dams of heifers were fed supplement during gestation for all years during this experiment except dams of heifers born in 2016. Heifers were born in the spring each year around March, and were grazed on pasture with dams until weaning in late October each year. At weaning, heifers were separated from dams and male calves and retained as replacement heifers on pasture at ENREC. The University of Nebraska-Lincoln Institutional Animal Care and Use Committee approved all procedures and facilities used in this experiment.

Weights and Body Condition Scores

Birth weights, weaning weights, and yearling weights were collected for heifers each year. Adjusted birth weights were calculated using Cow Sense herd management software (Midwest Microsystems L.L.C., Lincoln, NE) and adjusted for age of dam. Adjusted weaning weights and adjusted yearling weights were also calculated using Cow

Sense software (Midwest Microsystems L.L.C., Lincoln, NE) and adjusted for dam age and calf age at weaning. Calf and yearling average daily gain (ADG) values and weight per day of age (WDA) were calculated for all heifers using Cow Sense software. Calf ADG represents ADG from birth to weaning. Yearling ADG represents ADG from weaning to yearling weight. WDA is the average weight per day of age from birth to yearling weight.

In addition, regular weights were recorded during sampling periods from weaning to breeding each year. In 2012-born heifers, weights were recorded in October, January, February, March, and May. In the 2013-born heifers, weights were recorded in October, February, April, and May. In the 2014-born heifers, weights were recorded every month from October to May except for the month of November. In the 2015-2017 born heifers, weights were recorded every month from October to May. Body condition scores (BCS) were also recorded monthly for the 2015-2017 born heifers on the same dates that weights were recorded.

Pre-breeding weight was considered to be the last weight before breeding recorded in May each year. Herd average weight for the physiology herd from 2012-2017 was calculated as the average weight of all cows greater than 1.5 years old and was found to be 1169 lbs (530 kg). Percent herd average weight (PHAW) at pre-breeding was calculated for heifers using pre-breeding weight divided by average herd weight times 100 percent. Herd average mature weight from 2012-2017 was calculated as average weight of all cows in the physiology herd greater than 4.5 years old and was found to be 1369 lbs (621 kg). Percent average mature body weight (PMBW) was calculated for

heifers using pre-breeding weight divided by average herd mature weight times 100 percent.

Average daily gain at time of puberty for each heifer was calculated by finding the monthly weights immediately before and after puberty date and dividing difference in weights during that period by number of days during that time period. Weight at puberty was found to be the initial monthly weight (before or on puberty date) + (ADG during that time period x number of days between initial monthly weight date and puberty date).

Blood Sample Collection

Blood samples were collected regularly during the sampling period from approximately weaning to breeding each year (Figures 2-1 and 2-2). For the 2012-born heifers, samples were collected monthly from October to December and twice monthly from January to June. In 2013, samples were collected monthly from October to December and twice monthly from January to May. In 2014, samples were collected monthly from October to December, twice in January, and weekly from February to May. Weekly blood samples were collected during the entire sampling period from October to May for the 2015-2017 born heifers (Table 2-1 and Figure 2-2).

Blood samples were collected using coccygeal venipuncture into glass vacutainer blood collection tubes (Becton, Dickinson and Company, Franklin Lakes, NJ) containing 12 mg EDTA and placed immediately on ice. Within several hours, blood samples were centrifuged at approximately 700 g and 4 degrees Celsius for 30 minutes. Immediately following, plasma was removed and stored in polypropylene tubes (Globe Scientific, Inc., Paramus, NJ) at -20 degrees Celsius.

Progesterone Measurements

Progesterone (P4) for each plasma sample was detected using radioimmunoassay (RIA). Each sample was run in duplicate, and the average value for the duplicates was used as the mean progesterone value for that sample. Progesterone values were recorded in ng/ml. Samples found to have a coefficient of variation (CV) greater than 15 percent between duplicates were re-run in another assay.

In years 2012 and 2013, the Coat-a-Count assay kit (Diagnostic Products Corporation, Los Angeles, CA) was used to determine progesterone concentrations. Intra-assay coefficient of variation averaged 6.1%, and inter-assay coefficient of variation was 18.6% for the Coat-a-Count assay. In years 2014-2017, progesterone concentrations were determined using the ImmuChem^{TM} Coated Tube Progesterone ^{125}I RIA kit (ICN Pharmaceuticals, Inc., Costa Mesa, CA). Intra-assay coefficient of variation averaged 2.3%, and inter-assay coefficient of variation was 14.0% for the ImmuChem^{TM} Coated Tube Progesterone ^{125}I RIA assay.

Pre-breeding Ultrasound

Prior to breeding each year, heifers were evaluated using transrectal ovarian ultrasound. Uterine horn diameter, ovary size, number and size of antral follicles on each ovary, and presence or absence of corpora lutea were recorded for each heifer. Antral follicle count was considered the total number of antral follicles recorded for both left and right ovaries. Uterine horn diameter was the diameter of the right uterine horn. A reproductive tract score was calculated for each heifer using the criteria outlined in Anderson et al. (1991).

Breeding

In May each year, heifers were synchronized with 2 injections of Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) 14 days apart and were fitted with estrus detection patches. After the second injection of $PGF_{2\alpha}$, heifers were detected for visual signs of estrus and/or patch activation at both a.m. and p.m. for approximately 1 week. All heifers that showed estrus were bred using artificially insemination (AI). After 10 days, all heifers were placed with herd cleanup bulls (Figure 2-1). Heifers were pregnancy checked using transrectal ultrasound at approximately 6 weeks after AI for AI pregnancy. Approximately 12 weeks after bull turnout, heifers were pregnancy checked for overall pregnancy. Overall pregnancy diagnosis was performed using manual palpation for 2012-2015-born heifers. For 2016-2017-born heifers, overall pregnancy diagnosis was performed using blood samples analyzed by GeneSeek (Neogen Genomics, Lincoln, NE) through the Bovine Early Stage Pregnancy Detection (BioPRYN) test, an enzyme-linked immunosorbent assay (ELISA) testing for the presence of pregnancy-specific protein B (PSPB). The first 21 days of the calving season was determined by the first date calved and the 21 days on/after that date.

Development of a SAS Analysis for Puberty Groups

Four Puberty Groups

The first occurrence of progesterone (P4) ≥ 1 ng/ml during the sampling period was considered puberty date. Continuous cyclicity was evaluated by percent of samples where P4 ≥ 1 ng/ml after puberty date during the sampling period; heifers with 50% or greater samples with P4 ≥ 1 ng/ml on and after puberty date were considered to have continuous cyclicity, and heifers with fewer than 50% of samples with P4 ≥ 1 ng/ml on and after puberty date were considered to have discontinuous cyclicity. A custom SAS

analysis was developed to detect four distinct puberty groups using puberty date and whether cyclicity continued over the sampling period: 1) Early – reached puberty before March 12 and had continuous cyclicity from puberty until breeding; 2) Typical – reached puberty after March 12 and had continuous cyclicity from puberty until breeding; 3) Start-Stop – reached P4 ≥ 1 ng/ml during the sampling period but had discontinuous cyclicity from "puberty" to breeding; and 4) Non-Cycling – had no occurrence of P4 ≥ 1 ng/ml prior to breeding.

March 12 was used to separate Early and Typical heifers because it marked the 25th percentile for puberty date in a previous analysis of puberty date distributions for heifers in the UNL physiology herd. A date was used to determine whether heifers were Early or Typical rather than separating groups by age at puberty because in a typical U.S. beef cow-calf operation, heifers must reach puberty at a certain point in the season regardless of their age and birth date in order to achieve maximum reproductive efficiency (R. Funston, Musgrave, Meyer, & Larson, 2012; Perry & Cushman, 2013; Vraspir, Summers, Roberts, & Funston, 2014).

P4 concentrations from samples collected on the date of weaning were not included in the dataset when determining puberty group. Samples with P4 ≥ 1 ng/ml were commonly seen on the date of the first blood sample collected at weaning but not on a sample taken immediately after weaning (Figure 2-3). Elevated P4 concentrations at weaning could be attributed to weaning stress.

Two Subgroups of Start-Stop Heifers

Start-Stop heifers born in 2012-2016 were further classified into two subgroups based on whether they showed evidence of reinitiating continuous cyclicity prior to

breeding: 1) Start-Stop-Start (SSS) heifers showed evidence of continuous cyclicity prior to breeding whereas 2) Start-Stop--Discontinuous (SSD) heifers did not regain continuous cyclicity prior to breeding. A SAS code was used to separate Start-Stop heifers into these two subgroups by considering progesterone values for Start-Stop heifers in the month of May only (samples most immediately prior to breeding). Heifers with at least one occurrence of P4 ≥ 1 ng/ml in the month of May followed by 50% or greater samples where P4 ≥ 1 ng/ml until breeding were considered SSS heifers. Heifers with no occurrences of P4 ≥ 1 ng/ml in the month of May, or heifers with at least one occurrence of P4 ≥ 1 ng/ml in May followed by fewer than 50% of samples where P4 ≥ 1 ng/ml, were considered SSD heifers.

Statistical Analyses

Data analyses were conducted using SAS v9.4. All data are presented as mean ± standard error of the mean (SEM). For all analyses, differences were considered significant when $P \leq 0.05$ and a tendency when $P \leq 0.10$ and > 0.05.

Puberty group characteristics (age at puberty, average progesterone concentrations) were analyzed using the MIXED procedure of SAS with year and group*year included in the model statements. For average progesterone concentrations, animal was included in the class statement with random animal within group used to account for repeated measures. Percent of samples where P4 ≥ 1 ng/ml was evaluated using the GLIMMIX procedure of SAS with a binomial distribution and logit link and year and group*year interaction included in the model.

Weight data for comparisons between the four puberty groups (birth weight, weaning weight, adjusted birth weight, adjusted weaning weight, adjusted yearling

weight, pre-breeding weight, age at weaning, average daily gain, and weight per day of age) were analyzed using the MIXED procedure of SAS with year and group*year included in the model statement. For comparisons between the four puberty groups, if there was no significant group*year interaction this term was dropped from the model. For comparisons between the Start-Stop subgroups with a low n for each subgroup, group*year interactions were included in the model. PHAW and PMBW were analyzed using the GLIMMIX procedure of SAS with a binomial distribution and logit link and year and group*year interaction included in the model.

Antral follicle count and uterine horn diameter were analyzed using the MIXED procedure of SAS with year and group*year included in the model statements. If no significant group*year interaction was observed, this term was dropped from the model. Reproductive tract score data were analyzed as a percent of heifers with a score of 5. Data were distributed binomially and analyzed using the GLIMMIX procedure of SAS. No significant group*year interactions were observed for reproductive tract score and this interaction term was dropped from the model.

Pregnancy and calving data (percent showed estrus in response to prostaglandin, percent pregnancy to AI, percent overall pregnancy, and percent calved in the first 21 days) were analyzed using the GLIMMIX procedure of SAS with a binomial distribution and logit link. For comparisons between puberty groups, model included year, group, and group*year interaction. If there was no significant group*year interaction, this term was dropped from the model. For comparisons between Start-Stop subgroups, year was not included in the model.

RESULTS

Number of Heifers in Puberty Groups by Year

Across all six years of data collection and 611 total heifers, 23.4% of heifers were Early, 45.7% were Typical, 14.9% were Start-Stop, and 16.0% were Non-Cycling (Table 2-1). This trend was similar in percentage of heifers in each puberty group for the 2015-born heifers. In years 2012 and 2014, there were more Early and fewer Non-Cycling heifers. In 2013 and 2016, there were fewer Early and more Non-Cycling heifers. There was a higher percentage of Start-Stop heifers in 2014 compared to the percentage of Start-Stop heifers across all six years of data collection. There was a higher percentage of Typical heifers in 2016, and a higher percentage of Early heifers in 2017 compared to percentages across all six years (Table 2-1).

Puberty Date Distributions

Heifers reaching puberty prior to March 12 were either classified as Early or Start-Stop. From October – January, more heifers reaching the first occurrence of P4 $\geq$ 1 ng/ml were Start-Stop than Early (Figures 2-4 and 2-6). From February – March 11, more heifers reaching the first occurrence of P4 $\geq$ 1 ng/ml were Early than Start-Stop (Figure 2-4 and 2-6). Average date at first P4 $\geq$ 1 ng/ml was earliest for 2014-born heifers, followed by 2012-born heifers, 2015-born heifers, 2017-born heifers, 2013-born heifers, and 2016-born heifers (Figure 2-5 and Table 2-4). Figures 2-7 and 2-8 show distribution of puberty dates by year.

Average age at first P4 $\geq$ 1 ng/ml was lowest in 2014-born (298 d) heifers, followed by 2015-born heifers (316 d), 2016-born (318 d) and 2017-born heifers (324 d), and was greatest in 2012-born (331 d) and 2013-born (334 d) heifers (Table 2-4).

Differences in Plasma Progesterone Among Puberty Groups

Typical heifers showed patterns of regular cyclicity beginning after March 12 from puberty date until breeding (Figure 2-9). Early heifers showed patterns of regular cyclicity from puberty date (before March 12) until breeding (Figure 2-10). Start-Stop heifers had different patterns of cyclicity, including: 1) 1-3 isolated incidences of P4 ≥ 1 ng/ml (Figure 2-11-A,B) with no regular cyclicity, 2) one incidence of P4 ≥ 1 ng/ml in November – December followed by a long period of no cyclicity and regular cyclicity initiated prior to breeding (Figure 2-11-C,D), and 3) several consecutive occurrences of P4 ≥ 1 ng/ml followed by a long period of no cyclicity and regular cyclicity initiated prior to breeding (Figure 2-11-E,F). All Non-Cycling heifers had no occurrence of P4 ≥ 1 ng/ml from weaning to breeding (Figure 2-12).

Early (208 d) heifers were the oldest puberty group at weaning, followed by Typical (203 d) heifers which were not significantly older than Start-Stop (200 d) heifers but were older than Non-Cycling (198 d) heifers (Table 2-2).

There was a significant group*year interaction for average age at first occurrence of P4 ≥ 1 ng/ml, average weekly progesterone, and average progesterone concentration for all samples where P4 ≥ 1 ng/ml (Table 2-2). Average age at first P4 ≥ 1 ng/ml was lowest in Start-Stop heifers (265 d), followed by Early (317 d) and Typical (378 d) heifers (Table 2-2). Typical (82.7%) and Early (74.2%) heifers had a greater percent of samples from weaning to breeding where P4 ≥ 1 ng/ml compared to Start-Stop (27.6%) heifers (Table 2-2). Average progesterone concentration across the entire sampling period was greatest in Early heifers (2.58 ng/ml), followed by Typical (1.42 ng/ml), Start-Stop (1.05 ng/ml), and Non-Cycling (0.06 ng/ml) heifers (Table 2-2). The average

progesterone concentration of the initial sample measured where P4 ≥ 1 ng/ml was highest in Typical heifers (3.90 ng/ml), followed by Early (3.31 ng/ml) and then Start-Stop (1.88 ng/ml) heifers (Table 2-2). The average progesterone concentration in samples collected after initial P4 ≥ 1 ng/ml was greatest in Typical (4.47 ng/ml) heifers, followed by Early (4.10 ng/ml) and Start-Stop (1.24 ng/ml) heifers (Table 2-2). For all samples where P4 ≥ 1 ng/ml, average progesterone concentrations were higher in Typical (5.41 ng/ml) and Early (5.45 ng/ml) heifers than in Start-Stop (4.09 ng/ml) heifers (Table 2-2).

Weight and Average Daily Gain

Adjusted birth weight was not different between puberty groups (Figure 2-13-A). However, average adjusted weaning weight was significantly greater in Typical (246 kg) and Early (246 kg) compared to Start-Stop (240 kg) heifers but was not different from Non-Cycling (242 kg/d) heifers (Figure 2-13-B). Average adjusted yearling weight was higher in Typical (335 kg) and Early (334 kg) compared to Start-Stop (326 kg) and Non-Cycling (326 kg) heifers (Figure 2-13-C). There was a tendency for a significant interaction in group*year for weight at pre-breeding (P=0.074); Typical and Early heifers were significantly heavier at pre-breeding than Start-Stop heifers most years (Figure 2-13-D). Average daily gain from birth to weaning was greater in Typical (0.98 kg/d) and Early (0.98 kg/d) than in Start-Stop (0.94 kg/d) heifers but was not different from Non-Cycling (0.96 kg/d) heifers (Figure 2-13-E). Average daily gain from weaning to yearling weight was not different between groups (Figure 2-13-F). Overall weight per day of age gained from birth to yearling weight was significantly higher in Typical (1.07 kg/d), Early (1.05 kg/d), and Non-Cycling (1.05 kg/d) compared to Start-Stop (1.01 kg/d) heifers (Figure 2-13-G).

