EFFECT OF OVULATORY FOLLICLE SIZE ON BOVINE PREGNANCY ASSOCIATED GLYCOPROTEINS IN BEEF CATTLE

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by

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The undersigned, appointed by the Dean of the Graduate School, have examined the thesis entitled

EFFECT OF OVULATORY FOLLICLE SIZE ON BOVINE PREGNANCY ASSOCIATED GLYCOPROTEINS IN BEEF CATTLE.

Presented by Cia Leeanne Johnson

A candidate for the degree of Master of Science

And hereby certify that in their opinion it is worthy of acceptance

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DEDICATION

It is my pleasure to dedicate this thesis to some of the most important people in my life. First and foremost to my husband, Robert, who refused to let me give up! No matter how much I wanted to or how mad I got. To my mom who was always just a phone call away when I needed to draw on her unending support. My mom also did an incredible job assisting me with the editing of my thesis before its submission to my committee. To Melissa, who kept me positive when everything seemed to be going downhill fast. Melissa also played a key role in creating the first draft of this thesis. To Jason and Sara who were a never-ending fountain of support to me during the writing process. To Courtney, for cracking the whip when it was time to write my literature review. To Hannah, who recorded the lectures in block 5 of veterinary school for me and AJ who took notes for me during that same block. And finally, to all of my friends and family for their love and support throughout the years. Thank you all so much!

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LIST OF ABBREVIATIONS

Ab	Antibody
AI	Artificial insemination
BCS	Body condition score
bPAG	Bovine pregnancy associated glycoprotein (s)
bPL	Bovine placental lactogen
BNC	Binucleate cell (s)
C	Celsius
cAMP	Cyclic adenosine monophosphate
CEO	Cumulus-enclosed oocytes
cDNA	Complimentary Deoxyribonucleic acid
CDO	Cumulus-denuded oocytes
CIDR	Controlled Internal Drug Release
CL	Corpus luteum/Corpora lutea
CST3	Cystatin C
CTSL	Capethesin L
d	Day (s)
E2	Estradiol-17β
ELISA	Enzyme linked immunosorbent assay
ESR1	Estrogen receptor alpha

ESR2	Estrogen receptor beta
FSH	Follicle stimulating hormone
GH	Growth hormone
GLYCAM1	Glycosylation-dependent cell adhesion molecule-1
GnRH	Gonadotropin releasing hormone
h	Hour
IgG	Immunoglobulin
i.m.	Intramuscular
IFN-t	Interfuron tau
LE	Luminal epithelium
LGALS15	Galectin-15
LH	Luteinizing hormone
mg	milligram
MGA	Melengestrol acetate
MHz	Megahertz
mRNA	Messenger ribonucleic acid
min	Minute(s)
mL	Milliliter
mm	Millimeter
ng	Nanograms
NFDM	Non-fat dry milk
NT	Nuclear transfer
OGPs	Oviduct-specific estrogen-dependent glycoproteins

oPL	Ovine placental lactogen
OTR	Oxytocin receptor
P4	Progesterone
PAG	Pregnancy associated glycoprotein (s)
PG	Prostaglandin $F_{2\alpha}$
pg	Picogram (s)
PGE_2	Prostaglandin E_2
PL	Placental lactogen
PNPP	Para-nitrophenyl phosphate
PR	Progesterone receptor
PSP60	Pregnancy-specific-protein 60
PSPB	Pregnancy-specific-protein B
RIA	Radioimmunoassay
SAS	Statistical analysis system
SEM	Standard error of the mean
sGE	Superficial ductal glandular epithelium
SPP1	Osteopontin
TAI	Fixed time artificial insemination
trt	Treatment
μg	Microgram
μL	Microliter
U.S.	United States
yr	Year

EFFECT OF OVULATORY FOLLICLE SIZE ON BOVINE PREGNANCY ASSOCIATED GLYCOPROTEINS IN BEEF CATTLE

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ABSTRACT

The gonadotropin releasing hormone (GnRH) induced ovulation of small dominant follicles was associated with reduced pregnancy rates and late embryonic/fetal survival around the time of embryo-uterine attachment. Pregnancy associated glycoproteins (PAG) are secreted by binucleated trophoblast cells into the maternal circulation and have been used to monitor placental function and embryo/fetal mortality. The overall objective was to examine the relationship between ovulatory follicle size and circulating concentrations of bovine pregnancy associated glycoproteins (bPAG). Postpartum cows (n = 69) were treated with the CO-Synch protocol (GnRH-1, followed by prostaglandin $F_{2\alpha}$ [PG] 7 d later, and a second injection of GnRH [GnRH-2] plus artificial insemination (AI) 48 h after PG [d 0]) and classified into one of four groups based on GnRH-induced ovulation of the following follicle sizes at insemination: 1) small dominant follicle (≤ 12 mm; n = 9), 2) small to medium follicle (12 to 13 mm; n = 20), 3) medium to large follicle (14 to 15 mm; n = 28) or 4) large follicle (≥ 16 mm; n = 12). Serum samples were collected every other day from d 20 to 60 with daily samples from d 26 to 30 (d 0 = GnRH-2). After d 60, serum samples were collected monthly until

calving. Bovine PAG were detected in serum by using an antibody mixture (Ab 1 or Ab 2), which detected different bPAG gene products in an ELISA. There was an effect ($P < 0.05$) of treatment on pregnancy rates at d 30 post insemination with pregnancy rate being higher ($P < 0.05$) following GnRH-induced ovulation of 14 to 15 mm compared to 12 to 13 mm follicles. The first increase ($P < 0.0001$) in bPAG occurred on d 24 with both Ab 1 and Ab 2. There was no difference between the two antibody mixtures in the pattern of serum concentrations of bPAG from d 20 to 60; however, there was a difference in the quantity of bPAG with concentrations of bPAG being greater with Ab 2 compared to Ab 1. There was an effect of day ($P < 0.0001$) on bPAG but no effect of ovulatory follicle size or ovulatory follicle size by day interaction from d 20 to 60 with either antibody. Furthermore, there was an effect of month ($P < 0.0001$) on bPAG but no effect of treatment or treatment by month interaction from 3 months of gestation to calving. Estradiol (E2) increased ($P < 0.001$) from PG (d -2) injection to insemination (d 0) regardless of ovulatory follicle size. Animals that showed estrus had higher ($P < 0.001$) serum concentrations of estradiol at insemination than the animals that did not show estrus. Pregnancy rates were higher ($P < 0.05$) in cows that showed estrus compared to cows that did not show estrus. Whether or not the animals expressed estrus on d 0 did not effect serum concentrations of bPAG from d 20 to 60. Furthermore, estrus expression did not effect the serum concentrations of bPAG from three months of gestation to calving. There was no correlation between the change in E2 from d -2 to 0 and the release of bPAG from d 20 to 60 ($r^2 = 0.01$). There was an effect of day ($P < 0.0001$) on serum concentrations of progesterone (P4) from d 20 to 60 but no effect of treatment or treatment by day interaction. In addition, there was no relationship between

mean circulating concentrations of P4 and the release of bPAG from d 20 to 60 (r^2 = 0.05). In summary, there was no effect of ovulatory follicle size on serum concentrations of bPAG or P4 in pregnant cows. Cows that exhibited standing estrus had higher serum E2 concentrations prior to insemination and were more likely to become pregnant after insemination.

CHAPTER I

INTRODUCTION

Artificial insemination (AI) is the most effective method for genetic improvement in beef cattle. Rapid genetic improvement can be made by inseminating heifers and cows to sires proven to be genetically superior for economically relevant traits. Unfortunately, the time and labor required to detect estrus hampers the wide spread use of AI in the beef industry. Increased adoption of AI in beef cattle is dependent upon development of economical and effective methods of synchronizing estrus and ovulation such that cattle can be inseminated at a predetermined time. More specifically, an optimum protocol for synchronizing estrus and ovulation should meet the following criteria: 1) reduce or eliminate the time required for estrus detection, 2) result in conception rates that are equal to natural service, 3) allow cycling animals to conceive earlier in the breeding season, 4) induce a fertile estrus in anestrous females to allow them to conceive earlier in the breeding season, and 5) minimize the number of times the animals are handled.

The ability to precisely synchronize estrus and ovulation has been facilitated by recent advances in our understanding of patterns of follicular growth in cattle. Bovine ovarian follicles grow in distinct wave-like patterns, with new waves occurring every 10 d (ranging from 6 to 15 d; Sirois and Fortune, 1988). This knowledge combined with the ability to induce premature regression of a corpus luteum (CL) has allowed the

development of synchronization protocols that control both follicular waves and luteal lifespan (Thatcher and Santos, 2007). Many of the current protocols for fixed-time artificial insemination (TAI) utilize the gonadotropin-releasing hormone (GnRH) – prostaglandin $F_{2\alpha}$ (PG) – GnRH method for synchronization of estrus and ovulation (see Figure 1.1; Lamb et al., 2009).

The purpose of the initial GnRH injection is to induce ovulation of a dominant follicle and synchronize a new follicular wave. Ovulation in response to GnRH induces the formation of luteal tissue which can increase the proportion of peripuberal and anestrous cows that begin to cycle (Lamb et al., 2009) An injection of PG is administered 7 d after the first GnRH injection to regress luteal tissue followed 48 to 72 h later by a second injection of GnRH . With the CO-Synch protocol AI occurs at GnRH injection; whereas, insemination occurs 16 h after GnRH with the Ovsynch protocol (Figure 1.1).

