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South Dakota State University

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THE EFFECT OF TRIPTORELIN ON OVULATION RATE AND CONCEPTION  
RATE IN GILTS AND THE ENDOCRINE PROFILE IN NON-PREGNANT AND  
EARLY PREGNANT GILTS

By

Joseph Wollbrink

A thesis submitted in partial fulfillment of the requirements for the

Master of Science

Master of Animal Science

South Dakota State University

2020

## THESIS ACCEPTANCE PAGE

Joseph Wollbrink

This thesis is approved as a creditable and independent investigation by a candidate for the master's degree and is acceptable for meeting the thesis requirements for this degree.

Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

Jeffrey Clapper

Advisor

Date

Joseph P Cassady

Department Head

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Dean, Graduate School

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## ABSTRACT

THE EFFECT OF TRIPTORELIN ON OVULATION RATE AND CONCEPTION  
RATE IN GILTS AND THE ENDOCRINE PROFILE IN NON-PREGNANT AND  
EARLY PREGNANT GILTS

Joseph Wollbrink

2020

Triptorelin is a gonadotropin releasing hormone agonist that has been shown to be effective in weaned sow single fixed time artificial insemination protocols.

Administration of triptorelin 96 h after weaning has been shown to be effective in synchronizing sows to be inseminated one time 24 h later without reducing pregnancy rate or the number of pigs born. In the current experiment, 23 crossbred gilts (249 d, 123 kg) were administered an intramuscular injection of PG600. Nine days after PG600 administration, gilts were fed 15 mg of altrenogest once daily for 16 d. Twelve gilts (OVU) were administered 200 µg triptorelin 96 h after the last altrenogest feeding. A single artificial insemination was then performed regardless the expression of estrus 126 h after the last altrenogest feeding. Eleven gilts (CON) were inseminated upon the expression of standing estrus and received a second insemination 24 h later. Blood was collected on d 0 (day of triptorelin administration), 1, and 2 after the administration of triptorelin for the measurement of serum concentrations of estrogen and progesterone by RIA. Estrus detection was performed daily beginning 3 d following the last altrenogest administration and ended 7 d after the last altrenogest administration. Gilts were

slaughtered on d 33 when reproductive tracts were collected. Reproductive tracts were evaluated for the number of fetuses in the uterus and the number of CL on the ovary. No differences ( $P>0.05$ ) were found in pregnancy rate, number of fetuses, number of CL, and the ratio of fetuses to CL in all animals. When non-pregnant gilts were removed from the analysis there was a trend ( $P=0.08$ ) for the CON group to have a greater ratio of fetuses to CL. Expression of standing estrus was also greater ( $P<0.05$ ) in the CON group than OVU group. No differences ( $P>0.05$ ) were found in serum concentrations of estrogen between OVU and CON gilts. The CON group's expression of estrus occurred around the time when progesterone was at the lowest in the group. The OVU had its lowest serum concentration of progesterone earlier than CON group. This may indicate that the OVU group could have ovulated earlier than the CON group. Data from the first experiment has demonstrated that even though conception rates of fixed timed AI protocols involving GnRH agonists are similar to standard AI, the conception rate versus ovulation rate may show differences in the two protocols. When comparing the ovulation rate with conception rate, our experiment has shown that GnRH agonists, accompanied by a single fixed timed AI, may not be effective in maximizing the number of conceptuses in gilts. Many of the gilts in this experiment failed to become pregnant even though standing estrus was exhibited in the CON group.

Anterior pituitary and blood samples from these gilts were collected to determine serum concentrations of insulin like growth factor (IGF) and luteinizing hormone. Anterior pituitaries were also used to determine the expression of IGF receptor, gonadotropin releasing hormone (GnRH) receptor, luteinizing hormone beta ( $LH-\beta$ ), IGF binding protein -2, 3, and 5. Anterior pituitaries and serum samples were collected from

15 pregnant and 8 non-pregnant gilts on d 33 after AI. Serum concentrations of estradiol and progesterone and AP concentrations of LH and IGF-1 were determined by RIA. Relative expression of GnRHR, LH- $\beta$ , IGF-1, IGFBP-2, IGFBP-3, and IGFBP-5 were determined using real time reverse transcriptase PCR. Fold changes in relative expression were determined using the Relative Expression Software Tool. Non-pregnant gilts were assumed to be undergoing the luteal phase of their reproductive cycle. Our data supported this because no differences ( $P>0.05$ ) were determined in serum concentrations of progesterone or estradiol between non-pregnant and pregnant gilts. Mean AP concentrations of LH were greater ( $P<0.05$ ) in the pregnant gilts than non-pregnant gilts. Mean AP concentrations of IGF-1 were not different ( $P>0.05$ ) between pregnant and non-pregnant gilts. Mean relative expression of LH- $\beta$  was .8 fold lower ( $P<0.05$ ) and IGFBP-2 tended to be 0.8 fold lower ( $P=0.095$ ) in pregnant gilts compared to non-pregnant gilts. No differences were found ( $P>0.05$ ) between pregnant and non-pregnant gilts in relative expression of AP GnRHR, IGF-1, IGFBP-3, and IGFBP-5.

Data from the second experiment have shown that changes in multiple endocrine factors, such as LH and the IGF system, may play a crucial part in maintaining early pregnancy but further investigation is needed.

Keywords: Triptorelin, Ovulation rate, Conception rate, Insulin like growth factor, Luteinizing hormone, Swine.

## Chapter 1

### Literature Review

#### Fixed Timed Artificial Insemination Background

Fixed timed artificial insemination (FTAI) is an emerging technology of interest to the swine industry. This technology could greatly benefit the swine industry; particularly swine units utilizing batch farrowing systems and niche markets (Brussow et al., 2009; Knox et al., 2018). The beef and dairy industries have used this technique for over 40 years and have reported variations in pregnancy rates. Use of FTAI in the cattle industry has led to the reduction of estrus detection protocols and has improved genetics in dairy and beef herds (Suadsong, 2011; Baruselli et al., 2018; Wiltbank and Pursley, 2014). The use of FTAI would guarantee that sows and gilts have been bred and placed into groups, thus reducing labor and semen costs while maintaining good reproductive performance (Knox et al., 2018; Stewart et al., 2010).

Labor is one of the major expenses encountered in the swine industry (Sharma et al., 1997). If labor required for artificial insemination (AI) could be reduced by 50% this would allow for more skilled and trained AI technicians to perform AI; resulting in better conception rates and reproductive performance (Knox et al., 2018). Another advantage of FTAI, is the possibility of a safer environment for employees because boars would not be needed during the insemination process (Ulguim, 2018). Removal of boars would also lead to savings in terms of space, feed and associated veterinary supplies (Knox et al., 2018).

Producers employing batch farrowing systems could also benefit by using FTAI. The use of batch farrowing systems is increasing because larger weaned groups are needed. It also enables all in/all out procedures on the sow farms for better biosecurity (Bown, 2006). Batch farrowing management systems require a group of estrus synchronized sows and gilts be available for artificial insemination at scheduled times (De Rensis and Kirkwood, 2016). One of the disadvantages of batch farrowing is if estrus is not observed and AI is not performed, the producer must then wait until the next standing estrus (Knox et al., 2018). The swine industry has attempted to solve this problem through the use of synthetic hormones and GnRH (gonadotropin releasing hormone) agonists. Some examples of these hormones are progestogens, GnRH agonists, equine chorionic gonadotropin (eCG), human chorionic gonadotropin (hCG) and porcine luteinizing hormone (pLH). Most of these hormones have been shown to be very effective in the synchronization of estrus and ovulation (Stevenson and Davis, 1982; Davis et al., 1979; Kraeling et al., 1981; Davis et al., 1985; Estienne and Hartsock, 1998; Bates et al., 1991; Knox and Tudor, 1999). In order for FTAI to be effective, synchronization of ovulation must be accomplished. Currently one of the most effective ways to synchronize ovulation in swine is through the use of GnRH agonists.

### **Timed AI in Cattle**

The use of FTAI in cattle has resulted in more desirable genetics and the ability to match the demands of beef and dairy products on a per herd basis. There are two types of FTAI protocols currently being used in the cattle industry; progesterone (P4) releasing device used with either an estradiol (E2) (South America and Australia) or GnRH (North America, Europe, New Zealand) administration (Bo et al., 2016). Two common protocols

used in North America are OV-synch in the dairy industry and Co-synch in the beef industry. Both methods use an initial GnRH injection, then a prostaglandin injection 5 days later, and lastly a GnRH injection either at FTAI which is 72 h later for Co-Synch protocol (Bridges et al., 2008) or a GnRH injection at 56 and FTAI 16 h later for Ov-Synch protocol (Wiltbank and Pursley, 2014). The objective of these protocols is to synchronize the follicular waves, thus synchronizing estrus and ovulation and eliminating the need for estrus detection (Martinez et al., 2000; Pursley et al., 1995; Wiltbank and Pursley, 2014). The use of GnRH has been demonstrated to induce ovulation in both dairy and beef cattle. Results vary as to when ovulation occurs in cattle but it has been previously reported that ovulation occurs between 24 to 32 h after administration of various GnRH agonists (Rajamahendran et al, 1998; Mattos et al., 2001; Pursley et al., 1995). One major concern for the cattle industry is the variable conception rates associated with the use of FTAI. Several case studies across the world have demonstrated that FTAI conception rates in both cows and heifers can range anywhere from 5-100% with averages being approximately 40-60% (Geary et al., 2001; Bormann et al., 2006; Taponen, 2009; Sani et al., 2011; Bo et al., 2016).

The use of conventional AI in the swine industry has yielded greater results in conception rates compared to that of the cattle industry. This protocol involves once or twice daily estrus detection with inseminations occurring at estrus detection and then every 24 h until standing estrus is no longer observed (Knox et al., 2013). Use of this method has led to farrowing rates anywhere from 65-91% with averages of 77- 85% over the past 10 years (Knox et al., 2013; Koketsu et al., 1997; Knox, 2014; King et al., 1998).

### **GnRH Agonist use in Swine Industry**

Gonadotropin releasing hormone agonists are used in the swine industry to control the initiation of the LH (luteinizing hormone) surge to time when ovulation occurs (Schneider and Brussow, 2006; Esbenshade and Britt, 1985; Brussow et al., 1996; Möllerholtkamp et al., 1995). Accurate timing of ovulation is vital for successful planning of artificial insemination resulting in pregnancy (Knox, 2015).

A goal of swine producers using GnRH agonists, is to farrow more pigs per sow per year. The first GnRH agonist reported to induce ovulation in gilts was Gonadorelin (Fetagyl) (Baker et al., 1973). One possible method to produce more pigs per sow per year, is by the use of Gonadorelin to induce ovulation and conception of prepubertal gilts. When GnRH was administered in combination with pregnant mare serum gonadotropin (PMSG) to 160 d old gilts, all gilts ovulated (Guthrie, 1977). Gilts injected with GnRH at this same age, yet determined to be pubertal, were also found to have an increased ovulation rate compared to controls (Archibong et al., 1987). Another method to produce more pigs per sow per year, is with the administration of GnRH during lactation. Ovulation occurred in response to a single 500 µg injection of Gonadorelin on day 14-17 postpartum (Guthrie et al., 1978). Standing estrus was also observed in 50, and 100% when giving GnRH in an intravenous pulsatile manner every 2 h to lactating sows. These experiments were, however, conducted at 24 d and 30 d post parturition respectively (Cox and Britt, 1982).

The timing of the GnRH agonist administration is key to the timing of ovulation in sows and gilts. Martinat-Botte et al. (2010) found that an intramuscular injection of 10 µg of Buserelin to sows at 94 h post weaning resulted in a decrease in the variability of the timing of ovulation to 142±1.9 h. It was also found that gilts given Buserelin 104-



120 h after the last dose of a progestogen resulted in ovulation 144-168 h later (Martinat- Botte et al., 2010). Another study concluded that administration of Buserelin earlier at 77 h post weaning led to ovulation in sows at 109-121 h (Driancourt et al., 2013). In the same study, the dosage was validated by comparing 6, 10 and 16 µg of Buserelin. No difference in ovulation rate was detected in sows or gilts regardless of dosage.

Goserelin (Fertilan) is another generic GnRH agonist used in Germany for the induction of ovulation in multiple farm species. Studies performed by Brussow et al. (2007) have revealed that administration of 20 µg of Goserelin led to gilts ovulating within 34 to 42 h post administration. This study was accompanied by the administration of eCG 24 h after last dose of progestogen (Brussow et al., 2007). Goserelin has also been shown to induce a preovulatory like surge of LH (Brussow et al., 2007; Peltoniemi et al., 1995) but under chronic administration led to LH suppression due to GnRH receptor down regulation (Peltoniemi et al., 1995).

Porcine luteinizing hormone is not a GnRH agonist but is an endogenous hormone that can be administered to induce ovulation. Exogenous administration of LH in sows and gilts has resulted in the shortening of the timing of ovulation for the use of single FTAI successfully. Research has shown that administration of 5 mg pLH to sows and gilts 72-80 h after receiving 600 IU of eCG shortened the span of ovulation to 34-42 h (Garcia et al., 2007; Gama et al., 2005; Casser et al., 2005; Bennett-Steward et al., 2007; Viana et al., 2002). Administration of pLH after synchronization with an oral progestogen and eCG still led to ovulation at 35-45 h after pLH administration (Degenstein et al., 2008). When pLH was administered at the onset of estrus with two FTAI or single FTAI there were no differences in farrowing and pregnancy rates (Zak et

al., 2009; Fontana et al., 2014) and there was also an increase in litter size and litter weight (Zak et al., 2009). Ulguim et al. (2014), also tested a submucosal administration of pLH in the vulva of gilts followed by a single FTAI at 16 h after administration. Pregnancy rates have varied throughout these experiments in which it was first reported that no difference occurred in pregnancy (Ulguim et al., 2014) and then in a subsequent experiment resulted in lower farrowing rate in pLH gilts (Ulguim et al., 2016).

In general, GnRH agonists and pLH could be useful for single FTAI protocols in swine production however, many are not approved for use in the United States. In the United State OvuGel is currently the only ovulation inducing hormone approved for use in the swine industry.

### **OvuGel and Triptorelin**

Intravaginal triptorelin has now been tested in numerous species other than the pig for the induction of ovulation (Viudes-de-Castro et al., 2007; Sinclair et al., 2017). Rabbits are induced ovulators but the exact mechanism of induction is unknown (Dal Bosco et al., 2011; Morrell, 1995). Administration of Buserelin has been reported to induce ovulation in does and increase pregnancy rates (Quintela et al., 2004; Dal Bosco et al., 2011). The use of an intravaginal GnRH was first tested using buserelin as a substitute for semen extender in rabbit does. There was no difference between intramuscular and intravaginal administration except the intravaginal dosage required a greater dose of Buserelin (Quintela et al., 2004). Intravaginal triptorelin administration was then compared with the intramuscular Buserelin and was reported no differences in ovulation or pregnancy rates were observed. Triptorelin was reported to have a high ovulation induction (70-74%) and a pregnancy rate (74%) greater than the control group

which received just extender and semen (32.5%). (Viudes-de-Castro, 2007). These studies have demonstrated that addition of GnRH agonists to semen doses are effective in the induction of ovulation in does.

When replacement gilts enter the herd depends on the sow barns gilt flow. In a 20 group (weekly farrowing) system, replacement gilts are brought into herd as they exhibit standing estrus. In batch farrowing systems however, gilts undergo synchronization of estrus to match weaned sow groups (Terlouw and Johnston, 2017). The best method to date is the use of an oral progestogen for 10-14 d or more to synchronize standing estrus within 3-10 days after last feeding of progestogen (Davis et al., 1979; Stevenson and Davis, 1982; Estienne et al., 2001; Wood et al., 1992; Kraeling et al., 1981). Researchers have reported success with the use of progestogens and FTAI breeding on days 5, 6, 7 after last progestogen (Davis et al., 1985). The goal is to have a single FTAI for utilization of superior genetics and timing of AI (Terlouw and Johnston, 2017; Knox et al., 2017).

Triptorelin is a GnRH agonist used for the induction of ovulation in swine and humans. The first experiments using a triptorelin analog were performed on male chimps and showed an increase in both LH and testosterone (Doering et al., 1980). Tests then involving the administration of triptorelin to human females with ovulation disorders resulted in the increased induction of ovulation (Canales et al., 1980; Itskovitz-Eldor et al., 2000). The swine industry developed a product known as OvuGel containing the active ingredient triptorelin acetate. The product is unique, because it is administered intravaginally and is the only FDA approved product in the USA for the induction of ovulation in swine (Dillard et al., 2018).

OvuGel (triptorelin acetate) was shown to have contributed to greater reproductive success in weaned sows using a FTAI method (Ulguim et al., 2018; Knox et al., 2017; Knox et al., 2018). This product has been labeled for administration at 96 h post weaning and a subsequent single fixed time artificial insemination 22 +/-2 h regardless of whether the sow exhibits estrus (Knox, 2014). Results from these trials have shown no ill effects on pregnancy rates or number of piglets born using a single FTAI (Ulguim et al., 2018; Knox et al., 2017; Knox et al., 2018) while the use of two doses has shown to be effective as well (Knox et al., 2011). A key difference between the synchronization of sows using OvuGel and traditional synchronization with weaning is that more sows ovulated in the OvuGel group between the time of 96h after weaning (when OvuGel is administered) to 144h (Knox et al., 2018; Knox et al., 2011).

