

Utilization of Early Weaning and Intrafollicular Insemination as Methods to  
Improve the Reproductive Performance of Cattle

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Thesis submitted to the faculty of the Virginia Polytechnic Institute and  
State University in partial fulfillment of the requirements for the degree of

Masters of Science

In

Animal and Poultry Sciences

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December 8, 2014  
Blacksburg, Virginia

Keywords: early weaning, heifer, glucose tolerance test, epinephrine  
challenge, progesterone clearance test, intrafollicular insemination

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## ABSTRACT

Optimization of reproductive efficiency of both beef and dairy herds is critical for sustainability and profitability. Two separate experiments were performed to test the reproductive outcomes following early weaning of beef heifers and intrafollicular insemination in dairy cows.

Early weaning is a proven way to induce precocious puberty in heifers. Heifers will experience more estrous cycles before breeding, which is associated with increased fertility. In this experiment, heifers were either subjected to early weaning and a high concentrate diet (EW;  $106.5 \pm 3.4$  days of age) or normal weaning (NW;  $231.7 \pm 3.33$  days of age) treatments. Despite no effect ( $P > 0.15$ ) of weaning treatment on age at puberty, EW heifers tended to have higher pregnancy rates than NW heifers. A progesterone clearance analysis revealed that EW heifers also have greater ability to metabolize progesterone. This altered progesterone metabolism could be a direct result of changes in metabolism caused by feeding a high concentrate diet after early weaning.

Pregnancy rates in cattle are often lower than desired. New reproductive advances are constantly developed to improve reproductive function. A recently described possible technique is intrafollicular insemination (IFI). The objective of the second experiment was to investigate whether IFI can cause fertilization. Abattoir ovaries with dominant follicles injected with semen and incubated overnight displayed sperm in close association with granulosa cells. When synchronized cows were subjected to IFI, no

pregnancies resulted. While other studies have demonstrated success with IFI, it is still unknown if fertilization is possible within the follicle of the ovary.

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## List of Abbreviations

ADG	Average daily gain
AI	Artificial insemination
ARC	Arcuate nucleus
ART	Assisted reproductive technique
AUC	Area under the curve
AVPV	Anteroventral periventricular nucleus
COC	Cumulus oocyte complex
E2	Estradiol
ER	Estrogen receptor
EW	Early Weaning
FGF2	Fibroblast growth factor 2
FSH	Follicle stimulating hormone
GH	Growth hormone
GHR-1A	Growth hormone receptor 1A
GnRH	Gonadotropin releasing hormone
GPR	G-protein coupled receptor
HPO	Hypothalamic-pituitary-ovarian
IFI	Intrafollicular insemination
IFNG	Interferon gamma
IGF-BP	Insulin like growth factor binding protein
IGF-I	Insulin like growth factor I
LH	Lutenizing hormone

ME	Median eminence
MPF	Maturation promoting factor
NEFA	Nonesterified fatty acid
NO	Nitric oxide
NPY	Neuropeptide Y
NW	Normal weaning
OT	Oxytocin
P4	Progesterone
PDE3	Phosphodiesterase 3
PGF2a	Prostaglandin F2a
PKA	Phosphokinase A
PKC	Phosphokinase C
POA	Preoptic area
RDP	Rumen degradable protein
SEM	Scanning electron microscopy
StAR	Steroidogenic acute regulatory protein
TNF- $\alpha$	Tumor necrosis factor alpha
VEGF	Vascular endothelin growth factor

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## **Chapter 1**

### **Introduction**

Puberty in beef heifers is a widely researched event that directly affects on-farm productivity and profitability. Management strategies that hasten the onset of puberty can lead to shorter generation intervals and improve lifetime reproductive performance of the heifer. The target age for breeding beef heifers is 14 months, followed by calving at 24 months (Perry and Cushman, 2013). Heifers that are pregnant early in the breeding season, calve early, and rebreed quickly have increased productivity throughout their lives (Engelken, 2008).

There are a multitude of factors that influence age at puberty, with nutrition arguably being the most important. Traditionally, it was recommended that heifers be 65% of their mature body weight at breeding (Funston and Deutscher, 2004). Recent research suggests that intense nutritional management of heifers can lead to puberty at lower body weights. Heifers that reach puberty and cycle multiple times before the breeding season have higher first breeding conception rates (Engelken, 2008). But in traditional management systems, heifers bred on their third estrous cycle were heavier and older at breeding compared to those bred on their first cycle (Byerley et al., 1987). Managing heifers to achieve puberty early is a way for producers to increase the likelihood that heifers will enter the breeding season having completed multiple estrous cycles. This will cause them to conceive earlier in the breeding season, thereby improving multiple aspects of productivity.



Early weaning followed by feeding a concentrate diet is a practice that has proven effective to increase marbling scores in beef steers (Scheffler et al., 2014). Researchers have also examined the effects of early weaning on replacement beef heifers. When heifers are early weaned and fed a high concentrate diet, they exhibit puberty at a younger age compared to their conventionally weaned counterparts (Moriel et al., 2014). Therefore, early weaning can be a way for producers to induce puberty before the breeding season to allow heifers multiple cycles before first service. Objectives of the first study were to evaluate if an early weaning strategy affects puberty, pregnancy rates, and metabolic function of replacement heifers.

Attainment of puberty is only one important aspect to managing a successful herd. Producers can have low herd pregnancy rates, even though cows are pubertal and cycling. Innovations in reproductive techniques such as artificial insemination, *in vitro* fertilization, and embryo transfer have been successful ways to increase pregnancy rates in breeding herds. A new technique, intrafollicular insemination, may offer an option to cause successful pregnancies in cattle and humans with fertility problems (López-Gatius and Hunter, 2011). Therefore, the aims of the second study were to elucidate if IFI is a successful method to achieve pregnancies in dairy cows, and to hopefully decipher if sperm is capable of fertilizing an oocyte in the follicle.

## Chapter 2

### Review of the Literature

#### *Attainment of Puberty*

For heifers to maximize lifetime productivity, heifers should conceive early in the first breeding season and calve at 24 months of age (Lesmeister et al., 1973). One potential setback to lifetime productivity is if heifers fail to attain puberty by the start of the desired breeding season. Puberty is broadly defined as the time at which an animal is able to reproduce successfully (Moran et al., 1989; Senger, 2012). Most researchers, however, characterize puberty as the time of first ovulation followed by a normal luteal phase (Byerley et al., 1987; Funston and Deutscher, 2004; Martin et al., 2008; Atkins et al., 2013; Moriel et al., 2014). Since ovulation itself is relatively difficult to detect in a non-invasive manner, circulating P4 concentrations are frequently used as an indicator of ovulation and successful luteal development. Heifers are considered pubertal when blood P4 levels remain  $>1\text{ng/mL}$  for 2 consecutive weeks (Byerley et al., 1987; Gasser et al., 2006a; Gasser et al., 2006b; Gasser et al., 2006c; Gasser et al., 2006d; Martin et al., 2008). In the beef heifer, the onset of puberty can occur between 9 and 24 months of age, with an average of 11 months. Weight, body size, nutritional status, and genetics all affect attainment of puberty in beef heifers (Lesmeister et al., 1973). Each of these factors can be influenced so that replacement heifers are pubertal by the start of the breeding season.

In a beef production system, it is advantageous for a heifer to reach puberty and breed early so that following calving she has a longer rebreeding season than multiparous cows. Primiparous heifers take longer to resume cyclicity than older cows (Rice and Wiltbank, 1972). First calf heifers are still growing, so in addition to the nutritional

demands of a suckling calf, heifers are partitioning energy to their own growth. Since the most influential factor affecting the interval from calving to conception is body condition (Wathes et al., 2007), it is critical that the nutritional status of primiparous heifers be closely monitored during the periparturient period. Compared to cows, heifers are also more prone to dystocia problems, as they have smaller pelvic areas than their multiparous counterparts. Dystocia is problematic because it is associated with a longer interval to rebreeding. These differences show that cows and heifers must be managed differently to hasten rebreeding for optimal breeding and calving seasons.

Another factor influencing age at puberty is breed. *Bos indicus*-influenced heifers are older and heavier at puberty compared to *Bos taurus* breeds (Bagley, 1993). Even within the British breeds, weight and age at puberty differ. Larger breeds, such as Simmental and Charolais, are larger and heavier at puberty compared to Hereford or Angus. Therefore, it is important for growing heifers to be nutritionally managed to reach their target breeding weight prior to the desired breeding season. Weight, rather than age, has the most influence on puberty in beef heifers. Heifers fed diets formulated to increase average daily gain came into puberty earlier than heifers with lower average daily gains, implying that body weight is a primary factor affecting age at puberty (Short and Bellows, 1971). The traditional industry standard is that heifers should reach 60-65% of their mature body weight by breeding (Funston et al., 2012). Recent research, however, suggests that the target body weight for puberty is actually lower. When heifers were managed to reach 55% of mature body weight (low) compared to 60% of mature body weight (high), 85% of the high gain heifers were cycling at breeding compared to only 74% of the low gain heifers (Funston and Deutscher, 2004). Interestingly, despite the

difference in cyclicity, there were no differences in pelvic size, pregnancy rate, or calving ease. This allows producers to develop heifers to a lower target breeding weight while not adversely affecting reproductive outcomes.

Feeding heifers to a lower target weight prior to the breeding season (compared to the traditional 60-65% of mature body weight) provides an economic advantage, in that it saves producers money to feed heifers for less gain without damaging reproductive parameters. However, if taken to the extreme, dietary restriction before puberty negatively influences age at puberty. In one study, heifers were fed two different diets for 30 weeks during the prepubertal period. Those that were fed a diet targeting 0.3 kg of gain/day reached puberty 4 weeks later than heifers fed for 0.6 kg of gain/day (Luna-Pinto and Cronje, 2000). The difference was influenced not by target weight, as the heifers weighed the same at puberty, but instead by the rate of gain during the prepubertal period.

While pregnancy rates at the end of the breeding season are not affected by developing heifers to a smaller target breeding weight, this practice may put them at a disadvantage the following year when they need to rebreed. Pubertal heifers developed to 50% of mature body weight followed by a 60-day breeding season calved later in the calving season (Martin et al., 2008). This shows that developing heifers to a lower target body weight delays conception. This is because heifers inseminated during their first estrous cycle are less likely to become pregnant than heifers bred on their third estrous cycle (Byerley et al., 1987). To further complicate matters, around the time of puberty, 25% of heifers will display a non-ovulatory estrus characterized by a lack of luteal function. Therefore, the advantages and disadvantages of developing heifers to a lighter

pre-breeding weight must be considered prior to employing this practice. If the management system allows, simply implementing a longer breeding season for these heifers could improve reproductive outcomes, making this an economically advantageous strategy for producers to manage their animals (Funston et al., 2012).

### *Endocrine Regulation of Puberty*

Throughout a cow's reproductive lifetime, the hypothalamus, pituitary, and ovary will act together in an endocrine manner to form the hypothalamic-pituitary-ovarian (HPO) axis. In the female, the hypothalamus is responsible for releasing gonadotropin-releasing hormone (GnRH). Gonadotropin-releasing hormone then stimulates the anterior pituitary to release follicle stimulating hormone (FSH) and luteinizing hormone (LH). These gonadotropes act on the ovaries to cause a myriad of effects, including follicle development and ovulation.

The hypothalamus is the last part of the HPO axis to mature to induce puberty in the female (Kinder et al., 1995). The hypothalamus contains GnRH neurons, which are housed in two different hypothalamic nuclei: the surge center and the tonic center (Senger, 2012). This surge center of the hypothalamus is responsible for releasing GnRH in a surge like manner to ultimately cause ovulation, while the tonic center is responsible for the release of basal levels of GnRH before the surge.

Normally, GnRH secretion is suppressed via negative feedback from estradiol (E2) produced by the ovarian follicles (Gonzalez-Padilla et al., 1975). Gonzalez-Padilla and coworkers (1975) saw a decrease in E2 approximately 40 days before the onset of the pubertal LH surge, caused by increased negative inhibition of E2 (Gonzalez-

Padilla et al., 1975). This suggests that a decreased sensitivity to E2 and removing the E2 block on the hypothalamus allows for increased secretion of GnRH (Atkins et al., 2013). In an experiment by Day and coworkers (1984), ovariectomized heifers displayed a rapid increase in LH concentration and frequency, while ovariectomized heifers with an E2 implant did not. These findings show that E2 from the ovary directly affects timing and pubertal development of the LH surge. Studies from this same laboratory also show a decrease in estrogen receptor (ER)-positive neurons in the anterior hypothalamus and medial basal hypothalamus just prior to pubertal onset, allowing for decreased sensitivity to E2 and increased GnRH pulses (Day et al., 1987).

Gonadotropin releasing hormone receptor concentrations in the anterior pituitary vary depending on puberty status (Schoenemann et al., 1985). The basal concentrations of systemic LH and FSH increases before puberty, due to tonic release of GnRH from the hypothalamus (Schoenemann et al., 1985). During the peripubertal period (50-120 days before puberty), the negative inhibitory effects of estrogen begin to lessen (Atkins et al., 2013). As heifers approach the time of their first ovulation, the frequency of GnRH pulses increases due to decreased sensitivity to estrogen (Senger, 2012). The increase in GnRH signaling from the surge center gets strong enough to cause an LH release from the anterior pituitary sufficient in amplitude and frequency to cause ovulation. After puberty, this increase in GnRH occurs approximately every 21 days in conjunction with ovulation following estrus.

### *Insulin-like growth factor-I*

Insulin-like growth factor-I (IGF-I) is a peptide hormone produced throughout the body (although most IGF-I is produced in the liver) and plays an essential role in reproduction (Velazquez et al., 2008). Its production is controlled by growth hormone (GH) released from the anterior pituitary and is transported via IGF binding proteins (IGF-BP) (Adashi, 1998; Yuan et al., 1998). While there are 6 IGF-BPs, IGF-BP3 is the most abundant and binds 95% of IGFs in circulation (Zulu et al., 2002). The binding proteins act to increase or decrease the bioavailability of IGF-I, with different tissues utilizing different binding proteins (Yuan et al., 1998; Silva et al., 2009) Its two receptors (type I and type II) are similar to insulin receptors and act through the tyrosine kinase pathway. Insulin like growth factor receptor type I has a high affinity for IGF-I, but will bind insulin and IGF-II at lower affinities. Studies show disruption of IGF-I and IGF-I receptor causes failure to attain puberty (Silva et al., 2009). However, when GH signaling is blocked, IGF-I is still produced, indicating some IGF-I synthesis independent of GH stimulation.