Pre-breeding Reproductive Tract Development

There was a group*year interaction for pre-breeding antral follicle count, where most years antral follicle count tended to be higher in Non-Cycling heifers than in Typical or Start-Stop heifers (*P*=0.07; Figure 2-14-A). Pre-breeding uterine horn diameter was larger in Typical (10.02 mm) than in Non-Cycling (9.29 mm) heifers and not different from Early (10.00 mm) or Start-Stop (9.77 mm) heifers (Figure 2-14-B). At pre-breeding, the highest percent of Early (92.7%) heifers had a reproductive tract score of 5, followed by Typical (76.2%), Start-Stop (48.6%), and Non-Cycling (1.27%) heifers (Figure 2-14-C). Average reproductive tract scores were 4.92 in Early heifers, 4.71 in Typical heifers, 4.46 in Start-Stop heifers, and 3.98 in Non-Cycling heifers (Table 2-3).

Breeding

A greater percentage of Typical (80.3%) and Early (79.9%) heifers showed estrus in response to prostaglandin, followed by Start-Stop (49.7%) and Non-Cycling (9.71%) heifers (Figure 2-15-A). All heifers that showed estrus were artificially inseminated, and of the heifers that were artificially inseminated, there were no differences in conception rates (Figure 2-15-B). Following AI, all heifers were placed with a herd bull. Overall pregnancy rate did not differ between puberty groups (Figure 2-15-C). More Typical (57.9%), Early (51.0%), and Start-Stop (45.2%) heifers calved in the first 21 days of the calving season compared to Non-Cycling (20.9%) heifers (Figure 2-15-D).

Start-Stop Subgroups

Between the Start-Stop subgroups (SSS- n=40, SSD- n=34; Table 2-7), there were no differences in adjusted birth weight (Figure 2-16-A), but SSS (246 kg) heifers tended to have greater adjusted weaning weights than SSD (231 kg) heifers (*P=0.057,* Figure 2-

16-B). Prebreeding weight also tended to be greater in SSS (356 kg) than SSD (338 kg) heifers (Figure 2-16-D). There were no significant differences in adjusted yearling weight, ADG, or WDA between the subgroups. Age at weaning differed significantly between subgroups (SSS – 203 d, SSD – 190 d, P=0.015).

At pre-breeding, no differences in antral follicle count or uterine horn diameter were seen between subgroups, but a greater percent of SSS (71.3%) heifers had a reproductive tract score of 5 compared to SSD (24.0%) heifers (Figure 2-17). Average reproductive tract scores were 4.7 in SSS heifers and 4.2 in SSD heifers.

At breeding, a greater percent of SSS (79.5%) heifers showed estrus in response to prostaglandin than SSD (14.7%) heifers (Figure 2-18-A). All heifers that showed estrus were artificially inseminated. Following AI, all heifers were placed with a herd bull. Overall pregnancy rate tended (P=0.097) to be greater in SSS (84.6%) heifers than SSD (67.8%) heifers (Figure 2-18-B). More SSS (61.1%) heifers calved in the first 21 days of the calving season than SSD (20.8%) heifers (Figure 2-18-C).

DISCUSSION

Average puberty date as well as the distribution of puberty dates differed each year in this study. This was impacted by different frequency of sampling each year, with an increase in frequency of sample collection during years 2015-2017. Other factors such as dam supplementation or nutrient restriction also impacted average puberty date, as seen previously in other studies (Corah, Dunn, & Kaltenbach, 1975; R. N. Funston, Martin, Adams, & Larson, 2010). Heifers born in 2013 were gestating during the 2012 drought and had a later average puberty date. Dams of 2016-born heifers received no

supplement during pregnancy, and 2016-born heifers had a later average puberty date. Corresponding to this later puberty date distribution and later average puberty date, a smaller percentage of 2013-born and 2016-born heifers were Early, and more 2013-born and 2016-born heifers were Non-Cycling compared to the overall trend of puberty group distributions.

Start-Stop heifers showed the most variation in progesterone profiles prior to breeding (Figure 2-11). Most Start-Stop heifers had the first occurrence of 1 ng/ml progesterone during the months of November-January (Figures 2-6-E,F, 2-8-C). Thirty-seven of 77 Start-Stop heifers showed 1-3 incidences of 1 ng/ml progesterone but no evidence of cyclicity prior to breeding (See Figure 2-11-A,B and Table 2-7). These were considered Start-Stop-Discontinuous (SSD) heifers. The other 40 of 77 Start-Stop heifers had at least one occurrence of progesterone ≥ 1 ng/ml, discontinued cycling, and then began cycling continuously prior to breeding; these were categorized as Start-Stop-Start heifers (SSS; Figure 2-11-C,D,E,F and Table 2-7).

In this study, 1 ng/ml circulating plasma progesterone was used as a threshold to indicate puberty date. This level has been commonly used in previous research studies when identifying puberty date in beef cattle (Honaramooz et al., 1999; Piccolo et al., 2018). For Early and Typical heifers, this threshold appeared to accurately identify approximate date of puberty attainment as indicated by continuous cyclicity following first occurrence of progesterone ≥ 1 ng/ml. This threshold also appeared to accurately identify those heifers that were not cycling prior to puberty (Non-Cycling heifers). In contrast, the “puberty date” identified for Start-Stop heifers was not the date that marked the initiation of continuous cyclicity. Using the SAS analysis, we were able to identify

the group of Start-Stop heifers, which differed from their contemporaries and deviated from typical puberty attainment. For Start-Stop heifers, the first occurrence of progesterone ≥ 1 ng/ml was a questionable indicator of reproductive competency due to their discontinued cyclicity after initial "puberty date."

Interestingly, there seem to be similarities between the Start-Stop-Start heifers in this study and the precocious puberty heifers in the study discussed by Wehrman et al. (1996). These precocious puberty heifers showed evidence of temporary luteal function at an early age (similar to what is seen in most Start-Stop heifers), resumed anestrus, and then resumed cyclicity again later. This study hypothesized that precocious puberty occurs when the inhibitory effects of estradiol are delayed, and estradiol from follicles on the ovary could induce an LH surge, leading to ovulation and cyclicity without the negative feedback of estradiol which accounts for the transient occurrence of circulating progesterone at an early age (Wehrman et al., 1996). The paper suggested that when estradiol began to have an inhibitory effect on the HPG axis, this would cease cyclicity leading to temporary anestrus until at a normal weight and age signaling would be appropriate for cyclicity to resume (Wehrman et al., 1996). This suggested mechanism could explain one possibility for the irregularity in puberty attainment demonstrated in progesterone profiles of Start-Stop heifers.

Age at puberty attainment has a lasting effect on the lifetime reproductive performance of a cow. Early heifers in this study reached puberty before March 12 each year and continued cycling continuously until breeding, and Typical heifers reached puberty after March 12 each year and continued cycling. As a result, Early heifers had the highest average progesterone concentration during the sampling period, followed by

Typical, Start-Stop, and Non-Cycling heifers. Interestingly, for all samples where P4 ≥ 1 ng/ml, average progesterone concentrations were higher in Typical and Early heifers than in Start-Stop heifers (Table 2-2), suggesting that average progesterone concentrations in Start-Stop heifers during periods of cyclicity were decreased compared to Typical and Early heifers.

Weight, plane of nutrition, and body fat are important regulators of puberty attainment. There were no differences in adjusted birth weights between puberty groups, but Early and Typical had higher adjusted weaning weights than Start-Stop heifers, and average daily gain from birth to weaning was higher in Typical and Early heifers than in Start-Stop heifers (Figure 2-13-E). There were no differences in average daily gain between groups during the period after weaning until yearling weight, but Typical and Early heifers remained significantly heavier than both Start-Stop and Non-Cycling heifers at their adjusted yearling weights (Figure 2-13-C). These data show that the difference in adjusted yearling weights between Typical and Early vs. Start-Stop and Non-Cycling heifers was achieved primarily during the preweaning period, which corresponds to previous research showing that preweaning body weight gain has a larger impact on reducing age at puberty than postweaning body weight gain (Buskirk et al., 1995; Patterson et al., 1992; Perry, 2016). This period of accelerated preweaning rate of gain also coincides with the window from 4-8 months of age when leptin has the largest effect on regulating age at puberty in heifers (Allen et al., 2017; Cardoso et al., 2014).

The crucial time to evaluate reproductive competency is at prebreeding. Most producers aim for a target weight at breeding so that heifers are 55-65% mature body weight (R. N. Funston & Deutscher, 2004; Patterson et al., 1992; Perry, 2016). At

prebreeding, Typical and Early heifers were significantly heavier at prebreeding than Start-Stop heifers most years. [There was a tendency for a significant interaction in group*year for weight at prebreeding (P=0.074) (Figure 2-13-D).] Uterine horn diameter is correlated to reproductive tract development in heifers, and average prebreeding uterine horn diameter was greater in Typical heifers than in Non-Cycling heifers and not different from Early or Start-Stop heifers (Figure 2-14-B). At prebreeding, the highest percent of Early heifers had a reproductive tract score of 5, followed by Typical, Start-Stop, and Non-Cycling heifers.

After prostaglandin injection at breeding, a greater percentage of Typical and Early heifers displayed estrus followed by Start-Stop and Non-Cycling heifers. Because all heifers that showed estrus were artificially inseminated, a greater percent of Typical and Early heifers were artificially inseminated, followed by Start-Stop and Non-Cycling heifers. There were no differences in conception rate to artificial insemination between puberty groups. However, a greater number of Typical, Early, and Start-Stop heifers calved in the first 21 days of the calving season compared to Non-Cycling heifers. Females that calve earlier in the calving season are likely to have increased fertility over their lifespan and last a longer time in the herd (D. M. Larson & Funston, 2009; Perry & Cushman, 2013). In addition, research has shown that heifers born earlier in the calving season make better replacement heifers because they are more likely to reach puberty before breeding, calve earlier as heifers, and produce calves with heavier weaning weights (R. Funston et al., 2012; D. M. Larson & Funston, 2009). Early heifers were the oldest puberty group at weaning, followed by Typical heifers, with Start-Stop heifers not different than Typical heifers, and Non-Cycling the youngest on average at weaning.

Following AI, all heifers were placed with a herd bull. Overall pregnancy rate did not differ between puberty groups, showing that Start-Stop heifers and Non-Cycling heifers were able to become pregnant by a bull even without evidence of previous cyclicity during the sampling period. This could be due to an extended opportunity for attainment of puberty/regular cyclicity within a larger time window or as a result of bull exposure. Bull exposure has been known to influence puberty attainment in heifers (Roberson et al., 1991), and heifers at a certain weight range and between 12 to 14 months are most influenced by bull exposure prior to puberty (Rekwot et al., 2001; Roberson et al., 1991). This age range corresponds to the time of bull exposure for the heifers in this study. Bull exposure impacts the female hypothalamic-pituitary-gonadal axis through pheromones, which are air-borne chemicals released from the urine or feces of the male and taken up by the olfactory system of females. Pheromones can increase GnRH signaling in the hypothalamus, which may induce puberty in heifers or cause re-induction of cyclicity in cows after anestrus periods such as pregnancy (Rekwot et al., 2001).

Previous research has shown that heifers reaching puberty prior to breeding achieve a greater average pregnancy rate (Vraspir et al., 2014). In our study, we found no difference in overall pregnancy rates regardless of pubertal status before breeding. Previous research has also shown that that heifers reaching puberty earlier in the season do not have an advantage to those reaching puberty later in the season (Vraspir et al., 2014), which is also shown by our data with no differences in pregnancy rates between Early and Typical heifers.

When comparing heifers within the Start-Stop puberty group, heifers that began cycling regularly prior to breeding (SSS heifers) tended to have higher adjusted weaning weights than those that did not cycle regularly prior to breeding (SSD heifers), making SSS heifers similar in weight gain to Typical or Early heifers. SSS heifers were also significantly older than SSD heifers, similar to Typical or Early heifers. No differences were seen in calf ADG due to a low experimental n, but SSS heifers tended to be heavier than SSD heifers at prebreeding, and significantly more SSS heifers had a reproductive tract score of 5 than SSD heifers at prebreeding. Following the pattern of Typical or Early heifers, a greater percent of SSS heifers responded to prostaglandin by showing estrus, were AIed, and calved within the first 21 days of the calving period. Although all heifers categorized as Start-Stop reached a threshold of 1 ng/ml in the time between weaning and breeding, those that had evidence of continuous cyclicity especially in the month prior to breeding followed the patterns of Typical and Early heifers and are more likely to become cows with greater lifetime fertility and reproductive longevity.

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Table 2-1. Number of blood samples collected and number of heifers in each puberty group by year

	Year						
	2012	2013	2014	2015	2016	2017	**2012-2017**
Number of samples/heifer	14	13	22	30	31	31	
Number of heifers per year (N)	68	99	105	106	119	114	611
Puberty Group, N (%)							
Early	24 (35.3)	6 (6.1)	38 (36.2)	30 (28.3)	8 (6.7)	37 (32.4)	143 (23.4)
Typical	25 (36.7)	44 (44.4)	31 (29.5)	48 (45.3)	77 (64.7)	54 (47.4)	279 (45.7)
Start-Stop	15 (22.1)	22 (22.2)	28 (26.7)	13 (12.3)	7 (5.9)	6 (5.3)	91 (14.9)
Non-Cycling	4 (5.9)	27 (27.3)	8 (7.6)	15 (14.1)	27 (22.7)	17 (14.9)	98 (16.0)

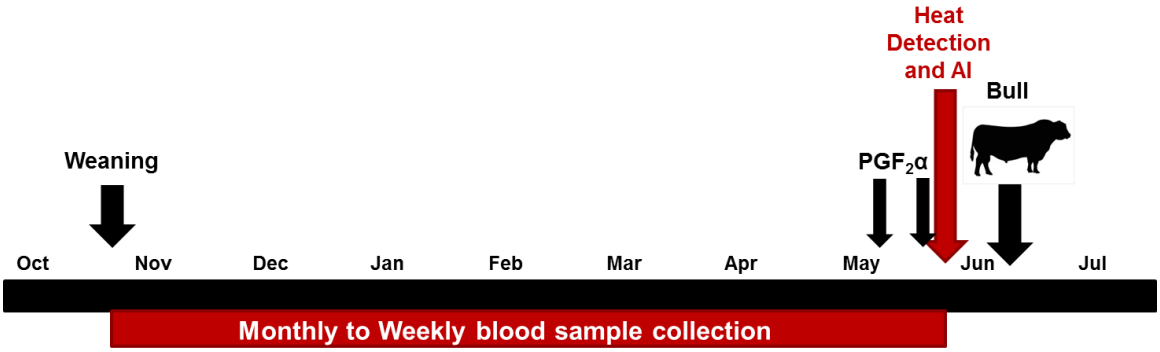


Figure 2-1. Experimental Timeline

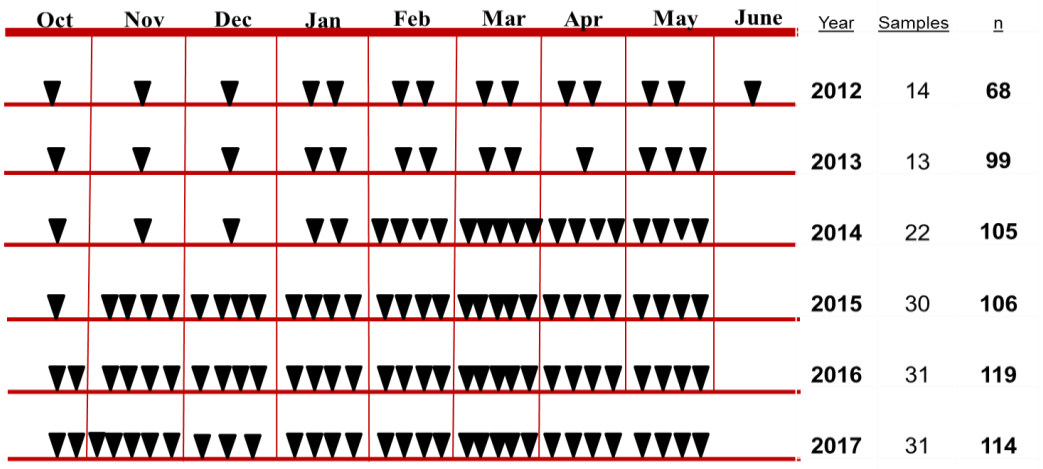


Figure 2-2. Blood samples were collected monthly to weekly from weaning to breeding across 6 years