Ovulatory follicle size at the second injection of GnRH (CO-Synch protocol) has been shown to affect pregnancy rates in beef cows. Cows that had a dominant follicle > 12 mm in diameter at the second GnRH-induced ovulation had higher pregnancy rates compared to cows induced to ovulate follicles $\leq$ 12 mm (Lamb et al., 2001). Cows (Perry et al., 2005) and heifers (Perry et al., 2007) that were induced to ovulate a small ($\leq$ 11 mm) follicle had decreased pregnancy rates in comparison to those that ovulated a large (> 11 mm) follicle following CO-Synch. However, cows that showed estrus and spontaneously ovulated small dominant follicles had pregnancy rates comparable to cows that were induced to ovulate a large follicle (Perry et al., 2005). Therefore, it appears that the physiological maturity of a follicle (i.e. ability to initiate estrus) and not the absolute

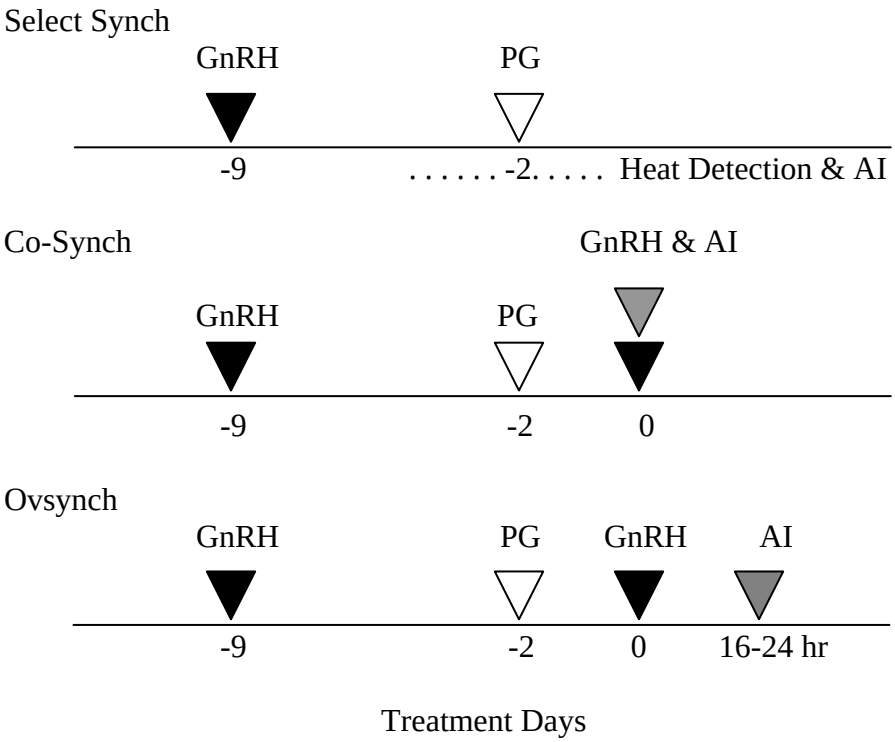


Figure 1.1. Methods used to synchronize ovulation (Ovsynch, CO-Synch) or estrus (Select Synch). Gonadotropin releasing hormone (GnRH) injected on d -9 of treatment will induce ovulation of a dominant (> 10 mm) follicle and initiate a new follicular wave. Injection of prostaglandin $F_{2\alpha}$ (PG) on d -2 will induce luteolysis (primary and accessory CL). GnRH injection on d 0 will induce ovulation in fixed-time AI protocols.

diameter is important for the establishment and maintenance of pregnancy (Perry et al., 2005).

The physiological mechanisms responsible for the decreased pregnancy rates and increased late embryonic/fetal mortality following GnRH-induced ovulation a of small dominant follicle are not known. Late embryonic/fetal loss in cattle frequently occurs around the time of embryo uterine attachment (Vasconcelos et al., 1997; 1999; Cartmill et al., 2001; Moreira et al., 2001), and this was the case following GnRH-induced ovulation of small dominant follicles in postpartum beef cows (Perry et al., 2005). In order to investigate mechanisms associated with late embryonic/fetal mortality it would be helpful to identify a marker to monitor conceptus viability over time. Bovine pregnancy associated glycoproteins (bPAG) are expressed in the binucleate cells of the placenta, detected in the maternal circulation around d 24 to 26 after insemination (Green et al., 2000), and may serve as a marker in the maternal circulation for placental function (Perry et al., 2005). A preliminary study, from our laboratory, indicated that GnRH-induced ovulation of small follicles resulted in reduced secretion of bPAG from d 24 to 60 compared to GnRH-induced ovulation of large follicles (Busch, 2005). Therefore, we wanted to further examine the relationship between ovulatory follicle size and serum concentrations of PAG from d 20 to 60.

The objectives of this study were to: 1) Determine when the first statistically significant increase in bPAG occurs in pregnant cows following insemination; 2) Examine the effect of late embryonic/fetal mortality on serum concentrations of bPAG; 3) Compare the pattern of secretion of bPAG in serum samples in which bPAG were detected with two different mixtures of monoclonal antibodies; 4) Determine the effect of

ovulatory follicle diameter at insemination on the circulating concentrations of bPAG from d 20 to 60 post insemination and from three months to calving; 5) Determine the relationship between preovulatory serum concentrations of estradiol (E2; d -2 and 0) and serum bPAG levels from d 20 to 60 post insemination (d 0) and from three months to calving; and 6) Determine the relationship between serum concentrations of progesterone (P4) and bPAG from d 20 to 60 post insemination (d 0) and from three months to calving.

CHAPTER II

REVIEW OF LITERATURE

INTRODUCTION

Artificial Insemination (AI) is an efficient and economical method for genetic improvement in cattle. However, detecting spontaneous estrus in cattle in which estrus has not been synchronized is time consuming and labor intensive. To offset the time and labor involved in estrus detection, protocols for synchronizing estrus and/or ovulation have been developed. Many available estrus synchronization protocols utilize the gonadotropin-releasing hormone (GnRH) – prostaglandin $F_{2\alpha}$ (PG) – GnRH method (Figure 1.1). These protocols follow a schedule of an initial GnRH injection followed 7 d later with an injection of PG, followed 48-72 h later by a second injection of GnRH. In beef heifers and postpartum cows insemination generally occurs at the second GnRH injection. The purpose of the initial injection of GnRH is to induce ovulation of a dominant follicle and to synchronize of a new follicular wave. In prepubertal heifers and anestrous cows induction of luteal tissue following the first GnRH injection increases the proportion that initiate a normal cycle. PG induces luteolysis of the corpus luteum (CL) from the preceding estrus as well as any accessory CL that form in response to GnRH-induced ovulation. The second GnRH injection is administered to synchronize insemination with induction of ovulation of the dominant follicle.

Previous studies reported variation in the size of the dominant follicle induced to ovulate at the second GnRH injection in beef heifers (Perry et al., 2007) and postpartum cows (Perry et al., 2005). Furthermore, cows (Perry et al., 2005) and heifers (Perry et al., 2007) that were induced to ovulate a small (≤ 11.3 mm) follicle had decreased pregnancy rates in comparison to those that ovulated a large (> 11.3 mm) follicle following the CO-Synch protocol. However, cows that showed estrus and spontaneously ovulated a small dominant follicle had pregnancy rates comparable to cows that were induced to ovulate a large follicle. Therefore, it appears that the physiological maturity of a follicle (i.e. ability to initiate estrus) and rather than the absolute diameter is important for the establishment and maintenance of pregnancy (Perry et al., 2005).

The purpose of this chapter is to review the literature in the following areas: 1) follicular, oocyte, and luteal determinants of fertility, and 2) products of the bovine placenta with particular emphasis on pregnancy associated glycoproteins (PAG).

DETERMINANTS OF FERTILITY

For this review fertility is defined as the ability of a female to conceive and maintain pregnancy to parturition. There are numerous factors that influence fertility including nutrition, environment (e.g. heat stress), seminal traits, and expression of estrus. However, the focus of this section of the review will be on follicular, oocyte, and luteal factors that may affect fertility, with emphasis on ruminants in general and cattle in particular.

Follicular Determinants of Fertility

The preovulatory follicle may affect the establishment and maintenance of pregnancy via an effect of follicular hormones on the oviductal/uterine environment and (or) an effect of the follicular environment on oocyte competence. Each of these possibilities will be discussed below.

Effect of Follicular Hormones on the Oviductal/Uterine Environment

Cattle that exhibit standing estrus within 24 h of TAI have greater serum concentrations of estradiol (E2) and greater pregnancy success compared to animals that do not exhibit estrus (Perry et al., 2005, 2007; Perry and Perry, 2008a). Increased pregnancy rates in cattle that express estrus may be due to effects of follicular steroids on the oviductal and (or) uterine environment or possibly the oocyte.

Oviductal Environment

During late follicular development and estrus the oviduct undergoes physiological and biochemical changes that optimize the microenvironment for fertilization and early cleavage-state embryonic development (Buhi et al., 2000). These changes appear to be regulated by ovarian steroids, the most important of which being estrogen (reviewed in Buhi et al., 1997, 2000). The oviduct is a biologically active tissue that synthesizes and releases protein macromolecules (Geisert et al., 1987). Oviductal biosynthetic activity is greater under estrogen as opposed to progesterone (P4) domination in the cow (Geisert et al., 1987).

One group of macromolecules that has been described in a variety of species is the oviduct-specific, estrogen-dependent glycoproteins (OGPs; Buhi, 2002). The presence of OGPs is maximized at the time of ovulation, fertilization and the early

cleavage-stage embryo (Buhi et al., 2000). The apparent targets of the OGPs are the zona pellucida, perivitelline spaces of oviductal oocytes and membranes of oocytes or blastomeres (reviewed by Buhi, 2002); however, the functional role of OGPs remains a mystery. In the bovine, OGPs bind to the sperm membrane and may facilitate capacitation (King et al., 1994).

Uterine Environment

Preovulatory concentrations of E2 may impact the uterine environment via several mechanisms: increasing sperm longevity within the uterus via a decrease in uterine pH, adequate preparation of granulosa cells for luteinization, and (or) preparation of the endometrium for pregnancy establishment and maintenance.

Effect of Uterine pH: Estradiol may impact sperm longevity by altering the uterine environment prior to fertilization (Perry and Perry, 2008a). The motility of sperm collected from the caudal epididymis was reduced following a decrease in pH (Acott and Carr, 1984). More specifically, the motility of bull sperm was suppressed as pH decreased from 6.9 to 6.4 (Goltz et al., 1988) and increased as pH also increased (Jones and Bavister, 2000). The preceding suppression of sperm motility was reversible for up to 24 h by increasing pH.

Frozen thawed spermatozoa frequently have a reduced lifespan compared to a fresh ejaculate due to premature capacitation during the freezing and thawing process (Bailey et al., 2000). Consequently, a transient decrease in uterine pH may increase sperm lifespan by causing a transient decrease in motility. With TAI protocols in beef cattle, semen deposition normally occurs at GnRH injection and the interval from the second GnRH injection(or initiation of estrus) to ovulation is approximately 30 h (Pursley

et al., 1995; Vasconcelos et al., 1999). When cattle spontaneously express estrus and ovulate the cows are artificially inseminated approximately 8 to 12 h after the onset of estrus which translates into approximately 18 to 22 h preceding ovulation. In the preceding situation, frozen thawed sperm don't need to survive as long as with fixed-time AI since the spontaneous preovulatory gonadotropin surge (signal for ovulation) occurs before or at the onset of estrus and the interval from the luteinizing hormone (LH) surge to ovulation is similar to the interval from a GnRH-induced LH surge to ovulation (Pursley et al., 1998).

Perry and Perry (2008a) reported a decrease in uterine pH at the second GnRH injection in cows that had elevated concentrations of E2. Furthermore, uterine pH was lower for cows in estrus compared to cows that did not express estrus (Perry and Perry, 2008b). A decrease in uterine pH may initially decrease sperm motility and thus increase sperm longevity leading to increased fertility of cows that have elevated serum concentrations of E2.

Effect of Follicular Environment on Subsequent Luteinization of Granulosa Cells: Estradiol may have a role in the preparation of follicular cells for luteal formation and function. Granulosa cells convert androgens to E2 via aromatase and intrafollicular concentrations of E2 can attain concentrations of ≥ 1 μg/ml. In addition, granulosa cells contain the genomic E2 receptor (ESR2; Rosenfeld et al., 1999) as well as nongenomic receptors (Wang et al., 2007). Therefore, E2 can act directly on granulosa cells via intracrine, autocrine, or paracrine communication. The ability of luteinized human granulosa cells to secrete P4 was increased when cells were collected from follicles that had increased follicular fluid concentrations of E2 compared to the granulosa cells

collected from follicles with a lower concentration of E2 (McNatty et al., 1979). Also, the postovulatory secretion of P4 in ewes given an aromatase inhibitor before induced ovulation was delayed (Benoit et al., 1992).