One of the most important roles of IGF-I in reproduction is its effect on follicular development (Velazquez et al., 2008; Silva et al., 2009). The ovary produces and has receptors for IGF-I, thereby affecting the ovary in an autocrine and paracrine fashion (Adashi, 1998). It acts on the granulosa cells to stimulate proliferation, as well as stimulating steroidogenesis and progesterone release in luteal cells (Yuan et al., 1998; Zulu et al., 2002). As the follicle develops, IGF-I receptor concentration increases in the follicle granulosa and thecal cells (Silva et

al., 2009). It acts to stimulate aromatase and increase the sensitivity of cells in the follicle to FSH and LH (Zulu et al., 2002). Insulin-like growth factor-I and IGFbps have also been found in follicular fluid (Yuan et al., 1998). A study by Yuan and co-workers (1998) showed that IGF-I, IGF-II, and IGFBP-2 mRNAs are most prominent in follicles during early- and mid-dominance. They did not find IGFBP-2 in late dominant follicles, suggesting that the granulosa cells of dominant follicles do not produce mRNA for IGFBP-2. These dominant follicles did, however, have increased levels of IGF-I mRNA compared to subordinate follicles. High levels of plasma IGF-I correlate to high levels of follicular fluid IGF-I, most likely transported by IGFBP-3. *In vitro* studies have shown an increase in follicle diameter and E2 production when whole preantral follicles were cultured with IGF-I supplemented to the media (Thomas et al., 2007). Walters and co-workers (2006) found that adding IGF-I to whole antral follicle culture media increased E2 concentration in the follicle, increased follicle size, and improved oocyte competency. The ratio between free IGF-I and bound IGF-I in follicle dominance is important. Within 8 hours of the dominant follicle deviating from the subordinant follicles of the cohort, increases in free IGF-I and reduction of IGFBP are detectable (Ginther et al., 2002).

The previously described effects of IGF-I on follicle development are pertinent to the discussion of puberty because body weight and IGF-I are closely related during prepubertal growth (Velazquez et al., 2008). A study in sheep found that early pubertal lambs had higher circulating concentrations of IGF-I compared to lambs that reached puberty later (Shirley et al., 2001). Other researchers have found that in heifers circulating IGF-I concentrations increased as puberty approached



(Velazquez et al., 2008). Garcia and coworkers (2003), however, found decreasing levels in IGF-I in the weeks leading to puberty attainment (Garcia et al., 2003).

Nutritional development of heifers can cause differences in IGF-I (Velazquez et al., 2008). Feed-restricted heifers had lower levels of circulating IGF-I along with decreased LH (Yelich et al., 1996). Prepubertal heifers immunized against growth hormone releasing factor had decreased serum IGF-I concentrations and delay of puberty (Simpson et al., 1991). This suggests IGF-I is an important metabolic mediator of puberty, and is capable of affecting follicular development and the LH surge (Velazquez et al., 2008).

### *Leptin*

Plane of nutrition, body weight, and body adiposity are important factors that are capable of affecting the age at puberty. Leptin, a protein hormone synthesized by adipose cells, may be involved in communicating metabolic status to the HPO axis, thereby altering puberty attainment. Discovered in 1994, leptin is a product of the adipose obese gene and has receptors found on the median eminence (ME), arcuate nucleus (ARC) and ventromedial nucleus of the hypothalamus. These are areas that are involved in reproduction, feeding behavior, and growth (Williams et al., 2002). Leptin was originally found to play a role in GnRH signaling in *ob/ob* mice. These mice, which lack a functional leptin gene, have impaired fertility, which is restored after treatment with exogenous leptin (Zieba et al., 2005). Leptin regulates LH secretion through neuropeptide Y (NPY). During fasting, leptin is suppressed and NPY is stimulated, leading to decreased pulses in LH and increased

feeding behavior. Thus high leptin levels act by suppressing NPY and stimulating POMC neurons, suppressing feeding and increasing satiety (Gao and Horvath, 2008). Neuropeptide Y may act through both the hypothalamus and anterior pituitary to affect LH secretion (Williams et al., 2002).

Leptin is thought to be a key regulator in the onset of puberty in both males and females. Changing an animal's plane of nutrition can change the amounts of circulating leptin available, as well as IGF-I, insulin, and GH (Garcia et al., 2002). Circulating concentrations of leptin linearly increase with body weight 16 weeks before the onset of puberty. In heifers fed a restricted diet, leptin was 70% lower than that found in heifers fed a control diet (Zieba et al., 2004). Prior to the attainment of puberty, the HPO axis becomes hypersensitive to leptin secretions. However, heifers given exogenous leptin did not experience changes in LH pulse frequencies (Maciel et al., 2004). When comparing heifers fed a high plane of nutrition to those fed a lower plane of nutrition with exogenous leptin, the heifers fed the higher plane of nutrition experienced puberty earlier than the restricted heifers given leptin (Carvalho et al., 2013). There has been no evidence to suggest that prepubertal administration of leptin can cause elicit GnRH secretions similar to that of a sexually mature animal (Zieba et al., 2005).

As previously stated, giving exogenous leptin to prepubertal heifers is not an effective method for inducing puberty (Maciel et al., 2004). There are many theories as to why leptin fails to do so. Leptin receptor concentration in the ARC and ventromedial nucleus and on the gonadotropes could be low during the prepubertal

period. Leptin may only stimulate LH in response to nutritional stress and not during normal growth and nutritional status (Amstalden et al., 2002; Amstalden et al., 2003). Leptin needs to be bound to a carrier binding protein to cross the blood brain barrier, so sufficient amounts of the binding protein may not be available in prepubertal heifers given exogenous leptin (Garcia et al., 2002). Therefore, even though endogenous leptin may be a key factor in signaling metabolic status to the HPO axis to cause induction of puberty, there are limited opportunities to accelerate puberty via exogenous leptin administration.

### *Kisspeptin*

Kisspeptin, a neuropeptide produced by the *Kiss1* gene in the hypothalamus, works through G-protein coupled receptor 54 (GPR54) to mediate the GnRH response to E2 (Chaikhun et al., 2013; Okamura et al., 2013; Sanchez-Garrido and Tena-Sempere, 2013). Kisspeptin neurons are found in the ARC, anteroventral periventricular nucleus (AVPV), the preoptic area (POA), and the ME in the hypothalamus (Chaikhun et al., 2013; Okamura et al., 2013). The kisspeptin neurons co-localize with GnRH neurons in the ME and ARC in ruminants (Pompolo et al., 2006). Kisspeptin neurons in the ARC and POA express ER $\alpha$ , and neurons in the ARC express P4 receptor in ewes (Franceschini et al., 2006; Smith et al., 2008). It is widely known that ER $\alpha$  is a key player in estrogen negative and positive feedback on GnRH secretion. Gonadotropin-releasing hormone neurons, however, do not contain ER $\alpha$  (Lehman et al., 1993; Clarke, 2011). Ninety percent of GnRH neurons are found to express GPR54 in the mouse (Han et al., 2005; Sanchez-Garrido and Tena-Sempere, 2013). During proestrus, kisspeptin is upregulated in the anteroventral periventricular nucleus (AVPV) and downregulated in

the ARC (Clarke, 2011). The co-localization of kisspeptin neurons with GnRH neurons, the expression of ERα on kisspeptin neurons, as well as the expression of GPR54 on GnRH neurons shows that kisspeptin acts as the intermediate between E2 feedback and GnRH release.

Kisspeptin is also thought to be a key player in the onset of puberty in mammals. Mice that lack the *Kiss1* gene or GPR54 do not exhibit puberty (Sanchez-Garrido and Tena-Sempere, 2013). A study by Kadokawa and co-workers (2008) observed increases in GH and LH concentration in prepubertal heifers given kisspeptin compared to heifers who did not receive kisspeptin. This suggests that exogenous kisspeptin can work to activate GnRH neurons to release LH from the pituitary. The expression of *Kiss1* has also been linked to the metabolic status in females. There is less *Kiss1* expression in the hypothalamus of fasted rats, which corresponds with decreased LH levels (Castellano et al., 2005; Castellano et al., 2010).

### *Steroidogenesis*

Theca cells and granulosa cells play a very important role in E2 synthesis. Granulosa cells are located inside the follicle basement membrane and surround the oocyte. Theca cells, specifically theca interna cells, are located around the growing follicle, outside the basement membrane. These two cell types work together to form the “2-cell, 2-gonadotropin model” of E2 production (Senger, 2012). Briefly, theca interna cells have GPR for LH located on their cell membranes. Once LH from the anterior pituitary binds to its receptor on the cell membrane, it activates protein kinase A (PKA), which in turn activates enzymes CYP11A1, CYP17, and 3BHS. Cholesterol, the

precursor of steroids, is transported into the mitochondria of the cell via steroidogenic acute regulatory protein (StAR). Cholesterol, with the help of the activated enzymes, is converted to androstenedione. Androstenedione then moves across the basement membrane of the follicle and enters the granulosa cell. Granulosa cells have GPRs for FSH located on the cell membrane. Similar to the theca interna cell, FSH activates cAMP, which activates PKA. Protein kinase A activates P450aromatase, which converts androstenedione to estrone. Estrone is converted to E2 by 17BHS and is released into the body. A study by Orisaka and co-workers (2006) demonstrated this model when culturing granulosa cell. Granulosa cells cultured with pure FSH at different levels did not show any changes in E2 production. When cultured with theca cells for 24 hours, however, E2 levels increased 6-fold (Orisaka et al., 2006). Therefore, theca interna cells and granulosa cells work in conjunction to produce E2.

### *The Estrous Cycle*

The bovine estrous cycle occurs approximately every 21 days throughout an animal's reproductive lifespan. It is divided into two phases: the follicular phase and the luteal phase (Senger, 2012). The follicular phase lasts approximately 3-4 days and is characterized by final maturation of a dominant follicle and subsequent ovulation. The luteal phase lasts for the remainder of the cycle and is characterized by the presence of the corpus luteum (CL) on the ovary.

### *Follicle growth*

Follicles grow on the bovine ovary in a wave-like pattern during the entire estrous cycle. Researchers have found that most cows have between two and three follicular

waves, although as few as one and as many as four have been reported (Savio et al., 1988; Ginther et al., 1989; Knopf et al., 1989). During this time, follicles undergo stages of recruitment, selection, and dominance (Lucy et al., 1992; Senger, 2012). If not able to undergo ovulation, selected dominant follicles will undergo growing, static, and atresia phases.

During the primordial, primary, and secondary stages of development, follicular growth is independent of gonadotropin secretion (Orisaka et al., 2006). Next, the antral (tertiary) follicle becomes somewhat, but not absolutely, dependent on FSH secretion through 4mm in size. Follicle stimulating hormone and LH becomes crucial thereafter for growth and recruitment of the cohort and development of the wave.

At the beginning of the follicle wave, a small number of follicles will be recruited to grow out of the now gonadotropin-sensitive pool of small antral follicles on the ovary. The first wave will start on the day of ovulation, or day 0 (Ginther et al., 1989). The emergence of a follicular wave corresponds with an increase in systemic FSH levels, which return to basal levels after follicles >5mm emerge (Ginther et al., 1998). The E2 produced by the growing follicles will stimulate the production of inhibin, which causes a subsequent decrease in circulating FSH concentration. The 4 to 6 mm follicles will grow in diameter until one follicle is selected by day 7 to be the largest dominant follicle on the ovary (greater than 10 mm) (Lucy et al., 1992). If FSH is blocked, then there will be no follicle development on the ovary (Mihm and Bleach, 2003). The nondominant follicles on the ovary will undergo atresia.

The dominant antral follicle will shift from FSH dependency to LH dependency as the granulosa cells acquire LH receptors and the follicle deviates from the cohort. This dependence upon LH will persist through post-selection growth through ovulation and is crucial for follicles to grow larger than 9 mm in size (Ginther et al., 2001; Mihm and Austin, 2002). It is important for the growing cohort to be responsive to LH in order to produce E2 (Mihm and Bleach, 2003). Large anovulatory follicles that develop will secrete E2, but GnRH concentrations will remain low due to progesterone dominance from the corpus luteum (CL), thereby preventing the rise needed in LH for ovulation.

A new follicular wave will start following the regression of the first dominant follicle in response to decreased levels of E2, which causes levels of FSH and LH to rise again. This new follicular wave will either undergo atresia after recruitment and selection (3-wave cycle) or become the ovulatory follicular wave (2-wave cycle). Dominant follicles of the first and second follicular wave have the ability to ovulate if exogenous PGF2a is administered to regress the CL. The sizes of the anovulatory dominant follicles and ovulatory dominant follicle are similar (Ginther et al., 1989).

The dominant follicle has greater aromatase activity in the granulosa compared to subordinate follicles (Badinga et al., 1992). Recall that androstenedione is converted to E2 by aromatase. More aromatase activity allows more E2 to be converted from androstenedione. As a result, the E2 concentration in the fluid of the dominant follicle is greater than in subordinate follicles. Higher levels of P4 are found in subordinate follicles compared to dominant follicles. Concentrations of E2 in the blood will change as the ovarian follicles increase and decrease in size. The E2:P4 ratio is higher in the dominant

follicle's follicular fluid. An estrogen active follicle has a E2:P4 ratio >1 in the follicular fluid (Mihm and Bleach, 2003).

Dominant follicle development also relies on the interplay of FSH, inhibin, activin, and follistatin. Increasing E2 levels will increase the responsiveness to FSH and LH and also promote P450aromatase efficacy (Beg et al., 2003). Follicle stimulating hormone added in culture will increase the expression of mRNA for P450aromatase, LH receptor, and StAR, which is important for E2 production (Orisaka et al., 2006). Inhibin, from the granulosa cells, is a dimeric protein that suppresses FSH secretion by 50% from its peak concentration (Lucy et al., 1992; Mihm and Austin, 2002; Senger, 2012). Activin, the homodimer of inhibin, increases granulosa proliferation and enhances steroidogenesis. It also increases FSH receptor number on the granulosa cell, thereby increasing inhibin synthesis. Activin is regulated by follistatin, a binding protein for activin (Mihm and Bleach, 2003). Increased E2 and increased high molecular weight inhibins occur in the beginning of the follicular wave (Mihm and Austin, 2002). This coincides with high levels of activin and low levels of follistatin in the largest follicle. High levels of activin mean high levels of inhibin, which restrict FSH circulation and keeps the subordinate follicles from growing, thereby assisting with dominant follicle selection.

### *Ovulation*

The follicular phase of the estrous cycle includes proestrus and estrus. The HPO axis regulates the follicular phase. The start of the follicular phase begins with regression of the CL via luteolysis. The recruitment and selection of the ovulatory follicular wave



will occur just prior to luteolysis. As this dominant follicle develops, estradiol levels will increase, and progesterone levels will decrease due to the regressing CL. The pulse frequency of LH will increase to 1 per hour at the time of ovulation (Senger, 2012). The climax of the LH frequency and amplitude will stimulate ovulation.

In order for ovulation to occur after the LH surge, elevated blood flow, connective tissue breakdown, and ovarian contractions must all occur. The preovulatory LH surge will cause an increase in prostaglandin E2 production by the ovary, which will cause elevated blood flow to increase pressure on the follicle wall. The LH surge will also cause the dominant follicle to switch from E2 to P4 production. Progesterone causes collagenase release from the theca interna cells. This will cause the thinning of the follicle wall at the apex, and eventual rupture at the stigma (LeMaire, 1989). Enzymes act to breakdown the basement membranes that separate granulosa from theca cells and germinal epithelium from tunica albuginea before ovulation can occur (LeMaire, 1989). The ovary will also produce prostaglandin F2a (PGF2a) (Senger, 2012). Prostaglandin F2a will cause smooth muscle contractions in the ovary. All of these events will cause the stigma of the follicle wall to weaken and ovulation. The oocyte will be released from the ovary and transported into the oviduct to await fertilization.

### *Luteal Development*

Immediately after ovulation, theca cells and granulosa cells begin the process of luteinization. Granulosa cells become large luteal cells, and theca cells become small luteal cells (Niswender et al., 2000). This structure is known as the CL, and its main function is to secrete P4. Reorganization of thecal, granulosa, fibroblasts, and endothelin

cells characterizes luteal development. The luteinized granulosa cells undergo hypertrophy, while fibroblasts undergo hyperplasty (Sangha et al., 2002). Theca cells luteinize to form small luteal cells and epithelium reorganizes to intersperse the large luteal cells. These factors all contribute to the growing size of the CL.