The use of oral progestogens is a common method to synchronize gilts into sow herds. Knox et al. (2017a) examined when ovulation occurred in gilts after the last administration of progestogen, and they concluded that the range in the number of days after last progestogen administration prevents the application of a single FTAI protocol. Knox et al. (2017) reported that ovulation can occur from 136 h to 200 h after last progestogen feeding regardless of when OvuGel was administered. However, their results demonstrated that OvuGel administered at 120 h after last progestogen feeding resulted in the synchrony of ovulation to occur 24-48 h post treatment in 81% of gilts (Knox et al., 2017). Kraeling et al (2013) reported higher ovulation rates though prior to this study. Their study involved gilts given different doses of triptorelin at 120 h and had a 95% ovulation rate within 48 h regardless of treatment (Kraeling et al., 2013)

Studies using the GnRH agonist triptorelin has been reported to be effective in both sows and rabbit does. More research is, however, necessary to develop a protocol for gilts in order to better induce the exact timing of ovulation.

### **Ovulatory Factors**

Under normal conditions the sow and gilt will ovulate on average 70% of the way through standing estrus (Soede and Kemp, 1997). The use of GnRH agonists has been shown to decrease the variability of the timing of ovulation in sows (Martinat-Botte et al., 2010, Knox et al., 2011) and gilts (Brussow et al., 2007; Knox et al., 2017) however, this does not ensure that ovulation will occur during these designated times. This can be due to a sow's or gilt's follicles failing to reaching a preovulatory size at time of GnRH agonist administration (Bracken et al., 2006; Knox et al., 2018) but other factors have been shown to affect the estrus to ovulation interval and ovulation rate.

One factor that has been well documented to affect the estrus to ovulation interval (EOI) in sows is the wean to estrus interval. Numerous researchers have found a correlation between an extended wean to estrus interval and a shorter EOI (Kemp and Soede, 1996; Nissen et al., 1997; Knox et al., 2002; Belstra et al., 2004). Knox and Zas (2001) demonstrated that sows exhibiting estrus day 6-8 post weaning had an EOI 16 h shorter than sows exhibiting estrus on day 3 post weaning. The reason behind longer wean to estrus intervals is not fully understood but some factors that may play a role are ovarian cysts, a lack of ovarian activity (Knox and Probst-Miller, 2004) and poor nutrition (Aherne and Williams, 1992).

A major external factor that may alter the estrus to ovulation interval (EOI) in the sow is season changes. During the summer months, EOI was shown to increase by an average of 8 h (Belstra et al., 2004) and an increase as high as 19 h in multiparous sows (Knox and Tudor, 1999). These effects have also been demonstrated in cattle as heat stress caused low ovulation rates in lactating cows (Wilson et al., 1998). It has also been demonstrated that no differences occurred in swine EOI during the various seasons (Knox and Zas, 2001). In a study conducted by Belstra et al. (2004), one of the three farms on trial exhibited no differences even though sows were exposed to a similar barn setting and environmental conditions. Seasonal changes may then play a role in the EOI but results may vary based on other factors.

Today's genetic lines are selected for the desires of the consumer and the producer for the best quality hogs and pork (Robinson and Buhr, 2005). The selection for both maternal and paternal traits may be linked to variability in EOI. Belstra et al., (2004) found that a maternal crossbreed ( $\frac{1}{2}$  Yorkshire,  $\frac{1}{2}$  Landrace) had a shorter EOI by 4 h compared to a maternal breed crossed with a paternal ( $\frac{1}{2}$  Yorkshire,  $\frac{1}{4}$  Landrace,  $\frac{1}{4}$  Duroc) (Belstra et al., 2004). Various cattle breeds have also been shown to have some variability in EOI across breeds. The bos indicus breed has a 3 h shorter EOI compared to the Bos taurus. Belstra et al (2004) concluded that genotype may impact the EOI however more research is necessary.

Factors that can be managed, such as parity and lactation length, have no effect on EOI (Knox and Zas, 2001; Belstra et al., 2004). Genetics, wean to estrus interval, and seasonality may cause major variability of EOI across farms. These factors could then cause greater variability in the timing of single FTAI after administration of GnRH

agonists. If differences are too variable then this could result in low conception rates and a decrease in number of sows becoming pregnant.

The goal of intravaginal triptorelin in the swine industry is to induce the release of the LH surge from the anterior pituitary (AP) thus inducing ovulation to time when to perform AI in order for the highest pregnancy rate (Knox et al., 2018). Several factors, such as genotype, seasonality, and estrus to ovulation, have been linked to causes of unsuccessful pregnancy (Belstra et al., 2004) yet other factors associated with endocrinology could affect pregnancy. The pituitary secretes essential hormones required for pregnancy in several species including the pig (Kraeling and Davis, 1974; Anderson et al., 1967) yet information relating to anterior pituitary function during early pregnancy is limited.

### **The hypothalamus and pituitary**

The hypothalamus and anterior pituitary gland are key controllers in the maintenance of pregnancy in swine. Kraeling and Davis (1974) found that hypophysectomy on day 70-90 of gestation resulted in abortion. Other studies demonstrated that hormones coming from these glands, such as GnRH and LH, to be of vital importance in order for pregnancy to be maintained (Tast et al., 2000; Anderson et al., 1967). During the estrous cycle and pregnancy, expression and concentration of hormones change depending on the gilt or sow's physiological state (Guthrie et al, 1972). Changes occurring in tissue concentration and expression of hormones during pregnancy in the hypothalamus and pituitary have, however, not been well established in the porcine species.

### **Progesterone and Estradiol**

Two hormones associated with maintenance of pregnancy are E2 and P4. Conceptus derived E2 is associated with the maternal recognition of pregnancy in the sow and is necessary in order for pregnancy to commence 11 days after fertilization (Geisert et al., 1990). In the sow, serum concentrations of E2 are low throughout the luteal phase and increased at estrus (Guthrie et al., 1972; Magness et al., 1983). Similar serum concentrations of E2 were present during the first 12 days after insemination as that of the luteal phase and remain low throughout the sow's gestation (Guthrie et al., 1972).

Serum concentrations of P4 change throughout the estrous cycle and are associated with the formation and establishment of the corpus luteum (Masuda et al., 1967; Guthrie et al., 1972). During the first 12 days after insemination, serum concentrations of P4 are similar to the levels exhibited during the luteal phase of the estrous cycle (Guthrie et al., 1972). It was demonstrated that an increase in serum concentrations of P4 occurred up to a peak at day 12 in both pregnant and non-pregnant gilts. The only difference between the estrous cycle and pregnancy is that during pregnancy a gradual decrease occurred until nearing parturition; while during the estrous cycle a sharp decrease to low levels occurs due to luteolysis of the CL (Guthrie et al., 1972).

Serum concentrations of P4 and E2 during the luteal phase of the estrous cycle are similar to levels during the pregnancy. Differences between pregnant and luteal phase levels may exist due to the long term levels and anatomical changes that occur during pregnancy.

### **GnRH protein and receptors**



Gonadotropin releasing hormone is a neuropeptide that regulates gonadotropin biosynthesis and release from the AP using G-protein coupled receptors (Ciftci, 2015; Kim et al., 2011). Researchers found an increase in the porcine species (Clapper and Taylor, 2011) or a controversial decrease (Wylot et al., 2008) in the expression of AP gonadotropin releasing hormone receptor (GnRHR) occurred during the follicular phase compared to the other stages of the estrous cycle. Research in other species demonstrated that the expression of GnRHR in the rodent and bovine was not different during pregnancy and the luteal phase of the estrous cycle (Reeves et al., 1982; Schoenemann et al., 1985). Similar to results found in swine (Clapper and Taylor, 2011), the bovine and rodent species had greater GnRHR expression in the AP during the follicular phase (Reeves et al., 1982; Schoenemann et al., 1985) and at estrus (Schoenemann et al., 1985) than other stages of the estrous cycle. Transcriptional activity of GnRHR was documented to occur in the AP of the pregnant pig (Siawrys and Buchowski, 2018) yet to the best of our knowledge no research has been conducted regarding how pregnancy affects the expression of GnRHR in swine.

### **Luteinizing Hormone**

Luteinizing hormone is the luteotrophic agent in the pig and is responsible for the support of the CL to produce P4 during pregnancy and during the estrous cycle (Tast et al., 2002; Virolainen et al., 2003; Panasiewicz et al., 2004). Parlow et al. (1964) examined pituitary concentrations of LH during various days of the estrous cycle and at 25 d of pregnancy in the pig. They found low levels of LH at estrus (d 1) and the early luteal phase (d 4) and, an increase in LH concentration in the AP during the luteal phase (d 10) that continued to increase during the follicular phase (d 18) (Parlow et al., 1964). Similar

results have been found in other studies conducted on pigs throughout the estrous cycle (Wylot et al., 2008; Clapper and Taylor, 2011). Anterior pituitary gland concentrations of LH in pregnant gilts (d 18-25) was found to be elevated two fold greater than any stage of the estrous cycle (Parlow et al., 1964; Melampy et al., 1966) and a steady decrease continued for the remainder of gestation (Melampy et al., 1966). Results from these swine studies that demonstrated a two fold increase in LH expression during pregnancy are, however, conflicting to what have been shown in other species. Researchers found that in the bovine, ovine, and rat species, AP LH content was either unchanged or was decreased during pregnancy as compared to during the estrous cycle (Chamley et al., 1976; Schoenemann et al., 1985; Schwartz and Bartosik, 1962; Greenwald, 1966).

### **IGF system**

Research has shown that the IGF system has played a critical role in reproduction for both males and females (Neirijnck et al., 2019). Components of the insulin like growth factor (IGF) system have been found in many tissues and cells and have been linked to many different physiological functions (Le Roith, 1999). The IGF system is made up of several components including the IGF polypeptides (IGF-1 and IGF-2), IGF receptors (IGFR type 1 and IGFR type 2), and the IGF binding proteins (IGFBP) (Hwa et al., 1999).

### **IGF-1**

Insulin like growth factor 1 (IGF-1) is a polypeptide with insulin like metabolic effects that is primarily expressed and secreted from the liver yet its mRNA (messenger ribonucleic acid) expression has also been found in many other tissues such as the heart, kidney, and AP (D'Ercole et al., 1984; Escalada et al., 1997; Murphy et al., 1987). The

IGF-1 system is complex as more evidence is presented on its physiological roles and the actions of IGF-1 (Le Roith et al., 2001). One of the first hypotheses for its physiological roles was demonstrated by D'Ercole et al. (1984), in which they presented that IGF-1 is a growth hormone (GH) dependant peptide. Insulin like growth factor 1 concentrations increased in the heart, lung, liver, kidney, and serum of hypophysectomized rats that were injected with ovine GH (D'Ercole et al., 1984).

Insulin like growth factor-1 has also been demonstrated to be a GH independent factor. One way that this has been shown is with the use of gene knockout rodents. Insulin like growth factor 1 plays a critical role in growth and development at the embryonic stage, as shown in IGF-1 knockout mice having birth weights 60% less than their wild-type littermates (Liu, et al., 1993). When the GH gene was knocked out both in mice and the pig there were no differences in birth weights compared to control animals (Zhou et al., 1997; Hinrichs et al., 2018) suggesting a GH independent effect of IGF-1 during embryonic growth (Le Roith et al., 2001). There is also evidence that fertility is dependent on IGF-1 in a GH independent manner. This was demonstrated in the same way as IGF-1 gene knockout mice were infertile while GH gene knockout mice were still fertile (Zhou et al., 1997; Baker et al., 1996).

Other studies have also shown that IGF-1 also has a role in regulating GH production and secretion in a negative feedback loop (Yamashita and Melmed, 1986; Yamashita and Melmed, 1987; Simes et al., 1991). Simes et al. (1991), found that low concentrations of IGF-1 caused less feedback of GH secretion but at higher concentrations of IGF-1, GH secretion was then decreased. Yamashita and Melmed

(1986) supported this by demonstrating pituitary cells exposed to IGF-1 in a dose dependent manner decreased GH secretion and GH gene transcription.

The source of IGF-1 has also been shown to play a crucial role in growth and development (Ohlsson et al., 2000). Most serum IGF-1 was found to come from the liver as a source of endocrine action that may play a role in negative feedback on GH (Ohlsson et al., 2000). The actions of IGF-1 for growth may, however, be from other IGF sources in an autocrine/paracrine fashion (Isaksson et al., 1982; Schlechter et al., 1986). Some of the first research on this hypothesis was conducted by injecting human GH into the tibial epiphyseal cartilage plate of hypophysectomized rats which led to increased growth of the cartilage plate (Isaksson et al., 1982, Schlechter et al., 1986). In support of the autocrine/paracrine action, Schlechter et al., (1986) injected anti-IGF serum and human GH simultaneously into the tibial epiphyseal cartilage plate and found that the growth of this cartilage plate was completely halted. This led them to believe there is a source of autocrine/paracrine actions involved in bone growth (Schlechter et al., 1986). Researchers have also conducted numerous other experiments to support that there is autocrine/paracrine action in numerous species and tissues such as during porcine ovarian follicular growth (Zhou et al., 1996) and tumors (Yoshida et al., 2014).

There are two types of receptors that have been shown specifically to bind to IGFs. The first, IGF type-1 receptor, is a transmembrane tyrosine kinase receptor that mediates the effects of primarily IGF-1 (Adams et al., 2000). The other, IGF type-2 receptor, has been linked specifically to binding with IGF-2 only (Firth and Baxter, 2002). Both of these receptors have a role in the regulation in the binding of IGF to cause

an effect, however, the IGFBP are involved in the regulation of IGF binding to its receptor.

The IGF binding proteins role is essential for the regulation of IGF (Ranke and Elmlinger, 1997). There are at least six IGFBP that have been found to function in the transportation of IGF in plasma, prolong half-life of IGF (Guler et al, 1989), localize tissue specific regions for IGF, and modulate the presentation of IGF to its receptor (Ranke and Elmlinger, 1997; Mohan and Baylink, 2002; Bach, 2018). Many of these IGFBP have been found to inhibit the actions of IGF by inhibiting the binding of IGF to the IGF receptor (Firth and Baxter, 2002; Kim et al., 1997). Insulin like growth factor binding proteins -1, IGFBP-2, IGFBP-3, and IGFBP-5 have been shown to also potentiate the actions of IGF by decreasing the binding affinity for IGF (Firth and Baxter, 2002). These actions of potentiation and inhibition of IGF activity may be dependent upon the tissue and specific IGFBP. In mouse osteoblasts, IGFBP-5 was found to stimulate the mitogenic effect of IGF in the osteoblasts yet IGFBP-3 was found to have an inhibitory effect in the same study (Andress and Birnbaum, 1992). Insulin like growth factor binding protein-5 was also found to have an inhibitory effect on IGF stimulated steroidogenesis in granulosa cells (Liu et al., 1993).

One role that is still being researched is the importance of IGFBP involvement in mediating pituitary function (Roberts et al., 2001). In cattle, it was found that IGFBP in the anterior pituitary had a higher binding intensity for IGF-1 than in the hypothalamus and liver (Funston et al., 1993). It has also been demonstrated that the AP of many species may synthesize and secrete IGFBP-2, -3, and -5 (Roberts et al., 2001; Simes et al., 1991). These secretions may be altered due to the stage of the estrous cycle and the

physiological state of the animal. Funston et al., (1995) documented that there was a positive correlation with serum concentrations of P4 and AP IGFBP-2, -3, -5 in cattle.

## **IGFBP-2**

Insulin like growth factor binding protein -2 was shown to be primarily expressed in the liver, adipocytes, reproductive system, and central nervous system in adults (Wheatcroft and Kearney, 2009). Insulin like growth factor binding protein-2 expression has also been documented in the AP of the pig, rodent, cow, and sheep (Funston et al., 1995; Michels et al., 1993; Clapper and Taylor, 2011; Clapper et al., 1998). All of these species have shown differences in expression of AP IGFBP-2 due to hormonal changes occurring during different physiological states or during the administration of hormones. In the cow, it was shown that during the preovulatory stage levels of AP IGFBP-2 were greater compared to those during the early luteal phase (Roberts et al. 2001). In support of this study, Funston et al., (1995) also found that the binding intensity for AP IGFBP-2 was greater during the early and late follicular phase (11-21 d) compared to early luteal phase (1-5 d) (Funston et al., 1995). The results also coincided with mRNA expression of AP IGFBP-2 in the rodent (Michels et al., 1993) and the relative amounts of the protein in the pig (Clapper and Taylor, 2011). Roberts et al., (2001) also demonstrated that there was an increase in IGFBP-2 secretion from the AP when treated with LHRH in vitro, however, there was no correlation with AP FSH or LH.