Effect of Estradiol on Uterine Environment: In dairy cows, uterine environment on d 7 after breeding differed between cows with excellent to good embryos compared to cows with fair to poor embryos (Wiebold, 1988) and the pattern of secretion of preovulatory concentrations of E2 and postovulatory concentrations of P4 has a critical role in the preparation of uterus for pregnancy. Moore (1985) reviewed a series of elegant studies in which the effect of supplementation of ovariectomized ewes with E2 and (or) P4 on the establishment and maintenance of pregnancy following embryo transfer in sheep was examined. In the review, he reported that ovariectomized ewes that did not receive a period of P4 priming (P4 administration that mimicked a luteal phase), but did receive subsequent E2 administration to mimic the preovulatory follicular phase followed by P4 supplementation were unable to maintain pregnancy following embryo transfer (Moore, 1985). However, when ewes received P4 priming for 1, 4, or 8 d the proportion of ewes that had a viable embryo at 17 to 18 d post embryo transfer was 6/20, 14/21, and 15/55, respectively (Moore, 1985). Miller et al. (1977) also reported that when ovariectomized ewes received P4 priming followed by E2 and then P4 that 21 of the 27 ewes had a viable embryo at 10 to 13 d after induced estrus. However, embryos transferred to ewes that did not receive either preovulatory E2 or P4 priming to simulate endogenous ovarian secretions ceased to develop normally 1 to 2 d after the time of transfer (Miller et al., 1977). Interestingly, when only E2 administration was omitted from the preceding sequence of steroid injections, the amounts of endometrial E2 and P4

receptors at the time of embryo transfer were reduced (Miller et al., 1977). E2 has also been associated with the induction of endometrial P4 receptors in sheep (PR: Miller et al., 1977; Zelinski et al., 1980; Ing and Tornesi, 1997).

Mechanisms Affecting Oocyte Competence

Oocyte competence is defined as the ability of an oocyte to resume meiosis following gonadotropin stimulation; undergo cleavage divisions after fertilization; develop to the blastocyst stage; and result in birth of live and healthy young (Sirard et al., 2006). Oocyte competence may be directly affected by the follicular environment (e.g. ovarian steroids) since the murine oocyte is reported to express the estrogen receptor (Wu et al., 1992). It is well known that oocyte competence is affected by cumulus cells during nuclear and cytoplasmic maturation. Oocyte competence has an important affect of fertility since it has been linked to embryo quality (Hutt and Albertini, 2007).

Effect of the Pre-Ovulatory Period on Oocyte Competence

The role of follicle stimulating hormone (FSH) in acquisition of oocyte competence has been associated with its effect on follicular maturation (Sirard, 2001). In cattle, FSH stimulates granulosa cell proliferation in the preantral phase, initiates recruitment of a follicular cohort at the onset of a follicular wave, prevents atresia, and induces the synthesis of luteinizing hormone (LH) receptors in combination with E2. Previous studies have shown that exogenous FSH administration can increase the number of viable oocytes per collection from heifers or cows (Sirard and Lambert, 1985; Armstrong et al., 1994). When heifers were treated with an optimal FSH protocol, prior to oocyte collection, more than 75% of the immature oocytes that were fertilized, in vitro, developed to the blastocyst stage (Blondin et al., 2002). However, when no FSH was

administered, only 40% of the oocytes developed to the blastocyst stage (Blondin et al., 2002). These results support the concept that culture conditions for in vitro oocyte maturation/fertilization and cleavage division are adequate. Furthermore, oocyte competence is acquired prior to germinal vesicle breakdown and influenced by the follicular microenvironment (Sirard et al., 2007).

Role of the Cumulus Cells in Nuclear Maturation of the Oocyte

Within an oocyte, induction of nuclear maturation is mediated by the somatic compartment of the follicle (Buccione et al., 1990). Cumulus cells contain LH receptors (Baltar et al., 2000) and Hyttel (1987) hypothesized that gonadotropins stimulate meiotic resumption by disrupting gap junctional communication between cumulus cells and the oocyte. Furthermore, down regulation of gap junctional communication is a prerequisite for cumulus expansion (Chen et al., 1990; Sutovsky et al., 1995).

Cumulus cells play an important role in oocyte maturation by maintaining the oocyte under meiotic arrest (Buccione et al., 1990), participating in the induction of meiotic resumption (Buccione et al., 1990), and supporting cytoplasmic maturation (Chian et al., 1994). Furthermore, removal of bovine cumulus cells inhibited oocyte nuclear maturation, in vitro (Fukui and Sakuma, 1980). The direct transfer of substances between cumulus cells and the oocyte is required for maintenance of meiotic arrest in mammals (Buccione et al., 1990). This transfer is mediated by gap junctions allowing the exchange of ions and small molecules among cells (Tanghe et al., 2002). Several substances are suspected to act as meiotic inhibitors, such as purines (hypoxanthine and adenosine [Eppig et al., 1985]) and cyclic adenosine monophosphatase (cAMP; Kumar and Gilula, 1996). It appears that cumulus cells control meiosis in bovine oocytes since

compounds that elevate intracellular concentrations of cAMP inhibit or delay meiotic resumption in cumulus-enclosed oocytes (CEO) but not in cumulus-denuded oocytes (CDO; Leibfried-Rutledge et al., 1989).

Role of the Cumulus Cells in Cytoplasmic Maturation of the Oocyte

The cumulus cells are also important for the bovine oocyte to complete cytoplasmic maturation, which is necessary to acquire the capacity to support male pronucleus formation, monospermic fertilization, and early embryonic development (Chian et al., 1994). Cytoplasmic maturation maybe modulated by the cumulus cells through secretion of soluble factors that induce developmental competence and (or) removal of an embryo development-suppressive component from the medium (Hashimoto et al., 1998).

Molecular maturation is also an important aspect of oocyte cytoplasmic maturation. Messenger ribonucleic acids (mRNA) synthesized in the oocyte during follicular maturation may be necessary for proper conceptus development. Piedrahita and others (2002) collected oocytes from 1 to 3 mm follicles and 6 to 12 mm follicles. Following somatic cell nuclear transfer of donor cells (EFC cell line) into the recipient cytoplasts originating from the preceding follicle groups, the embryos developed in vitro and were transferred to recipient females. On d 27 following embryo transfer, conceptuses derived from oocytes collected from the larger follicles had larger extraembryonic membranes compared to conceptuses derived from oocytes collected from the smaller follicles. Also, allantois/chorion fusion had not occurred in conceptuses derived from oocytes collected from the smaller follicles but had in conceptuses derived

from oocytes collected from the larger follicles (Piedrahita et al., 2002). These data provide evidence for an effect of the follicular microenvironment on oocyte competence.

Formation of persistent follicles in cattle provides another example of an effect of the follicular microenvironment on oocyte competence. Long-term treatment of heifers or postpartum beef cows with a low concentration of a progestin (e.g. melengestrol acetate [MGA]), in the absence of a CL, causes formation of a persistent ovarian follicle that results in low fertility after ovulation (Zimbelman and Smith, 1966; Patterson et al., 1989; Yelich et al., 1997). Persistent follicles have been characterized as having a prolonged lifespan, larger diameter, and increased E2 secretion compared to dominant follicles that develop during the luteal phase (Fralix, et al., 1996; Kojima et al., 1995; Sirois and Fortune, 1990). Furthermore, persistent follicles that contain aged oocytes have a normal fertilization rate, however, following fertilization fewer of the oocytes from persistent follicles developed to the morula stage compared to oocytes ovulated from growing follicles (Ahmad et al., 1995).

Relationship Between the Oocyte and Embryo Quality

The preceding sections focused on processes required to produce a developmentally competent oocyte as well as the growth and maturation phases of an oocyte. Recently, more emphasis has been placed on the final peri-ovulatory maturation of the oocyte (Hutt and Albertini, 2007). The reasons for this increased emphasis include the need for the oocyte to: achieve nuclear and cytoplasmic maturation stimulating modifications to the signaling pathways between the oocyte and the surrounding cumulus oophorus; undergo spontaneous resumption and completion of meiosis; and synthesize, process and secrete factors that impact the developmental potential of embryos (reviewed

in Hutt and Albertini, 2007). This stage highlights the link between oocyte competence and embryo quality.

Luteal Determinants of Fertility

Progesterone Profiles in Pregnant vs. Non-Pregnant Cows

Progesterone is required for the establishment and maintenance of pregnancy in numerous mammalian species. In cattle, numerous investigators have examined circulating concentrations of P4 in pregnant and nonpregnant cattle. Pregnant cows have greater serum concentrations of P4 beginning on d 9 after insemination compared to nonpregnant cows (Perry et al., 2005; Lopes et al., 2007) and differences in circulating concentrations of P4 in pregnant and non-pregnant cows have also been seen as early as d 6 post-insemination (Mann et al., 1999). Cows that underwent an earlier rise in P4 had embryos that were further developed and produced more of the antiluteolytic protein, interferon-tau (IFN-t), by d 16 than cows that had a delayed rise in circulating concentrations of P4 (Mann and Lamming, 2001).

Investigation of the effect of P4 supplementation on the establishment and maintenance of pregnancy in cattle has yielded mixed results. Progesterone has been administered to create an earlier postovulatory rise in P4 and several groups report improved bovine embryo development (Garret et al., 1988; Kerbler et al., 1997; Mann and Lamming, 2001; Carter et al., 2008). However, others report no consistent improvement in pregnancy rate following P4 supplementation in cattle (Funston et al., 2005; Hanlon et al., 2005; Galvao et al., 2006; Stevenson et al., 2007; 2008). After conducting a meta-analysis of 17 reports in which cattle received P4 supplementation,

Mann and Lamming (1999) concluded that P4 supplementation increased pregnancy rates by 5%.

Discrepancy in pregnancy rates following P4 supplementation in cattle may be partially explained by differences among studies in day of treatment and relative fertility of the cows that were treated (Mann and Lamming, 1999). P4 supplementation improved pregnancy rates when cows had low concentrations of P4 on d 5 (1 to 2 ng/ml; d 0 = AI) but did not improve pregnancy rates when cows had higher circulating concentrations of P4 on d 5 (Starbuck et al., 2001).