Progesterone is produced in the luteal cells from cholesterol (Senger, 2012). Similar to E2 synthesis, StAR brings cholesterol into the mitochondria, where P450aromatae converts it to pregnenolone. Pregnenolone leaves the mitochondrial and is converted to progesterone via 3BHSD, which then diffuses from the cell. While both small and large luteal cells produce P4, large luteal cells produce the majority of P4 in the CL (Rodgers et al., 1983). Interestingly, small luteal cells have more LH receptors are more LH responsive than large luteal cells (Pate, 1996). In a study by Rodgers and co-workers (1983) small and large luteal cells were cultured with and without LH (Rodgers et al., 1983). Small luteal cells demonstrated a significant increase in P4 production when LH was added to the media compared to the absence of LH. Large luteal cells did not alter progesterone production regardless of LH in the media. Small luteal cells are also more abundant than large luteal cells. In the cow, small luteal cells make up 26% of the luteal cells (by number), while large luteal cells only make up 3.5%. Large luteal cell, however, total 40% of luteal volume (Sangha et al., 2002). These large luteal cells have prostaglandin E2 receptors (PGE2), and P4 production is thought to be mediated through PGE2 (Weber et al., 1987).

The initial growth of the CL relies heavily on pituitary hormones. Hypophysectomized ewes had lower progesterone concentrations, luteal weights, and expression of P450scc and 3BHSD during days 5-12 of luteal development than intact

ewes (Juengel et al., 1995). Treatment of hypophysectomized ewes with GH and LH resulted in restoration of P4 values similar to that of control ewes. Growth hormone is responsible for the increase in size of the CL, while LH is in part responsible for P4 secretion. Interestingly, luteal size after day 8 does not effect P4 concentration (Mann, 2009). There is, however, a positive relationship between luteal weight and P4 concentration from days 5 to 8, suggesting an important role for early luteal development.

Luteinizing hormone acts through a GPR to activate the second messenger cAMP, which activates PKA cause the conversion of cholesterol to P4 in the small luteal cell (Niswender et al., 2000). Despite being responsible for producing most of the P4 coming from the CL, the large luteal cell is primarily LH unresponsive. Instead, it has receptors for the luteolytic hormone PGF2a which follows the PIP2/IP3/DAG second messenger system to activate protein kinase C (PKC) to inhibit P4 production. The large luteal cell also produces oxytocin (OT). Oxytocin is important in the pathway for luteal regression.

One of the most important events during luteal development is the development of the CL's capillary network (Niswender et al., 2000). The network is created by endothelial cells, which form next to luteal cells to transport progesterone. Angiogenesis of the CL is influenced by many factors. Vascular endothelin growth factor (VEGF) and fibroblast growth factor 2 (FGF2) are actively involved in the formation of blood vessels in the CL (Robinson et al., 2009; Galv et al., 2013). Blocking VEGF will halt angiogenesis and decrease P4 production. Fibroblast growth factor 2 inhibition will stop endothelial capillary formation. These two factors work together in the cow to cause growth and maturation of the CL's vascular network.

## *Luteolysis*

Luteolysis is the dynamic process of CL regression. If fertilization fails to occur, then luteal regression will take place on days 17 to 19 of the estrous cycle. The method of luteal regression in the cow revolves around the release of PGF<sub>2a</sub> from the endometrium. Prostaglandin F<sub>2a</sub> acts on endothelin-1, cytokines, and nitric oxide (NO) to cause luteal regression (Skarzynski et al., 2008). Prostaglandin was suggested to have a role in luteolysis in 1966 (Knickerbocker et al., 1988). It is converted from arachidonic acid by cyclooxygenase in the endometrium (McCracken et al., 1999). Luteolysis does not occur early in the luteal phase of the estrous cycle because animals must have exposure to P<sub>4</sub> for at least 10 days before luteolytic mechanisms are set into motion. In a previous experiment, ovariectomized cows displayed a normal response to OT 12 days after P<sub>4</sub> concentrations remained >1ng/ml (Lamming and Mann, 1995). Additionally, CL's younger than 5 days of age are not responsive to PGF<sub>2a</sub> because they have no yet formed receptors yet.

One regulator of PGF<sub>2a</sub> synthesis is E<sub>2</sub>, which acts to increase the amount of arachidonic acid available for conversion to PGF<sub>2a</sub>. When bovine endometrial explants are cultured with E<sub>2</sub>, the amount of basal PGF<sub>2a</sub> increased (Mann, 2001). Estradiol is also a stimulant of OT receptor formation. Throughout most of the diestrus phase of the estrous cycle, P<sub>4</sub> suppresses OT receptor formation (Mann, 2001). The ability of OT to initiate luteolysis is influenced by the concentration of OT receptors expressed in the endometrium (McCracken et al., 1999). Oxytocin is released from the posterior pituitary and the CL. It is released in pulses from the neurohypophysis, which causes a pulsatile release of PGF<sub>2a</sub> from the uterus (Tysseling et al., 1998). The pulsatile OT release is

caused by E2, and enhanced by pre-exposure to P4 (McCracken et al., 1996). The “pulse generator hypothesis” proposed by McCracken and co-workers (1996) hypothesizes that a high frequency of pulsatile OT causes a release of low levels of PGF2a, which then act to cause a release of luteal OT. The release of luteal OT causes increased secretion of PGF2a from the endometrium, which causes more luteal OT and formation of a positive feedback loop that eventually will end with luteolysis. The pulsatility of OT is important and results in a similarly pulsatile pattern of PGF2a release. If PGF2a is administered as a continuous infusion rather than in a pulsatile manner, a superphysiological dose is required to cause luteolysis (McCracken et al., 1996).

Prostaglandin F2a, when released by the endometrium, flows into the uterine vein, and then diffuses across to the ovarian artery. Blocking PGF2a will cause the CL to remain on the ovary (Niswender et al., 2000). One major way PGF2a blocks P4 synthesis is by affecting the amount of cholesterol entering the cell mitochondria. Prostaglandin F2a causes a decrease in the amount of StAR mRNA and StAR protein. If StAR protein is not available to move the cholesterol into the mitochondria, then progesterone production cannot occur.

The release of PGF2a triggers an increase in the concentration of other factors that contribute significantly to luteolysis. Recently, endothelin-1 has been suggested to play an important role in luteal degeneration. Endothelin-1 is released by endothelial cells and acts as a vasoconstrictor. Prostaglandin F2a stimulates endothelin-1 action to vasoconstrict the capillaries in the CL. A study by Girsh and co-workers (1996) showed cells cultured with PGF2a display an increase in endothelin-1. There is also a decrease in P4 when cells are cultured with endothelin-1. Luteolysis can be postponed by giving an

endothelin-1 receptor antagonist before administering PGF2a (Meidan and Levy, 2002). Choudhary and co-workers (2004) showed that luteal endothelin-1 is regulated by the amount of mRNA available to encode endothelin-1. Activity of the enzyme ECE-1, which controls the formation of endothelin-1 from its precursor, does not change throughout the estrous cycle. Therefore, PGF2a acts on endothelin cells to increase the amount of mRNA produced after day 10 of the estrous cycle (Choudhary et al., 2004).

Immune cells enter into the CL in the late luteal stage to produce pro-inflammatory cytokines (Yoshioka et al., 2012). Cytokines play a key role in luteolysis (Niswender et al., 2000). At the time of luteal regression, macrophage proliferation occurs in the CL. These macrophages are important in structural luteolysis. Macrophages will perform phagocytosis on the degenerating luteal cells, and stimulate PGF2a release from the CL. Tumor necrosis factor alpha (TNF- $\alpha$ ), produced by macrophages, and interferon gamma (IFNG), produced by T lymphocytes, reduce P4 production, induce apoptosis, and stimulate the production of luteal PGF2a (Niswender et al., 2000; Skarzynski et al., 2008). Interferon gamma treatment in bovine luteal cells causes an increase in MHC class 1 antigens, and upregulates TNF receptor (Suter et al., 2001). It also increases nuclear STAT-1 and phosphorylated STAT-3, which leads to increased levels of interferon regulatory factor 1. The movement of leukocytes into the luteal cells causes an increase in these cytokines, which work together through multiple pathways to cause luteal regression.

Another important regulator of luteolysis is NO. Nitric oxide, produced by nitric oxide synthase from L-arginine, binds to guanylate cyclase to increase intracellular cGMP (Motta et al., 2001). It also inhibits P4 secretion and induces apoptosis in the

steroidogenic cells (Yoshioka et al., 2012). A study found that TNF- $\alpha$  and IFNG regulate NO amounts by increasing the expression of NO synthase. The researchers also found that luteal cells cultured with P4 have lower NO production as a result of reduced NO synthase expression (Yoshioka et al., 2012). When luteal cells were cultured with a NO synthase inhibitor, P4 concentrations rose (Skarzynski and Okuda, 2000). Nitric oxide also stimulates PGF2 $\alpha$  production from the CL during the mid luteal phase. This evidence shows that NO is highly involved in the regulation of luteolysis. All of these factors combine to cause regression of the CL, which ultimately leads to a reduction in P4 and an increase in E2, which allows for dominant follicle ovulation and the start of the next cycle.

#### *Early Weaning and High Concentrate Feeding*

Early weaning is a widely studied strategy for enhancing carcass characteristics in beef calves. More recently, however, scientists have studied the effects of early weaning on heifer puberty attainment and development. Early weaning is defined as removing the calf from the dam at a time prior to the industry standard for weaning, or 7 months of age. It is associated with feeding a high concentrate diet during the time from early weaning to normal weaning. Early weaning can be a beneficial management tool for producers to utilize in certain situations (Rasby, 2007). It is most efficient when used before the start of the breeding season if nutrient quality or availability is low. This allows cows to either maintain or improve body condition prior to insemination; thereby improving the likelihood they will become pregnant.

Early weaning sometimes induces precocious puberty (puberty before 300 days of age) in beef heifers (W. J. Sexten, 2005; Gasser et al., 2006a; Gasser et al., 2006b; Gasser et al., 2006c; Gasser et al., 2006d; Waterman et al., 2012; Moriel et al., 2014). Attaining precocious puberty is beneficial to heifer calves because heifers that reach puberty early are able to complete more estrous cycles prior to breeding. This is important because heifer calves are more fertile on their third estrus than their pubertal estrus (Byerley et al., 1987). Attaining precocious puberty would allow heifers to cycle multiple times by the start of the heifer breeding season.

Researchers have induced precocious puberty in beef heifers by feeding a high concentrate diet following early weaning (W. J. Sexten, 2005; Gasser et al., 2006a; Gasser et al., 2006b; Gasser et al., 2006c; Gasser et al., 2006d; Moriel et al., 2014). Gasser and coworkers (2006a) demonstrated that 67% of beef heifers attained precocious puberty when early weaned at 112 days of age and then fed a diet of 60% corn. Only 20% of the heifers fed a control diet (30% corn) achieved precocious puberty. Feeding a high corn diet for a short period of time, followed by a low corn diet, and vice versa each resulted in 47% of heifers attaining precocious puberty.

Age at weaning and length of time on a high concentrate feed will influence the age at which heifers exhibit their first estrous. Early weaned heifers are always heavier than normal weaned heifers at time of normal weaning (Gasser et al., 2006a; Moriel et al., 2014). And even though these heifers reach puberty sooner, the differences in body weight are negligible or absent by breeding (W. J. Sexten, 2005; Waterman et al., 2012; Moriel et al., 2014). Interestingly, early weaned heifers fed a concentrate diet have average daily gain (ADG) rates similar to conventionally weaned calves after



conventional weaning and removal from feed (Moriel et al., 2014). Average daily gain can be influenced by a number of factors, such as stress due to changes in diet, environmental effects, and plane of nutrition prior to four months of age. Heifers weaned early and fed a lower targeted ADG corn silage diet followed by a constant ADG corn silage diet had similar body weights and average daily gain by the breeding season compared to their constant gain counterparts (Grings et al., 2007). There was no difference in precocious puberty, however, between the two groups. When heifers were early weaned and fed a diet consisting primarily of barley hay and soybean hulls, 90.8% of heifers were pubertal 39 days before the start of the breeding season, compared to 76.4% of their normal weaned counterparts (Waterman et al., 2012). As mentioned earlier, this allows a greater percentage of heifers to complete their first and second estrous cycles before breeding, which generally improves pregnancy rates (Byerley et al., 1987).

Heifers early weaned and fed high concentrate feed for 180 days tended to have a lighter body weight at puberty (Moriel et al., 2014). A study by Cicciooli and co-workers (2005) suggests that feeding light heifers a high starch feed before breeding can increase pregnancy rates. This could explain why heifers on the corn based diets exhibited precocious puberty while the heifers on a silage based diet did not.

Interestingly, most data indicate that early weaning does not improve overall pregnancy rates (W. J. Sexten, 2005; Grings et al., 2007; Waterman et al., 2012; Moriel et al., 2014). A low sample number in some of these studies could influence the pregnancy rate significance. More likely, however, is that conventionally weaned heifers did not experience precocious puberty conceived later in the breeding season. This causes

producers to question whether the practice of early weaning and feeding a high concentrate diet is worthwhile if there is no overall difference in pregnancy rate. There has been one exception in which early weaned heifers had a greater percentage pregnant compared to conventionally weaned heifers at the end of the breeding season (86.1 and 60.5% pregnant, respectively). Heifers were early weaned at 80 days of age and fed a ration containing 69% rumen digestible protein (RDP), which was followed by a 181 day heifer development period after normal weaning consisting of a 31% RDP ration (Waterman et al., 2012).

Weaning heifers early changes circulating metabolite levels (Waterman et al., 2012) and these changes may partially explain improved reproductive performance. Heifers were found to have higher insulin, nonesterified fatty acids (NEFA), and urea nitrogen levels after weaning at 80 days and feeding for 133 days compared to their conventionally weaned counterparts in one location. A replicate of this experiment at another location, however, only saw differences in urea nitrogen. Rumen undegradable protein levels in the diet, environment, and genetic differences offer explanations for these results. Only one study to date has investigated the weaning-induced differences in liver expression of GHR-IA, IGF-I, and IGFBP-3 (Moriel et al., 2014). The liver is the primary site of IGF-I synthesis, and is stimulated by the binding of GH to GHR-IA. Normal weaned heifers had increased levels of GHR-IA after 90 days compared to heifers weaned at day 70 and fed concentrate or put on pasture. By day 270, however, there were no treatment differences in hepatic GHR-IA, suggesting a short-term effect of feeding on GHR. Heifers early weaned and fed for 90 days had higher liver IGF-I by day 270. This indicates metabolic imprinting, caused by changing epigenetic status based on

early nutritional stressors, may have occurred during the feeding period following early weaning, causing long lasting effects on the heifers.

Early weaning can also be beneficial for dams when implemented before the start of the breeding season (Arthington and Kalmbacher, 2003). Removing calves 8 days before the start of the breeding season increases the occurrence of estrus by 29.0% (Laster et al., 1973) and reduces the postpartum anestrous period by 24 days (Houghton et al., 1990a). More importantly, early weaning improves behavioral estrus in young cows by 39.2% in the first 21 days of the breeding season (Laster et al., 1973). This enables faster rebreeding of the dams and a shortened breeding and subsequent calving season.