Another hormone that could be involved in the stimulation of IGFBP-2 is estrogen. Estrogen has been shown to increase mRNA expression of AP IGFBP-2 of the rodent both in-vitro and in-vivo (Michels et al., 1993) and a tendency to increase in ewes in vivo (Clapper et. al., 1998). The boar has also been shown to have greater serum

concentration of E2 and IGF-1 compared to the gilt and barrow (Clapper et al., 2000) and it was then found that greater relative amounts of IGFBP-2 were found in boar AP compared to barrows (Rempel and Clapper, 2002). In a supporting study, E2 was reduced by an aromatase inhibitor in boars which resulted in decreased relative amounts of AP IGFBP-2 (Hilleson-Gayne and Clapper, 2005).

The AP may secrete IGFBP-2 yet these secretions may not be what is primarily found in the blood. Roberts et al. (2001) documented that serum IGFBP-2 had a larger molecular weight compared to AP IGFBP-2. Evidence to support this is shown in that no differences have been found in serum IGFBP-2 binding activity and relative concentration throughout the estrous cycle and exposure to E2 in various female species (Funston et al., 1995; Molnar and Murphy, 1994; Clapper et al, 1998). Keller et al. (1998) found that during pregnancy serum concentrations of IGFBP-2 tended to be greater than in non-pregnant.

### **IGFBP-3**

IGFBP-3 has been shown to be the major carrier for IGF in serum (Jones and Clemmons, 1995) but the synthesis has been shown to occur primarily in the liver yet research has shown it is synthesized in the AP as well (Simes et al., 1991). During the bovine estrous cycle, the binding intensity of AP IGFBP-3 increased from standing estrus to the end of the luteal phase and then decreased during the follicular phase (Funston et al., 1995). Roberts et al., (2001) had a different outcome in which during the preovulatory period AP IGFBP-3 was highest compared to lowest during the early luteal phase.

Roberts et al. (2001) documented that the serum IGFBP-3 had a larger molecular weight compared to AP IGFBP-3. To the best of our knowledge, serum IGFBP-3 does not change throughout the estrous cycle in the cow and rodent (Funston et al., 1995; Molnar and Murphy, 1994) yet it has been shown to increase in ewes when E2 was administered (Clapper et al., 1998). Scientists have also demonstrated a tendency for pregnancy to decrease serum concentrations of IGFBP-3 in the cow (Keller et al., 1998) and the gilt (Yun et al., 2001).

### **IGFBP-5**

The binding activity of AP IGFBP-5 to radiolabeled IGF in the cow has been documented to be lowest during the early luteal phase with higher amounts throughout the rest of the estrous cycle (Roberts et al., 2001; Funston et al., 1995). Clapper and Taylor (2011) reported that relative amounts of porcine AP IGFBP-5 were greater during the preovulatory phase and then decreased throughout the rest of the estrous cycle. The effect of E2 administration did, however, promote differences to occur in the boar. The boar has more E2 than in the barrow and they had greater relative amounts of IGFBP-5 in the AP (Rempel and Clapper, 2002) and subsequently, when E2 synthesis was inhibited in boars they had less relative amounts of AP IGFBP-5 (Hilleson-Gayne and Clapper, 2005).

Roberts et al., (2001) found that serum IGFBP-5 was not readily detectable while other studies have shown that it does not change throughout the estrous in cattle, pigs, and mice (Funston et al., 1995; Molnar and Murphy 1994) There was no differences in serum IGFBP-5 when ovariectomized ewes were administered E2 (Clapper et al., 1998).



### **IGFBP on reproductive hormone secretion**

One of the functions of the IGFBP is to potentiate (Firth and Baxter 2002) or inhibit (Firth and Baxter, 2002; Kim et al., 1997) the presentation of IGF to its receptor. One of the actions of IGF-1 may be to stimulate the production of gonadotropins from the AP. Researchers found that the treatment of AP cells with IGF-1 in vitro, stimulated the release of the gonadotropins in the pig (Whitley et al., 1995; Barb and Hausman, 2009), rat (Soldani et al., 1994; Kanematsu et al., 1991; Pazos et al., 2004; Xia et al., 2001), and bovine (Hashizume et al., 2002).

During the follicular phase, E2 is increasing with follicular development while progesterone is decreasing as luteolysis occurs (Guthrie et al., 1972). To support the notion that gonadotropins may be influenced by IGFBP, researchers have shown that during the follicular phase and around the time of ovulation AP IGFBP-2, IGFBP-3, and IGFBP-5 expression and binding activity increases (Roberts et al., 2001; Funston et al., 1995; Clapper and Taylor 2011; Michels et al., 1993). The stimulation of IGF-1 activity may also be due to E2 and P4 concentrations. It has been documented in the mouse and ewe that treatment with E2 in vivo caused an increase in AP IGFBP-2 (Michels et al. 1993; Clapper et al., 1998; Roberts et al. 2001). This has also been shown in the pig however, E2 also increased the expression of AP IGFBP-5 in the pig (Rempel and Clapper, 2002; Hilleson-Gayne and Clapper, 2005). In cattle, correlations have also been made between increasing amounts of E2 and increasing IGFBP-2 while increased P4 was correlated with decreased AP IGFBP-3 and IGFBP-5 (Roberts et al., 2001).

### **Conclusion**

The use of a single FTAI protocol would be beneficial in order for pregnancy to be achieved as one of the main causes for non-pregnant sows is the failure of detect estrus (Kraeling and Webel, 2015). Other factors beyond the control of the technicians may also come into play including factors such as hormonal changes. Research has been conducted with several different species examining the changes that occur in the anterior pituitary expression of various hormones and receptors during the estrous cycle and pregnancy. Research on porcine species is limited and some have shown differences in the expression and concentrations of hormones when compared to other mammalian species (Parlow et al., 1964; Melampy et al., 1966). What can be determined is that the gilts or sows estrous cycle or hormonal status can change the hormone expression and concentration of hormones in the hypothalamic and pituitary tissues in order for optimal hormonal conditions for pregnancy to be maintained.

## Chapter 2

### The Effect of Triptorelin on Ovulation Rate and Conception Rate in Gilts

#### Introduction

The use of a single fixed timed AI protocol in the swine industry would be beneficial for scheduling labor (Sharma and Leung, 1996) and the use of superior genetics (Terlouw and Johnston, 2017). The use of fewer doses of semen and at decreased concentrations could, however, lead to a decrease in litter size. This will potentially lower the number of fetuses being conceived to CL count on the ovaries. Wu et al. (1995) reported during their experiment that the average percentage of fetus to CL count was roughly 77.4% on d30 of gestation. Knox et al. (2017) also found that the number of sperm cells used in a single fixed timed AI protocol decreased litter size and also tended to decrease pregnancy rates in sows.

The administration of triptorelin to sows at 96 h post weaning has been found to decrease the variability in the timing of ovulation in sows and thus could allow for a single fixed timed AI protocol to be used (Knox et al., 2017). This protocol has been very well documented to work with no differences in litter size or pregnancy rate with fixed timed AI systems in sows (Stewart et al., 2010; Knox et al, 2014) . The use of triptorelin in gilts for a single fixed timed AI protocol, however, has not been as successful due to the variation in the timing of ovulation. Knox et al. (2017a) demonstrated that 87.4% of gilts were exhibiting standing estrus on days 6-8 after last progestogen feeding and found no differences regardless if animals received triptorelin or the timing when triptorelin was

administered. The interval from triptorelin administration to ovulation was shorter in gilts administered OvuGel at 120 h, however, due to the range in the number of days that the onset of estrus occurred and timing of ovulation occurs would not allow for the application of a single fixed timed AI protocol. In order for a single fixed timed AI protocol to work in gilts the variation in the synchronization of ovulation must be reduced so that AI can take place from 0-24 hours before ovulation (Soede et al., 1995). Inseminations that take place other than in this time period have been shown to lead to decreased litter sizes and pregnancy rates in parities 1 and 2 (Rozeboom et al., 1997).

The hormone profile that occurs after administration of triptorelin is still under investigation. Triptorelin has been found to induce the onset of the LH surge in sows approximately 10 h after administration (Stewart et al., 2010). During this same experiment, estradiol levels were found to be highest 18 h prior to administration and then began to decrease at 26 hours post treatment. To the best of our knowledge, progesterone levels have not been evaluated during the timing of triptorelin administration. Other data has been presented that serum levels of progesterone increased sharply by one standard deviation around 40 h after the onset of estrus (Terqui et al., 2000). Terique et al. (2000) found that there was a linear relationship between an echosonography for detecting ovulation and the increase in progesterone levels.

The use of triptorelin has potential to significantly impact the swine industry in the synchronization of replacement gilts. The objective of this research was to determine the effect of triptorelin and artificial insemination using a single semen dose on the fetus to corpus luteum ratio, and on serum concentrations of estrogen and progesterone.

## Materials and Methods

All experimental procedures were reviewed and approved by the South Dakota State University Institutional Animal Care and Use Committee.

### Animals

Twenty-four crossbred (Duroc x Landrace x Large White) gilts of a similar age ( $249.9 \pm .3$  d) and weight ( $123 \pm 2$  kg) were used in this experiment. Twenty gilts were housed individually in (2.13 m x .61 m) stalls while four were housed in pairs in pens (1.83 m x 2.44 m). Gilts received 2.27 kg of a corn soybean meal based feed (3280 kcal ME/kg, 13.1% crude protein, 0.59% lysine) daily and water was provided ad libitum.

Each gilt was administered PG600 (400 IU equine chorionic gonadotropin and 200 IU human chorionic gonadotropin) five days after entering the designated room. Nine days after administration of PG600, gilts were orally administered 15 mg of altrenogest (Matrix; Merck Animal Health, Kenilworth, NJ) each day for 16 d to synchronize estrus. Gilts were exposed once daily to a mature boar beginning at d 4 after the last administration of altrenogest and continued for four days for the detection of standing estrus.

### Experimental design

Gilts were stratified based on weight and litter to the treatment groups. The control group (CON; n=11) were bred conventionally by artificial insemination conducted at detection of standing estrus and then a second artificial insemination

approximately 24h later. The OvuGel group (OVU; n=12) received 200 µg of triptorelin (OvuGel; United Animal Health, Sheridan, IN) administered into the anterior vagina 96 hours after last altrenogest feeding. OvuGel gilts then received a single artificial insemination 30 h later.

### **Sample collections**

On days 4 (1 h prior to OvuGel administration), 5, and 6 after last altrenogest feeding, blood was collected (10mL) from gilts via jugular venipuncture using a 17 g x 1.38 cm needle. Blood was stored overnight at 4°C then samples were centrifuged (1,500 x g for 30 min at -4°C) and serum collected and stored at -20°C.

All gilts were slaughtered at South Dakota State University Meat Lab  $33.2 \pm .1$  d after artificial insemination at which time reproductive tracts were collected and stored at 4°C until the next day. Blood was also collected (50 mL) at time of slaughter via jugular venipuncture. The number of CL were counted on each ovary, and the uterus was dissected to count the number of fetuses.

One control gilt was removed from the experiment due to an abscess on the left side of the neck prior to experiment being performed. Another control gilt was also removed from data due to serum P4 concentrations indicated that she had previously ovulated (Guthrie et al., 1972).

### **Estradiol-17β**

Serum concentrations of estradiol-17β (E2) were determined in duplicate in all blood samples by RIA (Jolitz et al., 2015). Estradiol-17β (E8875; Sigma Life Sciences, St. Louis, MO) was the standard and radioiodinated E2 (#07138228; MP Biomedicals,

Solon, OH) was the tracer. Antisera (GDN#244 anti-estradiol-17 $\beta$ -6-BSA; Fort Collins, CO) was used at a dilution of 1:425,000. Sera (100  $\mu$ l) was extracted with a 4 mL volume of methyl tert-butyl ether. Recovery of [125I] E2 added to porcine serum before extraction averaged  $92.2 \pm 3\%$ . Inhibition curves of increasing amounts of sample were parallel to standard curve. Intra-assay and inter-assay coefficients of variation were 26% and 22% respectively. Sensitivity of the assay was 0.2 pg/tube.

### **Progesterone**

Serum concentrations of progesterone were determined in duplicate in all blood samples by RIA (Jolitz et al., 2015). Progesterone (P0130; Sigma Life Science, St. Louis MO) was the standard and radioiodinated progesterone (#70-170126; MP Biomedical, Solon, OH) was used as the tracer. Antisera (#111.2C7.3; Enzo Life Sciences, Farmingdale, NY) was used at a dilution of 1:700,000. Samples were diluted 1:40 prior to assay in steroid buffer. Inhibition curves of increasing amounts of sample were parallel to standard curves. Intra-assay and inter-assay coefficient of variation were 7% and 15.4% respectively. Sensitivity of the assay was 3.3 pg/tube.

One control pig was removed from progesterone data due to serum levels of estradiol-17 $\beta$  and progesterone similar to that of the luteal phase (Guthrie et al., 1972).

### **Statistical Analysis**

To determine the effect of triptorelin on serum concentrations of estradiol-17 $\beta$  and progesterone statistical analyses were performed using the Mixed Procedure of SAS 9.4 (SAS 9.4, SAS). The model for determining differences in serum concentrations of estradiol-17 $\beta$  and progesterone was  $Y_{ijkl} = \mu$

+Pig<sub>i</sub>+Treatment<sub>j</sub>+Pig<sub>i</sub>(Treatment)<sub>j</sub>+Day<sub>k</sub>+Treatment<sub>j</sub> x Day<sub>k</sub> + Pig<sub>i</sub>(Treatment)<sub>j</sub> x Day<sub>k</sub> + e<sub>ijkl</sub> with repeated measures. Pig within treatment was used to test the effect of treatment. Pig within treatment was the subplot error term used to test pig, day, and treatment by day effects.

To determine the effect of treatment on estrus expression, day of estrus expression, pregnancy, number of fetuses and corpora lutea, and ratio of fetuses to corpora lutea, statistical analyses were performed using the GLIMMIX Procedure of SAS 9.4 (SAS 9.4, SAS) to compare pregnant and non-pregnant gilts. The model to determine differences in estrus, pregnancy, fetuses, corpora lutea, and ratio of fetuses to corpora lutea was  $Y_{ijk} = \mu + \text{Pig}_i + \text{Treatment}_j + \text{Pig}_i(\text{Treatment})_j + e_{ijk}$ . Pig within treatment was term used to test treatment.



## Results

The number of fetuses and CL, pregnancy rate, and ratio of fetuses to CL in all animals were not different ( $p>0.05$ ) at the time of slaughter (Table 2.1). Expression of estrus was observed more ( $P<0.01$ ) in CON group than in OVU group at the time of insemination (Table 2.1). The expression of estrus tended ( $p=0.08$ ) to be on a later date after last altrenogest in CON than OVU group.

**Table 2.1.** Reproductive performance of control (CON; n=11) and OvuGel (OVU; n=12) gilts

Group	Estrus expression, %	Day* of expressed estrus, n	Pregnancy, %	Fetus, n	Corpora lutea (CL), n	Ratio of fetus/CL, %
CON	81.81 ± 11.96 <sup>a</sup>	5.89 ± .19 <sup>c</sup>	72.72 ± 14.85	9.09 ± 1.86	14.27 ± .74	64.63 ± 12.54
OVU	16.67 ± 11.45 <sup>b</sup>	5.00 ± .40 <sup>d</sup>	58.33 ± 14.22	5.75 ± 1.78	13.17 ± .71	41.33 ± 12.00

Data presented are expressed as least-square means ± SEM. <sup>ab</sup> Values within a column lacking a common subscript differ ( $p<0.05$ ) between groups. <sup>cd</sup> Values within a column lacking common subscript tended to be different ( $p<0.1$ ) between groups. \*Day after last altrenogest feeding.

The number of fetuses, number of CL, and the day estrus was expressed in the gilts that were pregnant was not different regardless of treatment ( $p>0.05$ ; Table 2.2). The percentage of pregnant gilts in estrus at time of insemination was greater ( $p<0.01$ ) in the CON group than OVU group (Table 2.2). The ratio of fetus to CL had a tendency ( $p=0.08$ ) to be greater in the CON group compared to OVU group (Table 2.2).

**Table 2.2.** Reproductive performance of pregnant gilts in control (CON; n=8) and OvuGel (OVU; n=7) gilts

Group	N	Estrus expression, %	Day* of estrus, n	Fetus, n	Corpora lutea, n	Ratio of fetus/CL, %
CON	8	100 ± 11.72 <sup>a</sup>	5.88 ± .21	12.5 ± 1.30	13.88 ± .96	88.86 ± 6.53 <sup>c</sup>
OVU	7	28.57 ± 12.53 <sup>b</sup>	5.00 ± .42	9.86 ± 1.39	14 ± 1.02	70.85 ± 6.98 <sup>d</sup>

Data presented are expressed as least square means ± SEM. <sup>ab</sup> Values within a column lacking a common subscript differ ( $p<0.05$ ) between groups. <sup>cd</sup> Values within a column tend to differ ( $p<0.1$ ) between groups. \*Day after last altrenogest feeding.

Mean serum concentrations of E2 tended ( $p=0.06$ ) to be greater on d 5 than d 6 after last altrenogest feeding in CON group (Table 2.3). No other differences ( $p>0.05$ ) were detected based on treatment or day after last altrenogest feeding (Table 2.3).