Progesterone Regulation of the Uterine Environment

Domestic animals are spontaneous ovulators that undergo uterine-dependent estrous cycles until the establishment of pregnancy. The length of the estrous cycle is dependent on the endometrium as the source of the luteolysin, PG. During the estrous cycle, the epithelial lining of the endometrium releases oxytocin-induced luteolytic pulses of PG that result in functional and structural regression of the ovarian CL (Spencer et al., 2004). Following the rapid decrease in P4 at luteolysis, the preovulatory follicle secretes increasing concentrations of E2 that stimulate increased uterine estrogen receptor alpha (ESR1), progesterone receptor (PR), and oxytocin receptor (OTR) expression (Spencer et al., 2004) During early diestrus, P4 from the newly formed CL stimulates synthesis and secretion of PG (Spencer et al., 2004). During diestrus, circulating concentrations of P4 increase and act via PR to block expression of ESR1 and OTR in the endometrial luminal epithelium (LE) and superficial ductal glandular epithelium (sGE; Spencer et al., 2004). Continuous exposure of the uterus to P4 for 8 to 10 d down-regulates expression of PR in endometrial LE and sGE after d 11 and 12, allowing for a rapid increase in expression of

ESR1 on d 13 followed by OTR on d 14 in LE and sGE. (Spencer et al., 2004) Oxytocin, secreted beginning on d 9 of the estrous cycle, from the posterior pituitary and/or CL, induces release of luteolytic PG pulses from the endometrial LE and sGE on d 14 to 16 (Spencer et al., 2004). The CL undergoes regression, allowing for the return to estrus and completing the estrous cycle of the sheep (Spencer et al., 2004). In summary, P4 initially suppresses and subsequently induces development of the endometrial luteolytic mechanism during the estrous cycle (Spencer et al., 2004).

In addition to its preceding role in luteolysis, P4 stimulates expression of a number of genes in the endometrium that are important for embryo growth and survival. In dairy cattle blastocyst development followed by a successful pregnancy occurs in cows that have an early rise in P4 following mating (Mann and Lamming, 2001). The mechanisms by which P4 stimulates blastocyst growth are unknown; however, the process is thought to begin with P4 downregulating its receptor in the uterine epithelium (Spencer et al., 2007). This induces several epithelial genes including; cystatin C **(**CST3), capethesin L (CTSL), glycosylation-dependent cell adhesion molecule-1 (GLYCAM1), galectin-15 (LGALS15), and osteopontin (SPP1) (reviewed by Spencer et al., 2007). These genes are hypothesized to regulate the conceptus' development in the peri-implantation period of pregnancy (Spencer et al., 2007).

PRODUCTS OF THE PLACENTA

The establishment and maintenance of pregnancy is dependent upon the maintenance of the CL, endometrial differentiation in preparation for the arrival of the embryo, embryonic development/elongation/apposition, and placentation. This section of

the review will focus on changes in the embryo/blastocyst/ conceptus during the early stages of pregnancy (Table 2.1), and two placental gene products (pregnancy associated glycoproteins [PAG] and placental lactogen [PL]) that are produced by trophoblast binucleate cells (BNC) and might serve as markers of placental function.

Embryo-Maternal Interactions in the Establishment of Pregnancy

In cattle, fertilization occurs near the ampullary-isthmus junction of the oviduct and around the morula stage (d 5) the embryo enters the uterus (Shea, 1981). Blastocysts form on d 7 to 8, hatch from the zona pellucida on d 9 to 11, and elongate on d 12 to 13 (Shea, 1981). Maternal recognition of pregnancy occurs on d 15 to 17 (Bazer et al., 1997) which is followed by the formation of BNC around d 16 to 17 (Wooding and Wathes, 1980). Adhesion of the embryo to the endometrium occurs on d 21 to 22 (Peters 1996). BNC migrate to and fuse with the luminal epithelium to form trinucleate cells. These trinucleate cells form syncytial plaques (Spencer et al., 2007) which are part of the reason that placentation in cattle has been classified as syndesmochorial.

Implantation occurs in successive phases beginning with hatching from the zona pellucida (reviewed in Chavatte-Palmer and Guillomot, 2007; Spencer et al., 2007). This is followed by the pre-attachment phase, which is referred to by some as pre-contact and blastocyst orientation. At this stage there are no cell contacts observed between the trophoblast and the uterine epithelium (Chavatte-Palmer and Guillomot, 2007). Therefore, the blastocyst can be collected by flushing the uterus without damaging the embryo (Chavatte-Palmer and Guillomot, 2007). The blastocyst position within the uterine lumen is central in cattle and other species that are characterized by large

Table 2.1. Timing of important stages in the establishment of pregnancy in cattle.

ESTABLISHMENT OF PREGNANCY	**DAY OF GESTATION**
Maternal to Zygotic Transition	3 to 4
Morula	5
Enters Uterus	4 to 5
Blastocyst	7 to 8
Hatching from Zona Pellucida	9 to 11
Elongation of Trophoblast	12 to 13
Maternal Recognition of Pregnancy	15 to 17
Formation of BNC	16 to 17[$]
Placentation	20 to 90*
Adhesion	21 to 22

[$]As reviewed by Wooding and Wathes 1980
*As reviewed by King et al., 1981

blastocyst expansion (reviewed in Chavatte-Palmer and Guillomot, 2007). The blastocyst orientation is defined by the position of the embryonic disc relative to the mesometrium side of the uterus (to which the suspensory ligament is attached; reviewed in Chavatte-Palmer and Guillomot, 2007). In cattle the embryonic disc faces the antimesometrial pole and so its orientation is antimesometrial.

The pre-attachment phase is followed by apposition which is defined as the first cell to cell contact between the trophoblast and endometrial epithelium (Chavatte-Palmer and Guillomot, 2007). In ruminants, the first cell contacts begin in the vicinity of the embryonic disc and spread towards the ends of the extraembryonic membranes (Chavatte-Palmer and Guillomot, 2007). In the cow, the trophoblast attaches mainly to the caruncular epithelium and to a lesser extent, the intercaruncular mucosa (Chavatte-Palmer and Guillomot, 2007). In these areas transitory villi form on the trophoblast and invade the uterine glands (Chavatte-Palmer and Guillomot, 2007). This ensures anchorage of the conceptus in the uterine cavity and provides localized absorption sites of the glandular secretions (reviewed in Chavatte-Palmer and Guillomot, 2007).

The final stage of implantation is adhesion which can be subdivided into adhesion and invasion. For the purposes of this discussion we will refer to this as one stage. During adhesion the cellular contacts between the trophoblast and the uterine epithelium become closer (Chavatte-Palmer and Guillomot, 2007). Trophoblast invasion of the endometrium, to a greater or lesser extent, is species dependent; however, no true invasion is observed in ruminants (Chavatte-Palmer and Guillomot, 2007; Schlafer et al., 2000). Trophoblast invasiveness is primarily limited to BNC (Chavatte-Palmer and Guillomot, 2007). These cells and migrate between mononucleated cells in the

trophoblast and fuse with individual uterine epithelial cells to form syncytial plaques (Chavatte-Palmer and Guillomot, 2007). Binucleate cells represent around 20% of the trophoblastic cells in the ruminant placenta (Chavatte-Palmer and Guillomot, 2007). Binucleate cells originate from trophoblast cells through the process of acytokinesis in which the nucleus divides but the cytoplasm does not. During development, the BNC migrate from the chorionic epithelium to the endometrial epithelium (Wooding and Wathes, 1980). The BNC transfer complex molecules from the fetal to the maternal placenta (Schlafer et al., 2000). Two products that are secreted by BNC are PAG and PL (Schlafer et al., 2000).

Pregnancy Associated Glycoproteins

Pregnancy associated glycoproteins are placental proteins that belong to the aspartic proteinase family with more than 50% of their amino acid sequence identical to pepsin, cathepsin D and E (Beckers et al., 1998). Molecular modeling suggests that some PAG are unable to act as proteinases though, due to unusual amino acid substitutions near the catalytic center. Consequently, PAG possibly have other roles like peptide binding or immunosuppression (Dosogne et al, 1999).

History

Pregnancy associated glycoproteins were first discovered during attempts to develop an early pregnancy test in cattle. The first bovine PAG to be discovered was bovine pregnancy associated glycoprotein (bPAG)-1, which is closely related, possibly identical to, pregnancy-specific-protein B (PSPB), and pregnancy-specific protein-60 (PSP60; Wooding et al., 2005). Pregnancy-specific-protein B (bPAG-1) was isolated from bovine fetal cotyledons by Butler et al.(1982). In 1986, Sasser et al. developed a

specific radioimmunoassay (RIA) for PSPB. Since that time 22 distinct, full-length complimentary Deoxyribonucleic acid (cDNA) encoding different bPAG members have been cloned, but it is suspected that there are many more (Wooding et al., 2005; Telugu et al., 2009). Recently bPAG 22 was found to be a variant of bPAG 2 and not considered distinct enough to be classified as a separate bPAG (Telugu et al., 2009). The focus of the research on bPAG has been the development of a highly reliable, rapid, and economical test for pregnancy.

It is currently thought that there are two different types of ruminant PAG in existence and both are present from implantation to term. The ancient PAG are thought to have originated approximately 85 million years ago, corresponding to the time of the divergence of *Artiodactyla* and *Perissodactyla* lineages (Wooding, et al., 2005). These PAG exhibit more typical proteolytic activity and seem to be expressed throughout the trophectoderm (Green et al., 2000). The more modern PAG that are localized to the BNC of bovidae species seem to have diverged from the ancient grouping about 55 million years ago, corresponding with the separation of the lineages leading to modern day cattle and swine (Wooding et al., 2005). Although the exact function of either group of PAG has yet to be determined there are a few very plausible theories to consider for each and these are discussed in the functional implications section.

Ancient PAG

Out of the 21 bPAG that are known, only six are thought to be ancient PAG leaving the other 15 to the modern group (Wooding et al., 2005; Telugu et al., 2009). The ancient PAG are strategically oriented between the maternal and fetal epithelium of the placenta. This is an ideal position to serve several functions, one of which is adhesion

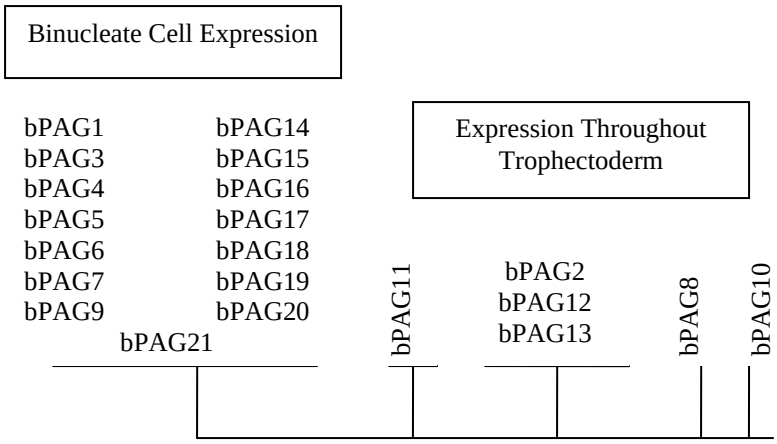


Figure 2.1. List of bPAG expressed by BNC and trophectoderm (Adapted from Green et al., 2000)

(Wooding et al., 2005). For efficient transport of gases and nutrients the gap between the trophoblast and uterine surface must be as narrow as possible. Another possible function of ancient PAG is that they possess highly specialized substrate specificity that allows them to proteolytically process and activate latent growth factors or other bioactive molecules secreted at the fetal-maternal interface (Wooding et al., 2005). Finally the ancient PAG could aid the modern PAG by destroying or sequestering harmful molecules and cells. Of the above mentioned functions adhesion had the most support as ancient PAG are either not expressed or expressed weakly in interplacentomal regions where there is less definitive contact (Wooding et al., 2005) until a recent examination of bPAG 2 and 12 by Telugu and others (2010). This study is the first to describe proteolytic activity in bPAG.