The observed improvements in reproductive performance of dams following early weaning are likely due to the removal of the suckling stimulus. Within a month after removal of calves, 90-100% of cows resume cyclicity (de Castro et al., 2011). Temporary weaning is a practice that causes cows to resume cyclicity while not permanently removing the calf from the dam. De Castro and co-workers (2011) observed, however, that removal of calves from dams for 5 days did not have an effect on ovarian activity. This shows that the removal of calves needs to last longer than 5 days in order for cows to have complete resumption of cyclicity.

Early weaning not only improves cyclicity status, but also increases the likelihood the cows will become pregnant. Young cows exhibited an increase in conception rate by 25.9% and 15.6% (for 2- and 3-year old cows, respectively) (Laster et al., 1973). Myers

and co-workers (1999) reported an increase in dam pregnancy rate by 18% when early weaning calves at 90 days.

Early weaning calves from their dams also results in an increase in body condition of the dams (Houghton et al., 1990b; Myers et al., 1999; Story et al., 2000; Odhiambo et al., 2009). Proper body condition of the cow before breeding is one of the main factors contributing to the resumption of cyclicity within the desired time of 60 days postpartum (Houghton et al., 1990b). A BCS of 5 or 6 on a 9-point scale is recommended for cows before breeding for their first or second calf, respectively (Rakestraw et al., 1986). Cows with a BCS less than 3 exhibited postpartum intervals 28 to 58 days longer than cows in moderate and fleshy body condition (Houghton et al., 1990b). Early weaning can be a useful tool for producers who need to increase the BCS of their cows prior to breeding. The reduction in lactation stress can cause cows to increase their BCS and as a result reduce the postpartum interval (Story et al., 2000; Odhiambo et al., 2009).

The producer should carefully evaluate the economics of early weaning and feeding a high concentrate diet before implementing the procedure. A study by Peterson and coworkers (1987) observed the cost of early weaning was less than the cost of normal weaning. The income on calf gain for early weaned calves fed a high concentrate diet was higher than that of normal weaned calves. The researchers also saw an increase in income based on cow gain for cows that had calves early weaned compared to a loss of income from cows that had calves normal weaned. Producers raising early weaned beef steers fed a high concentrate diet should consider retaining ownership through finishing (Rasby, 2007). These steers produce carcasses that have high quality grades, which can be economically rewarding to producers retaining ownership. Net income per early weaned

steer fed a high concentrate diet in 2000 was \$75.36, compared to a net income of normal weaned steers of \$62.16 (Story et al., 2000)

All economic considerations should be taken with caution. Factors such as cost of feed, price of live cattle, labor, facilities, forage availability, calving season and environmental effects all impact the producer economically on a year-to-year basis.

While early weaning reduces cow costs, these savings may be negated by increased heifer development costs for the early weaned calves (Rasby, 2007). A study by Mulliniks and coworkers (2013) found that net revenue for replacement heifers in early weaned heifers was greater than early weaned heifers revenue in 2013. Each production operation should consider all the costs and benefits of early weaning and feeding a high concentrate diet before carrying out the procedure.

In conclusion, age at puberty is a crucial event in a replacement heifer's life that affects lifelong reproductive productivity. It can be influenced by factors such as breed and body weight. Endocrine components, including leptin, IGF-I, and kisspeptin are key players in the onset of puberty. Different management strategies, such as early weaning have the capability of inducing precocious puberty. When heifers cycle before the breeding season, they are more fertile and more likely to get pregnant on their first service (Byerley et al., 1987). This ensures that heifer calve early in the calving season at 24 months of age, and subsequently have one calf every 12 months. Other positive effects, such as shortened post partum interval, are also seen in the dam when their calves are early weaned. Inducing precocious puberty by early weaning can be a useful management tool for producers with cow-calf herds. These benefits, however, should not

be considered without first deciding the economic gains and losses of early weaning and feeding a high concentrate diet in the production system.

### Chapter 3

The effects of early weaning on reproductive and metabolic parameters in replacement  
beef heifers

#### *Abstract*

Heifers early weaned and fed a high concentrate diet have the potential to experience precocious puberty (puberty < 300 days of age). When heifers experience precocious puberty, they have the opportunity to experience multiple estrous cycles before breeding, which has a positive influence on pregnancy rate. The objectives of this study were to: 1) during phase 1, determine if early weaning has an effect on reproductive performance, and 2) during phase 2, evaluate the effects of early weaning on selected metabolic functions. In phase 1, Angus x Simmental heifers (n=35) were stratified by sire and randomly assigned to normal weaning (NW, n=18) or early weaning (EW, n=17) treatments. Early weaning heifers were weaned at  $107 \pm 3$  days of age and transitioned to a high concentrate ad libitum diet containing 20% CP. Normal weaned heifers remained with their dams until  $232 \pm 3$  days of age, at which point both treatments were comingled and grazed on mixed summer pasture. Following NW, heifers were bled weekly for P4 to determine onset of puberty. Pelvic and ovary measurements were taken before breeding. All heifers were bred at  $436.8 \pm 4$  days of age. During phase two, pregnant heifers (n=16) were split into two replicates and subjected to a glucose tolerance test, epinephrine challenge, and progesterone clearance analysis. Early weaned heifers tended to ( $P < 0.15$ ) have higher pregnancy percentages compared to NW heifers. Age at puberty ( $P > 0.05$ ) was  $310 \pm 7$  days for NW heifers and  $306 \pm 7$  days for EW heifers. Body weight puberty

( $P>0.05$ ) for was  $650.2\pm 16.8$  kg for NW heifers and  $629.8\pm 16.8$  kg for EW heifers. Pelvic area ( $P>0.05$ ) for NW and EW heifers was  $174.6\pm 3.8$  and  $181.9\pm 3.9$  cm<sup>2</sup>, respectively. Ovary area ( $P>0.05$ ) was  $39.3\pm 1$  for NW heifers and  $38.3\pm 1.1$  for EW heifers. There was a treatment x time interaction ( $P<0.01$ ) for the progesterone clearance analysis. EW heifers had lower circulating P4 concentrations following the dose at the 15, 30, 45, and 120min timepoints compared to their normal weaned counterparts. Following the glucoses tolerance test, insulin area under the curve (AUC) tended to be different between treatments. These experiments suggest that early weaning heifers and feeding a concentrate diet could increase pregnancy rates within the breeding herd, and that there are possible long lasting effects acting to change P4 metabolism.

### *Introduction*

To maximize efficiency of the cow-calf herd, heifers should calve for the first time at 2 years of age (Bagley, 1993). Ideally, heifers should start cycling long before the start of the breeding season to increase the likelihood that they will conceive early in the breeding season. Heifers need to achieve puberty well in advance of the breeding season because it has been proven that when they are bred on their third estrus instead of their first, they are more likely to become pregnant (Byerley et al., 1987). Therefore, it is advantageous for heifers to attain puberty at 300 days of age or sooner, and management strategies that hasten puberty should improve the productivity of primiparous heifers.

Puberty is influenced by a number of factors, including body weight, age, and breed. Intensely managing heifers early in life can alter body weight and age needed to attain puberty. Moriel and co-workers (2014) altered age at puberty by weaning early at 70 days of age and feeding a high concentrate diet until 180 days of age. The heifers that



were early weaned and fed a high concentrate diet experienced puberty at a younger age than their conventionally weaned counterparts (292 and 397 days, respectively). Gasser and co-workers (2006a) were able to induce early puberty in heifers that were early weaned at 70 days and fed a high concentrate diet, but not in heifers that were early weaned and fed a control diet. These results show that feeding a high concentrate diet after early weaning is essential to achieving precocious puberty.

Manipulating the nutrition of these animals early in life is most likely altering their long-term metabolic status. This presumptive metabolic imprinting, or changes in the epigenetics of the animal due to nutritional stress early in life, could go on to affect the productivity of early weaned heifers (Moriel et al., 2014). It is also most certainly involved in their improved pubertal status. Unfortunately, there are many aspects of metabolism that have not yet been characterized in early weaned heifers. Therefore, the objectives of this study were to: 1) evaluate the effects of early weaning and feeding a high concentrate diet on indices of reproductive performance, and 2) to determine if early weaning and feeding a high concentrate diet alters the heifers' responses to metabolic challenges.

### *Materials and Methods*

All procedures were approved by the Virginia Tech Institutional Care and Use Committee (#14-039). Animals were housed at Smithfield Horse Center and Kentland Farm (Virginia Polytechnic Institute and State University, Blacksburg, VA) on pasture, dry lot, or bedded pens depending on the phase of the experiment.

### *Animals and Diets*

Thirty-five fall-born Angus x Simmental commercial heifers (born 8/5/12 to 10/13/12) were blocked by sire, stratified by weight, and randomly assigned to either an EW or NW management treatment. Early weaning heifers (n=17) were weaned at  $107\pm 3$  days of age and fed a high concentrate diet for 125 days. The diet consisted of 5 phases, with each phase targeting 0.63 kg/day ADG and constituting approximately 23 kg of weight gain (Table 3.1). Normal weaned heifers (n=18) remained with their dams and were placed on cool-season mixed pasture composed primarily of tall fescue (*Lolium arundinaceum*). They remained on pasture until normal weaning at  $232\pm 3$  days of age. Following normal weaning, EW and NW calves were placed together on pasture of the same forage type (cool-season mixed pasture) with a forage availability of 1,685 to 2,250 kg/ha (Scheffler et al., 2014).

#### *Data Collection Phase 1*

#### *Blood Collection and Analysis*

Blood was collected from all 35 heifers at the time of normal weaning and the week following to analyze for P4. Blood samples were collected from heifers weekly starting at  $261\pm 4$  days of age until they were considered pubertal, which was up to  $429\pm 4$  days of age. Blood was taken for the last time on all 35 heifers when they were  $427\pm 4$  days, at the time of estrous synchronization.

During weekly blood collection, heifers were caught, haltered, and tied, and the jugular vein punctured with a 20-gauge blood collection needle (Monoject; Covidien, Mansfield, MA). Blood was collected (Becton Dickson, Franklin Lakes, NJ) into two 10mL sodium heparin blood collection tubes (Tyco Healthcare Group LP, Mansfield,

MA). Tubes were inverted 5 to 8 times then immediately placed on ice. Blood was transported to the lab where it was centrifuged at 4°C for 20 minutes at 2500 RPM within 2 hours of collection. The plasma was removed, aliquoted in duplicates in labeled polystyrene tubes, and stored at -20°C until it was analyzed. Blood collectors were trained undergraduate and graduate students.

Blood was analyzed for puberty attainment using a Coat-A-Count solid-phase <sup>125</sup>I radioimmunoassay kit (Diagnostic Products Corporation, Los Angeles, CA). Heifers were considered pubertal if blood plasma P4 levels were > 1ng/mL for two consecutive weeks (Gasser et al., 2006a). Samples were allowed to thaw to room temperature. Once thawed, 1000µl of tracer and 100µl of sample were pipetted into propylene tube coated with antibody. Standards with known concentrations of P4 provided in the kits allowed for a standard curve. Samples were run in duplicate along with a high and low standard plasma sample. Samples incubated for three hours, then were placed on a gamma counter to quantify radio labeled P4 against the standard curve. The sensitivity of this assay for plasma is 0.1 ng/mL.

#### *Pelvic Measurements*

Pelvic measurements were taken at 406±3 days of age using a rice pelvimeter (Lane Manufacturing Inc., Denver, CO). One trained student performed pelvic measurements on all 35 heifers. Pelvic height was measured by resting the pelvimeter on the floor of the pubis symphysis and opening to the sacral vertebrae (Van Donkersgoed et al., 1990). Pelvic width was determined by measuring the distance between shafts of the ilium at the widest point. Pelvic height was multiplied by width to determine pelvic area.

### *Ovary Maps*

Ovary maps diagramming ovarian structures were made when heifers were  $409 \pm 3$  days of age. The heifers' left and right ovaries were located via ultrasound (Ibex Pro; EI Medical Imaging, Loveland, CO) and measurements were taken. Measurements that were recorded include: whole ovary height and width; follicle location, number, height, and width; CL height and width. Ovarian structures and measurements were drawn onto diagrams to create ovary maps. The same skilled person performed all the ultrasounding.

### *Breeding*

Two mL of Bovishield Gold (Zoetis, Florham Park, NJ) was administered IM 21 days before breeding. Heifers were synchronized using the Co-Synch + CIDR and timed artificial insemination (AI) protocol. All heifers received 2mL GnRH (Cystorelin; Merial LLC, Duluth, GA) intramuscularly and a vaginal insert (EAZI-BREED CIDR; Interag, Hamilton, New Zealand) containing 1.38 g progesterone. The CIDRs were removed one week later and heifers given 5mL of prostaglandin (Lutalyse; Zoetis Inc., Kalamazoo, MI). Heifers received 2mL GnRH at time of AI. All heifers not pregnant from the first AI were rebred. First service occurred at  $437 \pm 4$  days of age.

### *Data Collection Phase 2*

#### *Animals*

Sixteen pregnant heifers that conceived from the first AI were chosen for the second phase of the experiment. Heifers were separated by sire and weight into two groups of 8. During this time, heifers were fed 2.5% of BW in corn silage (approximately

11.3kg/head/day) and supplemented with free choice orchard grass hay. In preparation for the second phase of the experiment, heifers were haltered and tied in pens 3x/week for approximately 1 hour each time. Heifers were considered halter broke when they were calm while being handled by people and no longer fought restraint.

### *Catheterization*

Heifers were fitted with bilateral indwelling jugular vein catheters. The first replicate group was fitted with catheters, followed by the second group two weeks later. During catheterization, heifers were haltered and tied to the chute. The jugular area was clipped and scrubbed with betadine scrub (Providerm Iodine 10%; Butlerschein Animal Health, Dublin OH) followed by 70% alcohol spray. The jugular vein was punctured with a Medicut intravenous cannula (Tyco Healthcare UK, Northern Ireland, UK), needle removed, and gas sterilized catheter tubing measuring 80cm or 120cm threaded into the jugular vein. Blood was drawn into the catheter to ensure proper placement, then immediately flushed with saline. Catheters were secured to the neck using a butterfly tape and suture. The catheter line was locked with heparin (Sagent Pharmaceuticals, India) in 0.9% NaCl saline (Abbott Laboratories, North Chicago, IL) at 250 units/mL. Excess tubing was contained in a pouch made of mesh that was secured to the neck with suture, or in pouches made of Elastikon (Johnson and Johnson, New Brunswick, NJ). The neck was then wrapped in Elastikon and vetwrap (3M, St. Paul, MN) to protect and secure the pouches. A Velcro elastic girdle was wrapped around the neck for additional security.

Immediately following catheterization heifers were moved to Smithfield Horse Center into individual pens measuring 2.4x3.6 meters. Heifers were assigned to pens in

alternating treatments. Catheters were flushed and all heifers given penicillin. The flush protocol throughout the experiment was: 3mL drawn out of the line and discarded, followed by 4mL of saline flushed into the line, then locked with 4mL of high heparin saline (250 units/mL).

### *Metabolic Challenges*

Heifers were allowed a 3 day adjustment period following catheterization. Heifers were fed mixed orchard grass hay at 0700h, and the amount was adjusted daily based on refusal amounts from the previous day. Corn silage was fed at 1700h at 2.5% of body weight based on weight taken at catheterization. There were no refusals for corn silage. All catheters were flushed 1x/day, unless a catheter lost patency, in which case the opposing side was flushed 2-3x/day.