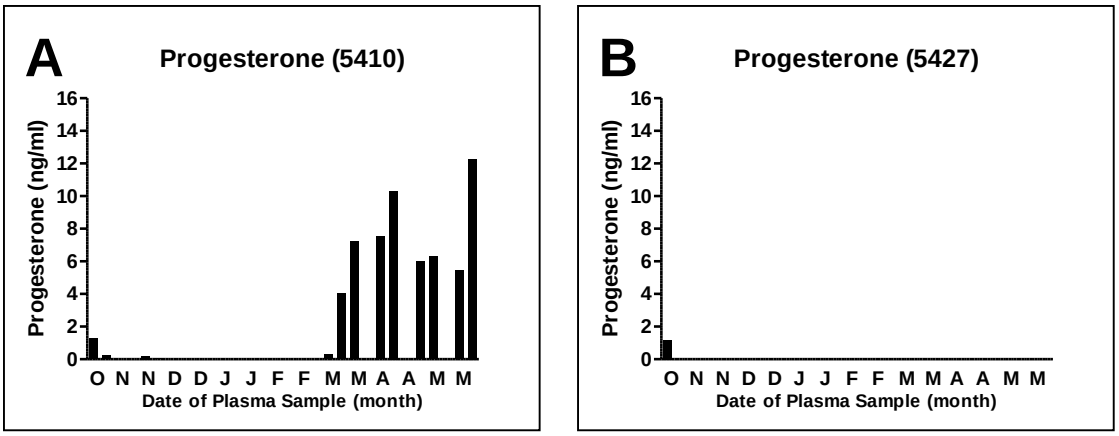


Figure 2-3. Examples of progesterone profiles of heifers with P4 ≥ 1 ng/ml occurring on date of weaning in October (A, B)

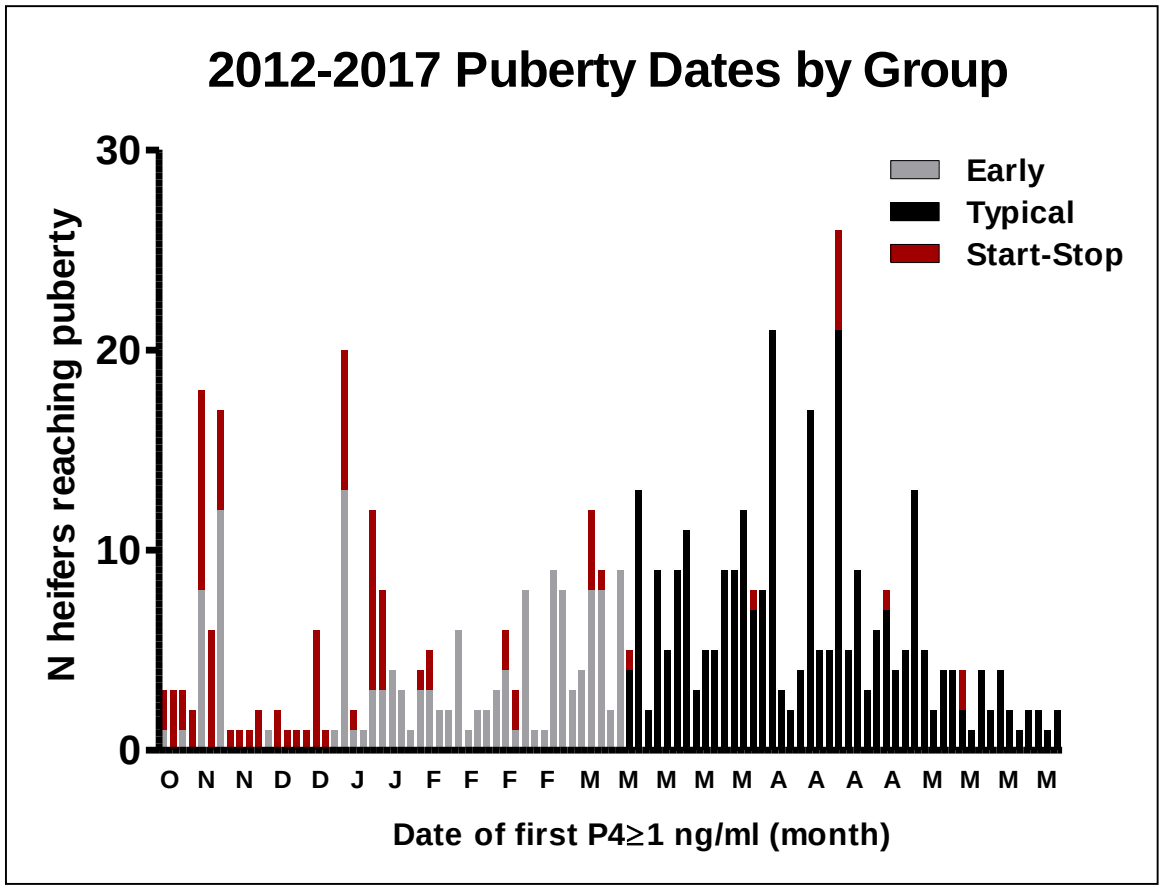


Figure 2-4. Distribution of puberty date (date of first P4 ≥ 1 ng/ml) for all 6 years (2012-2017 born heifers) by puberty group

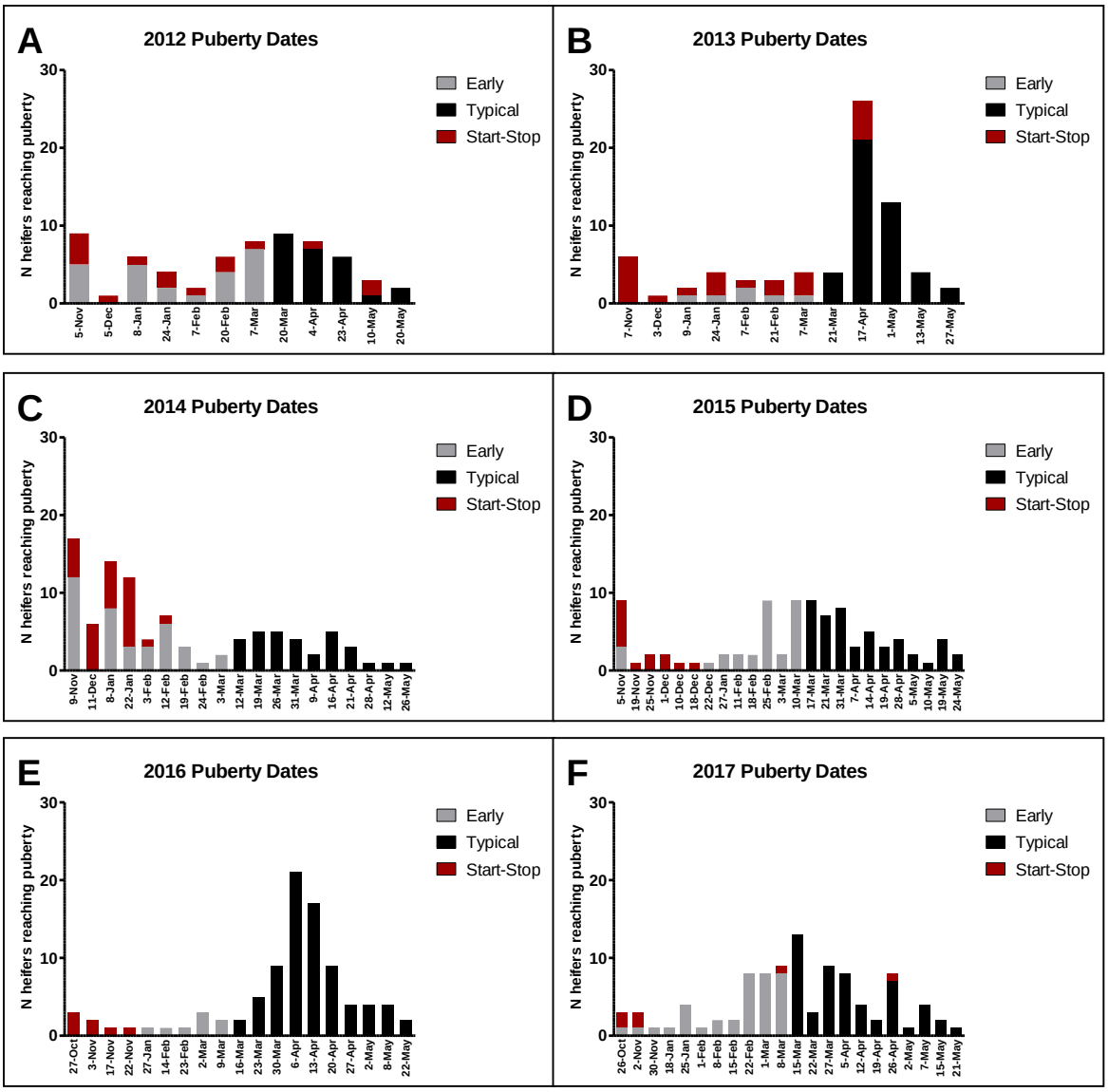


Figure 2-5. Distribution of puberty date (date of first P4 ≥ 1 ng/ml) in 2012-born heifers (A), 2013-born heifers (B), 2014-born heifers (C), 2015-born heifers (D), 2016-born heifers (E), and 2017-born heifers (F)

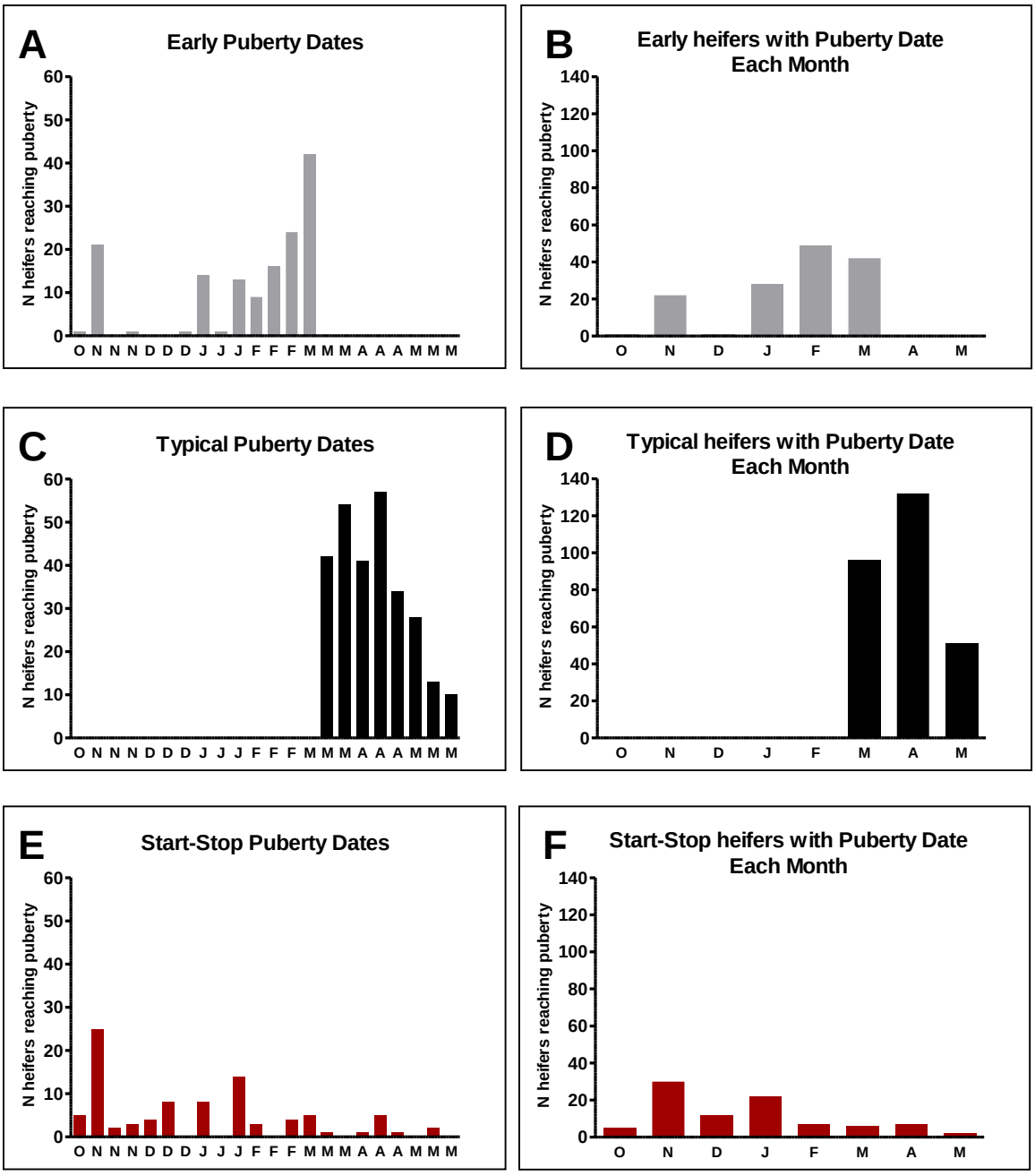


Figure 2-6. Puberty date (date of first P4 ≥ 1 ng/ml) distribution by month for Early (A, B), Typical (C, D), and Start-Stop (E, F) heifers

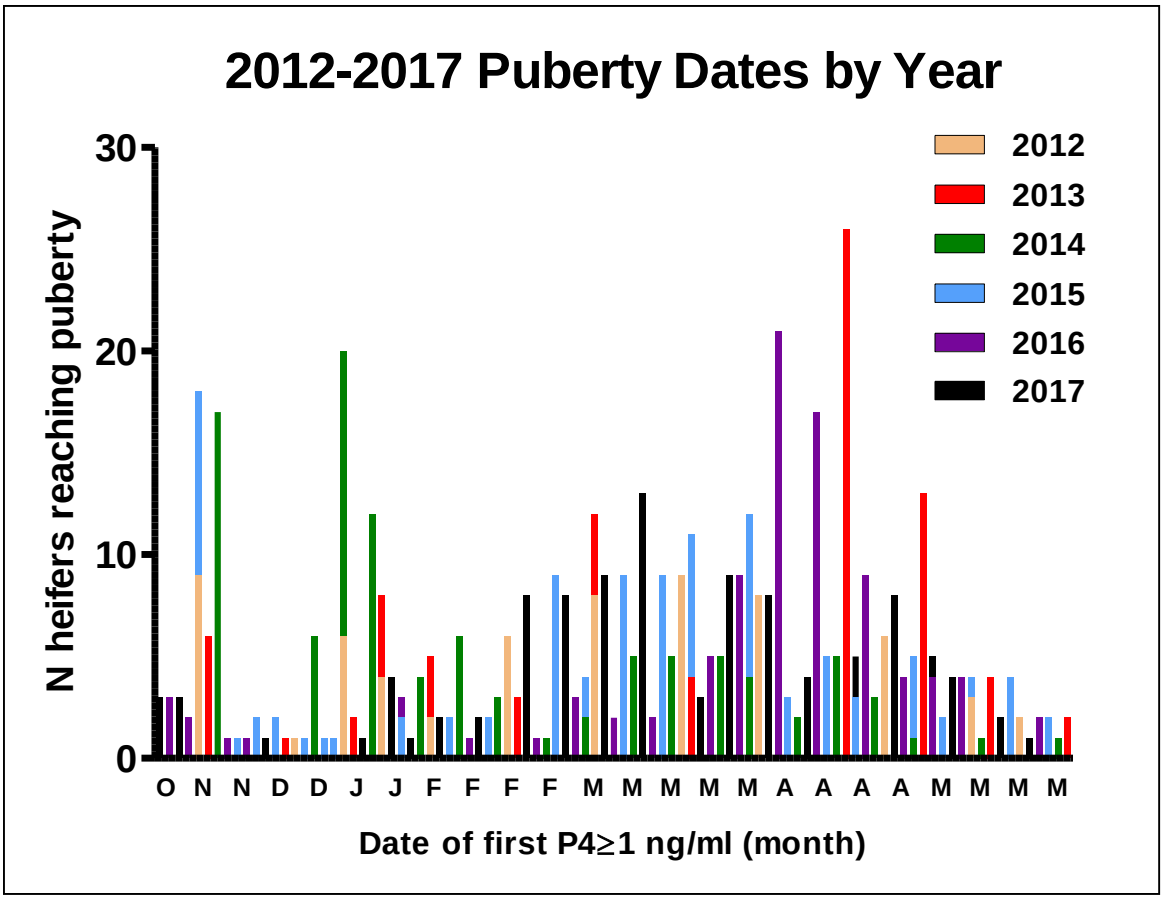
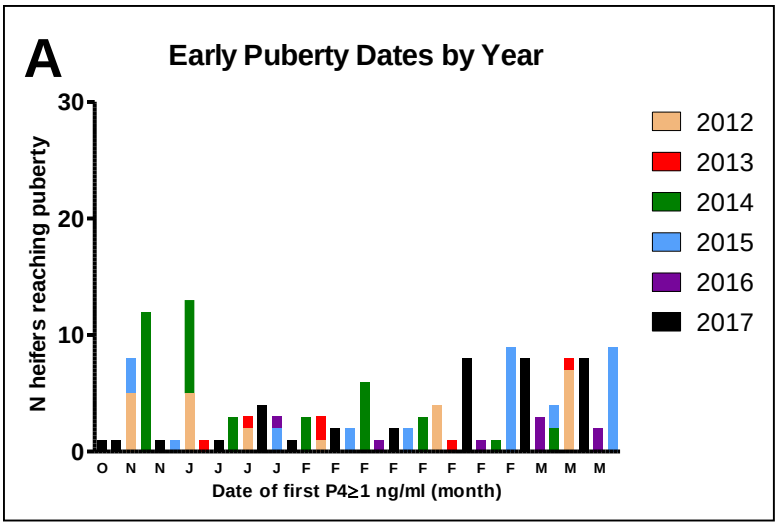
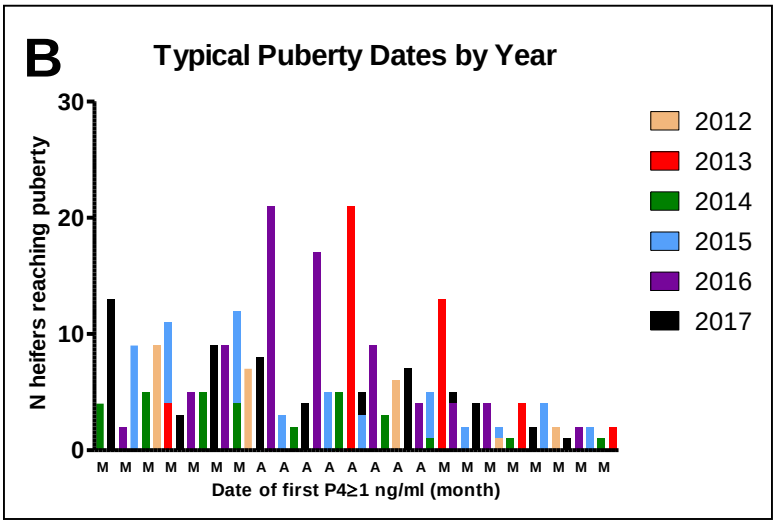