**Table 2.3.** Serum concentrations of estradiol-17 $\beta$  in control (CON; n=10) and OvuGel (OVU; n=12) groups

Day after altrenogest, d	CON (n=10)	OVU (n=12)
4	42.68 $\pm$ 4.35 <sup>ab</sup>	46.64 $\pm$ 3.88
5	54.35 $\pm$ 7.57 <sup>a</sup>	39.18 $\pm$ 6.79
6	39.34 $\pm$ 7.03 <sup>b</sup>	38.62 $\pm$ 6.30

Mean serum concentrations of estradiol-17 $\beta$  (pg/mL) in CON and OVU groups from 4 to 6 days after last altrenogest feeding. Data presented are expressed as least squares means  $\pm$  SEM.

<sup>ab</sup> Means with different letters within a column tended to be different between days ( $P<0.1$ )

Mean serum concentrations of P4 tended ( $p=0.06$ ) to be greater in CON group on day 4 than in CON group on day 6 (Table 2.4). Mean serum concentrations of P4 were also greater ( $p=0.02$ ) in CON group on day 5 than in OVU. (Table 2.4).

**Table 2.4.** Serum concentrations of progesterone in control (CON) and OvuGel (OVU) groups

Day after altrenogest, d	CON (n=10)	OVU (n=12)
4	4.51±1.23 <sup>a</sup>	2.03±1.12
5	3.59±0.72 <sup>abc</sup>	1.20±0.58 <sup>d</sup>
6	2.20±0.55 <sup>b</sup>	1.46±.51

Mean serum concentrations of progesterone (ng/mL) in CON and OVU groups from 4 to 6 days after last altrenogest feeding. Data presented are expressed as least squares means ± SEM.

<sup>ab</sup> Means with different letters within a column tended to be different between days ( $P < 0.1$ ).

<sup>cd</sup> Means with different letters within a row differ by treatment ( $P < 0.05$ ).

## **Discussion**

### **Triptorelin Intro**

The administration of the intravaginal GnRH agonist, triptorelin, has been shown to induce the LH surge and ovulation in both weaned sows (Stewart et al, 2010) and synchronized gilts (Knox et al., 2018; Kraeling et al., 2013). A single FTAI protocol in sows has been shown to be effective using a 96 h OvuGel administration post-weaning and then a subsequent insemination approximately 22 h later (Knox et al., 2018; Rostagno et al., 2016). There has been no differences in litter size and pregnancy rates using this single FTAI protocol compared to a double insemination protocol in which sows are bred at estrus and then 24 h later (Knox et al., 2018; Rostagno et al., 2016). Rostagno et al., (2016) reported there was no difference in farrowing rate or litter size in data gathered from 14 sow test trials conducted using a single FTAI protocol. The sow, however, has been shown to produce more ova per ovulation making the chance of a fertilization greater than the gilt (Clark et al., 1972). The protocol for a single FTAI in replacement gilts is still under investigation (Knox et al., 2018) and has not yet been established (Knox et al., 2018; Gesing, 2015; Knox et al., 2017; Quick, 2015).

### **Reproductive Characteristics**

In the present study, CL and fetus count, and pregnancy percentage were not different between the CON and OVU groups. This is similar to what numerous researchers have shown in sows, that the administration of the vaginal triptorelin with a single FTAI system has no effect on total born and pregnancy rates when using the 96 h post-wean protocol (Rostagno et al., 2016; Knox et al., 2014; Fabi et al., 2017; Knox et

al., 2011; Stewart et al., 2010). The ratio of fetuses to CL of pregnant gilts tended to be less in the OVU group, however numerous factors could be influencing this.

One key factor could be the number of semen doses administered to gilts. The protocol for the use of OvuGel (OvuGel; United Animal Health LLC, Sheridan, IN) states to use a single dose of semen and this has been proven to be effective in numerous sow studies (Knox et al., 2014; Rostagno et al., 2016). This new technology was designed to decrease the cost of labor by eliminating estrus detection and cost of semen doses by only using one dose (Knox, 2013; Fabi, 2017). In order to balance the cost of OvuGel (\$6.75/dose) in the present study, a single insemination was performed in OVU group as compared to two inseminations in CON group. Knox et al. (2017) found that sows administered lower number of sperm cells with a single FTAI protocol led to lower farrowing rates and litter sizes. Other studies have also confirmed that administering less than 3-5 billion cells per dose of semen resulted in fewer total born (Watson and Benan, 2002; Bracken et al., 2003). New technology has been developed in swine production for administering semen directly into the uterus using a specialized catheter without the need for uterine contractions to propel the semen to the site of fertilization (Sbardella et al., 2014; Garcia-Vazquez et al., 2019). This technology could be beneficial as the chance of backflow is decreased and there is less reliance on uterine contractions for semen movement (Sbardella et al., 2014).

The timing of when triptorelin and semen are administered relative to the timing of ovulation has been demonstrated to impact reproductive performance and the time period in which ovulation occurs. In the current study, 96 h after last altrenogest a single dose of triptorelin was administered followed by a single FTAI at 30 h. This was used to

prevent ovulation occurring before the optimal time for semen to be administered. This was similar to data presented by Knox et al., (2017) which showed a sharp increase (approximately 57%) in the number of gilts ovulated when administered triptorelin at 96 h after last altrenogest feeding and then ovulated at 144 h after last altrenogest feeding. This study also showed that multiple ovulations were occurring prior to AI in groups administered triptorelin at 120 and 144h after last altrenogest feeding (Knox et al., 2017). In order for pregnancy to be successful, administration of semen within 24 h of ovulation has been shown to produce the best results (Soede et al., 1995). Gesing (2015) conducted a study in which gilts were administered OvuGel at 142 h after last altrenogest feeding and then inseminated 24 h after and found no differences in total born compared to controls that received multiple doses. When triptorelin was administered at 118 h after last altrenogest feeding with the same protocol there was a decrease in total born of the triptorelin group (Gesing, 2015). Knox et al., (2017) also reported that the greatest proportion (81-89%) of gilts that ovulated within a 24-48 h window were administered triptorelin at 120 h. The recent studies show that during the current experiment, the administration of OvuGel may have been too early for the greatest synchrony of ovulation among gilts.

The genetic line being used has also been found to affect timing of ovulation in swine species (Belstra et al., 2004). To the best of our knowledge, all previous studies involving the use of triptorelin on synchronized gilts used breeds designated for their maternal characteristics (Gesing, 2015; Knox et al., 2017; Quick, 2015). In these studies, the timing from last altrenogest feeding to ovulation without the use of triptorelin was primarily between 144-196 h (Knox et al., 2017) and averaged 135 +/- 13.4 h (Quick,

2015). During these studies the optimal time for triptorelin administration was approximately 120 h (Knox et al., 2017; Quick, 2015). Belstra et al., (2004) found that in gilts that were composed of a three breed cross (including a terminal boar) they had a 4 h longer estrus to ovulation interval compared with a maternal breed sow (½ Yorkshire x ½ Landrace). The gilts in the present study fell into the category of the three breed cross and could have ovulated too late for the semen to be effective in fertilizing the most ovum within the 24 h window (Soede et al., 1995).

### **Progesterone**

Many researchers have examined the effects of GnRH analogs in numerous species on serum concentration of P4 and the results are dependent on the timing of administration during the estrous cycle (Ryan et al., 1994, Brussow et al., 2011). Ryan et al., (1994) found that when GnRH was administered at the time of AI, serum concentrations of P4 were suppressed in cows on days 3-5 of the estrous cycle, however, when cows were administered GnRH at day 12 post AI progesterone levels increased. The hypothesis behind this is that administration of GnRH increases LH secretion from the AP thus causing a LH surge and ovulation (Stewart et al., 2010). Administration of GnRH agonists should also cause LH to increase during pregnancy (Virolainen et al., 2003). The female pig did demonstrate this theory, in which LH did increase at estrus (Stewart et al., 2011) and Day 12 after insemination (Brussow et al., 2011) yet no difference in serum concentrations of P4 occurred in pregnant pigs (Brussow et al., 2011). Data presented here showed that serum concentrations of P4 did decrease in all but one gilt and, thus, all gilts need to be synchronized in order for triptorelin to be effective in inducing ovulation.



Determination of serum concentrations of P4 has been used previously to determine if ovulation has occurred and if a functional CL is present (Mann and Lamming, 2006). Soede et al. (1994) found detectable amounts of P4 were observed at the time of ovulation. After ovulation occurred, a rise in serum concentrations of P4 was observed by 1.0 ng/ml within 13 h post ovulation (Soede et al., 1994). In the present study, no increase in serum concentrations of P4 was observed to base when ovulation occurred yet progesterone concentrations tended to be lower in OVU group on day 4 and day 5 compared to the CON group. The present study showed that estrus was exhibited less in the OVU group during AI, however, the mean expression of estrus regardless of treatment was found to be prior to day 6 after last altrenogest feeding. The CON group tended to exhibit estrus later around the time when P4 was at its lowest. This could mean that ovulation did occur earlier in the OVU group compared to the CON group, however, could not be confirmed without the use of transrectal ultrasonography which was not present for the current experiment (Soede et al., 1994).

### **Estradiol and Estrus**

Serum concentrations of E2 in the current study were similar to results found in previous studies (Hunter et al., 1993; Guthrie et al., 1972; Soede et al., 1994). Estradiol has been shown to peak 1-3 h prior to standing estrus (Guthrie et al., 1972; Soede et al., 1994) and will also peak approximately 41 h prior to ovulation in the pig (Soede et al., 1994). It has well been accepted that E2 causes the external signs of estrus (Brandt et al., 2007; Andersson et al., 1984). The current study also demonstrated this by the tendency of the CON gilts to exhibit standing estrus around the time when E2 was greatest.

Gonadotropin releasing hormone agonists have previously been shown to decrease serum concentrations of E2 in sows 8 h after treatment (Stewart et al., 2010) and a day after treatment in cattle (Mann and Lamming, 1995). Stewart et al., (2010) stated that this may decrease the intensity of the standing reflex in sows and gilts. In this study, standing estrus was exhibited less in the OVU group at mating compared to CON even though serum concentrations of E2 were not different. The OVU group also tended to exhibit estrus earlier than CON group. During the current study there was no difference in serum concentrations of E2 on days 4, 5, and 6 after GnRH administration other than a tendency for CON to be less on day 6 than 5. The administration of triptorelin might have been early enough that the induction of ovulation could have altered the production of follicular estradiol. This may have caused the CL to luteinize and start to produce progesterone instead of estradiol causing fewer gilts to exhibit estrus (Stewart et al., 2010; Van de wiel et al., 1981).

### **Conclusion**

The use of triptorelin with a single FTAI protocol did not produce any differences in reproductive performance other than a trend for a lower percentage of fetuses to CL. The timing of administration has been determined in replacement gilts to have an effect on ovulation (Knox et al., 2017) and, thus, more data are needed to determine when the optimal time for administration will yield the highest reproductive performance. Triptorelin may alter the endocrine profile of gilts and the observance of estrus (Stewart et al., 2011) and, its use could contribute to the protocols that make estrus detection obsolete (Knox et al., 2018).

## **Chapter 3**

### **The Endocrine Profile in Non-Pregnant and Early Pregnant Gilts**

#### **Introduction**

The AP has been shown to produce and secrete many different hormones that have been associated with reproductive function (Roberts et al., 2001). Kraeling and Davis (1974) documented when a sow undergoes a hypophysectomy between 70-90 d of gestation the result is an abortion that may be due to loss of luteotrophic support. This is not the case in all species, however, during the late term of pregnancy in the mouse a hypophysectomy has no effect on pregnancy due to the placenta producing an adequate supply of the luteotrophic factor, placental lactogen, to maintain the CL and produce adequate P4 (Galosy and Talamantes, 1995; Thordarson et al., 1997). Some of the components produced and secreted by the AP of swine that may be involved in the maintenance of pregnancy are components of the IGF system and the gonadotropin LH.

Gonadotropin releasing hormone is not produced by the AP yet is responsible for the production of the two gonadotropins from the AP, in which GnRH will bind to its pituitary receptor (GnRHR), activating the G-protein signal transduction system (Millar et al., 2004). In the pig, GnRH was found to be vital in order for the maintenance of pregnancy beyond the maternal recognition of pregnancy in order to produce LH to maintain the CL (Tast et al, 2000). No differences have been found in AP GnRHR expression between the luteal phase and pregnancy in both cattle and mice (Reeves et al., 1982; Schoenemann et al., 1985) however, new data has been presented that documents a

decrease in AP GnRHR expression occurred during mid-gestation in mice (Proietto et al., 2019). Expression of AP GnRHR has, however, been shown to change throughout the estrous cycle with an increase in expression during the follicular phase in both of these species (Reeves et al., 1982; Schoenemann et al., 1985) and in the swine as well (Clapper and Taylor, 2011). Limited data has been presented yet on the effect of pregnancy on expression of porcine AP GnRHR yet data has been presented on the effect of LH expression and concentration (Parlow et al., 1964; Melampy et al., 1966).

The gonadotropin, LH, is produced in the AP of swine and, is the luteotropic agent in the pig that maintains CL function (Peltoniemi et al, 1995). Researchers found that pregnancy caused a twofold increase in AP concentration of LH as compared to any other stage of the estrous cycle in nonpregnant pigs (Parlow et al., 1964; Melampy et al., 1966). In other species, AP concentrations of LH has been shown to be lower or not change compared to other stages of the estrous cycle. (Chamley et al., 1976; Schoenemann et al., 1985; Schwartz and Bartosik, 1962; Greenwald, 1966). Luteinizing hormone synthesis is stimulated by GnRH, however, IGF-1 has been shown to stimulate LH synthesis (Barb and Hausman, 2009)

The IGF system has been found in many tissues and cells throughout the body (Le Roith, 1999). In the AP, IGF plays a critical role in the negative feedback release of GH (Simes et al., 1991) yet it also has a function in reproduction (Zhou et al., 1997) . Barb and Hausman (2009) found that by adding IGF-1, independent of GnRH, to prepubescent gilt AP cells in-vitro there was increased release of LH. The pituitary has also been shown to synthesize IGFBP-2, IGFBP-3, and IGFBP-5 (Roberts et al., 2001; Simes et al., 1991) which play a critical role in the transport, prolonging the half life of IGF, and may

play a role in gonadotropin release from the AP (Guler et al, 1989; Roberts et al., 2001; Clapper et al., 1998). The IGF system as well as the GnRH and LH hormones may have a synergistic effect in continuing pregnancy. Yuan and Lucy (1996) found that when IGF-1 and LH were administered to the large luteal cells and small luteal cells, respectively, there is an increase in progesterone secreted in-vitro from these cells respectively. Limited data has currently been presented on the effects that pregnancy has on the porcine AP IGF system but, researchers have demonstrated a fluctuation occurs in its expression and concentration dependent on stage of estrous and circulating hormone concentration (Clapper and Taylor, 2011; Rempel and Clapper, 2002).

Limited data is present on the effects of pregnancy on AP expression and AP concentrations in the pig. There are numerous data, however, on the effects of the luteal phase, which mimic serum E2 and P4 concentrations, on the hormonal expression and concentration in the anterior pituitary. The changes that occur both physiologically and hormonally during pregnancy may have a different effect than the luteal phase. The objective of this research was to determine the effects of pregnancy on serum concentrations of E2 and P4, anterior pituitary gland concentrations of IGF-1 and LH, and the expression of anterior pituitary GnRHR, LH- $\beta$ , IGF-1, IGF-1R, IGFBP-2, IGFBP-3, IGFBP-5 in gilts.

## Materials and Methods

### Samples

Gilts from the previous experiment were used as the source of tissue and blood. All gilts were slaughtered at South Dakota State University Meat Lab  $33.2 \pm .1$  d after artificial insemination at which time blood (50 mL) and anterior pituitaries were collected. Blood was stored overnight at 4°C the samples were centrifuged (1,500 x g for 30 min at -4°C), serum was collected, and then stored at -20°C. Anterior pituitaries were trimmed of connective tissue, bisected midsagittally, wrapped in aluminum foil, flash frozen in liquid nitrogen, and then stored at -80°C. Pregnancy of gilts was determined based on reproductive tracts containing fetuses and corpora lutea. Two of the non-pregnant gilts hypothalami and anterior pituitaries were unsalvageable during the course of the slaughter practice.

### IGF-1

Anterior pituitary gland concentrations of IGF-I were determined in duplicate by RIA (Echternkamp et al., 1990; Funston et al., 1995). One half of each anterior pituitary gland was homogenized in a 15-mL polypropylene tube with 1-mL of homogenization buffer (1% cholic acid, 0.1% SDS, 200  $\mu$ M phenylmethylsulfonylfluoride, 100  $\mu$ M EDTA, 1  $\mu$ M leupeptin, and 1  $\mu$ M pepstatin) and homogenized on ice with a T25 Ultra-27 Turrax tissue homogenizer (IKA Works, Wilmington, NC) for 30 s at 20,500 rpm. Anterior pituitary glands were then diluted to 100 mg of AP tissue/mL with homogenization buffer. Homogenates were centrifuged at 12,000 x g for 10 min at 4°C

and the supernatant was removed and stored at -20°C. Protein content of the AP homogenates (1:20 dilution) was determined by the Bradford method using reagents provided by BioRad (Hercules, CA). Insulin-like growth factor binding proteins were extracted from all homogenized anterior pituitary gland samples with a 1:17 ratio of sample to acidified ethanol (12.5% 2 N HCl:87.5% absolute ethanol; Daughaday et al., 1980). Recombinant human IGF-I (GF-050; Austral Biological, San Ramon, CA) was used as the standard and radioiodinated antigen. Antisera (AFP4892898; National Hormone and Peptide Program, NIDDK) was used at a dilution of 1:62,500. Recovery of [125I]IGF-I added to porcine pituitary samples before extraction averaged  $91 \pm 3.2\%$ . Inhibition curves of increasing amounts of sample were parallel to standard curves. Intra-assay coefficient of variation was 8.7%. Sensitivity of the assay was 10 pg/tube.