Three days after catheterization, heifers were administered metabolic challenges on alternate days for 6 days. Heifers were fed the night before and morning of each challenge with no water restriction. Approximately an hour before the challenges began, heifers were secured in their pens and had no access to hay or water. All challenges were performed with the heifers tied in their pen. Samples were all taken from one catheter, however if the catheter lost patency, the opposing side would be utilized. In the second replicate, 3 heifers were sampled in a freestanding alley capable of holding 3 heifers at a time. All challenges started between 0800 and 0900. For every challenge, handlers started sampling heifers in alternating order EW followed by NW.

Each sampling time point followed the same protocol. A waste sample of 4mL was taken and discarded. A 10mL sample was then taken using a different syringe. The

catheter was then flushed with 4mL saline, and then locked with 3mL of low heparin saline (50units/mL) until the next time point. The sample was put into blood plasma collection tubes and immediately placed on ice. For every challenge, samples were taken back to the lab throughout the challenge and centrifuged at 4<sup>0</sup>C 2500 RPM for 20 minutes. Samples were then split into aliquots and stored at -20<sup>0</sup>C until further analysis. Following the last sample, catheters were locked with 4mL of high heparin saline.

The first challenge performed was a glucose tolerance test. Blood samples were collected at -30, -20, -10, 0, 5, 7.5, 10, 15, 20, 30, 45, 60, and 90min relative to glucose administration (Wheelock et al., 2010). At time point 0, heifers were given a 50% dextrose 50% solution (MWI, Boise, ID) at 250 mg/kg of body weight that was immediately chased with 20mL of saline. Ten mL of sample was taken and divided into a sodium heparin coated tube for insulin analysis and a sodium fluoride coated tube for glucose analysis.

An epinephrine challenge was performed two days following the glucose tolerance test (5/10/2014 and 5/24/2014). Blood samples were collected at -30, -20, -10, 0, 2.5, 5, 7.5, 10, 20, 30, 45, 60, 90, and 120min relative to epinephrine administration (Rhoads et al., 2009). At time point 0, heifers were given epinephrine HCl (International Medical Systems, So. El Monte, CA) at 1.4 $\mu$ g/kg BW, which was chased with 20mL of saline. Ten mL of sample was taken via syringe and placed into a sodium heparin coated tube.

A P4 challenge was administered 2 days following the epinephrine challenge. Blood samples were taken relative to P4 administration at -45, -30, -15, 0, 7.5, 15, 30, 45,

60, 90, 120, and 180min (Hutchinson et al., 2012). Progesterone (Watson Pharma, Inc., Parsippany, NJ) was given at the 0 time point at 350ng/kg BW and chased with 20mL of saline. Ten mL of sample was taken with a syringe then placed in a sodium heparin coated tube.

Catheters were removed from heifers immediately following the P4 challenge. Catheterization sites were sprayed with AluSpray (Neogen Corporation, Lexington, KY) and heifers removed from their pens and placed on pasture. Heifers were monitored for infection for 1 week following removal of catheters.

#### *Blood Analysis*

Blood plasma from the glucose tolerance challenge was analyzed for insulin and glucose. Plasma insulin was analyzed with a commercially available bovine insulin ELISA (Mercodia, Uppsala, Sweden). Glucose was measured with a colorimetric assay kit (Autokit Glucose C2; Wako Chemicals, Richmond, VA). Non-esterified fatty acids were measured in the plasma from the epinephrine challenge with a commercially available enzymatic kit (NEFA C kit; Wako Chemicals, Richmond, VA). Blood P4 was measured by commercially available RIA kit, as described above. The intra- and interassay CV values were, respectively, 5.7 and 4.5% for insulin, 4.8 and 5.4% for glucose, 24.3 and 35.9% for NEFA, and 3.7 and 4.4% for P4.

#### *Statistical Analyses*

Data with single observations (days of age at puberty, body weight at puberty, pelvic area, area under the challenge curves) were analyzed for effects of treatment with the GLM procedure of SAS (SAS Institute Inc., Cary, NC). Those with repeated



measurements (challenge time points) were analyzed for main effects of treatment, time and their interaction using the MIXED procedure of SAS. For the MIXED procedures, the compound symmetry, unstructured and autoregressive 1 covariance structures were tested and the most appropriate (lowest Akaike's information criterion, Akaike's information criterion with correction, and Bayesian information criterion values) was used for each analysis. Heifer was included in the model as a random effect. Pregnancy rates were analyzed using the FREQ procedure of SAS

Results of the P4, insulin, and epinephrine challenges indicated that further analyses of the responses were warranted. As such, the area under the curve (AUC) for each individual heifer during each challenge was calculated by use of linear trapezoidal summation between successive pairs of concentrations and time coordinates as described in Rhoads and co-workers, 2009.

Results are reported as least squares means  $\pm$  standard error of mean. Separation of means was conducted with the Tukey procedure of SAS. Statistical significance was noted at  $P < 0.05$  and tendencies were reported at  $P < 0.15$ .

### *Results and Discussion*

Recent research conducted by Gasser and co-workers (2006d) demonstrated precocious puberty attainment in heifers that were EW and fed a high concentrate diet. During the current experiment, both NW and EW heifers came close to precocious puberty ( $310 \pm 7$  days and  $306 \pm 7$  days, respectively) however all heifers reached puberty after 300 days of age. There were no differences between ages at puberty ( $P=0.6973$ ) or body weight at puberty ( $P=0.3993$ ) between treatments (Table 3.2). These results conflict with data presented by Gasser and co-workers (2006c), where age and body weight at

puberty was less for EW heifers fed a high concentrate diet compared to NW heifers and EW heifers fed a control diet. Moriel and co-workers (2014) found EW heifers tended to achieve puberty at lighter body weight than NW, which may be a result of metabolic imprinting and enhanced growth after weaning, instead of the early weaning practice itself. This is supported by a study where heifers were fed *ad libitum* or a restricted diet after normal weaning (Roberts et al., 2009). Heifers fed *ad libitum* achieved puberty at a lighter body weight and tended to achieve puberty at a younger age than those fed a restricted diet. Therefore, earlier age and body weight at puberty may be a direct result of feeding instead of strictly early weaning.

The overall pregnancy rate for all heifers was 77.14%. Early weaned heifers fed a high concentrated diet had a tendency ( $P<0.15$ ) to have higher pregnancy rates at the end of the breeding season in comparison to NW heifers (Table 3.2). This finding is interesting, because days to puberty were not different between treatments and the average age at puberty is well over 100 days before breeding. This is pertinent because heifers are more likely to conceive on their third estrous compared to their first (Byerley et al., 1987). Since there was no difference in age at puberty, EW heifers should not have had more cycles than NW heifers before breeding. Gasser and co-workers (2006c; 2006d) observed that EW heifers fed a high concentrate diet had larger follicle sizes and increased frequency of LH pulses. Prepubertal heifers fed a diet on a higher plane of nutrition had a larger follicle size compared to heifers fed a low plane of nutrition (Bergfeld et al., 1994). Ovulatory follicle size (>10.7mm and <15.7mm in diameter) positively influenced pregnancy rates in heifers induced to ovulate (Perry et al., 2007).

Therefore, it is possible that EW heifer had larger follicle sizes and increased LH frequency, causing increased pregnancy rates.

Despite a tendency for EW heifers to have higher pregnancy rates, no differences were found in whole ovary size ( $p=0.5104$ ) between treatments (Table 3.2). Gasser and co-workers (2006c) reported EW heifers fed a high concentrate diet had greater dominant follicle diameters between 196 and 224 days of age than EW heifers fed a control diet. It is unclear, however, if whole ovary size is an indicator of ovulatory follicle size. There were no significant differences in pelvic area ( $p=0.1991$ ) between treatments.

A treatment x time interaction was detected ( $P<0.01$ ) in the comparison of the means for the P4 clearance analysis (Figure 3.1). The 45-minute timepoint was significantly different ( $P<0.05$ ), and the 15, 30, and 120-minute timepoints tended to be different ( $P<0.15$ ) between EW and NW heifers. The AUC values do not differ, however it was approaching tendency ( $P=0.16$ ) (Table 3.3). Differences in the means could be an indication of changed liver function between the two treatment groups. The NW heifers did not metabolize administered P4 as quickly as the EW heifers. This could be indicative of altered hepatic enzyme concentrations, such as 5 $\alpha$ -reductase, which is responsible for metabolizing P4 to pregnanolone. Nonlactating dairy cows fed a 1.5 x maintenance diet for two weeks had increased liver blood flow and lower blood progesterone concentration 4 hours after progesterone administration (Sangsritavong et al., 2002). Thus, feeding a high concentrate diet following early weaning may cause metabolically-induced changes in both liver blood flow and function that may be responsible for the differences in progesterone concentrations observed during this experiment. Moriel and co-workers (2014) observed that EW *Bos indicus* heifers had decreased amounts of GH receptor 1A

(GHR-1A) compared to NW heifers 90 days after early weaning. There was no difference, however, in GHR-1A between EW and NW heifers at 270 days post early weaning. This supports the hypothesis that EW and feeding a high concentrate diet alters liver function, but to an unknown extent.

There were no significant treatment x time effects ( $P>0.05$ ) for the NEFA response to the epinephrine challenge (Figure 3.2) or glucose concentrations during the glucose tolerance test (Figure 3.4). There were also no differences in AUC (Table 3.3). There was a tendency ( $P<0.15$ ) for NW insulin AUC to be greater than EW insulin AUC ( $291.7 \pm 40.91$  and  $196.7 \pm 38.2$ , respectively), however there was no treatment x time interaction ( $P>0.05$ , Figure 3.3). This indicates that metabolic status for glucose, insulin, and NEFA metabolism is likely unchanged. Waterman and co-workers (2012) observed a tendency for NW heifers to have lower circulating insulin levels compared to EW heifers in the time between EW and NW. However, this tendency was only observed at one location of the experiment. Heifers in a separate location that underwent the same treatment showed similar insulin concentrations.

In conclusion, EW heifers fed a high concentrate diet had an advantage in reproductive function compared to early NW heifers. While EW heifers had slightly higher pregnancy rates, there were no differences in age at puberty, body weight at puberty, or pelvic and ovary size. However, EW heifers have increased P4 metabolism, indicating that EW could have long lasting effects on liver function. Furthermore, there were no differences between insulin, glucose, or NEFA concentrations. This indicates that EW does not likely affect other metabolic functions.

**Table 3.1. Composition of early weaning (EW) diets on DM basis<sup>2</sup>**

	Initial Diet <sup>1</sup>	Final Diet
Corn, cracked	35.0	41.0
Soybean Meal (48% CP)	14.0	-
Distillers grain	15.0	15.0
Wheat middlings	10.0	10.48
Cottonseed hulls	8.0	10.0
Limestone	2.0	2.0
Cane molasses	6.0	6.0
Salt	0.5	0.5
Gorn gluten feed	9.5	14.98
Vitamin ADE premix	0.02	0.0
Rumensin	-	-
Nutrient composition		
Dry matter, %	88.1	87.9
NEm, Mcal/kg	1.96	1.94
NEm, Mcal/kg	1.26	1.23
ADF, %	13.02	14.47
Crude protein, %	19.98	14.05
Calcium, %	0.97	0.96
Phosphorus, %	1.07	0.49

<sup>1</sup>Diets phase fed to early weaning (n=17) heifers from weaning at 107±3 days of age to 232±3 days of age. The early weaning period was divided into 5 phases, with each phase diet formulated for 0.63 kg/d ADG and based on BW of heifers at the start of the phase.

<sup>2</sup>Adapted from Scheffler et al., 2014.

**Table 3.2. Reproductive differences between treatments**

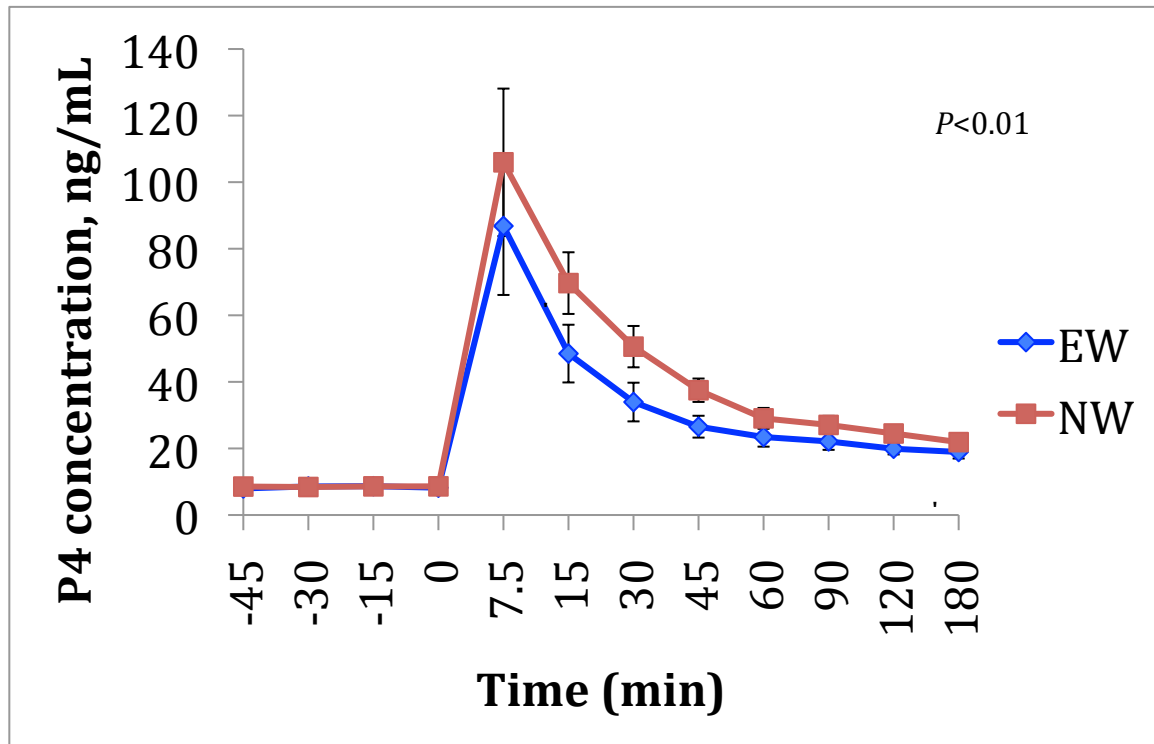
Item	Treatment <sup>1</sup>		P-value
	NW	EW	
Age at puberty, days	309.5 ± 6.8	305.7 ± 6.8	0.6973
BW at puberty, kg	650.2 ± 16.8	629.8 ± 16.8	0.3993
Pregnancy Rate, % <sup>2</sup>	66.67	88.24	0.1288 <sup>†</sup>
Pelvic area, cm <sup>2</sup>	174.6 ± 3.8	181.9 ± 3.9	0.1991
Ovary area, mm	39.3 ± 1.0	38.3 ± 1.1	0.5104

<sup>1</sup>Heifers were either weaned at 107±3 (EW, n=17) and fed a high concentrate diet until 232 days or remained with their dam and weaned at 232±3 days (NW, n=18), followed by comingling of the two treatment groups on pasture.

<sup>2</sup>Pregnancy rate determined at the end of the breeding season, after 2 AI attempts and a herd bull. Pregnancy rate reported as a % of total heifers.