Figure 2-7. Distribution of puberty date (date of first P4 ≥ 1 ng/ml) for all 6 years (2012-2017 born heifers) by year





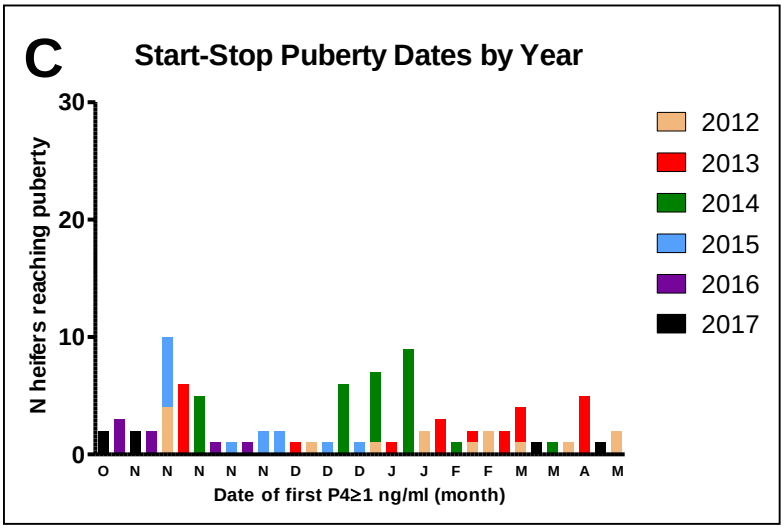


Figure 2-8. Distribution of puberty date (date of first P4 ≥ 1 ng/ml) for all 6 years (2012-2017 born heifers) for Early (A), Typical (B), and Start-Stop heifers (C)

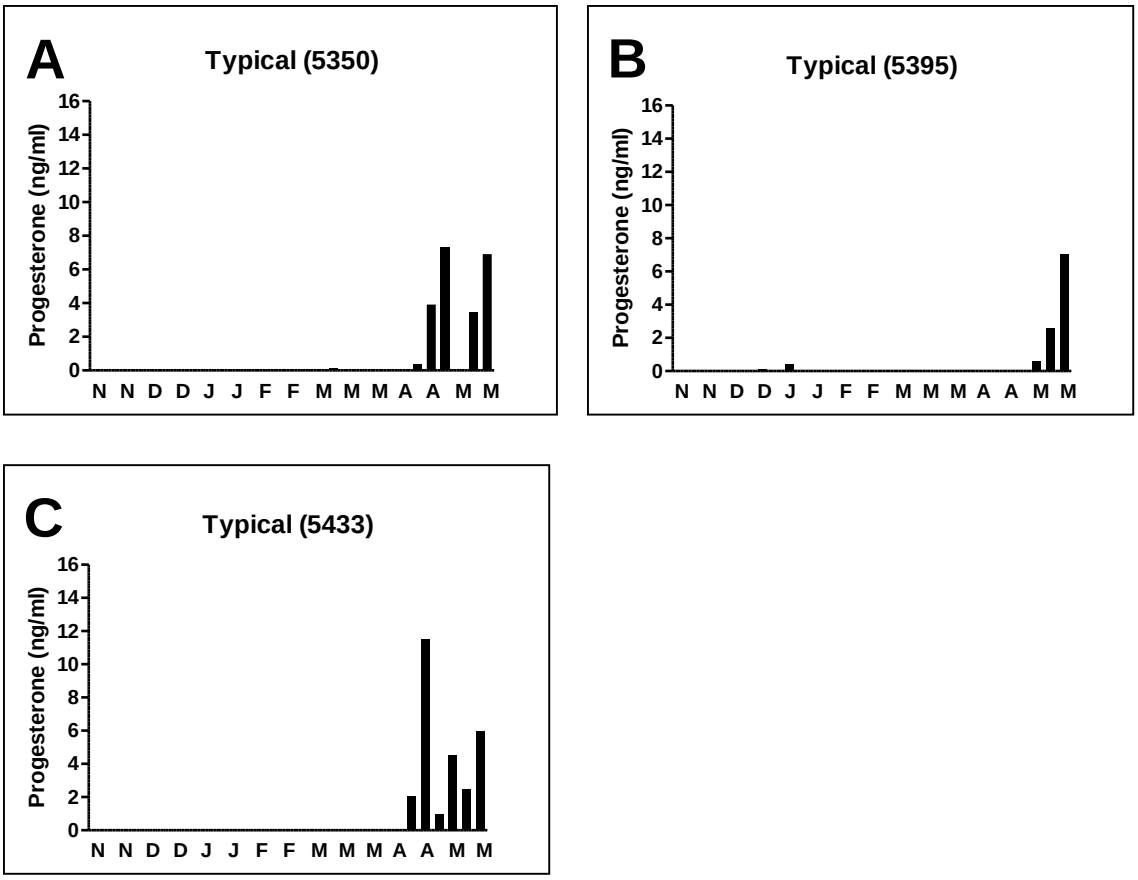


Figure 2-9. Progesterone profiles of representative Typical heifers (A, B, C)

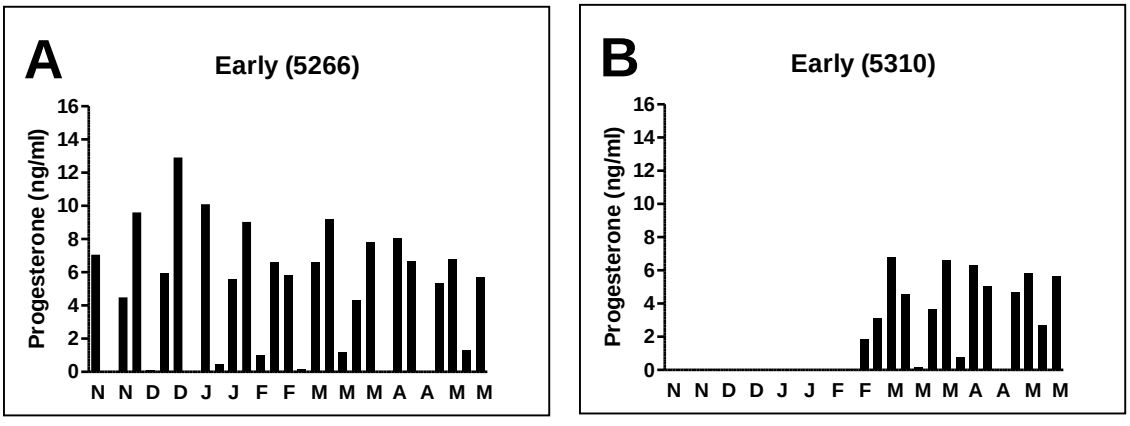


Figure 2-10. Progesterone profiles of representative Early heifers (A, B)

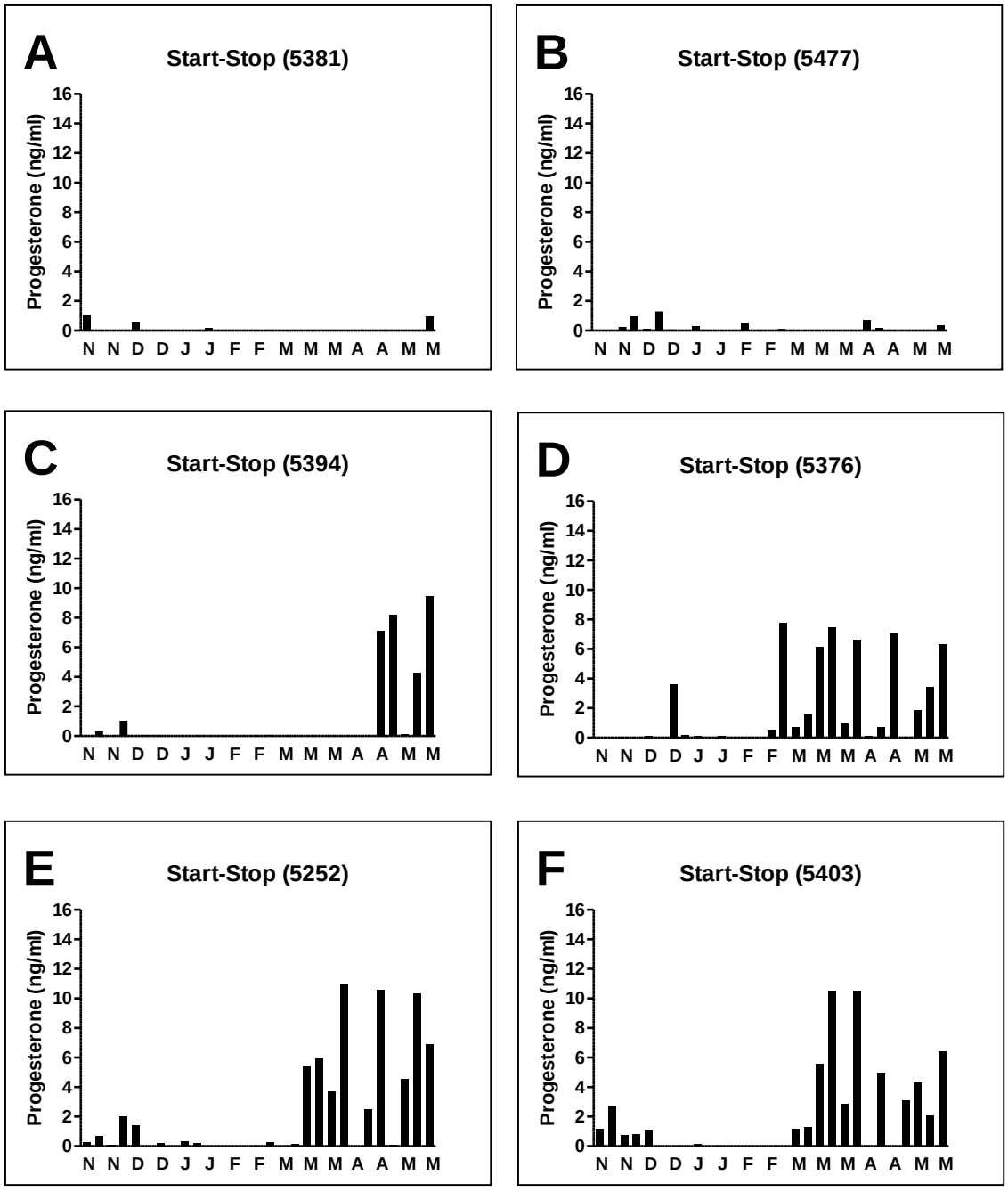


Figure 2-11. Progesterone profiles of representative Start-Stop heifers (A, B, C, D, E, F)

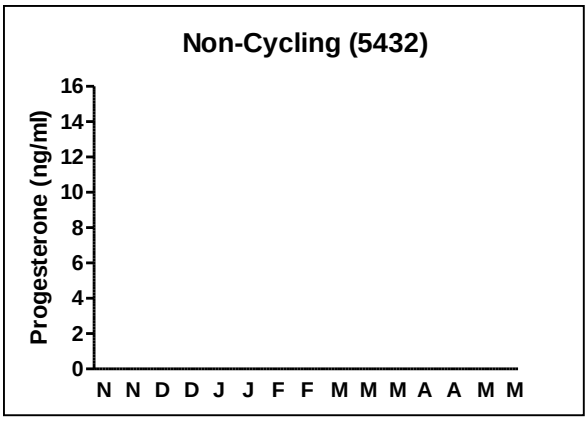


Figure 2-12. Progesterone profile of a representative Non-Cycling heifer

Table 2-2. Puberty Group Characteristics

	Puberty Group				*P*-value		
	Typical	**Early**	**Start-Stop**	**Non-Cycling**	Group x year	Group	Year
Total n from 2012-2017	279	143	91	98			
Average age at P4>1ng/ml, d	378 ± 2[a]	317 ± 4[b]	265 ± 4[c]	N/A	<0.0001	<0.0001	<0.0001
Avg age at Weaning, d	203 ± 1[b]	208 ± 2[a]	200 ± 2[bc]	198 ± 2[c]	0.0005	0.0014	<0.0001
Percent of samples where P4≥1 ng/ml after initial P4≥1 ng/ml	82.7 ± 2.4[a]	74.2 ± 4.7[a]	27.6 ± 5.6[b]	N/A	0.999	<0.0001	0.9858
Avg P4 of all samples	1.42 ± 0.05[b]	2.58 ± 0.09[a]	1.05 ± 0.09[c]	0.06 ± 0.10[d]	<0.0001	<0.0001	<0.0001
Avg P4 of initial sample where P4≥1 ng/ml	3.90 ± 0.13[a]	3.31 ± 0.22[b]	1.88 ± 0.25[c]	N/A	0.373	<0.0001	0.0055
Avg P4 after initial P4≥1 ng/ml	4.47 ± 0.10[a]	4.10 ± 0.14[b]	1.24 ± 0.14[c]	N/A	0.22	<0.0001	<0.0001
Avg P4 of all samples where P4≥1 ng/ml	5.41 ± 0.11[a]	5.45 ± 0.16[a]	4.09 ± 0.23[b]	N/A	0.0179	<0.0001	<0.0001

[a,b,c,d] Means in a row with different superscripts are different (*P*≤0.05).