Modern PAG

Modern PAG are primarily found in the trophoblast BNC and are thought to diffuse into the maternal circulation and localize in the maternal connective tissue. This localization places the modern PAG in a position to engage in immunological camouflage like blocking lymphocyte or polymorphonuclear leukocyte migration and activation (Wooding, et al., 2005). Alternatively, modern PAG have been reported to have a luteotrophic effect in that, when administered to cultured endometrial cells they promote increased prostaglandin E_2 (PGE_2), which is proposed to stimulate luteal P4 secretion (Wooding, et al., 2005; Weems et al., 1998). In 2007, Weems and others found concentrations of PGE_2 in ovine luteal tissue in vitro were increased by treatment with PSPB on d 40, 50, 60 and 90 of gestation. The CL becomes dependent on PGE_2 as a

luteotropin and the secretion of PGE_2 is proposed to be regulated by PSPB from d 50 to 90 in the ewe (Weems et al., 2007).

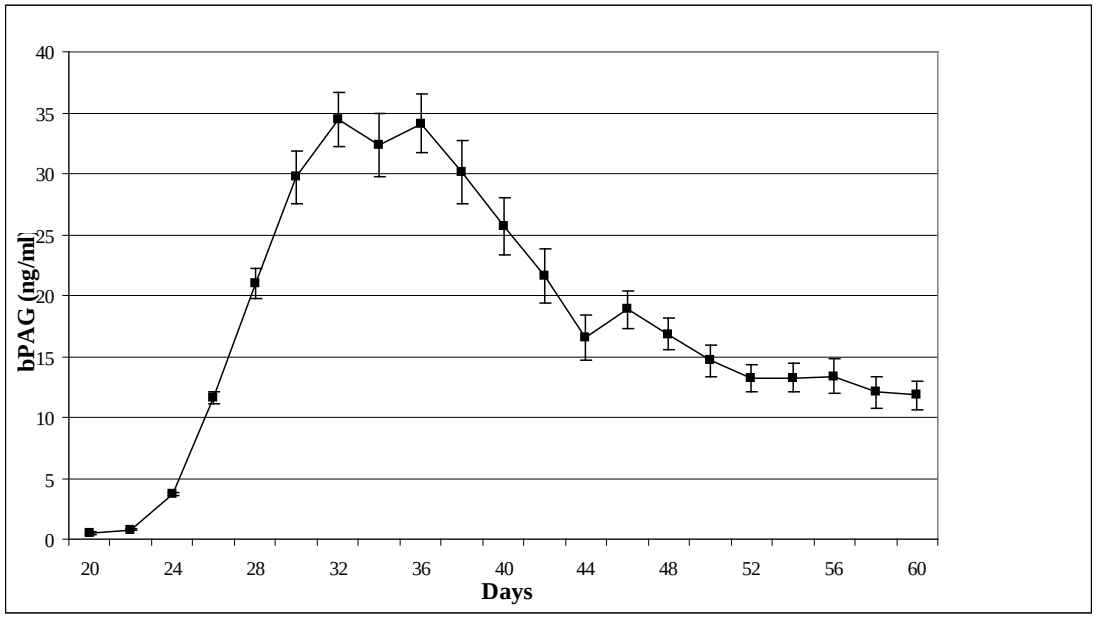
Expression Patterns Throughout Pregnancy

Detection of circulating concentrations of bPAG has been used to diagnose pregnancy in a variety of ruminant species including the cow, ewe, goat, buffalo, bison, moose and elk (Sousa et al., 2006). The half life of bPAG is an important factor affecting the effectiveness of bPAG for pregnancy diagnosis. For example, PSPB is a bPAG with a long half life (≈ 8 d) which has made it difficult to accurately detect pregnancy in cows that have a relatively short interval from calving to conception. The half lives of other members of the bPAG family are reported to be 1.7 to 4.3 d during early gestation (Busch 2005; Green et al., 2005).

Bovine PAG immunoreactivity rose rapidly between d 24 and 28 post insemination and subsequently rose steadily throughout pregnancy until a few weeks prior to parturition at which point bPAG concentrations rose rapidly, peaking during the last week of pregnancy (Green et al., 2005; also see Figure 2a, 2b). The increase in bPAG during the last trimester may be due to the release of sequestered bPAG from tissue or accessory sources of bPAG (Green et al., 2005). Animal to animal variation in circulating concentrations of bPAG is apparent and the cause of this variation is currently unknown.

Pregnancy stage, breed, number of parities, body weight of the dam, sex of the fetus and birth weight of the fetus have all been shown to affect bPAG concentrations during gestation (Lobago et al., 2009). Heifers, dams with low body weight, dams carrying a female fetus and dams that deliver a lower birth weight calf have higher serum

A



B

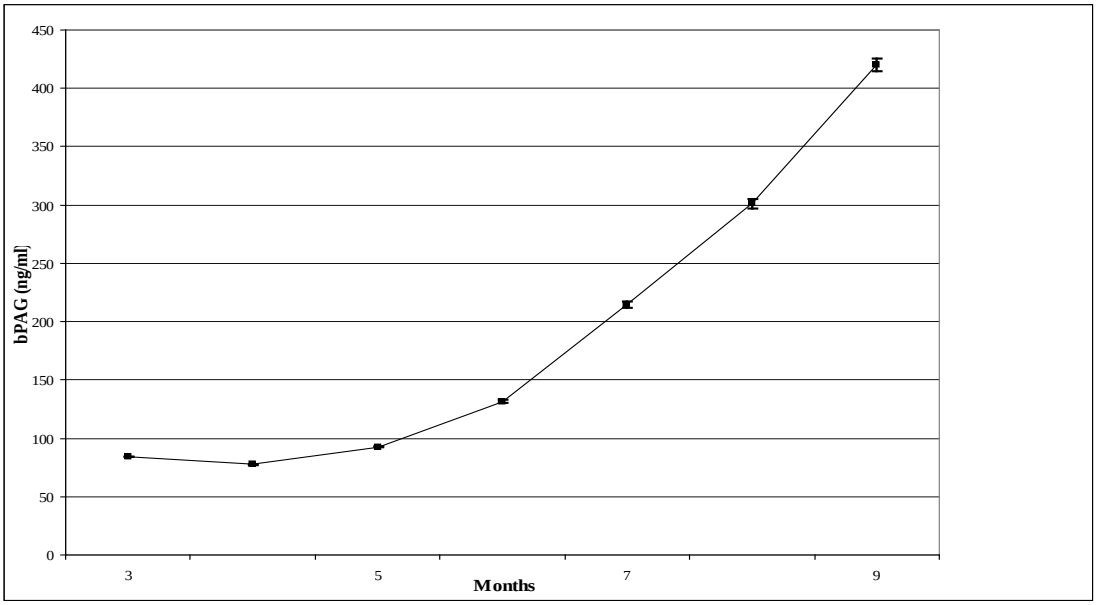


Figure 2.2. Serum concentrations of bovine pregnancy associated glycoproteins (bPAG), in postpartum beef cows detected with an enzyme linked immunosorbent assay from d 20 to 60 of gestation (panel A; d 0 = insemination). The first significant increase ($P < 0.0001$) in concentrations of bPAG occurred on d 24. Serum concentrations of bPAG in postpartum beef cows from three months of gestation to calving (panel B).

bPAG concentrations than their respective counterparts (Lobago et al., 2009). Dams with a low body condition score (BCS) have also been shown to have higher bPAG serum concentration levels (Sousa et al., 2003). Furthermore, it has been shown that the stage of gestation and fetal number influences serum bPAG concentrations (Patel et al., 1997). As a cow progresses through gestation serum bPAG concentrations increase and cows carrying twins have higher serum concentrations of bPAG than cows carrying singletons (Patel et al., 1997; Echternkamp et al., 2006). This is likely due to increased blood flow to the uterus (Ferrell and Reynolds, 1992) and increased placental mass for twins (Echternkamp, 1992). Low circulating concentrations of bPAG during late pregnancy and at the time of parturition have been associated with stillbirth and low calf viability (Kindahl et al., 2004). Breukelman et al. (2005) found that PAG reflect the viability of the trophoblast in ruminants, showing that when the fetal membranes separate from the endometrium, the trophoblastic BNC are no longer able to migrate to the maternal side of the intraplacentomal and extraplacentomal interface. Therefore, their contents cannot be expelled into the maternal blood and circulating PAG molecules will be metabolized resulting in declining maternal plasma concentrations of PAG. This finding supports the afore-mentioned study by Kindahl et al. (2004).

Cloning by nuclear transfer (NT) is associated with high rates of fetal loss in cows, mainly due to abnormal development of the extraembryonic membranes (Hill et al., 2000). When the placentomes from pregnancies generated by AI or somatic cell NT were compared, NT cows were noted to have poorly developed caruncles and fewer placentomes overall and reduced expression of PL and PAG genes in the NT cows (Hashizume et al., 2002). Increased serum concentrations of bPAG in heifers carrying

cloned embryos have been reported (reviewed in Sousa et al., 2008). Another study found the secretion of some bPAG is lower or higher by d 34 in NT cows as opposed to control cows as detected by RIA in maternal serum (Chavatte-Palmer et al., 2006).

Functional Implications Throughout Pregnancy

Although the role of bPAG during gestation is unclear, especially at parturition, there are a few theories. Local immunosuppression may be necessary in early pregnancy to reduce the maternal immune response and establish or maintain the histoincompatible feto-maternal unit (Green et al., 2005). This may make the peak values of bPAG in maternal plasma around calving seem contradictory, but this observation may be an effect of the exponential increase in placental blood flow as cows near parturition (Green et al., 2005). This could result in increased extraction of secretory products from the trophoblast by maternal circulation (Dosogne et al., 1999). This supports the theory that bPAG play a role in the initiation of parturition through remodeling of placental attachment or in the placental release mechanism (Zoli et al., 1992). PAG may also have luteotrophic effects in that, when administered to cultured endometrial cells they promote increased PGE_2 as described earlier (Wooding, et al., 2005).