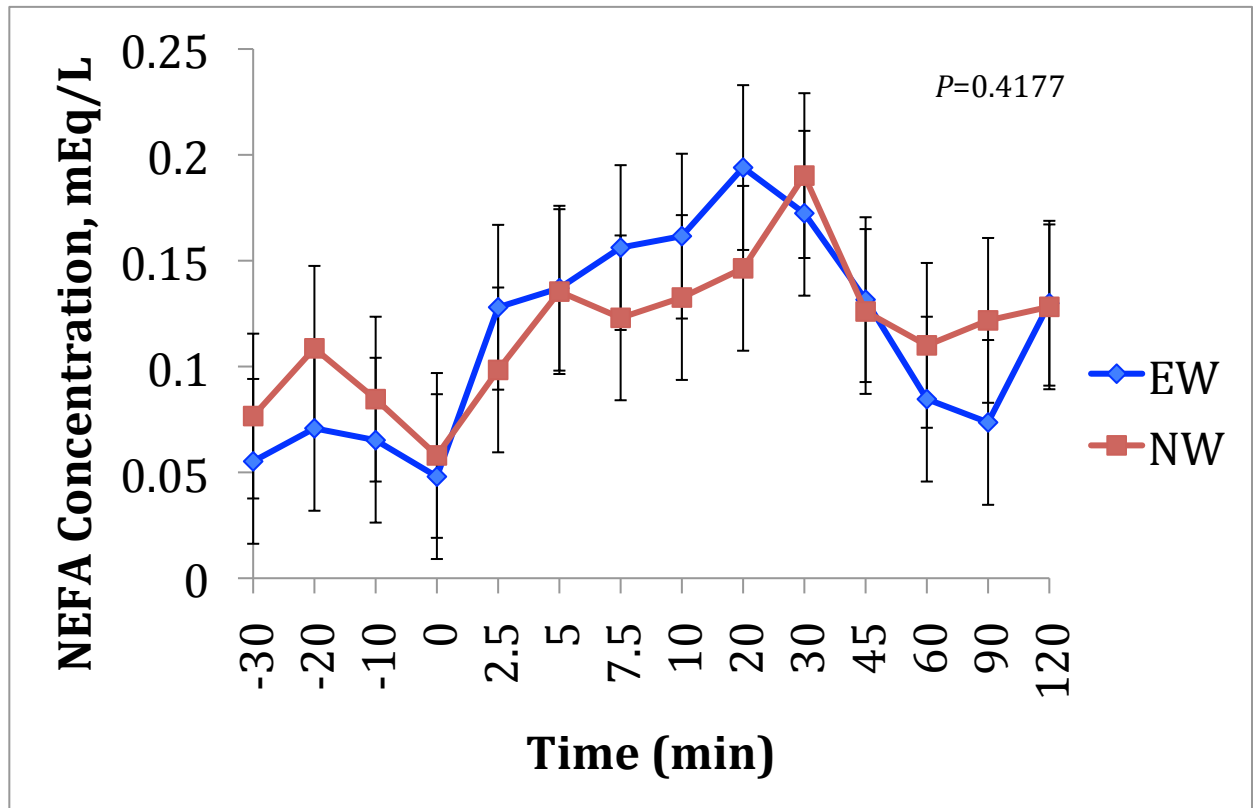
<sup>†</sup>Denotes a tendency ( $P < 0.15$ )

**Figure 3.1 Plasma P4 concentrations for early weaned (EW) and normal weaned (NW) heifers during the P4 clearance analysis.**



Plasma P4 concentrations during a P4 clearance challenge for early weaned heifers (EW, n=17) weaned at  $107 \pm 3$  days and fed a high concentrate diet and normal weaned heifers (NW, n=18) weaned at  $232 \pm 3$  days. Progesterone was administered at timepoint 0. Trt x time  $P < 0.01$ . Within a timepoint, † designates  $P < 0.15$  and \* designates  $P < 0.05$ .

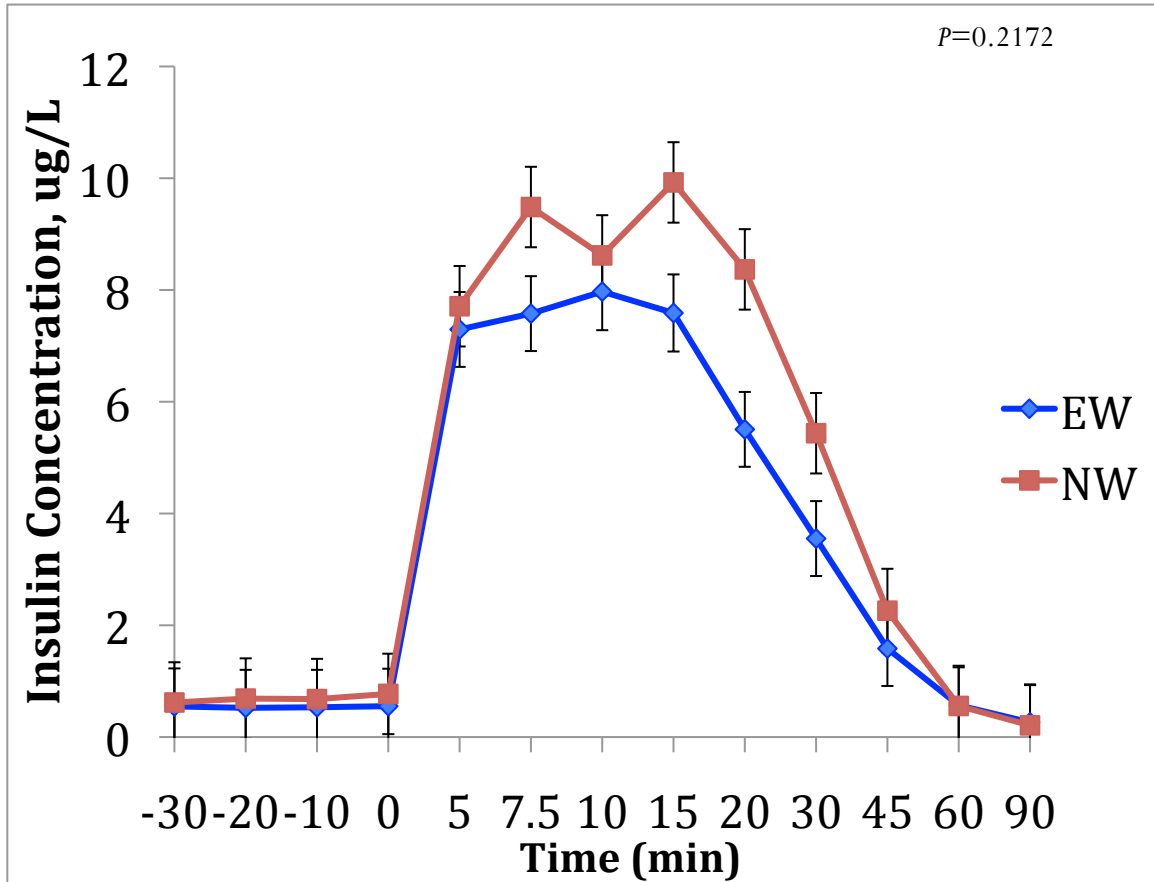
**Figure 3.2 Plasma NEFA concentrations for early weaned (EW) and normal weaned (NW) heifers during an epinephrine challenge.**



Plasma NEFA levels during an epinephrine challenge for early weaned heifers (EW, n=17) weaned at 107±3 days and fed a high concentrate diet and normal weaned heifers (NW, n=18) weaned at 232±3 days. Epinephrine was administered at timepoint 0. Trt x time  $P>0.05$ .

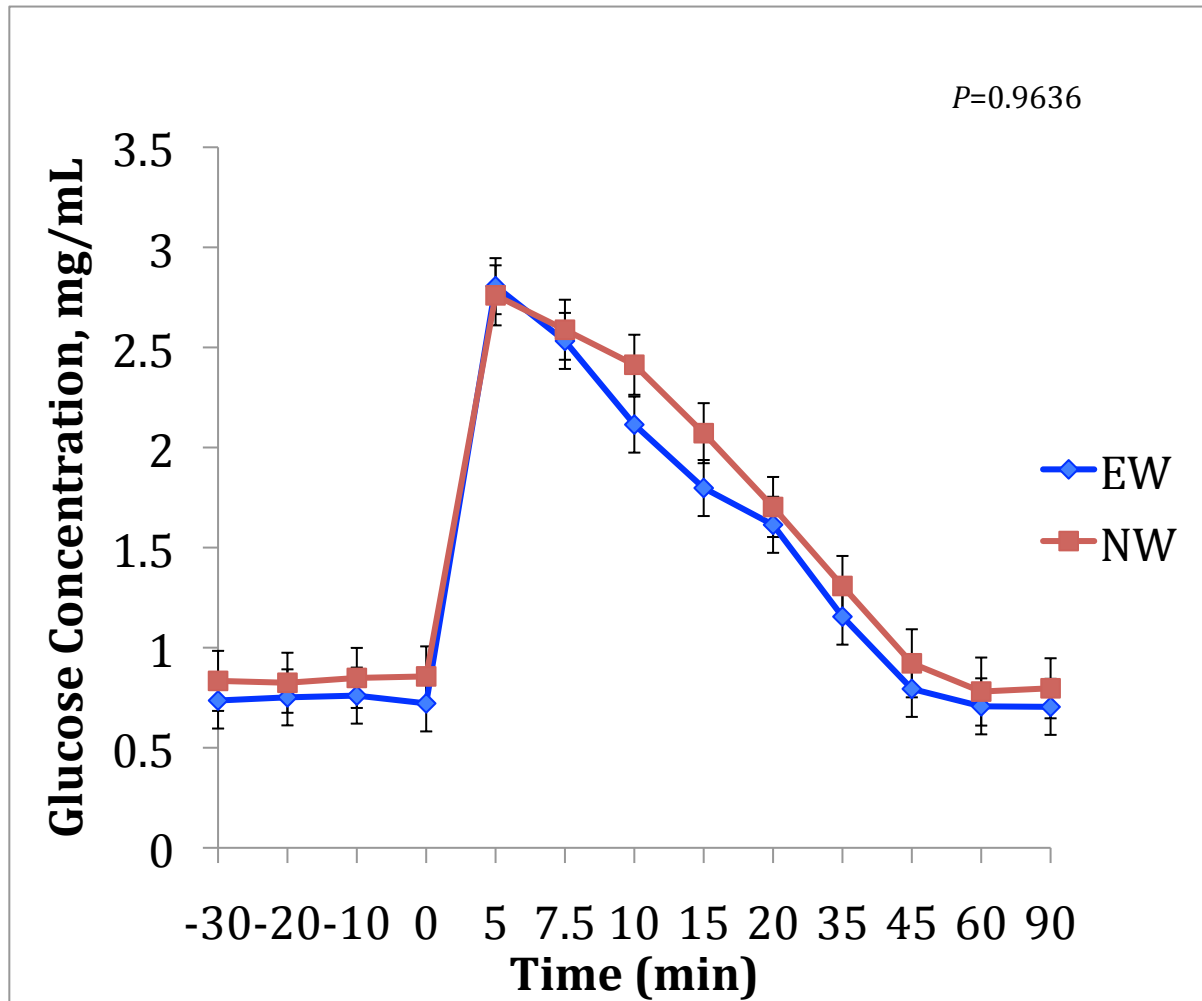


Figure 3.3 Plasma insulin concentrations for early weaned (EW) and normal weaned (NW) heifers during a glucose tolerance test.



Plasma insulin concentrations during a glucose tolerance challenge for early weaned heifers (EW, n=17) weaned at  $107\pm 3$  days and fed a high concentrate diet and normal weaned heifers (NW, n=18) weaned at  $232\pm 3$  days. Dextrose was administered at timepoint 0. Trt x time  $P>0.05$ .

**Figure 3.4 Plasma glucose concentrations for early weaned (EW) and normal weaned (NW) heifers during a glucose tolerance test.**



Plasma glucose concentration during a glucose tolerance challenge for early weaned heifers (EW, n=17) weaned at  $107\pm 3$  days and fed a high concentrate diet and normal weaned heifers (NW, n=18) weaned at  $232\pm 3$  days. Dextrose was administered at timepoint 0. Trt x time  $P>0.05$ .

**Table 3.3 Area under the curve (AUC) values for metabolic challenges**

Item <sup>2</sup>	Treatment <sup>1</sup>		P-value
	NW	EW	
P4	4704.0 ± 642.9	3405.7 ± 601.3	0.1641
Glucose	37.5 ± 6.09	43.8 ± 5.45	0.4701
NEFA	5.53 ± 0.88	6.86 ± 0.88	0.3189
Insulin	291.7 ± 40.91	196.7 ± 38.2	0.1136 <sup>†</sup>

<sup>1</sup>Heifers were either early weaned (EW, n=17) at 107±3 days of age and fed a high concentrate diet or normal weaned (NW, n=18) weaned at 232±3 days of age. Heifers were subjected to a P4 clearance test, glucose tolerance test to measure glucose and insulin response, or epinephrine challenge to measure NEFA response.

<sup>2</sup>Units for AUC are arbitrary

<sup>†</sup> denotes a tendency  $P < 0.15$

## Chapter 4

### Application of intrafollicular insemination *in situ* and *in vivo*

#### *Abstract*

A new assisted reproductive technique (ART), IFI, is the direct deposition of semen into the preovulatory follicle on the ovary. Intrafollicular insemination has proven possible in cattle and humans (López-Gatius and Hunter, 2011; Tocci and Lucchini, 2011). It is unknown, however, where fertilization in the reproductive tract takes place during this procedure. The objective of this experiment was to determine if fertilization is possible in the follicle and if successful pregnancies can result from this procedure. For experiment 1, ovaries were obtained from an abattoir and injected (n=12 and n=11 for each replicate) with frozen-thawed or processed semen with or without the addition of FSH. Ovaries incubated overnight and follicles were aspirated the following day. Oocytes were examined for possible fertilization under a phase contrast microscope or processed for scanning electron microscopy. Sperm was observed in close association with the granulosa cells, however it was unclear if penetration of the zona pellucida and fertilization occurred. In experiment two, non-lactating dairy cows of multiple breeds and parities (n=10) were synchronized in two replicates for estrus and underwent timed IFI. Pregnancy was evaluated with a transrectal ultrasound 30 days after insemination. One of the cows injected had her follicle aspirated 5 hours after injection. The oocyte was recovered, but no sperm were visible. None of the cows (0%) in this study became pregnant. Overall, the results from the experiments were inconclusive, and it remains

unknown whether IFI causes oocyte fertilization in the follicle and can result in successful pregnancies, despite previous reports of success in dairy cattle.

### *Introduction*

Intrafollicular insemination is a recently developed ART that has been utilized in humans (Tocci and Lucchini, 2011; Lucchini et al., 2012), horses (Eilts et al., 2002), and cattle (López-Gatius and Hunter, 2011). It is a method for directly delivering sperm to the oocyte by injecting it into the preovulatory follicle. In humans, it is an ART that is utilized to bypass certain infertility factors, such as endometriosis or male factor infertility (Lucchini et al., 2012). In livestock, it has been applied to circumnavigate certain subfertility problems, such as mating induced endometritis in the mare (Eilts et al., 2002) and heat stress in the cow (López-Gatius and Hunter, 2011).