### **Luteinizing Hormone**

Anterior pituitary gland concentrations of LH were determined in triplicate by RIA (Clapper et al., 1998). Porcine LH (AFP3881A; National Hormone and Peptide Program, NIDDK) was used as the radioiodinated antigen and standard. Luteinizing hormone antiserum (AFP15103194; National Hormone and Peptide Program, NIDDK) was used at a dilution of 1:150,000. Anterior pituitary homogenates were diluted 1:25,000 in 0.01 M PBS-0.1% gelatin prior to assay. Inhibition curves of increasing amounts of sample were parallel to standard curves. Intra-assay coefficient of variation was 5.7%. Sensitivity of the assay was 0.01 ng/tube.

### **Isolation of RNA**

Total RNA was isolated from one half of each AP using TriReagent (TR118; Molecular Research Company, Cincinnati, OH). Tissues were homogenized in a 15-mL polypropylene tube containing 1-mL of TriReagent with a T25 Ultra-Turrax tissue homogenizer (IKA Works, Wilmington, NC) for 30 s at 20,500 rpm. The resulting homogenates were diluted to 5-mL with TriReagent. One milliliter of the homogenate was removed from the 15-mL polypropylene tube and pipetted into a 1.5-mL polypropylene tube. One hundred microliters of 1-bromo-3-chloropropane (BCP; #BP151, Molecular Research Center, Cincinnati, OH) was added to the tubes, mixed, and incubated at room temperature for 5 min. Samples were centrifuged (12,000 x g for 15 min at 4°C) and the resulting supernatant was removed. Isopropanol (500 µL) was added to the supernatant to precipitate the RNA. The resulting RNA pellet was washed twice with 75% ethanol, centrifuged, dried, and suspended in nuclease-free water. Concentration of RNA was determined via spectrophotometer (Nanodrop, Thermo Scientific, Washington, DE). Purity of RNA was determined by measuring the A260/A280 ratio. The ratio of all samples ranged from 1.8 to 2.0. Any contaminating DNA was removed from 10 µg of AP RNA for each sample using the Turbo DNA-Free™ Kit (#AM1907; Ambion, Austin, TX) following the manufacturer's protocol. Two micrograms of the resulting RNA was reverse transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (#4374966; Applied Biosystems, Foster City, CA) following the manufacturer's protocol. Previously published mRNA sequences for the gene of interest and porcine  $\beta$ -actin were used to design specific forward and reverse primers. Primers were designed using software provided by Integrated DNA Technologies (IDT, Coralville, IA; Table 3.1).



**Table 3.1** Forward and reverse primers for Real-Time PCR for porcine mRNA

<b>Gene and Accession Number</b>	<b>Primer</b>	<b>Amplicon Size</b>
<b>IGF-I</b>		
(NM_214256.1) <sup>a</sup>		146 bp
<b>Forward</b>	5'-ATGCCCAAGGCTCAGAAGGAAGTA-3'	
<b>Reverse</b>	5'-AGGTAACTCGTGCAGAGCAAAGGA-3'	
<b>IGF-type-1 R</b>		
(NM_214172.1) <sup>b</sup>		105 bp
<b>Forward</b>	5'-CCAGGCCAAAACGACATATGA-3'	
<b>Reverse</b>	5'-GTGGAAGACGTACAACATTATAACCAA-3'	
<b>pIGFBP2</b>		
(AF120326.1) <sup>c</sup>		142 bp
<b>Forward</b>	5'-CGAGCAGGTTGCAGACAATG-3'	
<b>Reverse</b>	5'-GAACACAGCCAGCTCCTTCAT-3'	
<b>pIGFBP3</b>		
(AF085482.1) <sup>d</sup>		54 bp
<b>Forward</b>	5'-CATCCACATCCCCAACTG-3'	
<b>Reverse</b>	5'-CGGCACTGCTTTTTCTTGTAGAA-3'	
<b>pIGFBP5</b>		
(U41340.2) <sup>e</sup>		74 bp
<b>Forward</b>	5'-CGCAAGGGATTCTACAAGAGAAA-3'	
<b>Reverse</b>	5'-TCCACGCACCAGCAGATG-3'	
<b>GnRHR</b>		
(NM_214273.1) <sup>f</sup>		101 bp
<b>Forward</b>	5'-AGCCAACCTGTTGGAGACTCTGAT-3'	
<b>Reverse</b>	5'-AGCTGAGGACTTTGCAGAGGAACT-3'	
<b>LHβ</b>		
(NM_214080.1) <sup>g</sup>		151 bp
<b>Forward</b>	5'-ATGCTCCAGAGACTGCTGTTGT-3'	
<b>Reverse</b>	5'-TGCTGGTGGTAAAGGTGATGCAGA-3'	
<b>pβ-actin</b>		
(U07786.1) <sup>h</sup>		129 bp
<b>Forward</b>	5'-TCGCCGACAGGATGCAGAAGGA-3'	
<b>Reverse</b>	5'-AGGTGGACAGCGAGGCCAGGAT-3'	

<sup>a</sup>porcine IGF-1; <sup>b</sup>porcine IGF-1 receptor; <sup>c</sup>porcine IGFBP-2; <sup>d</sup>porcine IGFBP-3; <sup>e</sup>porcine IGFBP-5; <sup>f</sup>porcine GnRH receptor; <sup>g</sup>porcine LHβ; <sup>h</sup>porcine beta-actin

### **Real-Time PCR**

Real-time semi-quantitative PCR was used to measure the quantity of AP LH $\beta$ , GnRHR, IGF-1, IGF-1R, IGFBP-2, IGFBP-3, and IGFBP-5 mRNA relative to the amount of porcine  $\beta$ -actin mRNA in each sample. Measurements of the relative quantity of the cDNA of interest was carried out using RT2 Real-Time™ SYBR Green/ROX PCR Master Mix (SuperArray Bioscience Corp., Foster City, CA). Twenty-five microliter reactions were measured using the Stratagene MX3005P quantitative real-time PCR instrument (Agilent Technologies, Foster City, CA). Thermal cycling conditions recommended by the manufacturer (40 cycles of 30 sec at 95°C, 1 min at 55°C, and 1 min at 72°C) were used for all genes. Concentrations of forward and reverse primers used for the genes of interest and  $\beta$ -actin were 300 nM. A linear response was obtained when these concentrations of primer pairs were used with increasing amounts of cDNA. Dissociation curve analysis was performed after each real-time PCR run to confirm that a single amplicon was present. Additionally, all amplicons were electrophoresed through a 2% agarose gel and stained with ethidium bromide to visualize that only amplicons of the appropriate size were present in each sample.

### **Statistical Analysis**

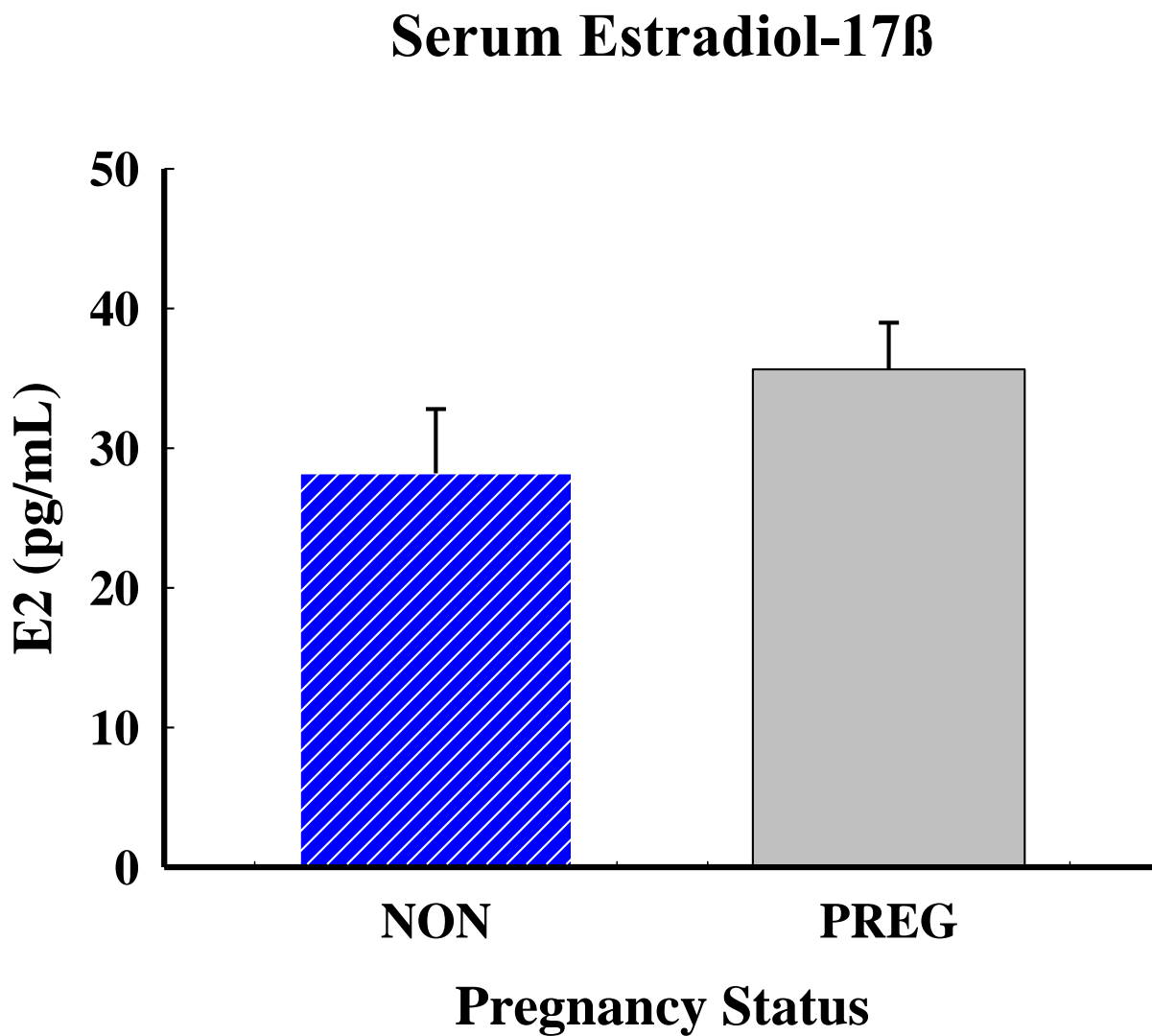
To determine the effect of pregnancy status on serum concentrations of estradiol and progesterone, and anterior pituitary concentrations of insulin like growth factor-1, and luteinizing hormone statistical analysis was performed using a GLIMMIX Procedure of SAS 9.4 (SAS 9.4, SAS) to compare pregnant and non-pregnant gilts. The model for

determining serum concentrations of estradiol-17 $\beta$  and progesterone, and anterior pituitary concentrations of lutenizing hormone and insulin like growth factor was  $Y_{ijk} = \mu + P_i + \text{Pregnancy Status}_j + P_i(\text{Pregnancy Status})_j + e_{ijk}$ . Pig within pregnancy status was the whole plot error term used to test the effect of treatment.

Fold differences in expression of AP LH $\beta$ , GnRHR, IGF-1, IGF-1R, IGFBP-2, IGFBP-3, and IGFBP-5 between treatments was determined using the Relative Expression Software Tool (REST; Corbett Research & M. Pfaffl, Technical University Munich). The expression of a target gene is standardized by a non-regulated gene. Relative expression is based on the expression ratio of a target gene versus a reference gene. The expression ratio results of the investigated transcripts were tested for significance by Pair Wise Fixed Reallocation Randomized Test (Pfaffl et al., 2002).

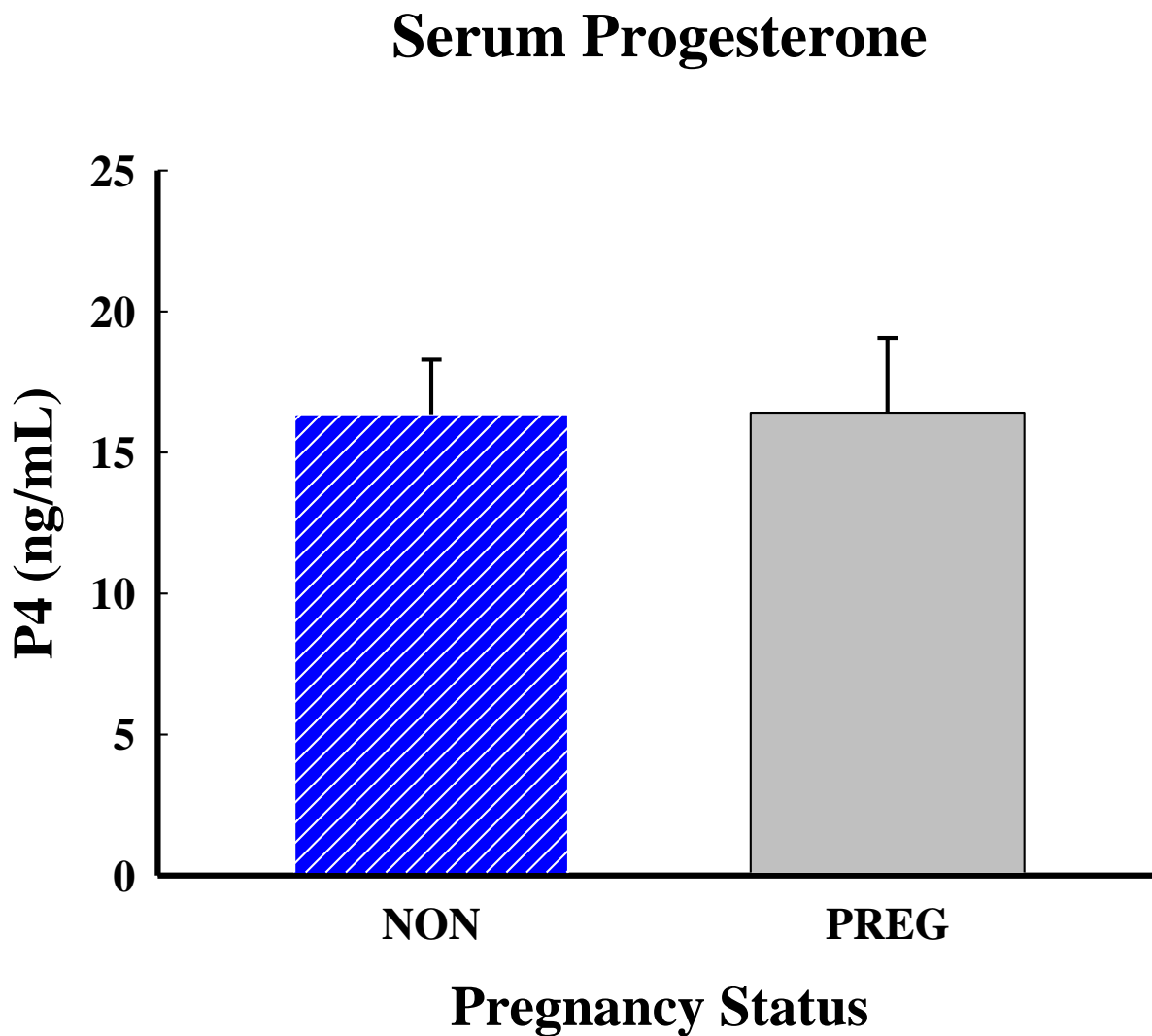
## Results

No differences were detected ( $P>0.05$ ) in mean serum concentrations of E2 between pregnant and non-pregnant gilts (Figure 3.1).