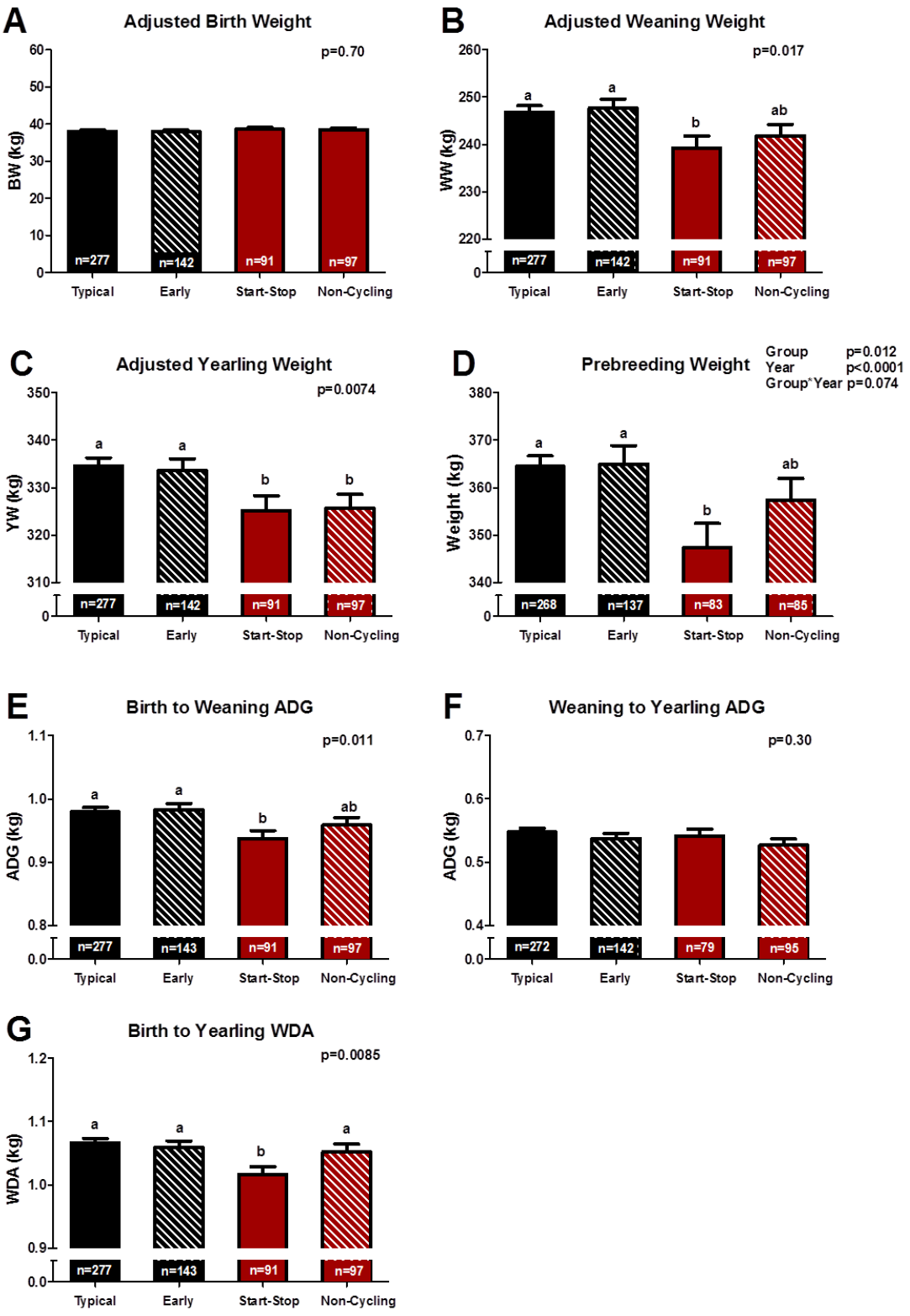


Figure 2-13. Effect of puberty group on adjusted birth weight (A), adjusted weaning weight (B), adjusted weaning weight (C), pre-breeding weight (D), calf average daily gain (E), yearling average daily gain (F), and weight per day of age (G)

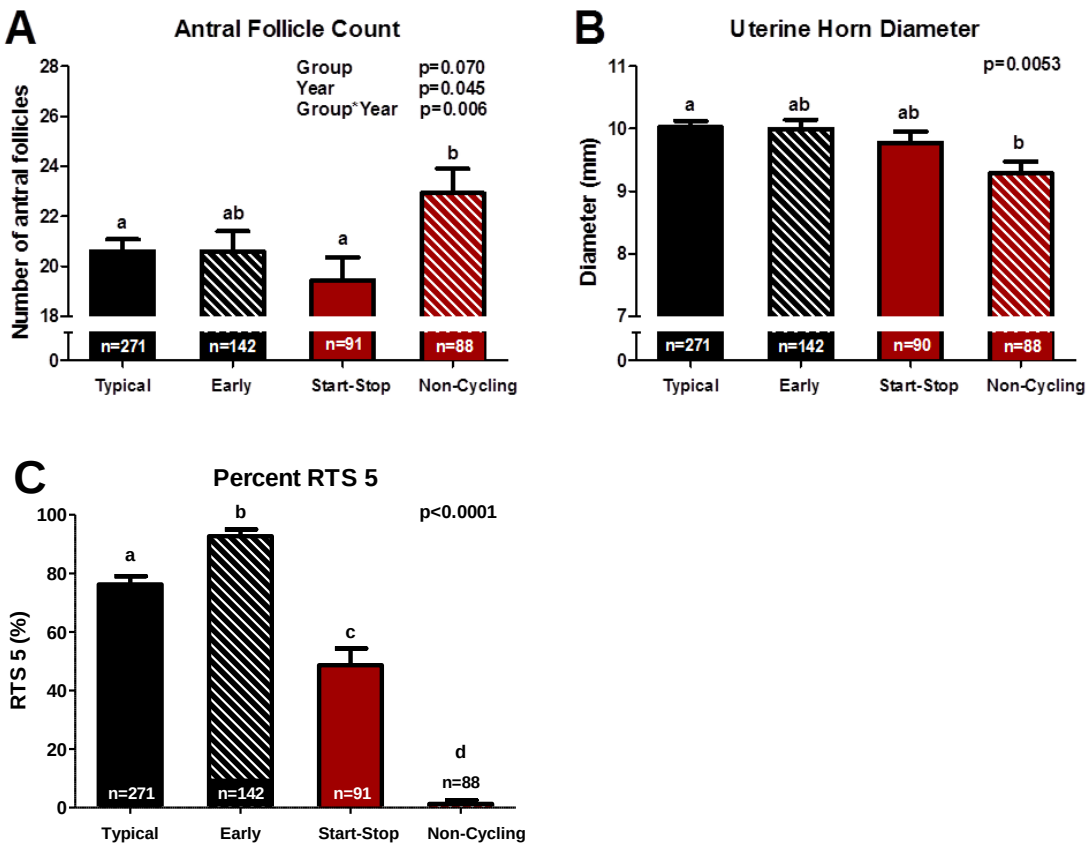


Figure 2-14. Effect of puberty group on total antral follicle count (A), uterine horn diameter (B), and percent reproductive tract score 5 (C)

Table 2-3. Average Reproductive Tract Score by group

	Puberty Group			
	Typical	**Early**	**Start-Stop**	**Non-Cycling**
Average RTS	4.71 ± 0.03	4.92 ± 0.04	4.46 ± 0.05	3.98 ± 0.05

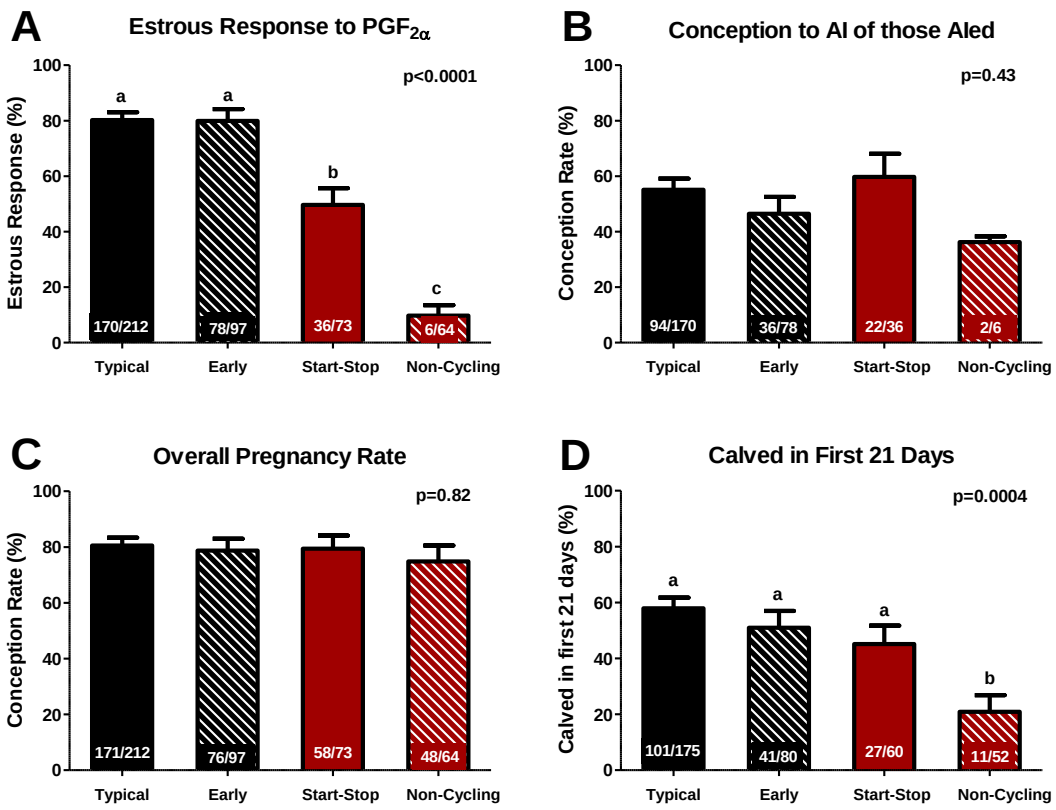


Figure 2-15. Percent showed estrus after PGF (A), conception to AI of those AIed (B), overall pregnancy rate (C), and percent calved in the first 21 days of the calving season (D)

Table 2-4. Average age and dates at puberty (first occurrence of P4≥1 ng/ml) and weaning by year

	Year						*P*-value		
	2012	**2013**	**2014**	**2015**	**2016**	**2017**	Year	Group	Group x year
N	68	99	105	106	119	114			
Avg age at first P4>1ng/ml, d	331 ± 4[a]	334 ± 5[a]	298 ± 3[c]	316 ± 4[b]	318 ± 6[ab]	324 ± 5[ab]	<0.0001	<0.0001	<0.0001
Avg age at Weaning, d	216 ± 3[a]	195 ± 2[c]	189 ± 2[d]	199 ± 2[c]	207 ± 2[b]	207 ± 2[b]	<0.0001	0.0014	0.0005
Avg date of first P4>1ng/ml*	2/21/13	3/22/14	2/1/15	3/2/16	3/27/17	3/11/18			
Avg date of Weaning	10/16/12	10/15/13	10/14/14	10/13/15	10/18/16	10/19/17			

[a,b,c,d] Means in a row with different superscripts are different (*P*≤0.05).

*Does not include Non-Cycling heifers

Table 2-5. Weight characteristics of heifers by puberty group

	Puberty Group				*P*-value
	Typical	**Early**	**Start-Stop**	**Non-Cycling**	
N	279	143	91	98	
BW[1], kg	34.03 ± 0.30	33.83 ± 0.41	34.26 ± 0.51	34.62 ± 0.50	0.64
Adj BW[2], kg	38.07 ± 0.30	37.93 ± 0.41	38.56 ± 0.51	38.49 ± 0.50	0.7
WW[3], kg	232.0 ± 1.7[a]	237.5 ± 2.3[a]	218.6 ± 2.9[b]	222.3 ± 2.8[b]	<0.0001
Adj WW[4], kg	246.8 ± 1.4[a]	247.6 ± 1.9[a]	239.2 ± 2.6[b]	241.9 ± 2.4[ab]	0.017
YW[5], kg	326.7 ± 2.0[a]	330.3 ± 2.8[a]	311.5 ± 3.4[b]	312.9 ± 3.3[b]	<0.0001
Adj YW[6], kg	334.6 ± 1.7[a]	333.7 ± 2.4[a]	325.2 ± 3.1[b]	325.7 ± 2.4[b]	0.0074
Calf ADG[7], kg	0.98 ± 0.01[a]	0.98 ± 0.01[a]	0.94 ± 0.01[b]	0.96 ± 0.01[ab]	0.011
Yrlg ADG[8], kg	0.55 ± 0.01	0.54 ± 0.01	0.54 ± 0.01	0.53 ± 0.01	0.3
WDA[9], kg	1.07 ± 0.01[a]	1.06 ± 0.01[a]	1.02 ± 0.01[b]	1.05 ± 0.01[a]	0.0085
PHAW[10], %	68.7 ± 2.9	68.5 ± 4.1	64.5 ± 5.4	66.5 ± 5.3	0.91
PMBW[11], %	58.6 ± 3.1	58.5 ± 4.4	55.1 ± 5.6	56.8 ± 5.5	0.95

[a,b,c,d] Means in a row with different superscripts are different (*P*≤0.05).

[1]BW = birth weight

[2]Adj BW = adjusted birth weight

[3]WW = weaning weight

[4]Adj WW = adjusted weaning weight

[5]YW = yearling weight

[6]Adj YW = adjusted yearling weight

[7]Calf ADG = average daily gain from birth to weaning

[8]Yrlg ADG = average daily gain from weaning to yearling weight

[9]WDA = weight per day of age from birth to yearling weight

[10]PHAW = percent herd average weight at prebreeding

[11]PMBW = percent herd mature body weight at prebreeding

Table 2-6. Weight characteristics of heifers by year

	Year						*P*-value
	2012	**2013**	**2014**	**2015**	**2016**	**2017**	
	68	99	105	106	119	114	
BW[1], kg	33.46 ± 0.60[a]	33.99 ± 0.49[a]	35.70 ± 0.48[b]	35.55 ± 0.48[b]	33.37 ± 0.47[a]	33.04 ± 0.47[a]	<0.0001
Adj BW[2], kg	37.16 ±0.60[a]	37.94 ± 0.49[a]	39.51 ± 0.48[b]	39.91 ± 0.48[b]	37.60 ± 0.47[a]	37.46 ± 0.47[a]	<0.0001
WW[3], kg	231.0 ± 3.3[a]	217.3 ± 2.8[b]	226.9 ± 2.7[a]	232.8 ± 2.7[a]	231.6 ± 2.7[a]	226.0 ± 2.7[a]	0.0007
Adj WW[4], kg	232.0 ± 2.8[a]	240.1 ± 2.4[b]	253.8 ± 2.4[c]	252.5 ± 2.3[c]	242.2 ± 2.2[b]	242.5 ± 2.2[b]	<0.0001
YW[5], kg	328.1 ± 4.0[ab]	303.2 ± 3.3[d]	319.1 ± 3.2[bc]	325.5 ± 3.2[ab]	331.9 ± 3.2[a]	314.3 ± 3.2[c]	<0.0001
Adj YW[6], kg	338.8 ± 3.4[a]	314.9 ± 2.9[b]	334.1 ± 2.9[a]	336.0 ± 2.7[a]	333.4 ± 2.7[a]	321.7 ± 2.8[b]	<0.0001
Calf ADG[7], kg	0.92 ± 0.01[a]	0.95 ± 0.01[a]	1.02 ± 0.01[b]	0.99 ± 0.01[b]	0.96 ± 0.01[a]	0.95 ± 0.01[a]	<0.0001
Yrlg ADG[8], kg	0.67 ± 0.01[a]	0.47 ± 0.01[d]	0.50 ± 0.01[c]	0.52 ± 0.01[c]	0.57 ± 0.01[b]	0.50 ± 0.01[c]	<0.0001
WDA[9], kg	0.91 ± 0.01[b]	1.12 ± 0.01[c]	0.86 ± 0.01[a]	1.17 ± 0.01[d]	1.11 ± 0.01[c]	1.11 ± 0.01[c]	<0.0001
PHAW[10], %	65.2 ± 6.0	66.6 ± 4.9	71.6 ± 4.5	65.7 ± 5.1	68.3 ± 4.9	64.8 ± 5.1	0.92
PMBW[11], %	55.7 ± 6.2	56.8 ± 5.1	61.1 ± 4.9	56.1 ± 5.3	58.3 ± 5.2	55.4 ± 5.2	0.97

[a,b,c,d] Means in a row with different superscripts are different (*P*≤0.05).

[1]BW = birth weight

[2]Adj BW = adjusted birth weight

[3]WW = weaning weight

[4]Adj WW = adjusted weaning weight

[5]YW = yearling weight

[6]Adj YW = adjusted yearling weight

[7]Calf ADG = average daily gain from birth to weaning

[8]Yrlg ADG = average daily gain from weaning to yearling weight

[9]WDA = weight per day of age from birth to yearling weight

[10]PHAW = percent herd average weight at prebreeding

[11]PMBW = percent herd mature body weight at prebreeding

Table 2-7. Number of heifers in each Start-Stop subgroup by year

	Year							
	2012	2013	2014	2015	2016	**2012-2016***	2017	2012-2017
Start-Stop heifers, N	15	22	22	10	5	**74**	3	77
Subgroup, N								
SSS	9	8	13	8	2	**40**	0	80
SSD	6	14	9	2	3	**34**	3	71

*Because all 2017-born Start-Stop heifers were SSD, only data from 2012-2016 born heifers were used in comparisons between SSS and SSD heifers.