Placental Lactogen

Placental lactogen (PL; or chorionic somatomammotropin), is also secreted by the BNC of the ruminant placenta. Placental lactogen is a placental polypeptide hormone that is structurally related to growth hormone (GH) and prolactin (Bolander and Fellows, 1976). Bovine placental lactogen (bPL) is glycosylated and secreted as multiple isoforms (Gootwine, 2004). This is likely due to differential splicing of bPL transcripts and allelic variants of the gene (Gootwine, 2004). Byatt and others (1986) found the molecular

weight of bPL to range from 31,000-33,000. Ruminant PL can bind to both prolactin and GH receptors (Anthony et al., 1995). In experiments with homologous mammary gland explants it was demonstrated that ovine placental lactogen (oPL) can mimic the action of ovine prolactin but not GH (reviewed in Gootwine, 2004). Ruminant PL antagonize GH activity in homologous systems because they cannot homodimerize the GH receptors (Gootwine, 2004).

Expression Patterns Throughout Pregnancy

The first ruminant PL to be described was that of the goat in 1972. Shortly thereafter, PL was discovered in sheep placental tissue (1974; reviewed in Byatt et al., 1992a). This was followed by the purification of PL in bovine by Bolander and Fellows (1975). PL is detectable in trophoblast tissue around d 16 in the ewe and d 36 in the cow (Gootwine, 2004). Serum concentrations of bPL vary widely throughout gestation (Wallace, 1993). Detectable serum concentrations of bPL have been noted as early as d 63, peak around d 215, remain at peak levels until just prior to parturition. Bovine PL decreases to undetectable concentrations within a few days following parturition (Wallace, 1993). In the ewe, oPL can be detected in maternal serum by d 50, peaks between d 120 and 140, then declines until parturition (Kappes et al., 1992). The serum half-life of recombinant bPL has been shown to be 7.25 min in dairy cattle (Byatt et al., 1992b).

Concentrations of oPL are positively correlated with fetal number (and therefore placental mass) during the last third of gestation (Gootwine, 2004). In ewes that are fed a high plane of nutrition total fetal cotyledon weight and oPL production was reduced compared to ewes fed a moderate diet (Wallace et al., 1997).

Functional Implications Throughout Pregnancy

Although the biological role of PL is not well described, Bolander et al. (1976) hypothesized that PL may induce prolactin receptors in mammary tissue. Interestingly, dairy cows had higher serum concentrations of bPL than beef cows (Bolander et al., 1976).

The birth weight of lambs born to ewes that were immunized against oPL were higher than those of non-immunized ewes with no effect on the establishment or maintenance of pregnancy (Leibovich et al., 2000). Furthermore, immunized ewes produced significantly more milk than non-immunized ewes with no effect on milk composition (Leibovich et al., 2000). This shows that alterations in oPL production do not affect the reproductive ability of the ewe, but, via some mechanism, may enhance fetal growth and mammogenesis prior to lambing and milk production during lactation (Gootwine, 2004).

CHAPTER III

EFFECT OF OVULATORY FOLLICLE SIZE ON BOVINE PREGNANCY ASSOCIATED GLYCOPROTEINS IN BEEF CATTLE

ABSTRACT

The overall objective was to examine the relationship between ovulatory follicle size and circulating concentrations of bovine pregnancy associated glycoproteins (bPAG). Postpartum cows (n = 69) were treated with the CO-Synch protocol (gonadotropin releasing hormone [GnRH-1], followed by prostaglandin F2α [PG] 7 d later, and a second injection of GnRH [GnRH-2] plus AI 48 h after PG [d 0]) and classified into one of four groups based on ovulatory follicle size at insemination: 1) GnRH-induced ovulation of a small dominant follicle (≤ 12 mm; n = 9), 2) GnRH-induced ovulation of a small-medium follicle (12 to 13 mm; n = 20), 3) GnRH-induced ovulation of a medium-large follicle (14 to 15 mm; n = 28) or 4) GnRH-induced ovulation of a large follicle (≥ 16 mm; n = 12). Serum samples were collected every other day from d 20 to 60 with daily samples from d 26 to 30. After d 60, serum samples were collected monthly until calving. bPAG were detected in serum by using two different antibody mixtures (Ab 1 or Ab 2) which detected different bPAG gene products in an ELISA. There was an effect ($P < 0.05$) of treatment on pregnancy rates at d 30 post insemination with pregnancy rate being higher ($P < 0.05$) following GnRH-induced ovulation of 14 to 15 mm compared to 12 to 13 mm follicles. The first increase ($P <$

0.0001) in bPAG occurred on d 24 with both Ab 1 and Ab 2. There was no difference between the two antibody mixtures in the pattern of serum concentrations of bPAG from d 20 to 60; however, there was a difference in the quantity of bPAG with concentrations of bPAG being greater with Ab 2 compared to Ab 1. There was an effect of day ($P < 0.0001$) on bPAG but no effect of ovulatory follicle size or ovulatory follicle size by day interaction from d 20 to 60 with either antibody. Furthermore, there was an effect of month ($P < 0.0001$) on bPAG but no effect of treatment or treatment by month interaction from 3 months of gestation to calving. Estradiol (E2) increased ($P < 0.001$) from PG (d -2) injection to insemination (d 0) regardless of ovulatory follicle size. Animals that showed estrus had higher ($P < 0.001$) serum concentrations of E2 at insemination than the animals that did not show estrus. Pregnancy rates were higher ($P < 0.05$) in cows that showed estrus compared to cows that did not show estrus. Whether or not the animals expressed estrus on d 0 did not effect serum concentrations of bPAG from d 20 to 60. Furthermore, estrus expression did not effect the serum concentrations of bPAG from 3 months of gestation to calving. There was no correlation between the change in estradiol from d -2 to 0 and the release of bPAG from d 20 to 60 ($r^2 = 0.01$). There was an effect of day ($P < 0.0001$) on serum concentrations of progesterone (P4) from d 20 to 60 but no effect of treatment or treatment by day interaction. In addition, there was no relationship between mean circulating concentrations of P4 and the release of bPAG from d 20 to 60 ($r^2 = 0.05$). In summary, there was no effect of ovulatory follicle size on serum concentrations of bPAG or P4 in pregnant cows. Cows that exhibited standing estrus had higher serum E2 concentrations prior to insemination and were more likely to become pregnant after insemination.

INTRODUCTION

The CO-Synch protocol is commonly used for synchronization of estrus and ovulation in beef cattle and consists of an injection of gonadotropin releasing hormone on d -9 (GnRH 1), prostaglandin $F_{2\alpha}$ (PG) on d -2, and a second injection of GnRH (GnRH 2) plus artificial insemination 48 h after PG (d 0). Ovulatory follicle size at the time of GnRH 2 in the CO-Synch protocol has been shown to affect pregnancy rates in dairy cows (Lamb et al., 2009), beef heifers, (Perry et.al., 2007), and beef cows (Perry et al., 2005). More specifically, induction of ovulation of a small follicle (< 12 mm) decreased pregnancy rates and increased late embryonic/fetal mortality in postpartum beef cows (Perry et al., 2005). However, there was no relationship between ovulatory follicle size and pregnancy rate or late embryonic/fetal mortality when cows spontaneously ovulated. Therefore, the physiological maturity of a dominant follicle rather than size seems to be the most important factor affecting the establishment and maintenance of pregnancy.

The physiological mechanisms responsible for the decreased pregnancy rates and increased late embryonic/fetal mortality following GnRH-induced ovulation of small dominant follicle are not known. The largest incidence of early embryonic mortality in cattle occurs by d 16 following insemination (Boyd et al., 1969; Diskin and Sreenan, 1980); however, late embryonic/fetal mortality around the time of embryo-uterine attachment has also been reported (Vasconcelos et al., 1997, 1999; Cartmill et al., 2001; Moreira et al., 2001). In addition, a significant portion of the late embryonic/fetal mortality associated with induced ovulation of small dominant follicles in postpartum beef cows occurred around the time of embryo-uterine attachment (Perry et al., 2005). Identification of a marker of conceptus viability in the maternal circulation would be

helpful in investigating mechanisms by which the physiological maturity of an ovulatory follicle might affect the incidence of late embryonic/fetal mortality. As an initial step, we chose to examine the relationship between ovulatory follicle size and PAG in postpartum beef cows.

Bovine PAG are secreted by trophoblast binucleate cells (BNC) and have been used to monitor the presence of the embryo/fetus and placental function. We were particularly interested in monitoring maternal concentrations of bPAG from d 28 to 60, which is the time when the greatest amount of late embryonic/fetal mortality has been detected. Importantly, this time period coincides with the time of placental attachment and development in cattle.

The objectives of this study were to: 1) Determine when the first statistically significant increase in bPAG occurs in pregnant cows following insemination; 2) Examine the effect of late embryonic/fetal mortality on serum concentrations of bPAG; 3) Compare the pattern of secretion of bPAG in serum samples in which bPAG were detected with two different mixtures of monoclonal antibodies; 4) Determine the effect of ovulatory follicle diameter at insemination on the circulating concentrations of bPAG from d 20 to 60 post insemination and from three months to calving; 5) Determine the relationship between preovulatory serum concentrations of estradiol (E2; d -2 and 0) and serum bPAG levels from d 20 to 60 post insemination (d 0) and from three months to calving; and 6) Determine the relationship between serum concentrations of progesterone (P4) and bPAG from d 20 to 60 post insemination (d 0) and from three months to calving.

MATERIALS AND METHODS

Experimental Design

Animals and treatments

All protocols and procedures were approved by the University of Missouri Animal Care and Use Committee (IACUC approval no. 3614). Postpartum suckled (2 to 16 yr old) crossbred beef cows (n = 98) at the University of Missouri-Columbia Beef Farm (mean days postpartum = 70.2; range 32 to 94 d) were treated with the CO-Synch protocol: GnRH (100 µg as 2 mL of Cystorelin (Merial) i.m. on d -9, prostaglandin $F_{2\alpha}$ (PG; 25 mg as 5 mL of Lutalyse [Pfizer] i.m.) on d -2, and a second injection of GnRH (100 µg of Cystorelin [Merial] i.m.) 48 h after PG) on d 0. Cows were artificially inseminated by an experienced technician with semen from a single bull at the second GnRH injection (d 0). The treatment groups consisted of GnRH-induced ovulation (d 0) of the following follicular sizes: small follicle (≤ 12 mm; n = 9), small-medium follicle (12 to 13 mm; n = 20), medium-large follicle (14 to 15 mm; n = 28) or large follicle (≥ 16 mm; n = 12). Any cows that showed estrus (as determined by visual observation and activation of a KMAR heat mount detector) on the day of the second GnRH injection (d 0) were artificially inseminated approximately 12 h after the onset of estrus and allotted into a separate group. Calves were maintained with cows at all times and allowed to suckle without restriction. Subsequent calving records were obtained from cows. Cows were removed from the four follicular size groups (n = 29) if they showed estrus prior to the second GnRH injection (n = 25), did not ovulate in response to the GnRH injection at the time of insemination (n = 3) or ovulated two follicles in response to the GnRH injection at the time of insemination (n = 1).