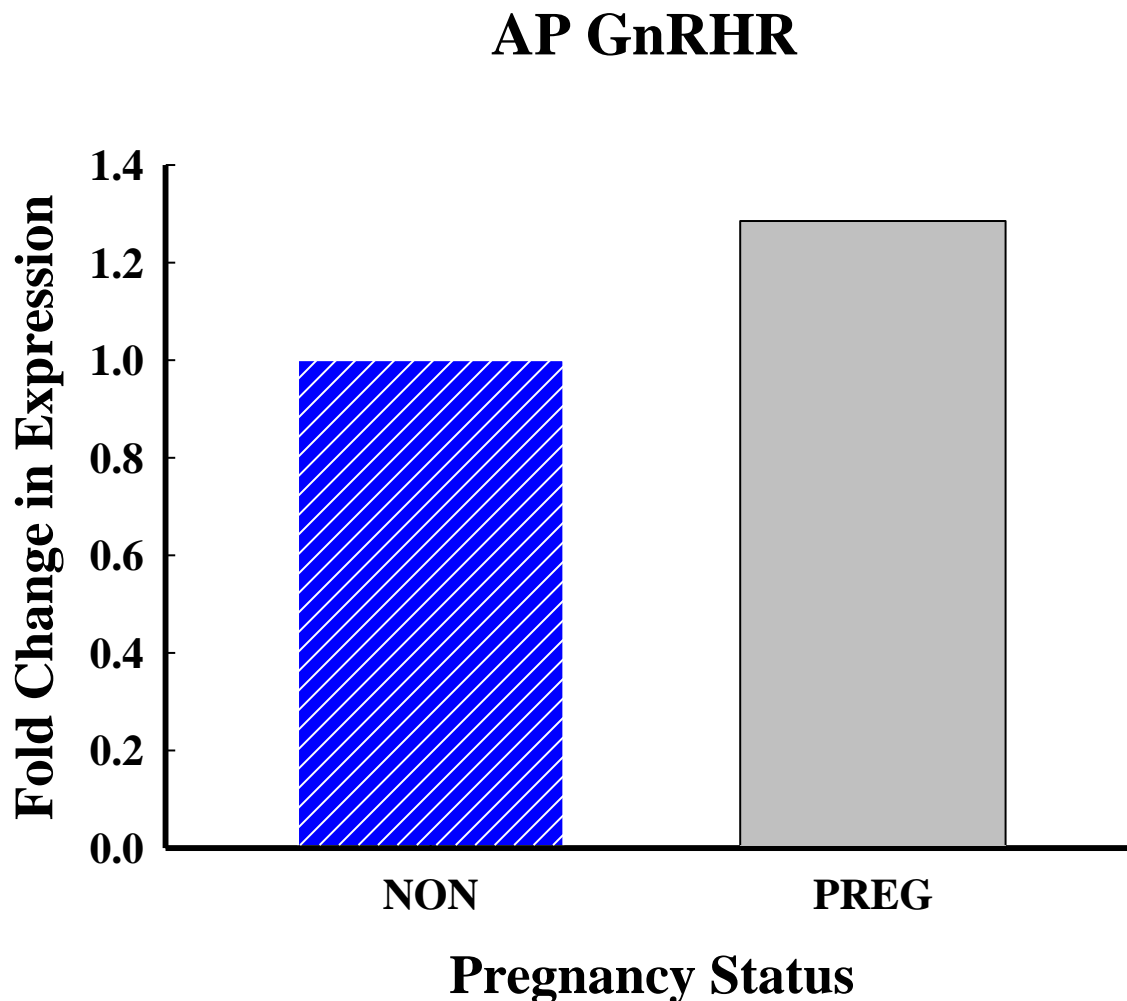
**Figure 3.1** Mean serum concentrations of estradiol-17 $\beta$  (E2) in gilts that were pregnant (PREG; n=15) or non-pregnant (NON; n=8). Data presented are expressed as least square-means  $\pm$  SEM.

No differences were detected ( $P>0.05$ ) in mean serum concentrations of P4 between pregnant and non-pregnant gilts (Figure 3.2).



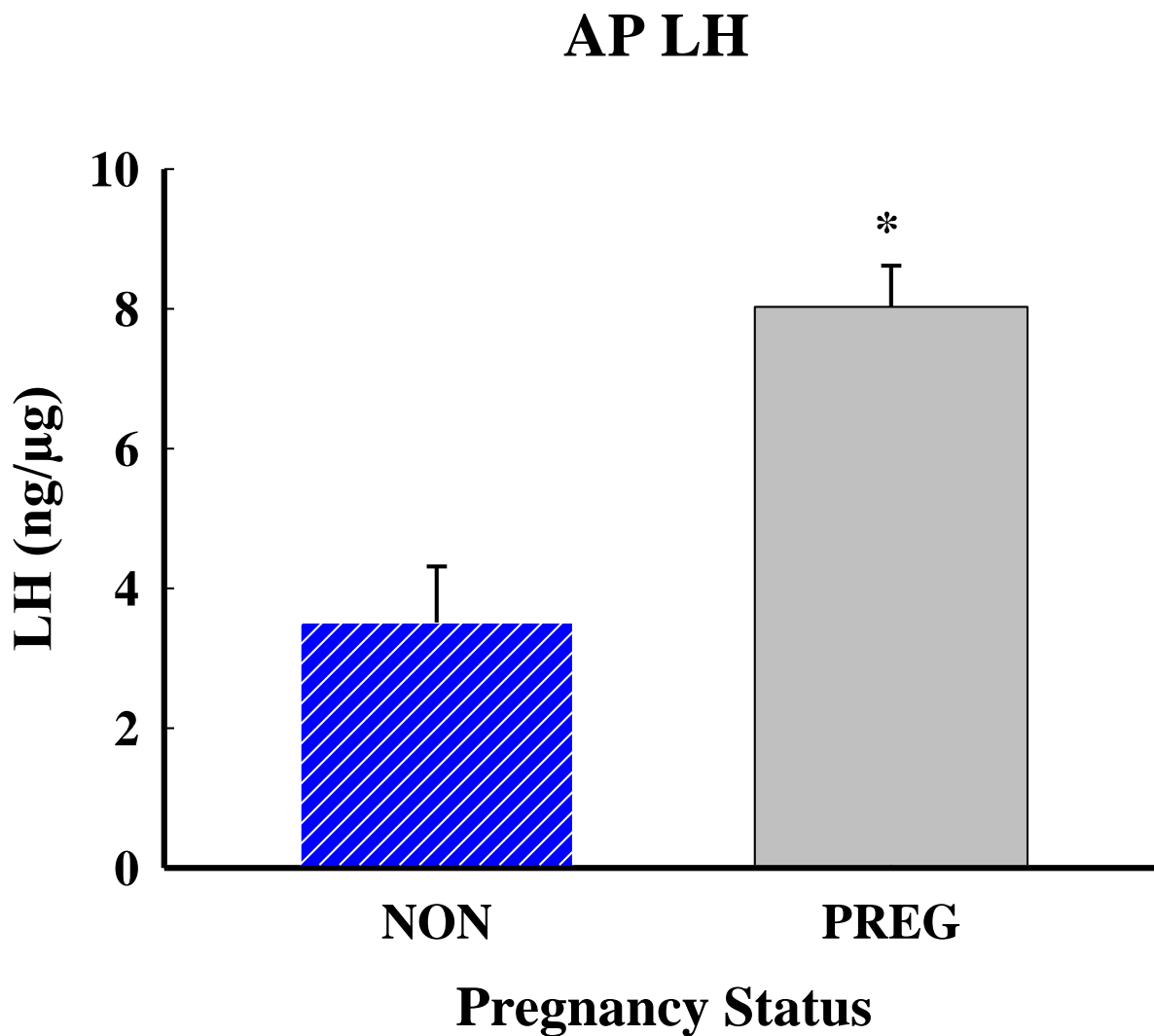
**Figure 3.2** Mean serum concentrations of progesterone (P4) in gilts that were pregnant (PREG;  $n=15$ ) or non-pregnant (NON;  $n=8$ ). Data presented are expressed as least square-means  $\pm$  SEM.

Mean relative expression of AP GnRHR did not differ ( $p>0.05$ ) in pregnant versus non-pregnant gilts (Fig 3.3).



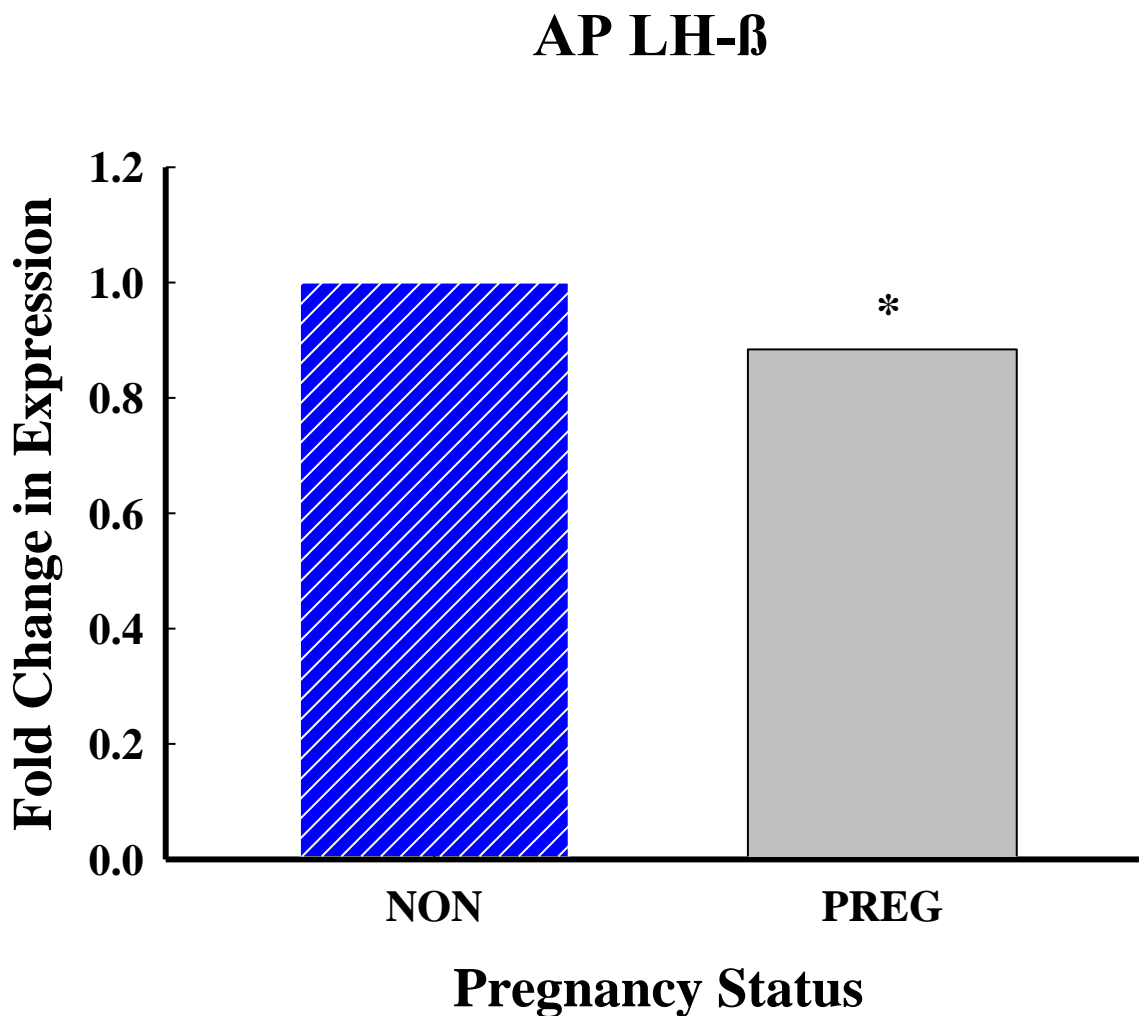
**Figure 3.3** Mean relative expression of anterior pituitary gonadotropin releasing hormone receptor (AP GnRHR) in gilts that were pregnant (PREG; n=15) or non-pregnant (NON; n=6). Total RNA was DNAase treated and analyzed for corresponding level of  $\beta$ -actin. Data presented are expressed as a fold change in expression relative to the pigs that were not pregnant.

Mean AP concentrations of LH was greater ( $p=0.0003$ ) in pregnant versus non-pregnant gilts (Figure 3.4).



**Figure 3.4** Mean anterior pituitary concentrations of luteinizing hormone (AP LH) in gilts that were pregnant (PREG;  $n=15$ ) and non-pregnant (NON;  $n=6$ ). Data presented are expressed as least-square means  $\pm$  SEM. \*Means with asterisks differ ( $p=0.0003$ ) by group.

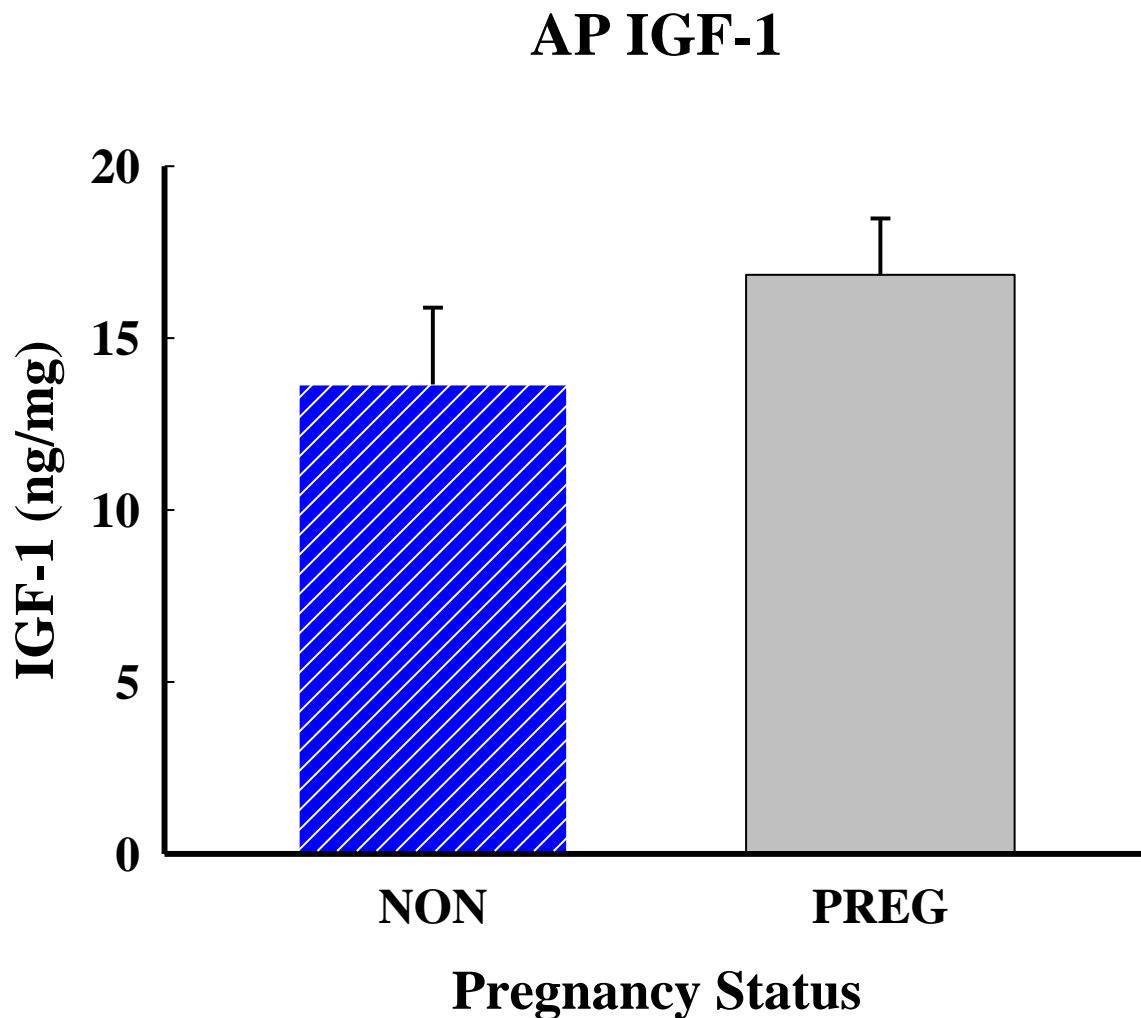
Mean relative expression of AP LH- $\beta$  was down regulated 0.813 fold in pregnant versus non-pregnant gilts ( $p=0.001$ ; Fig 3.5).



**Figure 3.5** Mean relative expression of anterior pituitary luteinizing hormone beta (AP LH- $\beta$ ) in gilts that were pregnant (PREG;  $n=15$ ) or non-pregnant (NON;  $n=6$ ). Total RNA was DNAase treated and analyzed for corresponding level of  $\beta$ -actin. Data presented are expressed as a fold change in expression relative to the pigs that were not pregnant. \*Means with asterisk differ ( $p=0.001$ ) by group.