The site of semen deposition during natural service in the cow is the fornix vagina (Senger, 2012). Traditional artificial insemination allows for semen deposition in the uterine body or deep in the uterine horn which, under optimal conditions, results in approximately the same rate of pregnancy success as natural service (López-Gatius, 2000). Traditional intrauterine artificial insemination is a straightforward procedure that is relatively non-invasive, and under most circumstances, there would be no need to employ more complex procedures. When breeding subfertile cattle, however, intrafollicular insemination has proven to be more successful than intrauterine insemination (pregnancy rates of 23.5% vs. 9%, respectively; (López-Gatius and Hunter, 2011). These cows were selected because of low fertility rates during the warm season (10% pregnancy rate with AI) compared to cool season breeding (33% pregnancy rate

with AI). Similar results have been observed in humans, with 29% of females becoming pregnant after IFI as opposed to 11% after uterine insemination (Tocci and Lucchini, 2011). In cattle, one successful pregnancy was even reported following intraperitoneal insemination (Lopez-Gatius et al.).

Despite having produced successful pregnancies, there are several aspects of this procedure that are not completely understood. Even though the sperm first encounter the oocyte in the ovarian follicle, it is unclear when and where fertilization actually occurs: are the sperm capable of fertilizing the oocyte in the follicle, or does fertilization instead take place after ovulation in the oviduct? Related questions include whether the follicle fluid is able to cause capacitation of the sperm, and if the sperm can penetrate the cumulus cell layer prior to ovulation.

Because of its unique method of sperm delivery, IFI can be utilized in both production and research applications. Intrafollicular insemination allows sperm to bypass travelling through the cervix, uterus, and oviduct, thereby removing sperm transport as a barrier to fertilization. As such, this technique could be developed to provide a novel research tool for scientists studying female fertility. The aim of this study was to determine whether, after IFI, sperm bind to the oocyte within the follicle and if we could successfully produce pregnancies using IFI.

## *Materials and Methods*

### *Overview of Experiment 1*

Intrafollicular fertilization was first attempted in abattoir ovaries. Bovine ovaries were obtained from an abattoir (Brown Packing Co; Gaffney, SC) located 3.5 hours away

and transported in warm saline with antibiotics. All ovaries were obtained during March, April, and May, reducing likelihood that the cattle had experienced heat stress prior to slaughter. Upon return to the lab, ovaries were washed in saline repeatedly until there was little blood left, and excess fat and tissue was trimmed. Ovaries with dominant follicles measuring <20mm were selected for injection. For every ovary, only one follicle was injected. Each injection went through the stroma of the ovary and the needle pushed into the antrum of the dominant follicle. After injection, ovaries were kept in individual containers in saline at 37°C for 24 hours. Needles were capped and remained in the ovaries to prevent leakage of the follicle fluid from the injection site. Follicles that had been injected were aspirated the following day and follicular fluid searched for the oocyte in phosphate buffered saline.

Twelve ovaries were split into 4 injection treatment groups. Six ovaries were injected with sperm that underwent the Percoll wash method (Swanson et al., 1995). Briefly, frozen semen straws (Select Sires; Plain City, OH) were thawed, emptied into a 15 mL conical, and underwent centrifugation on a 45%/90% Percoll gradient. The pellet was collected and resuspended in Sperm-TALP, then recentrifuged at 1000 X g for 5 minutes and suspended in IVF-TALP. The concentration was  $3 \times 10^7$  spermatozoa/mL. The spermatozoa were then split into 2 aliquots with one aliquot receiving 100ul of FSH. The dominant follicle of three ovaries was injected as previously described with Percoll-washed spermatozoa with FSH (P+FSH). The dominant follicle of three other ovaries was inseminated with Percoll washed spermatozoa with no added FSH (P). Each follicle was inseminated with 0.2mL of washed spermatozoa, or approximately 6 million sperm. The other six ovaries were treated with unprocessed frozen-thawed semen (Select Sires;

Plain City, OH) from proven bulls. The concentration was  $7.4 \times 10^7$  spermatozoa/mL. Fifty  $\mu$ L of FSH was added to one mL of semen (S+FSH), which was added evenly to three follicles, or approximately 24.6 million sperm per follicle. Frozen-thawed semen without FSH (S) was added to the remaining three ovaries. The three S ovaries received 1mL (74 billion sperm), 0.9mL (66.6 billion sperm), and 0.1mL (7.4 billion sperm) of semen. One ovary from each treatment group also received live dead sperm stain, which had no noticeable effect. Follicles were aspirated the following day and the oocytes were placed in a fixative of 2.5% gluteraldehyde and processed for scanning electron microscopy to evaluate sperm attachment (SEM).

Ovaries were obtained from an abattoir and a total of 11 ovaries were inseminated. All ovaries received frozen-thawed semen (Select Sires; Plain City, OH) from proven bulls. Follicle stimulating hormone was added to the semen at 100ul/mL. Nine ovaries received 0.25ml of semen and FSH (S+FSH). Two ovaries received 0.25ml semen and no FSH (S). All oocytes were photographed with a phase contrast microscope and then placed in fixative of 2.5% gluteraldehyde in phosphate buffered saline and processed for SEM.

### *Experiment 2*

The Virginia Tech Institutional Animal Care and Use Committee approved all procedures. The study was performed at the Virginia Tech Beef Barn in August of 2013. Ten non-lactating dairy cows of different parities, breeds, and ages from the Virginia Tech Dairy were used.

### *Synchronization*



The estrous cycles of dairy cows were synchronized in two replicates (n=10 and n=8, respectively) according to the ov-synch protocol (Pursley et al., 1997). The same cows were used for each replicate. Briefly, cows were given 2 mL of the GnRH analog Cystorelin (Merial LLC, Duluth, GA) on day 0. On day 7, cows received 5 mL of Lutalyse (Zoetis Inc., Kalamazoo, MI), followed by 2 mL of Cystorelin approximately 30 hours later. Cows were inseminated on day 9 by timed IFI.

### *Insemination*

Cows were trans-rectally ultrasounded with a 5-MHz probe for dominant follicles on the ovaries. Only cows with follicles greater than 11mm were used for insemination. The tail head was clipped and cleaned with iodine scrub and 70% ethanol in preparation for an epidural block. The proximal intercoccygeal space was punctured with a 20-gauge 1-inch needle at an approximate 45° angle. Five mL of lidocaine hydrochloride 2% was injected into the epidural space and time was allowed for the lidocaine to take effect (Rhoads et al., 2008).

Insemination of the follicle was guided with an Aloka 500V ultrasound scanner with a 7.5-MHz probe encased in a transvaginal custom made handle. The handle allowed the probe to sit at a 30° angle in relation to the needle guide, which ended at the base of the probe head. A 17-gauge aspiration needed was placed in the needle guide at time of insemination.

The vulva was washed clean with a betadine solution. The probe was coated in sterile lubricant, then inserted into the vagina and placed against the fornix vagina. The vaginal wall was pierced with the needle either to the left or right, depending on the

location of the dominant follicle. The needle was pushed through the stroma of the ovary and into the antrum of the dominant follicle. Each cow received approximately 0.1mL of semen deposited into the dominant follicle. The needle was withdrawn from the needle guide, and the transvaginal probe removed from the cow. In the first replicate, the injected follicle was aspirate 5 hours after injection and collected fluid was placed into a heparin blood collection tube. In the second replicate, pregnancy was diagnosed 30 days post insemination with transrectal ultrasonography. The same trained person did all ultrasound procedures.

## *Results*

### *Results from Experiment 1*

An oocyte was recovered from every follicle injected with semen. Oocytes were photographed under a phase contrast microscope at 20x or 40x to evaluate presence of sperm. The oocytes recovered from the P+FSH had a compact cumulus cell layer and did not show any visible sperm. Two of the oocytes from P treatment had no visible sperm, while 1 oocyte had one visible sperm.

Two of the oocytes from S+FSH had visible sperm present in the granulosa cells. All three oocytes recovered had a compact layer of cumulus cells. One of the S oocytes had no cumulus cells and no sperm present. Another from the same treatment had a compact cumulus layer and possible sperm visible. The third had an extremely dense cumulus layer and no sperm were apparent.

The only oocyte recovered from SEM was an S treated oocyte. There were too many granulosa cells to view the oocyte clearly and there were no visible sperm present.

All oocytes were recovered from the dominant follicles injected with sperm. Six of the 9 S+FSH had spermatozoal tails present in the cumulus cell layer (Figure 4.1). One of the S treated oocytes had visible spermatozoa, while the other had no cumulus cells and no spermatozoa present (Figure 4.2).

Only 3 (S+FSH) of the 11 oocytes were fully processed for SEM. The rest were lost in the process of preparing the samples. One oocyte appeared to be flattened and deflated under the scanning electron microscope, while another oocyte had no visible sperm present on the oocyte. The third sample had spermatozoa present in the granulosa cells of the oocyte (Figure 4.3).

### *Results from Experiment 2*

In the first replicate, only 2 of the 10 cows were successfully injected. Ultrasound difficulties occurred during 3 injections, which resulted in failed injections. Two of the cows did not respond to synchronization. Two of the cows did not respond to the Lidocaine epidural injections. One cow's follicle ruptured during injection. An oocyte was recovered from only one cow five hours after the follicle had been inseminated (Figure 4.4). There was no sperm found in the cumulus cells of the COC. The other successfully injected cow was not subjected to the attempted oocyte recovery five hours post insemination, but was not pregnant at the 30-day pregnancy check.

In the second replicate, five of the eight cows were successfully injected with semen into the follicle. Two cows did not respond to the synchronization treatment. One cow's vagina filled with air when inserting the transvaginal probe, making insemination impossible. None of the cows (0%) were pregnant 30 d post insemination.

## *Discussion*

Intrafollicular insemination has proven to be a successful ART for achieving pregnancy in cattle (López-Gatius and Hunter, 2011). It remains to be seen, however, whether sperm can successfully fertilize the oocyte in the follicle. The images taken during the *in situ* experiments show that sperm closely associate with the granulosa cells after insemination. The ability of this association to produce a successful embryo, however, remains to be seen. It was not confirmed whether or not the sperm bind to the zona pellucida in the follicle after IFI. While none of the cows in this study became pregnant, the number of successful injections was low, which could account for the 0% pregnancy rate.

The procedures for this study were slightly different from those done by Lopez-Gatius and co-workers (2011), which could account for the 0 percent pregnancy rate. There were also far fewer cows used (n=8 compared to n=50). Cows were bred off standing heat and the CL was less than 10 mm instead of using synchronization. For both studies, however, frozen thawed unprocessed semen was used.

An interesting question of this ART is where in the female reproductive tract the oocyte is fertilized. There are a number of possibilities. The first, most obvious possibility is that the oocyte is fertilized in the follicle before ovulation. When depositing a high concentration of sperm into the follicle, a concern for polyspermy arises. Since the oocyte is still protected by granulosa cells in the follicle, it is possible that this acted as a blockade and helped prevent polyspermy. Another option is the sperm follow the oocyte into the oviduct after ovulation. One possible way to determine if oocytes are fertilized in

the follicle or oviduct would be to aspirate the inseminated follicle after the LH surge but before ovulation and mature the oocyte using *in vitro* maturation. If the oocyte cleaves and matures to a blastocyst, then it is likely that the oocyte is fertilized in the follicle. One final option is that the sperm backflows out of the follicle during needle removal. Since intraperitoneal insemination has occurred, this also seems like a likely possibility (Lopez-Gatius et al.). Lopez-Gatius and coworkers (2011), however, noted there was no change in follicle size after the removal of the injection needle, suggesting that there was limited leaking of the sperm from the follicle.

Bovine follicular fluid contains compounds capable of causing capacitation and the acrosome reaction in sperm (McNutt and Killian, 1991; Thérien et al., 2001). Human follicular fluid has been shown to capacitate sperm as well (Fakih and Vijayakumar, 1991). Preliminary data from our laboratory shows that oocytes matured in bovine follicular fluid have expanded cumulus cells and when fertilized develop to the blastocyst stage. The LH surge causes cumulus cells surrounding the oocyte to expand (Assidi et al., 2010). This expansion causes increased competency of the oocyte. Sperm use chemotaxis to locate the cumulus cell oocyte complex (COC) during normal fertilization, and then penetrate the COC (Ikawa et al., 2010). This COC contains a number of useful factors that promote fertilization, such as TNF- $\alpha$ -induced protein 6 and pentraxin 3. When these factors are removed, reduced fertilization rates occur due to decreased COC integrity (Ikawa et al., 2010). Waiting until COC expansion and the LH surge for insemination of the semen into the follicle might help promote fertilization rates in IFI. There was no sperm present in the oocyte that was aspirated from one of the follicles successfully injected *in vivo*. There was only a 5-hour period from injection to aspiration, and it is

possible that the sperm were not capacitated in that time. If the sperm were not capacitated, they would be unable to fertilize the oocyte. Since IFI has proved more successful than intrauterine insemination in cattle and humans, understanding this process can help increase pregnancy rates in women and cows exhibiting infertility.

Some other factors to consider when applying IFI is the temporal relation between injection and the LH surge. Prior to the LH surge, oocytes are arrested in the diplotene stage of meiosis I (Romero-Arredondo and Seidel Jr, 1994). This arrest is characterized by germinal vesicle that contains a nucleolus with chromosomes partially condensed (Zhang and Xia, 2012). Once the LH surge occurs, the germinal vesicle breaks down and the oocyte resumes meiosis. Meiotic arrest is also dependent on high levels of cAMP within the oocyte. Before the LH surge, cAMP levels are high. This causes high activity in protein PKA, which blocks maturation promoting factor (MPF) and prevents meiosis from resuming. Lutenizing hormone acts to increase phosphodiesterase 3 (PDE3) by blocking cGMP. Increased PDE3 levels inhibit cAMP activity. This decreases PKA and allows MPF to activate and cause the resumption of meiosis (Gilchrist et al., 2008). When utilizing IFI, insemination needs to occur in the same time period as the resumption of meiosis.

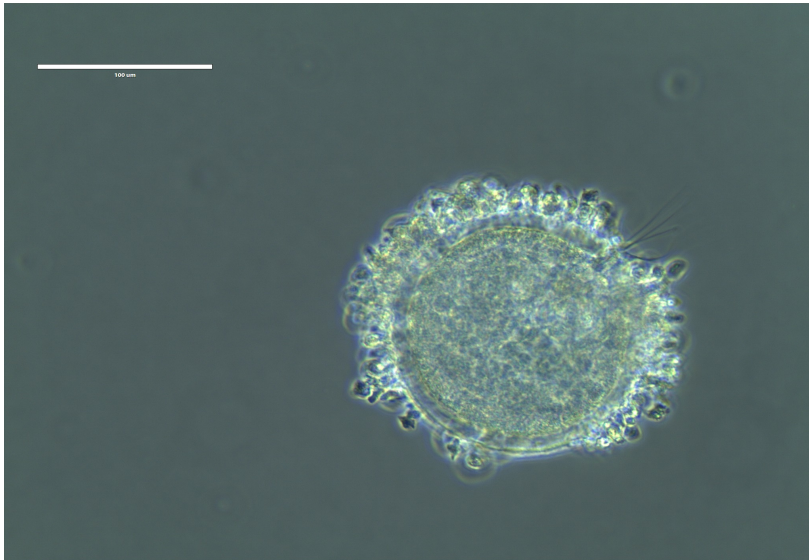
One other concern to consider is the possibility of an immune response from the cow caused by injecting sperm into the follicle. In one study in humans, serum was obtained at time of IFI and 4 weeks later to determine the presence of antisperm antibody (Zbella et al., 1992). Both samples tested negative. Lopez-Gatius and coworkers (2011) did not notice an immune response after IFI in cattle, even though blood vessels in the

follicle wall are exposed to sperm and seminal plasma proteins (López-Gatius and Hunter, 2011).