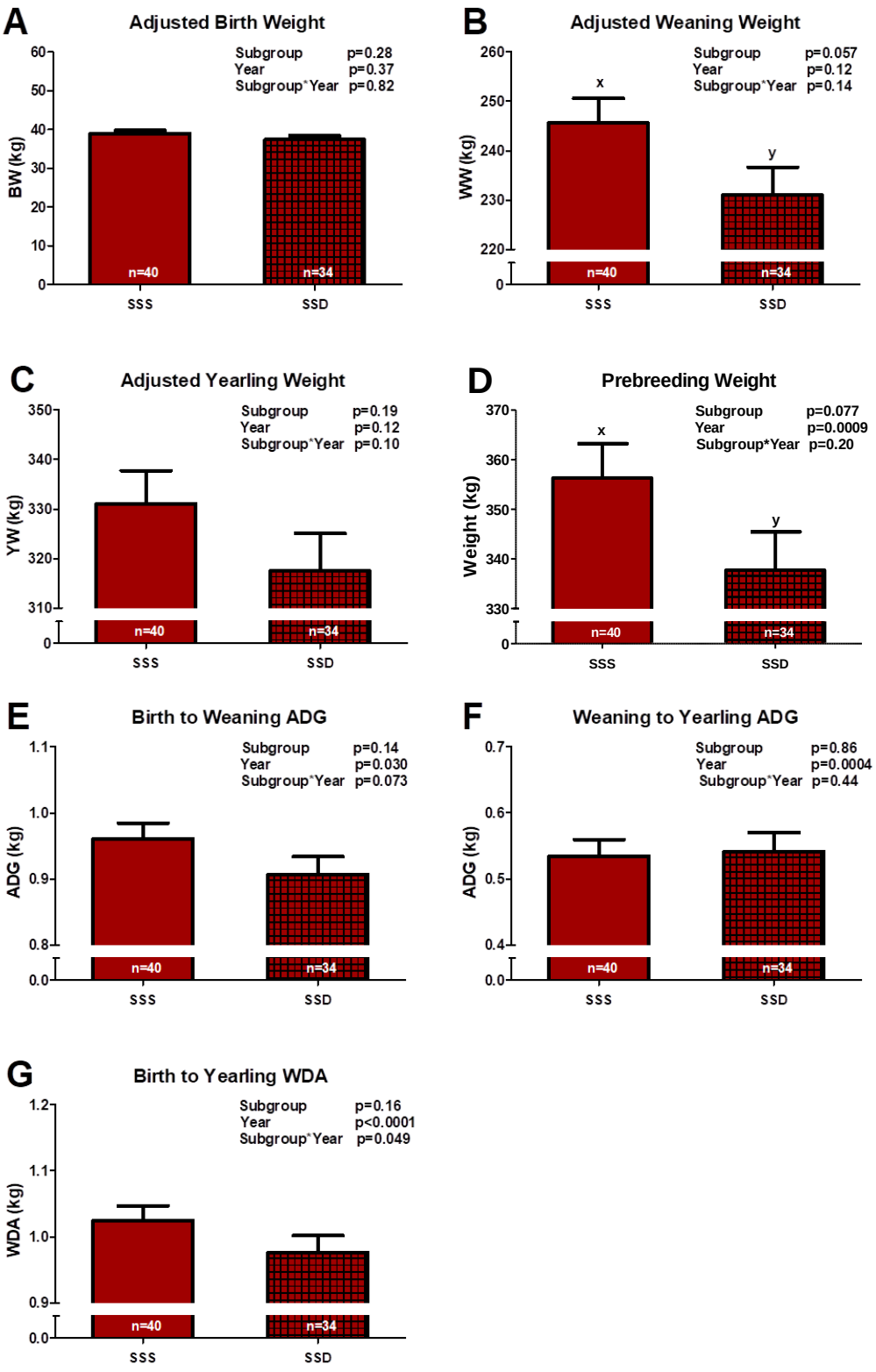


Figure 2-16. Effect of subgroup on adjusted birth weight (A), adjusted weaning weight (B), adjusted weaning weight (C), pre-breeding weight (D), calf average daily gain (E), yearling average daily gain (F), and weight per day of age (G)

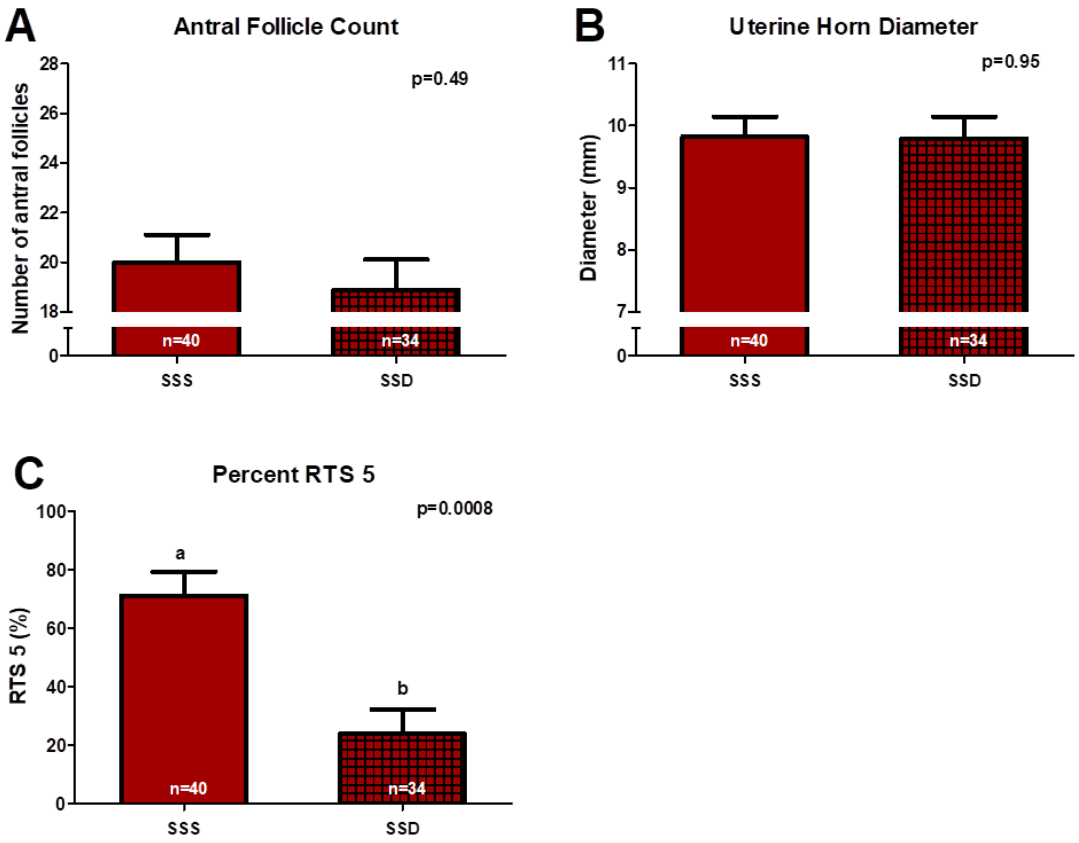


Figure 2-17. Effect of subgroup on total antral follicle count (A), uterine horn diameter (B), and percent reproductive tract score 5 (C)

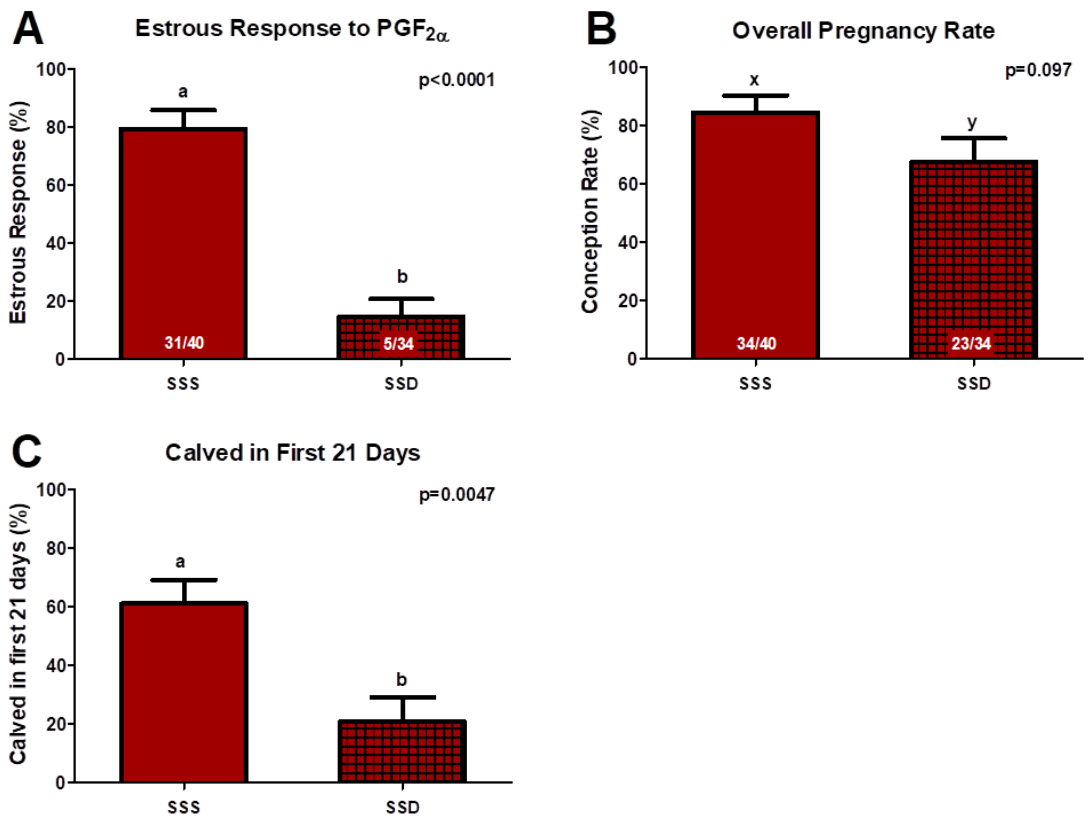


Figure 2-18. Percent showed estrus after PGF (A), overall pregnancy rate (B), and percent calved in the first 21 days of the calving season (C)

Chapter 3 : Start-Stop and Non-Cycling Heifers have Similar Characteristics as High A4 Cows

ABSTRACT

Several heifers from each puberty group were intensively studied during years 2014-2017 during the peripubertal period with every other day blood sample collections and ovarian ultrasound. Average progesterone concentrations during the intensive studies were lower in Non-Cycling than in Typical, Early, and Start-Stop heifers. Sex Hormone Binding Globulin (SHBG) appeared to be decreased in Start-Stop and Non-Cycling compared to Typical and Early heifers. A serial blood collection was performed during years 2015-2017. No differences in androstenedione during the serial blood collection were observed between puberty groups. Non-Cycling heifers had increased mean FSH during the serial blood collection, and Early heifers tended to have the greatest LH pulse amplitude compared to other puberty groups. During the intensive study, differences in estrus and ovulation were observed between heifers of different puberty groups. At the conclusion of the intensive study, heifers were stimulated with FSH and ovariectomized. No differences in ovarian characteristics were observed at ovariectomy between puberty groups. Interestingly, ovarian cortex from Non-Cycling secreted more androstenedione in culture media compared to Typical and Early heifers. Mature cows previously classified as Start-Stop or Non-Cycling also secreted increased androstenedione in culture media compared to Typical cows. Since ovarian cortex from High A4 cows secretes increased androstenedione in culture media compared to control cows, these findings lead us to predict that Start-Stop and Non-Cycling heifers are more likely to become High A4 with reduced fertility.

INTRODUCTION

As described in the introduction to Chapter 2, the UNL physiology herd has a population of High A4 cows that are characterized by secretion of excess androstenedione (A4) in follicular fluid. These High A4 cows are less fertile, have irregular cycles and are often anovulatory. Ovarian cortex from High A4 cows secretes 43 fold more A4 into culture media than ovarian cortex from control cows (Abedal-Majed, 2017).

Chapter 2 described the weekly blood sample collection from weaning to breeding, weight data, and conception rates used to investigate differences in heifer puberty attainment. To further examine puberty attainment mechanisms in these heifers and identify females that may be predisposed to become High A4 cows, several heifers from each puberty group in the 2014, 2015, 2016, and 2017-born heifers were more intensively evaluated at approximately 13-16 months of age during the peripubertal period. These heifers were evaluated every other day by blood sample collection and ovarian ultrasound, and at the completion of the intensive study each year heifers were ovariectomized.

MATERIALS AND METHODS

Animals

A total of 46 beef heifers across four years born in 2014-2017 were used in this experiment. (Table 3-1 shows the number of heifers in the experiment each year.) All heifers were from the physiology herd at the University of Nebraska-Lincoln and kept at

the Eastern Nebraska Research and Extension Center (ENREC). The physiology herd is made up of approximately 250 Red Angus composite cows. About 50% of cows were AI sired by Red Angus bulls, and about 50% were sired by Red Angus x Simmental composite herd bulls.

Dams of heifers were fed supplement during gestation for all years during this experiment except dams of heifers born in 2016. Heifers were born in the spring each year around March and grazed on pasture with dams until weaning in late October each year at approximately 7 months of age. At weaning, heifers were separated from dams and male calves and retained as replacement heifers on pasture at ENREC.

Heifers underwent monthly to weekly blood sampling with contemporaries as described in Chapter 2. Heifers were assigned puberty groups based on their weekly plasma progesterone concentrations from weaning until they were brought to Lincoln for the intensive study. The SAS program used to determine heifer puberty group is described in Chapter 2.

In late April or early May each year, prior to synchronization and breeding for the rest of the heifers, the subset of yearling heifers used in this intensive study was removed from the ENREC and brought to the Animal Science Complex at the University of Nebraska-Lincoln, where they were kept for 1-2 months for the duration of this experiment. Ovariectomies were performed on heifers at the conclusion of the experiment. The University of Nebraska-Lincoln Institutional Animal Care and Use Committee approved all procedures and facilities used in this experiment.

Estrus Synchronization

Heifers were brought to the Animal Science Complex in late April or early May each year. At the beginning of the intensive study, estrous synchronization was achieved by transvaginal ovum pick-up (OPU) followed by 2 injections of prostaglandin $F_{2\alpha}$. After an epidural injection of 3 ml 2% lidocaine was given, OPU was performed to ablate all follicles ≥7 mm in diameter using an ALOKA SSD-5000V ultrasound machine and a 5MHz transvaginal convex human transducer (ALOKA UST-981-5) adapted for beef cattle with an attached stainless steel needle guide and 18-gauge needle. A RocketCRAFT^{TM} oocyte aspiration pump (Rocket Medical, plc) was used to create suction on the other end of the needle. Following OPU, 2 injections of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) were administered to each heifer 24 hours apart (5 ml; 25mg/ml; Lutalyse, Pfizer Animal Health, New York, NY) to regress any corpora lutea present on the ovaries.

Ovarian Ultrasound and Estrus Detection

Following estrous synchronization which was performed at the beginning of the intensive study each year, transrectal ovarian ultrasound was performed approximately every other day during the intensive study for 1-2 estrous cycles (ALOKA SSD-5000V ultrasound machine, ALOKA UST-5541-7.5 probe). Follicles were identified on the ultrasound screen (Fricke & Lamb, 2002), and length and width of follicles ≥3 mm were measured on the ultrasound screen and recorded. Follicle diameter was calculated as the average of length and width. Locations of follicles were mapped at each ultrasound on a diagram of the ovaries. Using average diameter as well as location on the ovary, follicular waves were tracked. Ovulation was detected by ultrasound of dominant follicles followed by emergence of a developing corpus luteum.

Estrus detection patches (Estrotect™ Heat Detector, Rockway, Inc.) were applied to each heifer at the start of the intensive study and replaced after activation. A heifer was considered to have exhibited estrus if the estrous detection patch was activated or if visual observation of standing to be mounted was recorded.

Blood Sample Collection

Approximately every other day during the sampling period (Figure 3-1), blood samples were collected using coccygeal venipuncture into glass vacutainer blood collection tubes (Becton, Dickinson and Company, Franklin Lakes, NJ) containing 12 mg EDTA and placed immediately on ice. Within several hours, blood samples were centrifuged at approximately 700 g and 4 degrees Celsius for 30 minutes. Immediately following, plasma was collected and stored in polypropylene tubes (Globe Scientific, Inc., Paramus, NJ) at -20 degrees Celsius.

Serial Blood Collections

A serial blood collection was performed on several heifers in the intensive study each year for heifers born in 2015-2017 (Figure 3-1; Table 3-2). Heifers were halter-trained and acquainted with standing in stanchions in the Animal Science Building for approximately 10 days prior to the serial blood collection. One day prior to the serial blood collection, heifers underwent jugular cannulation. Either the right or left side of the neck was shaved and sterilized, and a 10-gauge needle was placed into the jugular vein. A sterile indwelling cannula made of Tygon® flexible plastic tubing (Saint-Gobain Performance Plastics) was inserted approximately 9 inches into the vein through the needle. The needle was removed, and the line was cleaned with 3.5% sodium citrate

solution, capped, and secured to the neck with a toggle suture repair button (JorVet™, Jorgensen Labs, Colorado), hip tag cement, and elastic adhesive tape.

The morning after jugular cannulation, heifers were haltered and placed in stanchions in the Animal Science Complex. Plasma samples were collected hourly, and serum samples were collected every 15 minutes. Blood was drawn through the indwelling cannulas at 15 minute intervals using a syringe and deposited into tubes. Following each sample collection, indwelling cannulas were flushed with a solution of 3.5% sodium citrate. Samples were collected over a period of 8 hours for the 2015-2016 born heifers and 9 hours for the 2017-born heifers.