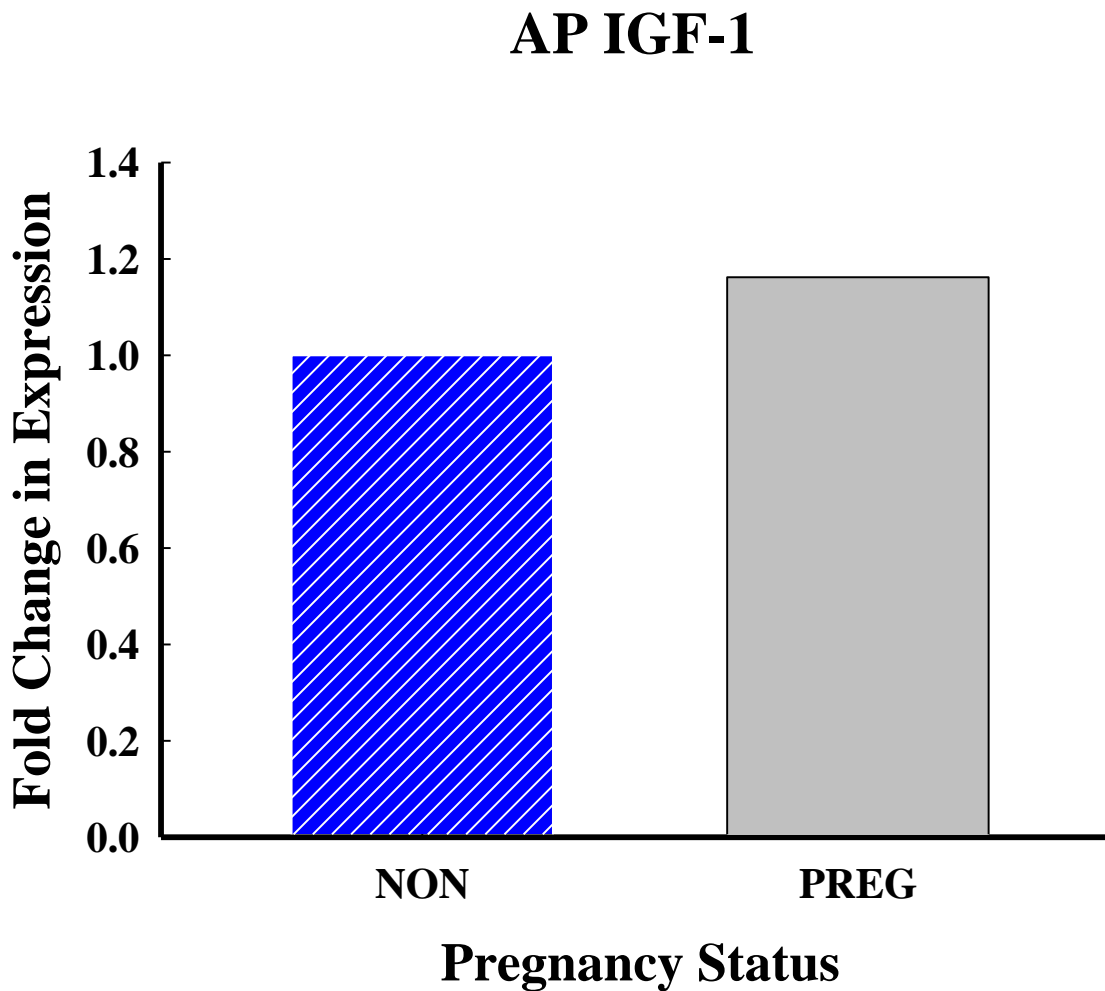


Mean AP concentrations of IGF-1 were not different ( $p>0.05$ ) in pregnant versus non-pregnant gilts (Fig 3.6).



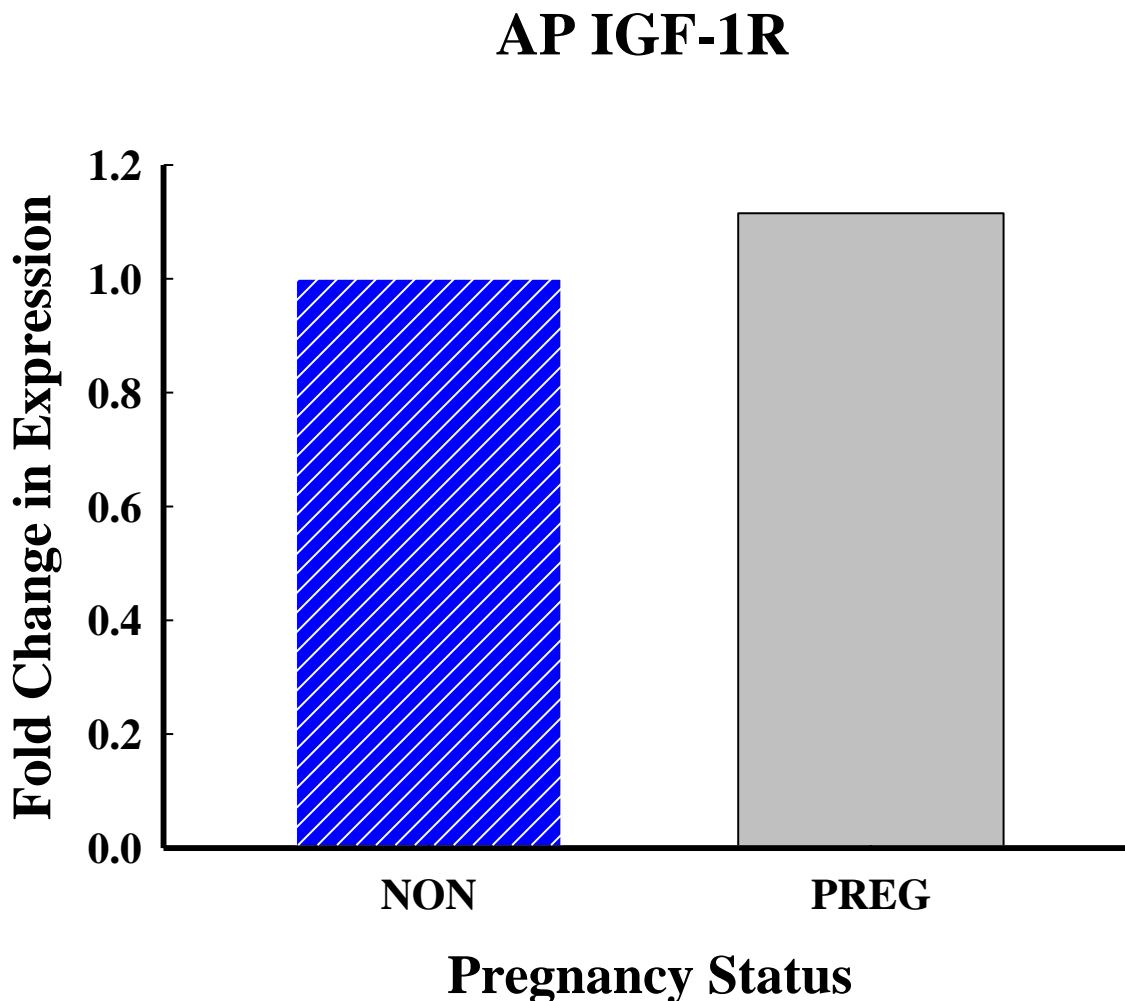
**Figure 3.6** Mean anterior pituitary concentrations of insulin like growth factor-1 (AP IGF-1) in gilts that were pregnant (PREG;  $n=15$ ) and non-pregnant (NON;  $n=6$ ). Data presented are expressed as least-square means  $\pm$  SEM.

Mean relative expression of AP IGF-1 were not different ( $p>0.05$ ) in pregnant versus non-pregnant gilts (Fig. 3.7).



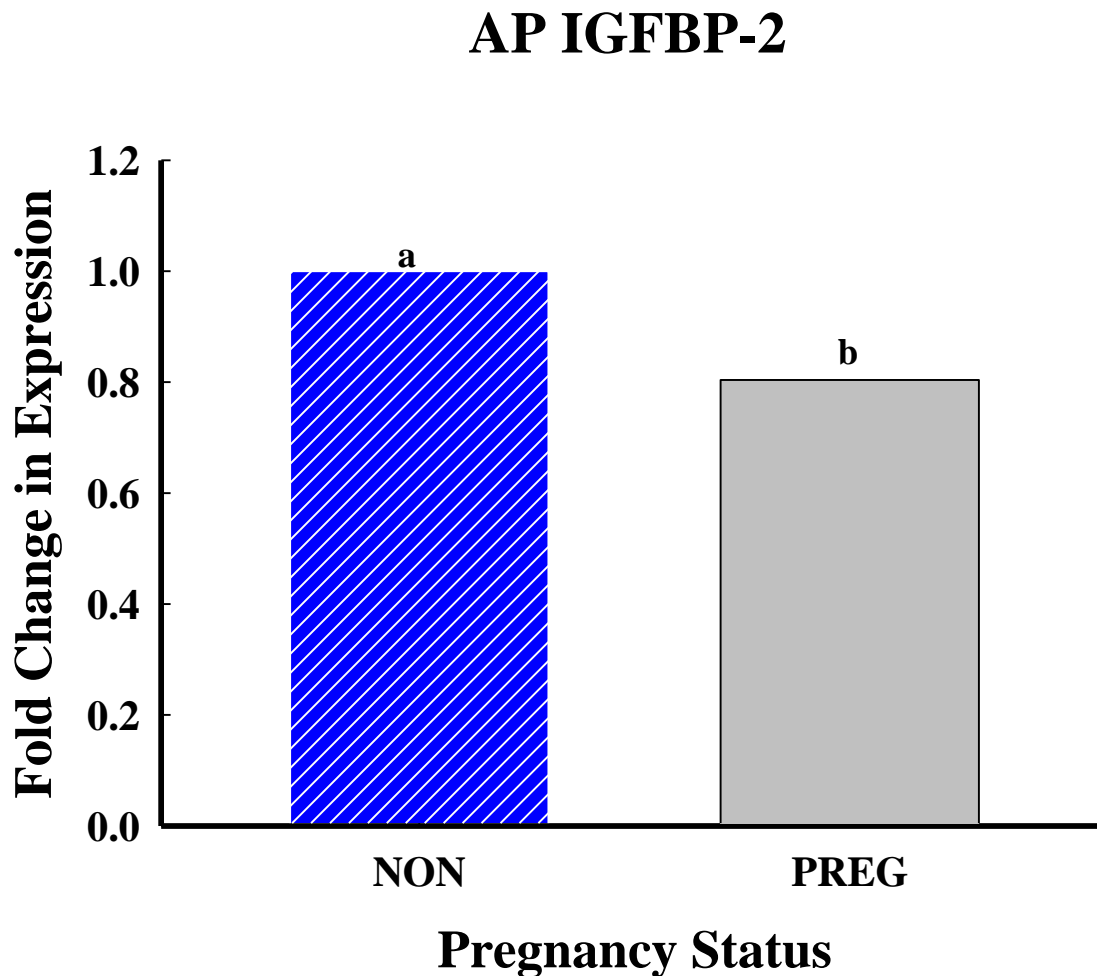
**Figure 3.7** Mean relative expression of anterior pituitary insulin like growth factor-1 (AP IGF-1) in gilts that were pregnant (PREG; n=15) or non-pregnant (NON; n=6). Total RNA was DNAase treated and analyzed for corresponding level of  $\beta$ -actin. Data presented are expressed as a fold change in expression relative to the pigs that were not pregnant.

Mean relative expression of AP IGF-1R were not different ( $p>0.05$ ) in pregnant versus non-pregnant gilts (Fig. 3.8).



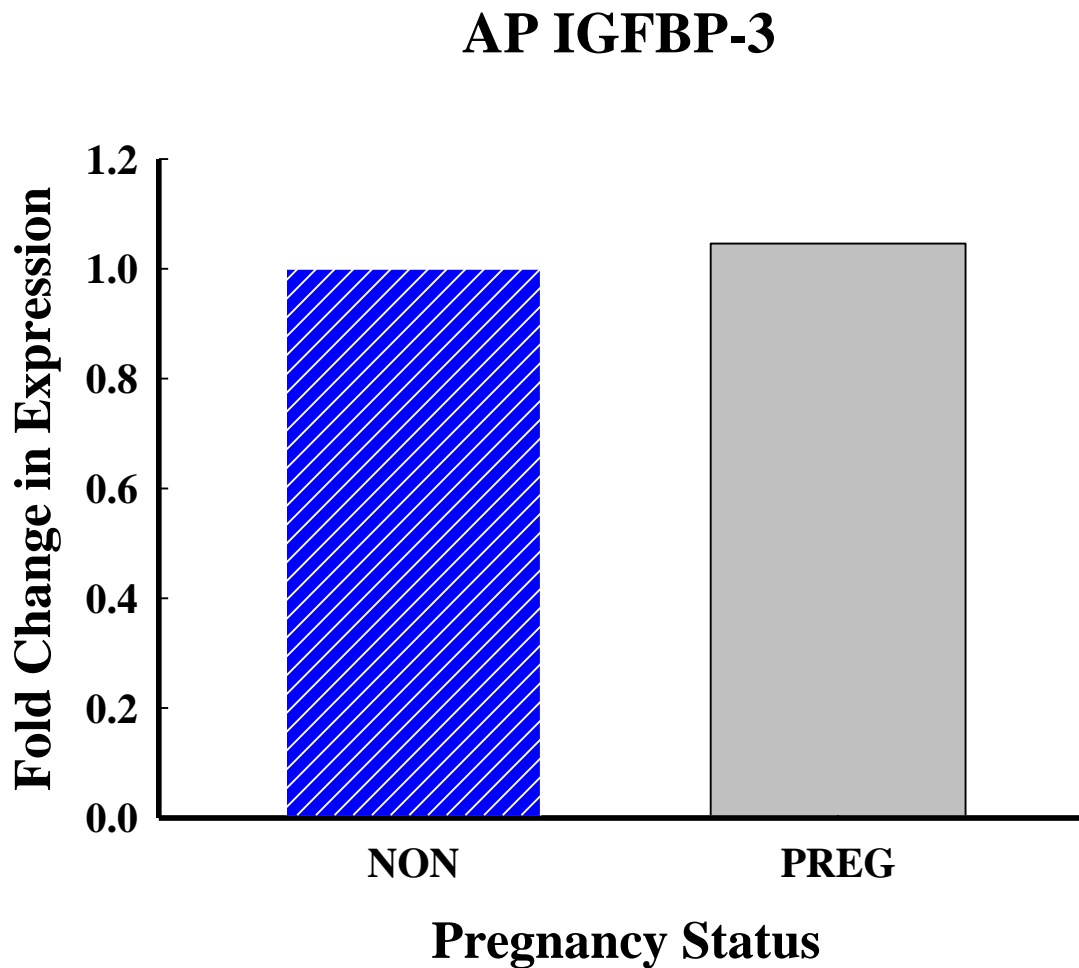
**Figure 3.8** Mean relative expression of anterior pituitary insulin like growth factor-1 receptor (AP IGF-1R) in gilts that were pregnant (PREG;  $n=15$ ) or non-pregnant (NON;  $n=6$ ). Total RNA was DNAase treated and analyzed for corresponding level of  $\beta$ -actin. Data presented are expressed as a fold change in expression relative to the pigs that were not pregnant.

Mean relative expression of AP IGFBP-2 tended to be down regulated by 0.804 in pregnant versus non-pregnant gilts ( $p=0.095$ ; Fig. 3.9)



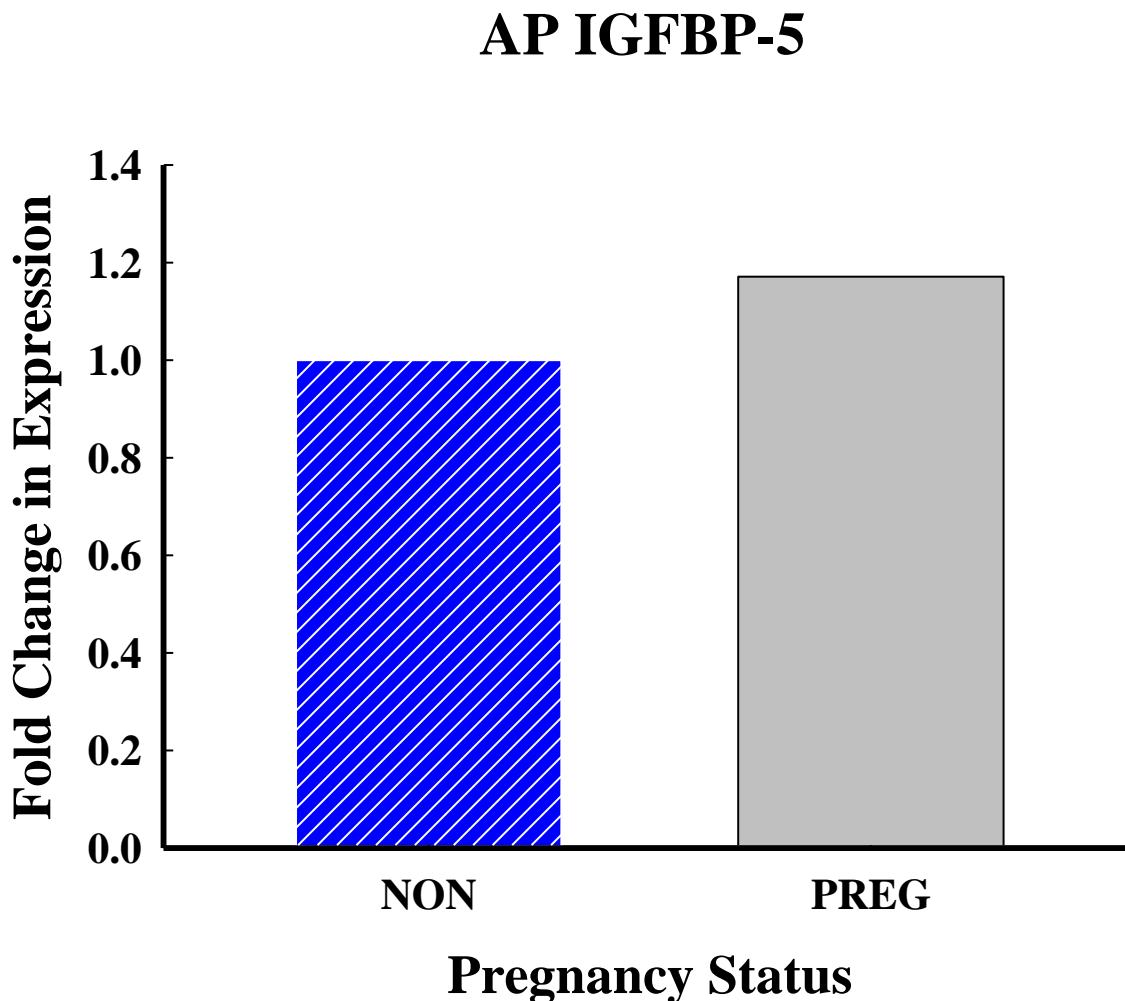
**Figure 3.9** Mean relative expression of anterior pituitary insulin like growth factor binding protein-2 (AP IGFBP-2) in gilts that were pregnant (PREG;  $n=15$ ) or non-pregnant (NON;  $n=6$ ). Total RNA was DNAase treated and analyzed for corresponding level of  $\beta$ -actin. Data presented are expressed as a fold change in expression relative to the pigs that were not pregnant. <sup>ab</sup> Means with different letters tended ( $p=0.095$ ) to differ by group.