Blood Sample Collection

Blood samples were collected via jugular venipuncture into 10 mL Vacutainer tubes (Fisher Scientific, Pittsburgh, Pennsylvania) on d -19 and -9 to determine estrous cycling status. Cows were considered anestrus if they had serum concentrations of progesterone less than 1 ng/mL at each of the preceding time points. Additional serum samples were also collected at PG (d -2), GnRH 2 (d 0), and every other d from d 20 to 60 with daily samples from d 26 to 30. Serum samples were also collected monthly from d 60 to calving. Serum concentrations of P4 and bPAG were determined at each of the preceding time points; whereas, serum concentrations of E2 were determined at PG and GnRH 2.

Blood was allowed to clot at room temperature for 1 h, and at 4°C for 24 h. Samples were centrifuged at 3,000 x g for 20 min, serum was decanted, and stored at -20°C until concentrations of P4, E2, and bPAG were determined via radioimmunoassay (RIA; P4 and E2) or enzyme-linked immumosorbant assay (ELSIA; bPAG).

Ultrasonography

Ovaries of all cows were examined by trans-rectal ultrasonography to characterize follicular development and ovulation (d -9, d -2, d 0 = AI, d 2) using an Aloka 500V ultrasound with a 7.5 megahertz (MHz) transrectal linear probe (Aloka, Wallingford, CT). All follicles ≥ 6 mm in diameter were recorded. Follicle size was determined by measuring follicular diameter at the widest point of the follicle and at a right angle to the first measurement by using the internal calipers on the Aloka 500V ultrasound. The two measurements were averaged to obtain average follicular diameter which was rounded to

the nearest 0.1 mm. Ovulation was defined as the disappearance of a large follicle from an ovary after GnRH administration (d 0).

The uteri of all cows were examined by trans-rectal ultrasonography on d 30, 45, 60 and 90 post AI to verify the presence of a viable embryo using an Aloka 500V ultrasound with a 5.0 MHz probe (Aloka, Wallingford, CT). If cows were determined to be not pregnant on d 30 (n = 22), d 45 (n = 3), d 60 (n = 0) or d 90 (n = 2) blood samples were no longer collected.

Assays

Serum concentrations of P4 and E2 were analyzed in all samples by RIA in duplicate 100 or 300 µL aliquots respectively, as described by Kirby et al.,1997. Intra- and interassay coefficients of variation for P4 assays were 2.5 and 11%, respectively. Intra- and interassay coefficients of variation for E2 assays were 8.5 and 12.5%, respectively. A value of $\leq$ 1 ng/mL of P4 was used as the point at which the corpus luteum (CL) was considered non-functional. Concentrations of bPAG in serum were determined by a monoclonal-based bPAG ELISA as described by Green et al. (2005; see Appendix A.1 and A.2).

Statistical Analysis

Serum concentrations of bPAG, P4, and E2 were analyzed by analysis of variance for repeated measures (Proc Mixed; Littell et al., 1998) in SAS (statistical analysis system; SAS Inst. Inc., Cary, NC). The statistical model consisted of the variable tested, day, and their interactions. Analysis of breakpoints were analyzed using (Proc NLIM; Robbins et al., 2006) in SAS. In addition, simple correlations were also determined in SAS.

The effects of treatment, age, d post-partum, body condition score (BCS), cyclicity, and expression of estrus on pregnancy rate were determined by categorical analysis of variance in SAS. When the F statistic was significant ($P < 0.05$) mean separation was performed using least significant differences (means ± SEM, Snedecor and Cochran, 1989).

RESULTS

There was no difference among treatments (< 12 mm follicles, 12 to 13 mm follicles, 14 to 15 mm follicles, $\geq$ 16 mm follicles) in cow age, days postpartum, BCS or estrous cyclicity status at the initiation of treatment (Table 3.1). However, there was an effect of treatment ($P < 0.05$; Table 3.1), expression of estrus ($P < 0.05$; Table 3.2), and cow age on pregnancy rates ($P < 0.05$; Table 3.3) at d 30 post insemination following AI (d 0).

Serum concentrations of bPAG were measured with a sandwich ELISA in which two combinations of monoclonal antibodies were employed. Antibody 1 (Ab 1) contained a mixture of two monoclonal antibodies (A6 and J2); whereas, antibody 2 (Ab 2) contained a mixture of three monoclonal antibodies (A6, J2, and L4). The major PAG bound by each mixture are Ab 1: A6 - bPAG 4, 6, 7, and 16; J2 - bPAG 20; and L4 - bPAG 21 (Green et al., 2005). These antibodies have a weak affinity for numerous other bPAG, the ones discussed above are the major bPAG recognized by each antibody. There was no difference between the two antibodies in the pattern of serum concentrations of bPAG from d 20 to 60; however, there was a difference in the quantity

Table 3.1. Number of cows, age, days postpartum, body condition score, and estrous cycling status for cows before initiation of treatment[a](<12 mm, n = 9; 12 to 13 mm, n = 20; 14 to 15 mm, n = 28; >16 mm, n = 12).

				Cows with elevated Progesterone[d]		Pregnancy Rate[e]	
Follicle Size	Age, yr	Days Postpartum[b]	BCS[c]	Proportion	%	Proportion	%
< 12 mm	8.6 ± 1.2	62.7 ± 4.1	5.9 ± 0.1	4/9	44	5/9	56[xy]
12 to 13 mm	7.4 ± 0.8	62.0 ± 3.4	5.9 ± 0.2	12/20	60	10/20	50[x]
14 to 15 mm	6.0 ± 0.6	60.1 ± 3.2	5.5 ± 0.2	16/28	57	24/28	86[y]
≥ 16 mm	6.6 ± 1.1	57.4 ± 4.5	5.7 ± 0.2	5/12	42	8/12	67[xy]

[a]Treatment groups were determined at the time follicular diameter was measured at artificial insemination (AI; d 0).

[b]Number (mean ± SEM) of days postpartum at the initiation of CO-Synch protocol.

[c]Body condition score (1 to 9 scale, where 1 = emaciated, and 9 = obese) of cows 30 d following timed AI.

[d]Estrous cyclicity = the percentage of cows with elevated (≥ 1 ng/ml) concentrations of progesterone in serum before treatment. Cows were considered to be cyclic if progesterone was elevated in either of two blood samples collected 10 d prior and at the initiation of treatment.

[e]Pregnancy Rate was determined at 30 d following timed artificial insemination

[xy]Numbers (mean ± SEM or proportion) within a column with different superscripts are different ($P < 0.05$)

Table 3.2. Effect of expression of estrus on pregnancy rate[a]

					Cows with elevated Progesterone[d]		Pregnancy Rate[e]	
Estrus	No.	Age, yr	Days Postpartum[b]	BCS[c]	Proportion	%	Proportion	%
Estrus	38	6.4 ± 0.6	61.6 ± 2.6	5.5 ± 0.1	20/38	53	30/38	79[x]
No Estrus	31	7.4 ± 0.6	59.7 ± 2.7	6.0 ± 0.1	16/31	52	17/31	55[y]

[a]Cows in the "estrus" group displayed standing estrus prior to PG or GnRH 2; whereas, cows in the "no estrus" group were not detected in estrus prior to GnRH 2.

[b]Number (mean ± SEM) of days postpartum at the initiation of CO-Synch protocol.

[c]Body condition score (1 to 9 scale, where 1 = emaciated, and 9 = obese) of cows 30 d following fixed timed AI.

[d]Estrous cyclicity = the percentage of cows with elevated (≥ 1 ng/ml) concentrations of progesterone in serum before treatment. Cows were considered to be cyclic if progesterone was elevated in either of two blood samples collected 10 d prior and at the initiation of treatment.

[e]Pregnancy Rate was determined at 30 d following timed artificial insemination

[xy] Numbers (mean ± SEM or proportion) within a column with different superscripts are different ($P < 0.05$)

Table 3.3. Effect of age, day postpartum, and pretreatment estrous cyclicity status on AI pregnancy rates at 30 d post insemination.

	AI pregnancy rates (30 d post insemination)
Age, yr	
2	7/8 (88%)[x]
3 to 5	13/19 (68%)[xy]
6 to 10	22/29 (76%)[x]
11 +	5/13 (38%)[y]
Days postpartum	
< 50 d	15/19 (79%)
50 to 70 d	15/25 (60%)
> 70 d	17/25 (68%)
Estrous cycling status	
Not cycling	21/32 (66%)
Cycling	26/37 (70%)

[xy] Proportions (%) within a column with different superscripts are different ($P < 0.05$)

of bPAG detected between the two antibodies, with higher circulating concentrations of bPAG detected with Ab 2 compared to Ab 1 (Figure 3.1). The correlation coefficient between serum concentrations of bPAG for Ab 1 and 2 from d 20 to 60 for Ab 1 and 2 was 0.88 ($P < 0.0001$).

In pregnant cows, bPAG remained elevated from d 24 to calving and there was a dramatic increase in bPAG from about 6 months of gestation until calving (Figure 3.3a). However, when serum concentrations of bPAG for individual cows were examined there was significant animal to animal variation (Figure 3.3b). Six individual cows that calved within one week of the nine month blood sample are represented in Figure 3.3b. All cows except for cow 38 reached the maximum value that the assay can accurately detect (565.73 ng/ml). Cow 38 (as well as the other 5 cows represented) carried her pregnancy to term and delivered an apparently healthy calf.

Bovine PAG can be used to diagnose pregnancy in cattle during the majority of gestation. The first increase in the slope of secretion of bPAG for Ab 1 and Ab 2 occurred on d 23.0 and d 22.9, respectively (Figure 3.2a) and the first significant increase ($P < 0.0001$) in serum concentrations of bPAG occurred on d 24 for both antibodies (Figure 3.2b). There was no difference between Ab 1 or Ab 2 in the sensitivity of the assay for purposes of pregnancy diagnosis.