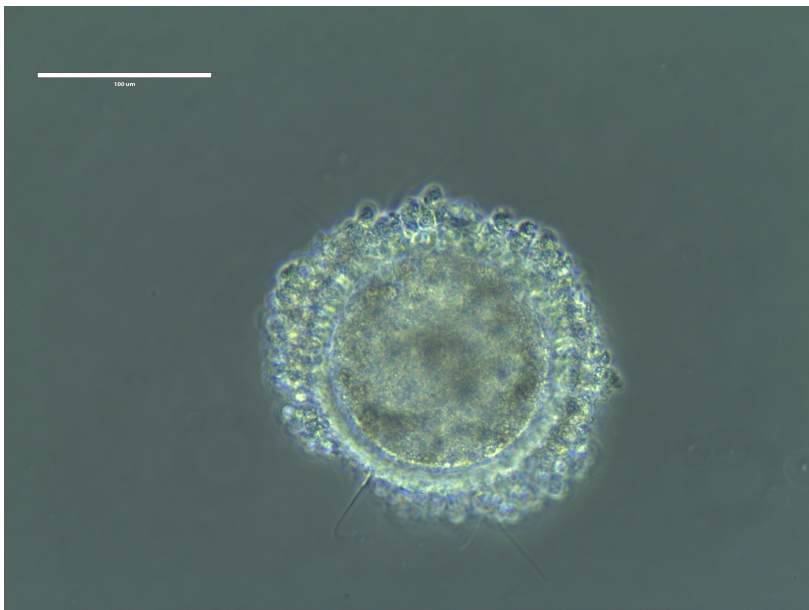
In conclusion, it remains unknown if sperm can bind to the zona pellucida and cause successful fertilization in the dominant follicle. Since successful pregnancies have been produced from this technique, it is possible the sperm bind to the oocyte before ovulation or simply follow the oocyte to the oviduct before fertilization. This process is beneficial to cows and women with low fertility, and a further understanding of this process is necessary to increase pregnancy rate in subfertile animals. It also would be a useful tool for scientists to study female infertility once the mechanisms become elucidated.

**Figure 4.1 Images of two oocytes recovered from treatment S+FSH.**

(a)



(b)

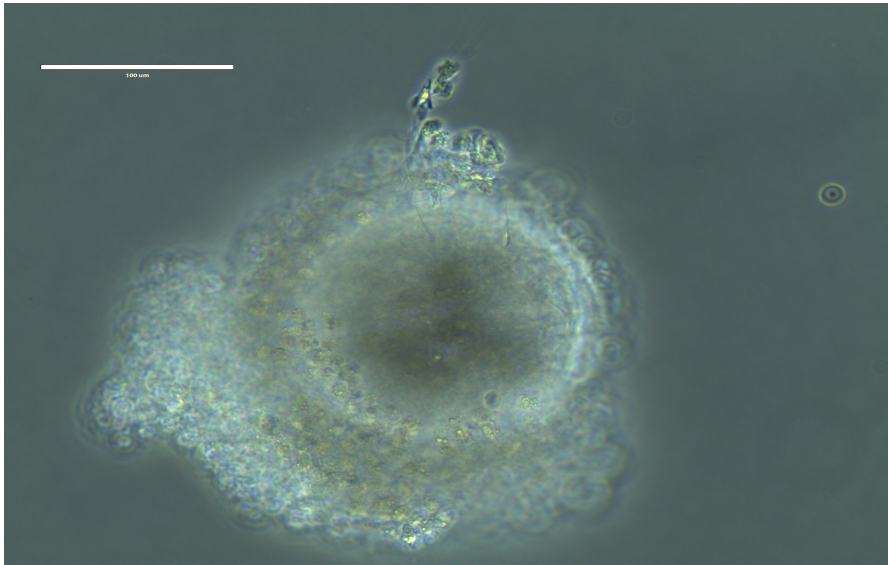


Phase contrast image of oocytes from follicles injected with semen and FSH. Sperm is visible in close association with the granulosa cells in image (a) and (b). Scale bar 100  $\mu$ m

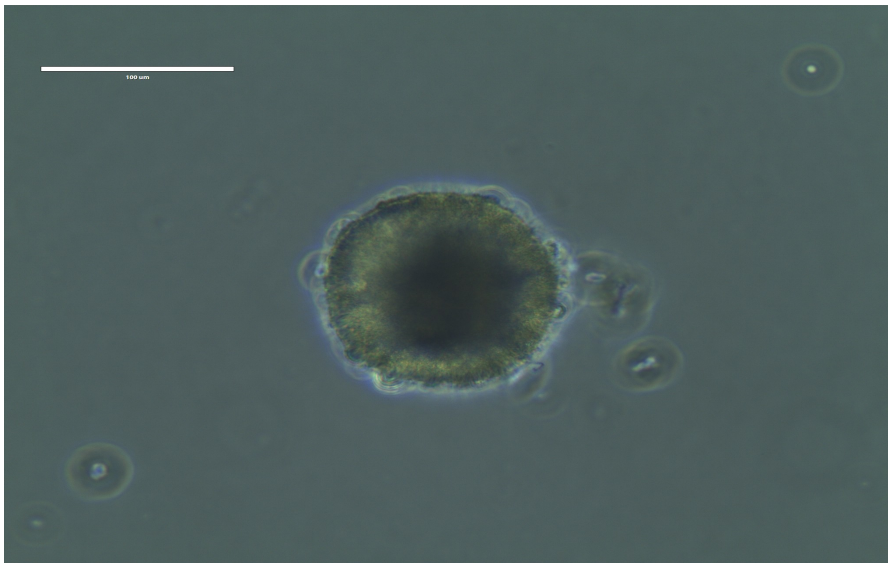


**Figure 4.2 Images of two oocytes recovered from treatment S.**

(a)



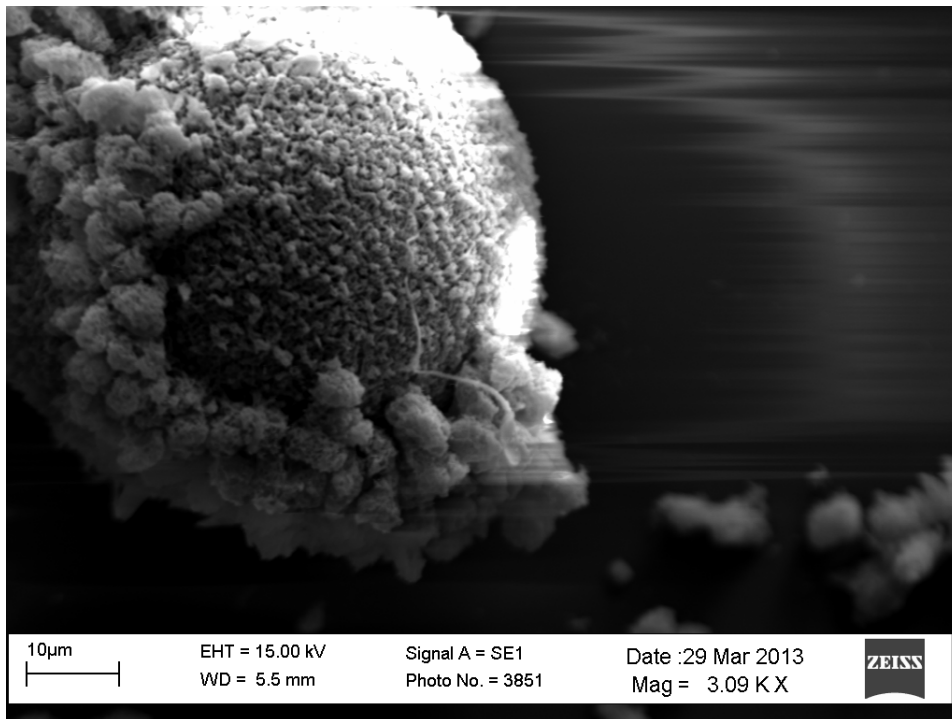
(b)



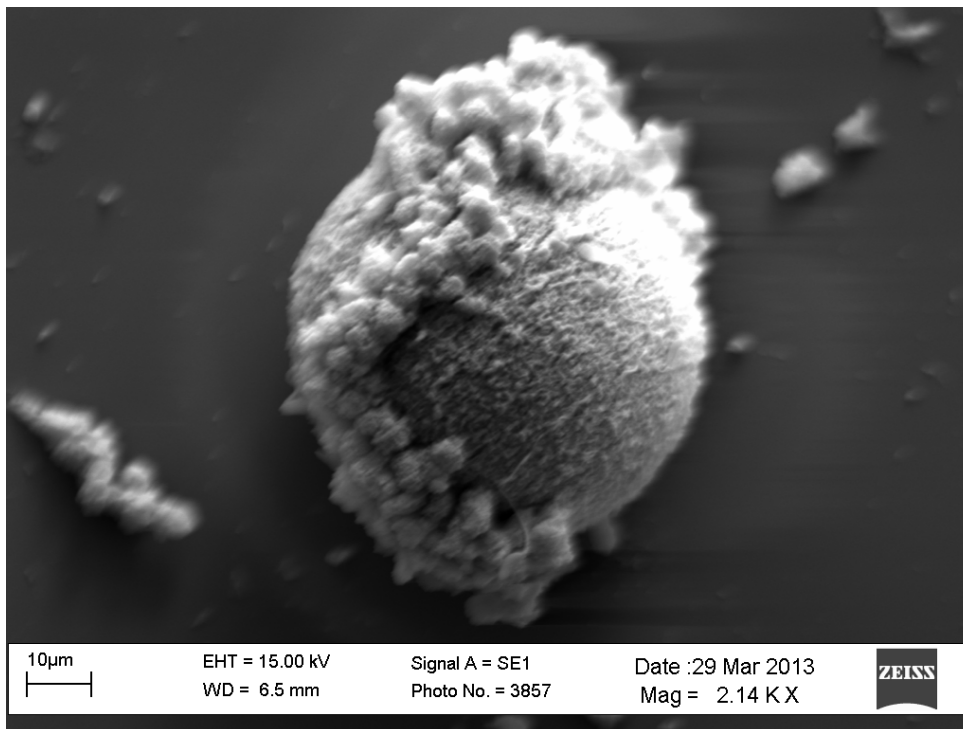
Phase contrast microscopy of oocyte incubated in the follicle after injection of semen without FSH. Image (a) shows sperm in close association with the granulosa cell. Image (b) shows no sperm and a degrading cumulus layer. Scale bar 100  $\mu\text{m}$ .

Figure 4.3 Scanning electron micrographs of Figure 4.1 (b).

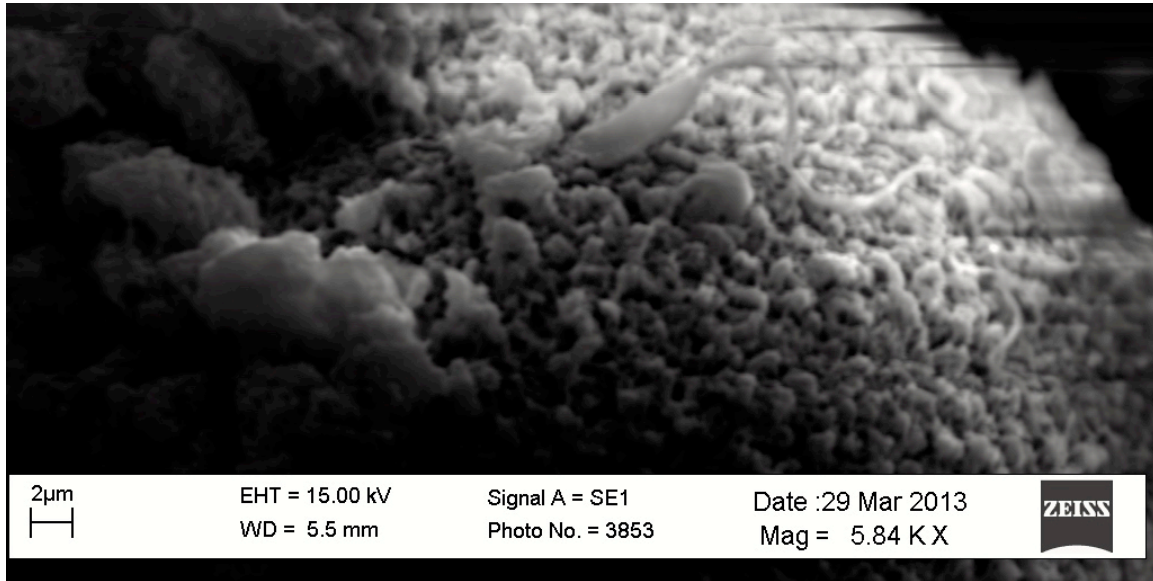
(a)



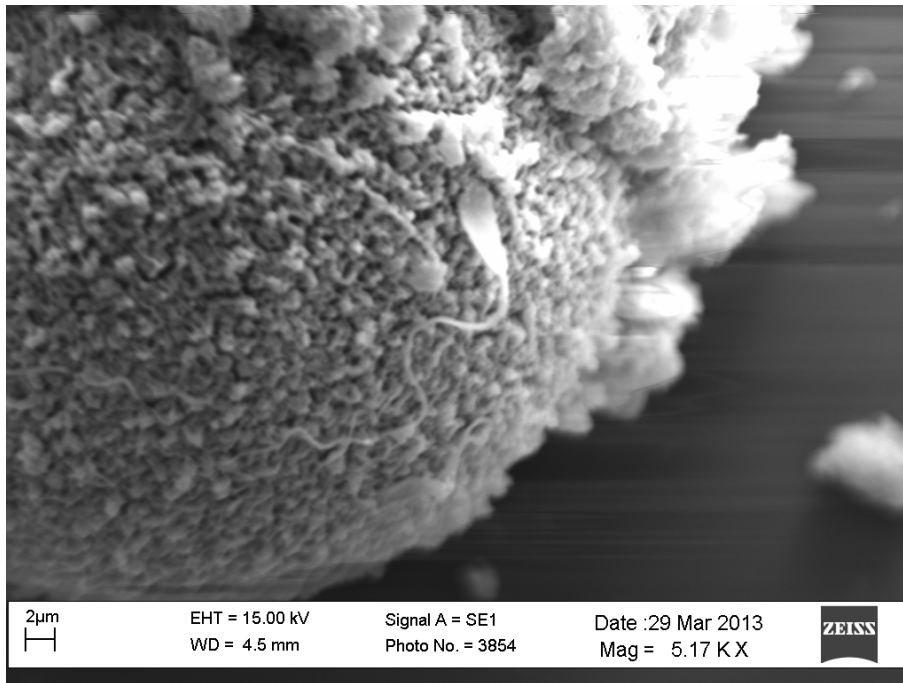
(b)



(c)



(d)



Images (a) and (b) show whole oocyte and possible sperm attachment. Scale bar 10  $\mu\text{m}$ .  
Images (c) and (d) are magnified images of sperm attachment. Scale bar 2  $\mu\text{m}$ .

**Figure 4.4 Image of an oocyte recovered after IFI and follicle aspiration.**



Phase contrast image of the oocyte recovered 5 hours after injection. There is no sign of sperm penetration in the expanded cumulus layer or zona pellucida. Scale bar 100  $\mu\text{m}$ .

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