Mean relative expression of AP IGFBP-3 were not different ( $p>0.05$ ) in pregnant versus non-pregnant gilts (Fig. 3.10).



**Figure 3.10** Mean relative expression of anterior pituitary insulin like growth factor binding protein -3 (AP IGFBP-3) in gilts that were pregnant (PREG; n=15) or non-pregnant (NON; n=6). Total RNA was DNAase treated and analyzed for corresponding level of  $\beta$ -actin. Data presented are expressed as a fold change in expression relative to the pigs that were not pregnant.

Mean relative expression of AP IGFBP-5 were not different ( $p>0.05$ ) in pregnant versus non-pregnant gilts (Fig. 3.11).



**Figure 3.11** Mean relative expression of anterior pituitary insulin like growth factor binding protein -5 (AP IGFBP-5) in gilts that were pregnant (PREG;  $n=15$ ) or non-pregnant (NON;  $n=6$ ). Total RNA was DNAase treated and analyzed for corresponding level of  $\beta$ -actin. Data presented are expressed as a fold change in expression relative to the pigs that were not pregnant.

## Discussion

Maternal recognition of pregnancy in the pig occurs due to conceptuses secreting adequate amounts of E2 around d 11-12 of pregnancy (Bazer and Thatcher, 1977, Bazer et al., 1992). This time period, between maternal recognition of pregnancy and day 30 of pregnancy has been shown to be the time when most fetuses are lost due to changes in uterine environment such as uterine spacing, genetic potential, and inadequate vascularization between the fetus and the sow (Kridli et al., 2016). Hormones secreted from the AP play a critical role in the maintenance of pregnancy in the pig (Tast et al., 2000; Kraeling and Davis, 1974). The expression and concentration of AP hormones has also been shown to change depending on the physiological status and the changing hormonal milieu that occurs throughout the estrous cycle (Hilleson-Gayne and Clapper, 2005; Rempel and Clapper, 2002; Roberts et al., 2001; Funston et al., 1995; Michels et al., 1993). There is limited information documenting the changes that occur in the AP during early pregnancy in the pig which may be of vital importance for the continuation of pregnancy.

Serum concentrations of P4 and E2 were not different between pregnant and non-pregnant gilts in the current study. Gilts that were not pregnant in this experiment were estimated to be in the luteal phase of the estrous cycle based upon the timing of their 21 day estrous cycle and in accordance with previous serum concentrations of E2 and P4 (Jolitz and Clapper, 2014; Guthrie et al., 1972; Clapper and Taylor, 2011). Serum concentrations of E2 and P4 in pregnant and non-pregnant gilts follow the same pattern from estrus until d 16 of the estrous cycle (Guthrie et al., 1972). After pregnancy recognition, serum concentrations of P4 remain elevated in pregnant swine, while serum

concentrations of P4 decrease at d 16 of the estrous cycle in non-pregnant gilts as the CL regress (Guthrie et al., 1972; Jolitz and Clapper, 2014). Serum concentrations of E2 during the estrous cycle remain at low levels until an increase occurs during the follicular phase and peaks just prior to estrus (Guthrie et al., 1972). Serum concentrations of E2 in pregnant gilts, however, remain low after pregnancy recognition (Guthrie et al., 1972). In the current experiment, it was found that the timing of sample collection may play a role in the differences that could occur during early pregnancy and non-pregnant gilts. More samples at different times throughout the estrous cycle and pregnancy may have shown differences in serum concentrations of E2 and P4 as has been previously demonstrated in earlier experiments (Guthrie et al., 1972; Jolitz and Clapper, 2014).

Gonadotropin releasing hormone receptor mRNA expression has been found to be localized in the pituitary of the gilt during pregnancy as well as when they are not pregnant (Siawrys and Buchowski, 2018) and its expression changes throughout the estrous cycle in the gilt and other species (Wylot et al., 2008; Clapper and Taylor, 2011). No differences were found in the expression of porcine AP GnRHR in the present study which is in accordance with what has been found in other species (Reeves et al., 1982; Schoenemann et al., 1985). This may, however, be due to the period of gestation when AP were collected. In the pregnant mouse, researchers have recently found a down regulation in GnRHR expression in the AP during mid gestation (Proietto et al., 2019) and a steady decrease throughout pregnancy in the ovary (Sengupta et al, 2008). Expression of GnRHR has also been shown to change due to changes in serum concentrations of E2 and P4 (Cheon et al., 2000; Turzillo et. al., 1998). Serum concentrations of E2 were positively correlated with AP GnRHR expression in the ewe



(Turzillo et al, 1998). Cheon et al. (2000) discovered that when AP cells were administered P4, there was a dose dependent decrease in expression of AP GnRHR mRNA. They also found that even when AP cells were primed with GnRH, P4 still caused a lower expression of AP GnRHR mRNA compared to those not primed with GnRH (Cheon et al., 2000). Day 12 of the porcine estrous cycle (luteal phase) mimics that of pregnancy in terms of serum concentrations of estradiol and progesterone (Guthrie et al., 1972). Throughout the luteal phase, no differences were found in AP GnRHR expression, however, there is evidence that during the follicular phase an increase in AP GnRHR expression occurs in multiple species including the pig (Clapper and Taylor, 2011; Reeves et al., 1982; Schoenemann et al., 1985). The increase is associated with the stimulation of AP production of GnRHR to enhance the responsiveness of the AP to GnRH (Karsch et al., 1997). Previous and present data have shown that the expression of AP GnRHR may be regulated in a timely, physiological, hormonal, and species dependent manner. Our study showed that no difference in expression of GnRHR occurred in pregnant versus non-pregnant. There is to the best of our knowledge no research examining the differences of GnRHR expression that occur during pregnancy in swine. Researchers have shown a difference in expression of AP GnRHR have occurred throughout pregnancy in the mouse (Proietto et al., 2019) and during the estrous cycle in the pig (Wylot et al., 2008; Clapper and Taylor, 2011). More samples gathered at different times of the estrous cycle and throughout gestation may reveal that the expression of GnRHR differs throughout these physiological stages.

Studies have shown that there is a positive correlation between AP IGF-1 and E2 in the pig (Hilleson-Gayne and Clapper, 2005; Rempel and Clapper, 2002). When

estradiol implants were administered to barrows AP concentrations of IGF-1 increased (Rempel and Clapper, 2002). During the gilt's follicular phase when serum E2 are elevated, AP concentrations of IGF-1 were also increased compared to the luteal phase (Clapper and Taylor, 2011; Jolitz and Clapper, 2014). Hilleson-Gayne and Clapper (2005) also demonstrated this theory by decreasing serum concentrations of E2 in the boar with an aromatase inhibitor which led to the subsequent decrease in serum concentrations of IGF-1. In the current experiment, serum concentrations of E2 were not different regardless of pregnancy status and, subsequently AP IGF-1 expression and concentrations of AP IGF-1 were not different. The timing in which AP were collected in relationship to length of pregnancy may, however, alter concentrations of AP IGF-1 and expression as has been demonstrated in the mouse. In the mouse, it was shown that an increase in AP concentrations of IGF-1 occurs throughout pregnancy while plasma and liver concentrations decreased (Escalada et al., 1997). This also may be due to the involvement of IGF-1 in gonadotropin release. Soldani et al., (1994) found that when rat AP cells were incubated with IGF-1 alone or administered GnRH while incubated with IGF-1 led to an increase in the release of AP LH. Porcine and bovine AP cells treated with IGF released more LH in response to GnRH than those AP cells not treated with IGF (Whitley et al., 1995; Hashizume et al., 2002). We found no differences in concentrations and expression of AP IGF-1 between early pregnant and nonpregnant gilts. This may be due to a combination of no difference in E2 between the two groups and the need for release of luteotropic factor at this time may be similar for pregnant and nonpregnant gilts in their luteal phase.

Insulin like growth factor-1 receptor has been localized in the AP primarily in cells that produce growth hormone, adrenocorticotrophic hormone, and gonadotropins in the mouse (Eppler et al., 2007). It has also been shown that in mice, AP IGF-1R expression does not change due to changes in serum concentrations of E2 (Michels et al., 1993). An experiment done in gilts, however, did show an increase in AP IGF-1R expression from early luteal phase to estrus (Clapper and Taylor, 2011). Our results demonstrated that no changes in expression occurred between pregnant and non pregnant gilts, however, the actions of IGF are not only regulated by the IGF-1R. The IGFBP have been shown to have a greater than or equal binding affinity for IGF-1 as the IGF-1R (Ranke and Elmlinger, 1997). The actions of IGFBP have been found to either inhibit or potentiate the actions of IGF-1 depending on the tissue and which IGFBP was involved (Firth and Baxter, 2002). The actions of the IGFBP may be involved in the regulation of IGF-1 during pregnancy and the estrous cycle.

In the present study, the expression of AP IGFBP-2 tended to be decreased in pregnant gilts compared to nonpregnant gilts. Based on previous research (Michels et al., 1993; Clapper et al., 1998; Rempel and Clapper, 2002), prolonged decreases in serum concentration of E2 may cause a decrease in the expression of AP IGFBP-2. A positive association between E2 and AP IGFBP-2 has been found in the rodent (Michels et al., 1993), ewe (Clapper et al., 1998) and pig (Rempel and Clapper, 2002). Serum concentrations of E2 and P4 on d 12 of the gilt estrous cycle mimics what occurs during early pregnancy (Guthrie et al., 1972). During this time of the luteal phase, it has been shown that the expression of AP IGFBP-2 was lower than that during the follicular phase when serum concentrations of E2 were elevated (Clapper and Taylor, 2011).

During the current experiment, no differences were found in the expression of AP IGFBP-3 or -5. It has been shown in cattle thecal cells that IGFBP-2 and IGFBP-3 inhibited IGF-1 induced production of androstenedione and P4 (Spicer et al., 1997). In the rodent, IGFBP-5 was found to stimulate the mitogenic effect of IGF in osteoblasts (Andress and Birnbaum, 1992). Clapper and Taylor (2011) hypothesized that the actions of IGFBP-2 and IGFBP-5 could enhance the IGF activity to release LH. This hypothesis could be valid because during pregnancy LH secretion from the AP is not as great as during the LH surge (Ziecik et al., 1982). The changes that occur in the expression of AP IGFBP may be regulated in a timely and physiological manner, however, they may also be regulated based on physiological needs for AP secretion of gonadotropins. Our findings show that IGFBP may be involved in maintaining pregnancy by the effect it has on the concentration of AP gonadotropins.

The secretion and production of the gonadotropins by the AP has been found to be necessary in order for pregnancy to be established and continue in the pig (Tast et al., 2000). Anterior pituitary LH is secreted into the bloodstream throughout pregnancy in a pulsatile manner and serum concentrations of and pattern of release are similar to that of the luteal phase (Ziecik et al., 1982). In the present study, decreased expression of LH- $\beta$  and increased AP concentrations of LH were found in pregnant when compared to non-pregnant gilts. Previous reports have documented that AP concentrations of LH doubled during pregnancy in the pig when compared to any phase of the estrous cycle (Parlow et al., 1964). Changes in AP concentrations of LH have been reported to occur throughout pregnancy in other species. Rahe et al., (1988) reported that AP LH concentrations were greatest at day 125 (midgestation) of pregnancy and decreased throughout the rest of

pregnancy in cattle. Melampy et al. (1966) speculated that during this time period of pregnancy, the AP may be either increasing the synthesis or regulating the release of LH. It has been shown that the synthesis of the LH $\beta$  subunit is the rate limiting step in the formation of LH (Leung et al., 1988; Fetherston and Biome, 1982; Godine et al., 1980). In the present study, expression of LH $\beta$  decreased yet an increase in concentration of AP LH occurred. The AP may then be regulating the release of LH into circulation and decreasing the synthesis of LH. This may be due to the constant feedback coming from high serum concentrations of P4, the decrease in expression of GnRHR, and low serum concentrations of E2. During the gilt's estrous cycle, AP concentrations of LH decrease at the time of estrus due to the release of the LH surge and then increase throughout the rest of the estrous cycle in preparation for the next LH surge (Jolitz and Clapper, 2014; Clapper and Taylor, 2011). Kasa-Vubu et al., (1992) conducted an experiment in sheep that showed that even in the presence of high circulating estradiol, progesterone blocks the GnRH and subsequent LH surge. Progesterone may then be the key limiting factor regulating the surge of LH released by the AP and with prolonged P4 during pregnancy causes the AP to store and regulate the release of AP LH.

### **Conclusion**

This is one of the first studies examining the effects of early pregnancy on the AP IGF-1 system in the pig. Other studies have shown that differences in the expression and synthesis of AP IGF system have effects on AP gonadotropin function and release in the pig (Clapper and Taylor, 2011; Whitley et al., 1995). Regulation of AP LH release may be caused by prolonged high levels of P4 but also by the down regulation of the IGFBP-

2. Further research is needed to determine what occurs throughout pregnancy in the porcine AP.

## Chapter 4

### Summary and Conclusions

The first experiment was conducted to compare ovulation rate with conception rate in swine when using the GnRH agonist triptorelin. Many studies have reported no difference in litter size using a single FTAI with triptorelin compared to the standard AI protocol. Those studies, however, did not compare ovulation rate with conception rate. In the present study, no differences were observed in litter size or pregnancy rate in CON pigs versus the OVU pigs, however, there was a tendency for the CON pigs to have a greater percentage of fetuses compared to the number of CL.

The serum hormonal profile during this experiment did show that the OVU group had a tendency to ovulate earlier than CON group. Serum concentrations of P4 in the OVU group tended to be lower on day 0 and day 1 post Ovugel administration compared to CON group. Serum concentrations of E2 were not different between OVU and CON gilts on any day, yet estrus was exhibited more at the time of AI in CON gilts. This may confirm previous reports stating that the administration of triptorelin may cause a decrease in the number of gilts exhibiting standing estrus due to ovulation occurring before the surge of E2 that has been shown to correspond with the onset of estrus.

The advantage that triptorelin could play in swine production is the ability to forecast the optimal time for AI to take place. This advantage will assist in achieving the highest reproductive performance, schedule labor needed for AI, and increase the ability to induce ovulation in gilts entering the herd regardless of whether they exhibit estrus. This study demonstrated that even though in previous studies the conception rate of

weaned sows and primiparous gilts were not different, there may be a difference when comparing the conception rate to the ovulation rate. More research is needed to determine the optimal time for administration of triptorelin in a FTAI protocol.

Results from the second experiment provide evidence that pregnancy may alter the concentration and expression of hormones in the AP compared to the luteal phase of non-pregnant gilts. The timing in which samples were collected could be of vital importance when characterizing the expression and concentration of hormones during early gestation.

In pregnant gilts, there was a decrease in AP LH- $\beta$  expression and an increase in AP LH concentration with no change in AP GnRHR expression. No differences in GnRHR expression between experimental groups could mean that the AP was not producing as great a quantity of gonadotropins during early pregnancy when compared to the luteal phase gilts. The feedback from progesterone may have also caused a decrease in the expression of GnRHR, thus reducing the production of gonadotropins during both the luteal phase and early pregnancy in gilts. The differences in LH concentration and LH- $\beta$  expression in pregnant versus non-pregnant gilts could be interpreted that LH is being stored in the AP thereby decreasing the release of LH into circulation. Previous research has shown that the actions of the gonadotropins are regulated by the IGF system as well. No differences occurred in pregnant versus non-pregnant for components of the IGF system in the current study other than a decrease in AP IGFBP-2 expression. This may support previous studies that have shown that IGFBP-2 may be involved in the release of LH from the AP.



In conclusion, this study supports the theory that during pregnancy the gilt AP is storing greater amounts of LH compared to the non-pregnant luteal phase gilts. This information may help to explain how changes in the endocrine system may control the synthesis or release of certain hormones affecting early pregnancy in the female pig.

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