Serum samples were collected every 15 minutes and were deposited into polypropylene tubes (Globe Scientific, Inc., Paramus, NJ) and stored at 4 degrees Celsius for 24 hours. Following, serum samples were centrifuged at 2800 g for 30 minutes, poured into polypropylene tubes, and stored at -20 degrees Celsius.

Plasma samples were collected hourly during the serial blood collection. Blood samples were collected using the indwelling cannula described above and deposited into a glass vacutainer blood collection tube containing 12 mg EDTA (Becton, Dickinson and Company, Franklin Lakes, NJ) and placed on ice. These blood samples were centrifuged at approximately 700 g and 4 degrees Celsius for 30 minutes, and plasma was collected and stored in polypropylene tubes (Globe Scientific, Inc., Paramus, NJ) at -20 degrees Celsius.

Progesterone Measurements

Progesterone (P4) concentrations for plasma samples collected every other day during the intensive studies for 2014-2017 born heifers and every hour during the serial

blood collections for the 2015-2017 born heifers were detected using radioimmunoassay (RIA). Each sample was run in duplicate, and the average value for the duplicates was used as the mean progesterone value for that sample. Progesterone values were recorded in ng/ml. Samples found to have a coefficient of variation (CV) greater than 15% were re-run in another assay.

Progesterone concentrations were detected using the ImmuChem^{TM} Coated Tube Progesterone ^{125}I RIA kit from ICN Pharmaceuticals, Inc., Costa Mesa, CA. Intra-assay coefficient of variation averaged 2.3%, and inter-assay coefficient of variation was 14.0%.

Androstenedione Measurements

Androstenedione (A4) concentrations were measured in the hourly plasma samples collected during the 2015-2017 born serial blood collections. Plasma A4 concentrations were detected using the ImmuChem^{TM} Double Antibody Androstenedione ^{125}I RIA kit from MP Biomedicals, LLC, Solon, OH. Steroids were extracted from the plasma samples using the protocol outlined in the kit and then reconstituted with steroid diluent. The radioimmunoassay was then performed on this extracted steroid phase according to kit protocol. Intra-assay coefficient of variation averaged 12.6%, and inter-assay coefficient of variation averaged 11.3%.

Sex Hormone Binding Globulin Measurements

Weekly and every-other-day plasma samples from the 2014- and several of the 2015-born heifers in the intensive study were analyzed for concentrations of sex hormone binding globulin (SHBG). SHBG concentrations were detected using a Bovine Sex Hormone Binding Globulin (SHBG) ELISA kit from MyBioSource, Inc. (San Diego,

CA). The average intra-assay coefficient of variation was 8.2%, and the inter-assay coefficient of variation was 12.0%.

LH and FSH Measurements

Concentrations of LH in serum (250 μl) were measured by Endolytics (Fort Collins, CO) in duplicate by radioimmunoassay, using NIDDK-oLH-26 as the standard (Vizcarra, Wettemann, Braden, Turzillo, & Nett, 1997). The average intra-assay CV was 12.9%, and the inter-assay CV was 11.2%.

Concentrations of FSH in serum were measured by Endolytics (Fort Collins, CO) in duplicate by radioimmunoassay (Vizcarra et al., 1997). This procedure is similar to that described by Bolt and Rollins (1983) and Crowe et al. (1997), using anti-ovine FSH serum (NIDDK-oFSH-RP-2) for standards. The average intra-assay CV was 13.5%, and the inter-assay CV was 11.5%.

Identification and analysis of LH pulses were performed using the approach of Goodman and Karsch (1980). Three criteria were used to determine a LH pulse: 1) a peak must occur within two samples of the previous nadir; 2) the amplitude must be greater than the sensitivity of the LH assay; and 3) the LH concentration at the peak must exceed the 95% confidence limits (based on overall assay variability) of the concentration at both the preceding and subsequent nadir. LH samples were analyzed for pulse frequency and amplitude by Dr. Stan Hileman and Michelle Bedenbaugh at the University of West Virginia.

Weights and Body Condition Scores

Initial weights and body condition scores (BCS) were recorded for each heifer at the beginning of the intensive study. Final weights and BCS were recorded at the completion of the intensive study on the day of ovariectomy.

Ovariectomies

At the conclusion of the study, ovaries were stimulated to induce follicle development and then collected using ovariectomy. To promote development of multiple follicles, transvaginal ovum pick-up (OPU) was performed to ablate all ovarian follicles ≥ 7mm in diameter 96 hours prior to ovariectomy using the procedure previously described. Following OPU, heifers were stimulated with 7 injections of follicle stimulating hormone (FSH) at 12 hour intervals (140mg/cow; 20mg/ml; Folltropin-V, Bioniche Animal Health USA, Inc., Athens, GA). 2 injections of $PGF_{2\alpha}$ were administered to each heifer 48 and 24 hours before ovariectomy (5 ml, 25mg/ml; Lutalyse, Pfizer Animal Health, New York, NY).

Bilateral ovariectomy was performed via right flank laparotomy (Youngquist, Garverick, & Keisler, 1995). Ovaries were weighed and measured immediately after removal. Average ovarian weight and area for each heifer was calculated as an average of the measurements of her right and left ovaries. Surface antral follicle count was recorded for each ovary, and total surface antral follicle count was calculated as the total number of antral follicles on the surfaces of both the right and left ovaries. All ovarian follicles ≥ 7 mm in diameter were measured (length + width/2 = follicle diameter). Numbers of surface antral follicles ≥ 7mm and < 7mm were recorded for each ovary, and total surface

antral follicles ≥ 7mm and < 7mm for each heifer were reported as a total between the right and left ovaries.

Androstenedione in Ovarian Cortex Culture Media

Ovarian cortical cultures were conducted on 2016-born heifers. On the day of ovariectomy, four 0.5-1mm^3 pieces of ovarian cortex were collected from each ovary. These ovarian cortex pieces were placed into culture well inserts (4 pieces/well; Millicell-CM, 0.4 µm pore size; Millipore Corporation, MA) in the wells of 24-well Costar culture plates (Corning, Inc., Corning, NY). Each well contained 300 µl serum-free Waymouth's medium MB 752/1 (Sigma; MO) supplemented with 75 µg/ml penicillin, 50 µg/ml streptomycin sulfate (Sigma; MO) and ITS (1000 mg insulin, 550 mg transferrin, 6.7 ng selenious acid) (Corning; Manassas, VA), and 1.25 mg BSA (Sigma; MO). Cortex pieces were cultured at 37 degrees Celsius in a humidified incubator with 5% CO_2. Culture medium was collected and replaced with fresh medium every day for seven days (Abedal-Majed, 2017).

The androstenedione concentration of cortex culture media was determined each day of culture for the 2015-born heifers in the intensive trial. A4 concentration was measured using the ImmuChem^{TM} Double Antibody Androstenedione ^{125}I RIA kit from MP Biomedicals, LLC, Solon, OH. Culture media was diluted 1:5 by using 100 µl of culture media and 400 µl of steroid diluent. These diluted samples were used in the assay steps according to the kit protocol. Sample concentration values were corrected for dilution factor (Abedal-Majed, 2017).

Statistical Analyses

Data analyses were conducted using SAS v9.4. All data are presented as mean ± standard error of the mean (SEM).

Average progesterone concentrations during the intensive study were analyzed using the MIXED procedure of SAS with year and group included in the model statements. There was no significant group*year interaction, so this term was dropped from the model. Animal was included in the class statement with random animal within group used to account for repeated measures.

Ovarian characteristics (ovary weight, ovary area, surface antral follicle count, and number of follicles ≥ or < 7mm) were analyzed using the MIXED procedure of SAS.

Average androstenedione and progesterone concentrations during the hourly samples of the serial blood collections were analyzed using the MIXED procedure of SAS with hour and hour*group included in the model statements. Animal was included in the class statement with random animal within group used to account for repeated measures. Because years 2015 and 2016 had hours 0-8 of plasma sample collection and year 2017 had hours 0-9, only hours 0-8 for all three years were used in this analysis.

Mean FSH, mean LH, and FSH:LH ratio for 2016-born heifers were analyzed using the MIXED procedure of SAS as a repeated measures analysis with compound symmetry structure. Time and time*group were included in the model statement, and the repeated measure was time with animal as the subject. LH pulses and LH pulse amplitude were analyzed using the GLIMMIX procedure of SAS.

Androstenedione produced into ovarian cortex culture media over seven days of culture was analyzed using the MIXED procedure of SAS with group, day of culture, and

group*day interaction included in the model statement. Day of culture was the repeated measure with animal as the subject.

RESULTS

Plasma Progesterone Concentrations

During the time that heifers were in Lincoln for the intensive studies and blood samples were collected approximately every other day, average plasma progesterone concentrations across the time period were significantly lower in Non-Cycling (0.99 ng/ml) compared to Typical (2.06 ng/ml), Early (2.32 ng/ml), and Start-Stop (2.18 ng/ml) heifers (Figure 3-2).

Serial Blood Collection Gonadotropins and Steroid Hormones

2015-born heifers serial blood collection serum samples were analyzed for FSH and LH concentration, FSH:LH ratio, number of LH pulses, and LH pulse amplitude (Table 3-3a). There were no group*time interactions for mean LH, mean FSH, or FSH:LH. Non-Cycling (541 ng/ml) heifers had significantly increased mean FSH concentrations in serum during the serial blood collection compared to Typical (292 ng/ml), Early (347 ng/ml), or Start-Stop (309 ng/ml) heifers ($p=0.023$). There were no differences in LH concentrations or FSH:LH. No difference in number of LH pulses was found between groups. LH pulse amplitude tended to be higher in Early (1678 ng/ml) and heifers compared to Non-Cycling (0 ng/ml) and Typical (308 ng/ml) heifers but was not different than Start-Stop (402 ng/ml) heifers ($p=0.078$; Table 3-3a).

Plasma samples collected hourly from 2015-2017-born heifers during the serial blood collections were analyzed for mean progesterone and mean androstenedione

concentrations (Table 3-3b). There was a group*hour interaction for average progesterone during the serial blood collections and no effect of group on average progesterone concentrations. Average androstenedione concentrations during the serial blood collection did not differ by group. Average androstenedione for all heifers with P4>1 ng/ml during the serial blood collection and for all heifers with P4<1ng/ml during the serial blood collection did not differ by group.

Sex Hormone Binding Globulin

Sex hormone binding globulin (SHBG) concentrations in 2014-2015-born heifers were analyzed in monthly/weekly plasma samples from weaning until the intensive studies and in daily plasma samples collected during the intensive studies. SHBG seemed to be decreased in Start-Stop and Non-Cycling heifers compared to Typical and Early heifers. SHBG in Typical and Early heifers appears to be increased before puberty and decrease around puberty initiation (Figure 3-3).

Occurrence of Estrus and Ovulation During the Intensive Studies

During all four years of intensive studies, estrus and ovulation were documented. All heifers categorized as Typical showed estrus during the intensive studies, while only 10 of 12 Early, 10 of 14 Start-Stop, and 9 of 10 Non-Cycling showed estrus during the intensive studies (Table 3-4). 9 of 10 Typical, 11 of 12 Early, 9 of 14 Start-Stop, and 7 of 10 Non-Cycling heifers ovulated during the intensive studies. 9 of 10 Typical, 9 of 12 Early, 9 of 14 Start-Stop, and 7 of 10 Non-Cycling heifers showed estrus followed by ovulation. 2 Early, 1 Start-Stop, and 1 Non-Cycling heifers ovulated without showing estrus, and 1 Typical, 1 Early, 1 Start-Stop, and 1 Non-Cycling heifer showed estrus

without ovulating during the intensive studies. 1 Early, 4 Start-Stop, and 1 Non-Cycling heifer had a complete lack of either estrus or ovulation during the entire intensive study.

Ovarian Characteristics at Ovariectomy

Ovarian characteristics at ovariectomy for 2014-2017-born intensive study heifers stimulated with FSH were compared (Table 3-5). There were no differences in ovary weight, ovary area, average surface antral follicle count, antral follicles < 7 mm, or antral follicles > 7 mm.

Androstenedione in Ovarian Cortex Culture Media

Androstenedione (A4) secreted into ovarian cortex culture media during 7 days of culture from 2016-born heifers was analyzed. Ovarian cortex from Non-Cycling (4.2 ng/ml) heifers produced significantly more A4 than Typical (0.062 ng/ml) or Early (0.091 ng/ml) heifers, but was not different from ovarian cortex from Start-Stop (1.8 ng/ml) heifers (Figure 3-4).

Changes in Cyclicity During the Intensive Studies

During the intensive study, changes in cyclicity occurred that did not always correspond with the puberty groups assigned prior to the intensive study (Table 3-6). 7 of 10 Non-Cycling heifers began cycling in Lincoln, 2 of 14 Start-Stop heifers began cycling regularly, and 1 of 10 Typical heifers and 1 of 12 Early heifers stopped cycling.

DISCUSSION

Several heifers each year born in 2014-2017 were brought to Lincoln during the peripubertal period for a more intensive study of cyclicity, follicular waves, hormones,

and sex hormone binding globulin, and ovarian cortex culture. Heifers were classified into one of four puberty groups using the SAS analysis described in Chapter 2 by their plasma progesterone profiles from the time they were weaned until they were brought to Lincoln for the intensive studies.

Several heifers demonstrated changes in cyclicity during the intensive study. 3 Non-Cycling heifers began cycling in Lincoln, and 2 Start-Stop heifers initiated regular cyclicity. Whether this initiation of cyclicity was induced from the stress of transportation, relocation, and increased handling, or as a natural attainment of puberty, is unknown. Heifers were transported for approximately 1 hour from ENREC to Lincoln and relocated into the Animal Science Complex for the duration of the intensive studies. In pigs, transportation and relocation have been shown to induce puberty attainment in gilts with delayed puberty and anestrous sows (Dalin, Nyberg, & Eliasson, 1988; Rojanasthien & Einarsson, 1988). Both the gilts and sows in these experiments had increased LH and estradiol following 1 hour of transportation and relocation (Dalin et al., 1988; Rojanasthien & Einarsson, 1988). Whether a similar phenomenon may also occur in heifers is unknown; however, it is known that stress and the adrenal system interacts with the hypothalamic-pituitary-gonadal axis and can impact signaling that affects reproduction. Another possible explanation for the initiation of cyclicity in these heifers during the intensive study is that they simply naturally reached puberty during the time they were in Lincoln.

Also during the intensive studies, 1 Typical heifer and 1 Early heifer stopped cycling. The Typical heifer had an extremely excitable temperament. The interruption in cyclicity in these two heifers was possibly a response to stress. Previous research has

shown that heifers with excitable temperaments have increased plasma cortisol concentrations and disrupted puberty attainment compared to animals with more moderate temperaments (Cooke et al., 2018).

Follicular waves, estrus detection, ovulation, and CL formation were documented for heifers during the intensive studies. Of the heifers in the intensive studies, 9 of 10 Typical, 9 of 12 Early, 9 of 14 Start-Stop, and 7 of 10 Non-Cycling heifers showed regular estrus followed by ovulation during the time they were in Lincoln (Table 3-4). 1 Early, 4 Start-Stop, and 1 Non-Cycling heifer had a complete lack of either estrus or ovulation during the entire intensive study. One heifer in each puberty group showed estrus without ovulating during the intensive studies. Estrus without ovulation commonly occurs in non-pubertal heifers and is documented to occur at a frequency of 17-63% (Nelson, Short, & Phelps, 1985; Rutter & Randel, 1986). During the intensive studies, 2 Early, 1 Start-Stop, and 1 Non-Cycling heifer ovulated without showing estrus. Ovulation without estrus can occur commonly in heifers prior to establishment of regular cyclicity, especially in prepubertal heifers 7-10 days before pubertal ovulation (Atkins et al., 2013). Our lab has demonstrated that High A4 cows show both behavioral estrus without ovulation and ovulation without behavioral estrus (Spuri Gomes et al., 2017).

Average progesterone concentrations during the intensive studies were lower in Non-Cycling than in Typical, Early, and Start-Stop heifers (Figure 3-2). Non-Cycling heifers also had significantly increased mean FSH concentrations during the serial blood collection compared to Typical, Early, or Start-Stop heifers ($p=0.023$). LH pulse amplitude tended to be higher in Early compared to Non-Cycling heifers ($p=0.078$). LH pulse amplitude increases as heifers mature toward puberty.

Although limited data prevented analysis for statistical significance, SHBG in Typical and Early heifers appears to be increased before puberty and decreased around puberty initiation. In girls, SHBG is greater before puberty and declines around the time of puberty (Pinkney et al., 2014). This would allow for an increase in bioavailable sex steroids. Along with that, increased adiposity and decreased circulating leptin were correlated with a prepubertal decrease in SHBG in girls (Pinkney et al., 2014). SHBG appeared to be decreased in Start-Stop and Non-Cycling compared to Typical and Early heifers. In humans, low SHBG concentrations are seen in metabolic disorders as well in Polycystic Ovary Syndrome (PCOS) where it is a likely contributor to excess androgens (Deswal et al., 2018).

Ovary size, ovary weight, average surface antral follicle count, antral follicles < 7 mm, or antral follicles > 7 mm did not differ between groups at ovariectomy. Prior to ovariectomy, heifers were stimulated with FSH. Unpublished data in our lab suggests that FSH stimulation masks ovarian dysfunction, which may account for the lack of differences seen in ovarian characteristics between puberty groups.

Ovarian cortex cultured over 7 days was analyzed for androstenedione secretion into culture media. Ovarian cortex from Non-Cycling heifers produced significantly more androstenedione than Typical or Early heifers and was not significantly different than Start-Stop heifers. Unpublished data from our lab also shows that ovarian cortex from cows previously classified as Start-Stop or Non-Cycling secretes increased androstenedione in culture media compared to Typical cows. This is a key finding since ovarian cortex from High A4 cows secretes increased androstenedione in culture media

compared to control cows, and leads us to hypothesize that Start-Stop and Non-Cycling heifers are more likely to become High A4 cows with reduced fertility.

The intensive studies provided a closer look at heifers during the peripubertal window and allowed us to add more substantial evidence to the reproductive tract score and heifer breeding data from Chapter 2 suggesting that Start-Stop and Non-Cycling heifers are more likely to become High A4 cows. In these intensive studies, we showed that Non-Cycling heifers had reduced progesterone concentrations and increased mean FSH concentrations. Sex hormone binding globulin appeared to be reduced in Start-Stop and Non-Cycling compared to Typical and Early heifers. A greater numerical percentage of Start-Stop and Non-Cycling heifers demonstrated irregularities in or complete lack of estrus or ovulation compared to Typical and Early heifers during the intensive studies, resembling previous documentation of estrus without ovulation and ovulation without estrus in High A4 cows. Most significantly, ovarian cortex from Non-Cycling heifers produced significantly more androstenedione than Typical or Early heifers in culture media, and ovarian cortex from cows previously classified as Start-Stop or Non-Cycling produced increased androstenedione in culture media compared to ovarian cortex from Typical cows. Because ovarian cortex from High A4 cows secretes increased androstenedione in culture media compared to control cows, this finding together with the other endocrine and fertility parameters leads us to hypothesize that Start-Stop and Non-Cycling heifers are more likely to become High A4 cows with reduced fertility.

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Table 3-1. Intensive study: number of samples collected and number of heifers in each puberty group by year

	Year				
	2014	2015	2016	2017	**2014-2017**
Number of samples/heifer	27	27	17	16	
Number of heifers per year (N)	10	12	12	12	46
Puberty Group, N					
Early	4	3	2	3	12
Typical	0	3	4	3	10
Start-Stop	6	3	2	3	14
Non-Cycling	0	3	4	3	10

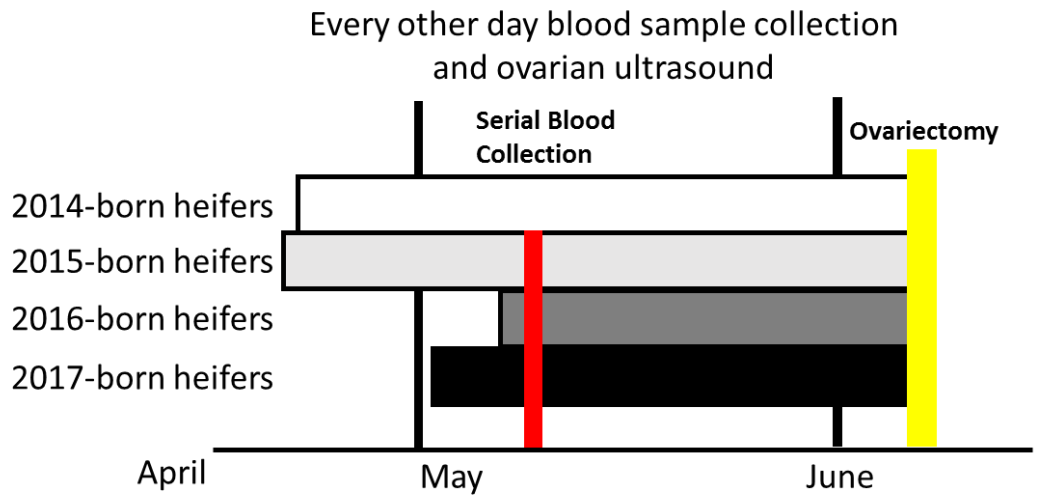

Figure 3-1. Experimental Timeline for the intensive studies

Table 3-2. Serial blood collections: number of samples collected and number of heifers in each puberty group by year

	Year			
	2015	2016	2017	**2015-2017**
Number of samples/heifer	9	9	10	
Number of heifers per year (N)	8	10	12	20
Puberty Group, N				
Early	2	1	3	6
Typical	2	3	3	8
Start-Stop	2	2	3	7
Non-Cycling	2	4	3	9

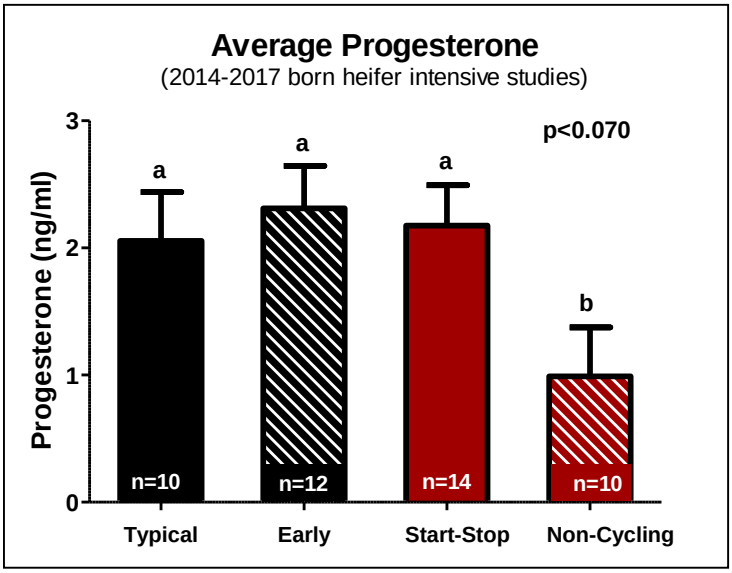

Figure 3-2. Average plasma progesterone during the intensive study for the 2014-2017 born heifers

Table 3-4. Serial blood collection mean FSH and LH, FSH:LH, LH pulses, LH pulse amplitude, average P4, and average A4

(a) 2015-born heifers	Puberty Group				*P*-value		
	Typical	**Early**	**Start-Stop**	**Non-Cycling**	Group	Time	Group*Time
N	2	2	2	2			
Mean FSH (ng/ml)	291.8 ± 37.9[a]	346.7 ± 37.9[a]	309.3 ± 37.9[a]	541.1 ± 37.9[b]	0.023	0.93	0.78
Mean LH (ng/ml)	153.3 ± 79.7	251.8 ± 79.7	94.3 ± 79.7	126.9 ± 79.7	0.59	0.43	0.68
FSH:LH	40.9 ± 1100	307 ± 1100	1305 ± 1100	2120 ± 1100	0.57	0.033	0.56
LH pulses	1.0 ± 0.6	1.5 ± 0.6	1.5 ± 0.6	0 ± 0.6	0.38	N/A	N/A
LH pulse amplitude (ng/ml)	308 ± 333[x]	1678 ± 333[y]	402 ± 333[xy]	0 ± 333[x]	0.078	N/A	N/A

(b) 2015-2017 born heifers							
	Typical	**Early**	**Start-Stop**	**Non-Cycling**	Group	Hour	Group*Hour
N	6	8	7	9			
Avg P4 (ng/ml)	2.06 ± 0.94	2.18 ± 1.10	3.16 ± 1.10	0.89 ± 0.88	0.46	0.0008	0.036
Avg A4 (ng/ml)	107 ± 14	95.3 ± 16	100 ± 15	124 ± 14	0.55	0.71	0.93
Avg A4 for all P4>1 ng/ml	109 ± 11	92.0 ± 9.3	82.4 ± 7.8	91.5 ± 12	0.35	0.56	0.016
Avg A4 for all P4<1 ng/ml	106 ± 20	98.9 ± 29	122 ± 28	132 ± 20	0.74	0.72	0.74

[a,b,c,d] Means in a row with different superscripts are different (*P*≤0.05).

[x,y] Means in a row with different superscripts tend to be different (*P≤0.10).*

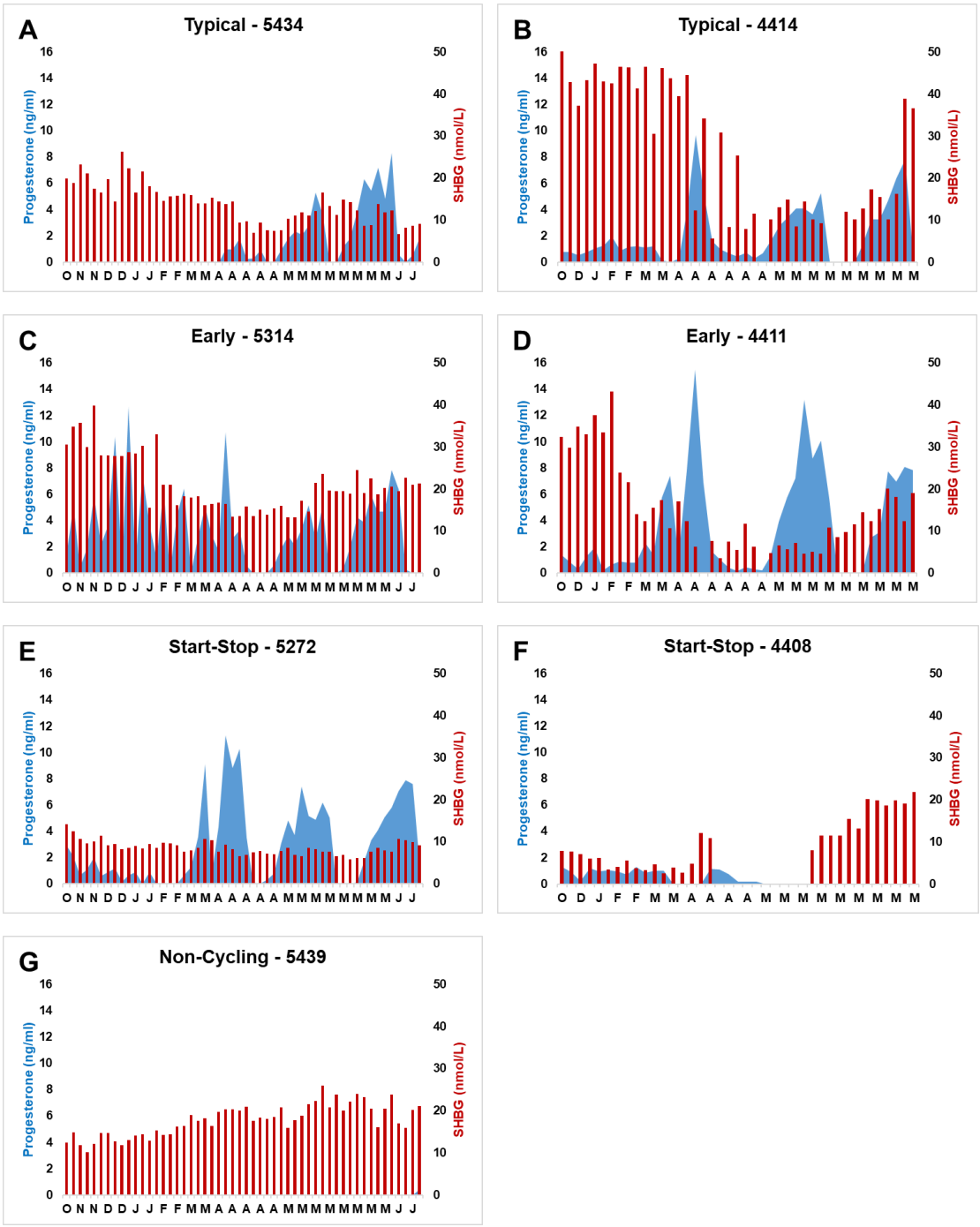

Figure 3-3. Profiles of sex hormone binding globulin (SHBG) and progesterone from samples collected from weaning through the intensive study period

Table 3-5. Occurrence of estrus and ovulation during the intensive studies from 2014-2017 born heifers

	Typical	Early	Start-Stop	Non-Cycling
Total n	10	12	14	10
Showed estrus	10	10	10	9
Ovulated	9	11	9	7
Estrus followed by ovulation	9	9	9	7
Ovulation without estrus	0	2	1	1
Estrus without ovulation	1	1	1	3
Neither estrus nor ovulation	0	1	4	1

Table 3-6. Ovarian characteristics at ovariectomy after FSH stimulation for 2014-2017 born intensive study heifers

	Puberty Group				*P*-value
	Typical	**Early**	**Start-Stop**	**Non-Cycling**	
N	12	10	14	10	
Ovary weight (g)	7.0 ± 0.9	8.7 ± 0.8	6.9 ± 0.8	7.8 ± 0.9	0.35
Ovary area (mm^2)	554 ± 50	690 ± 46	560 ± 44	581 ± 50	0.15
Average surface AFC	28.1 ± 3.7	26.8 ± 3.4	26.5 ± 3.3	23.7 ± 3.7	0.86
Antral follicles <7 mm	20.2 ± 3.5	17.2 ± 3.2	19.2 ± 3.1	15.5 ± 3.5	0.77
Antral follicles >7 mm	7.9 ± 1.5	9.6 ± 1.4	7.3 ± 1.3	8.2 ± 1.5	0.68

[a,b,c,d] Means in a row with different superscripts are different ($P \leq 0.05$).

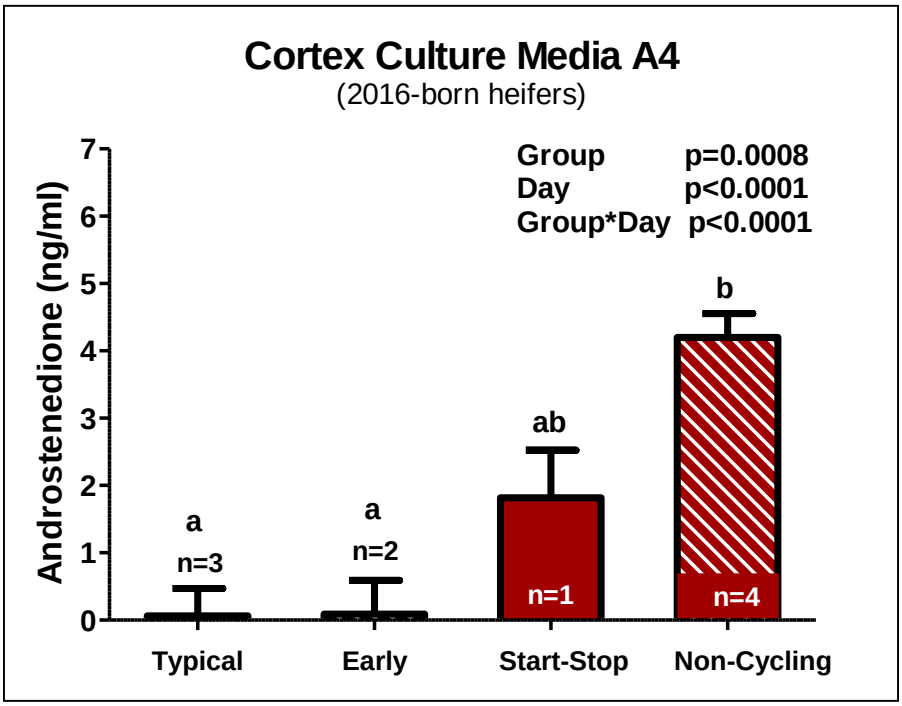

Figure 3-4. Ovarian cortex culture media A4 from 2016-born heifers in the intensive study

Table 3-7. Changes in Cyclicity that Occurred during the Intensive Trials (2014-2017-born heifers)

Animal	Year	Group	Changes in Cyclicity
6427	2016	Typical	Stopped cycling
4439	2014	Early	Stopped cycling
5336	2015	Early	Stopped and restarted cycling
4442	2014	Start-Stop	Began cycling normally
4451	2014	Start-Stop	Began cycling normally
5301	2015	Non-Cycling	Began cycling
5434	2015	Non-Cycling	Began cycling
6304	2016	Non-Cycling	Began cycling
6353	2016	Non-Cycling	Began cycling
6365	2016	Non-Cycling	Began cycling
7308	2017	Non-Cycling	began cycling
7441	2017	Non-Cycling	Began cycling