An important objective of the study was to examine the relationship between serum concentrations of bPAG and late embryonic/fetal mortality. The relationship between serum concentrations of bPAG and P4 is depicted for each of the cows that experienced late embryonic/fetal mortality (Figure 3.4). The last time a viable embryo

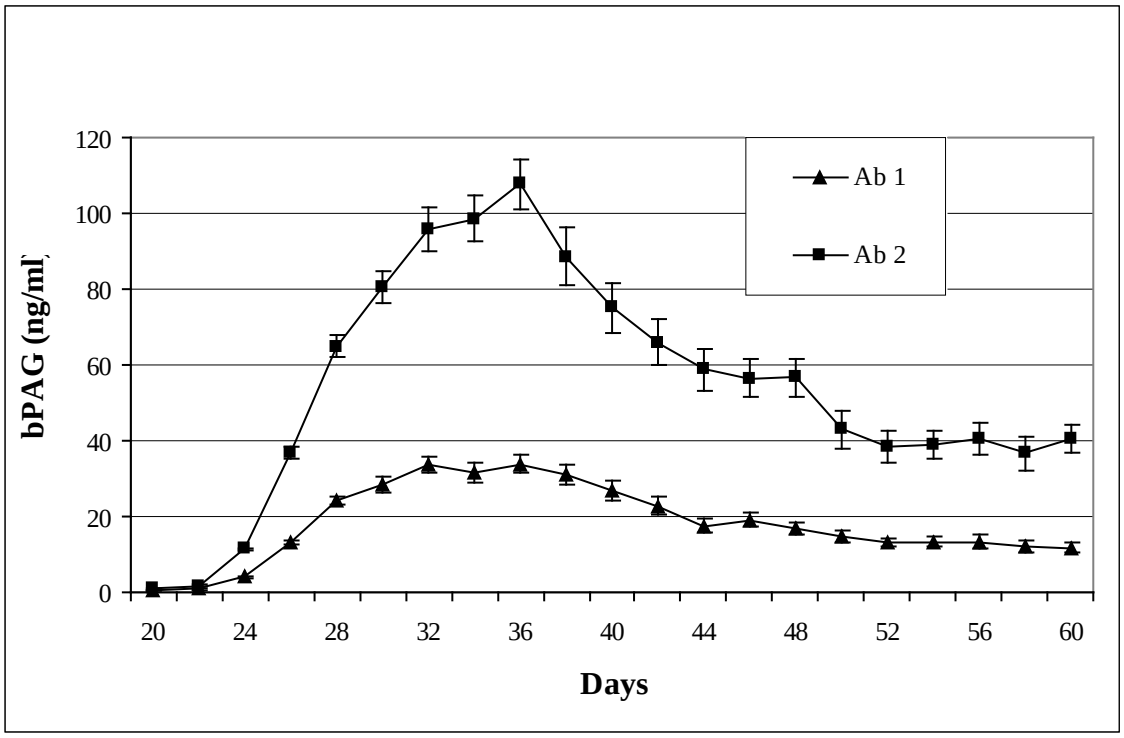
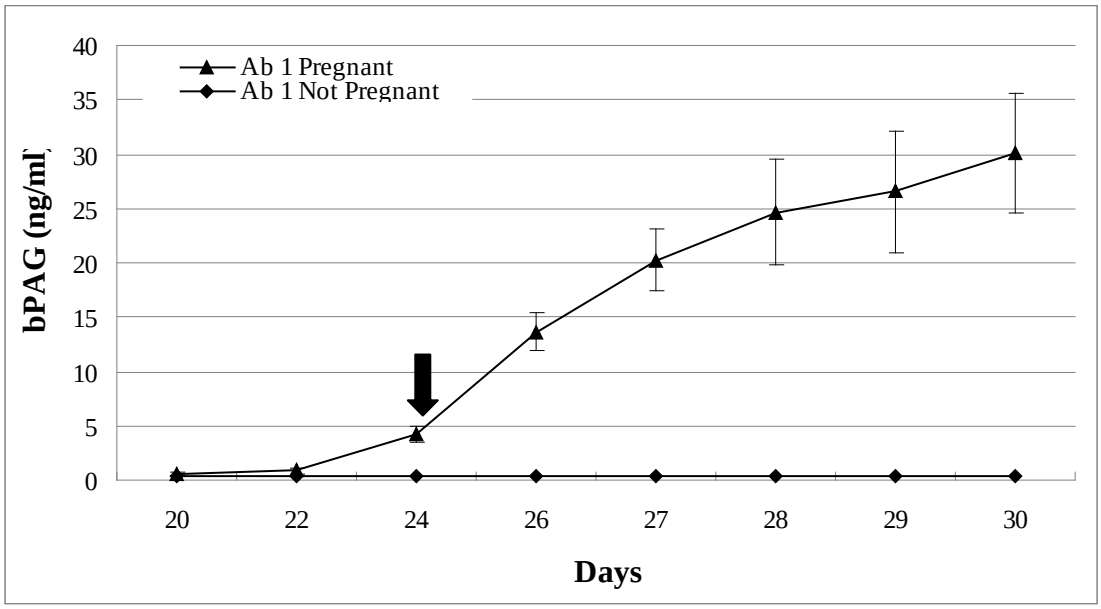


Figure 3.1. Comparison of serum concentrations of bovine pregnancy associated glycoproteins (bPAG) detected with antibody 1 (Ab1 antisera; n = 44) or antibody 2 (Ab 2 antisera; n = 44) in an enzyme linked immunosorbent assay (ELISA) from d 20 to 60 of gestation (d 0 = insemination). Correlation coefficient for the pattern of secretion of bPAG between the two antibodies was 0.88 ($P < 0.0001$).

A



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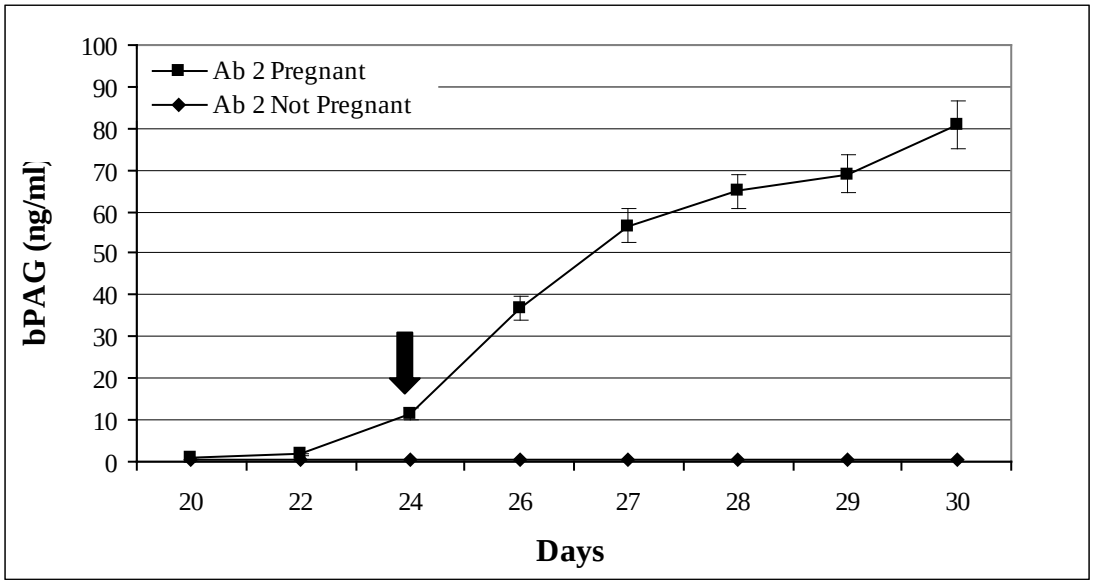
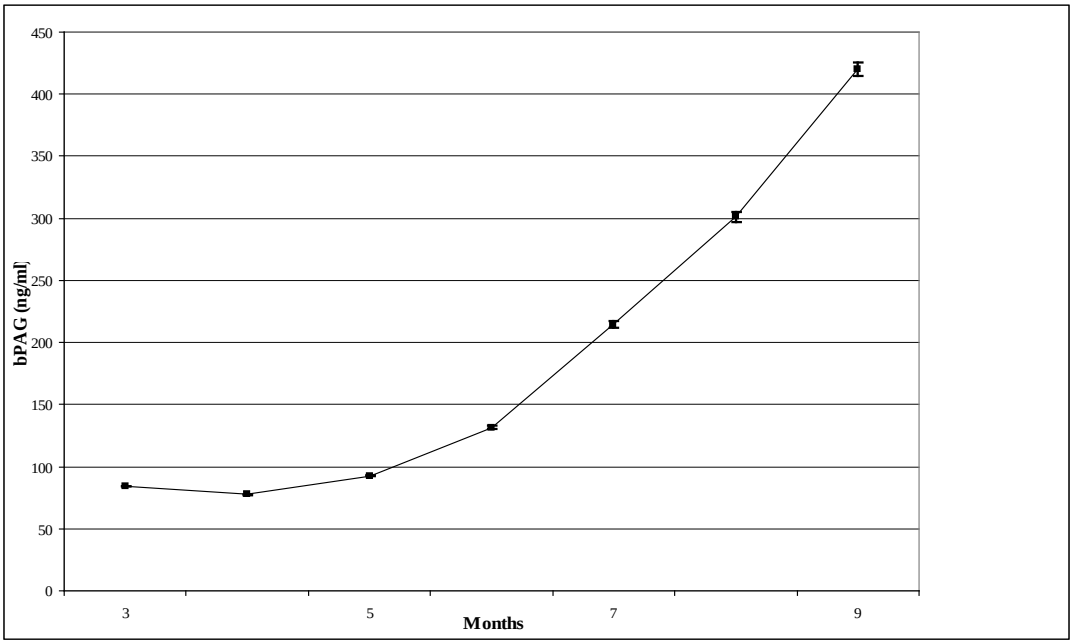


Figure 3.2. Serum concentrations of bovine pregnancy associated glycoproteins (bPAG) detected with antibody 1 (panel A; Ab 1 antisera; n = 44) or antibody 2 (panel B; Ab 2; Ab 2 antisera; n = 44) in an enzyme linked immunosorbent assay (ELISA) from d 20 to 30 of gestation (d 0 = insemination). The first increase in the slope of secretion of bPAG for Ab 1 and Ab 2 occurred on d 23.0 (A) and d 22.9 (B), respectively. The first significant increase ($P < 0.0001$; arrows) in concentrations of bPAG occurred on d 24 (A) and d 24 (B), respectively.

A



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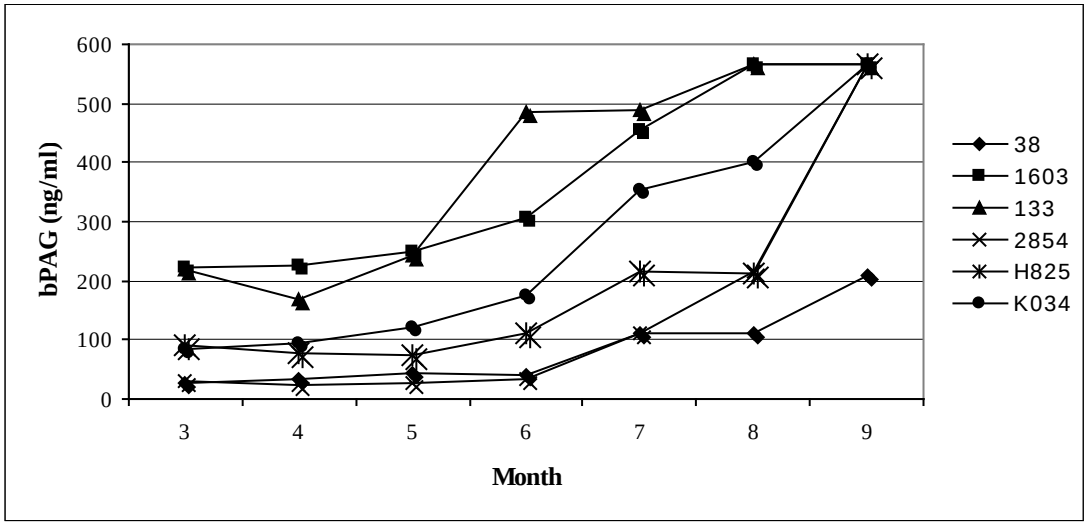
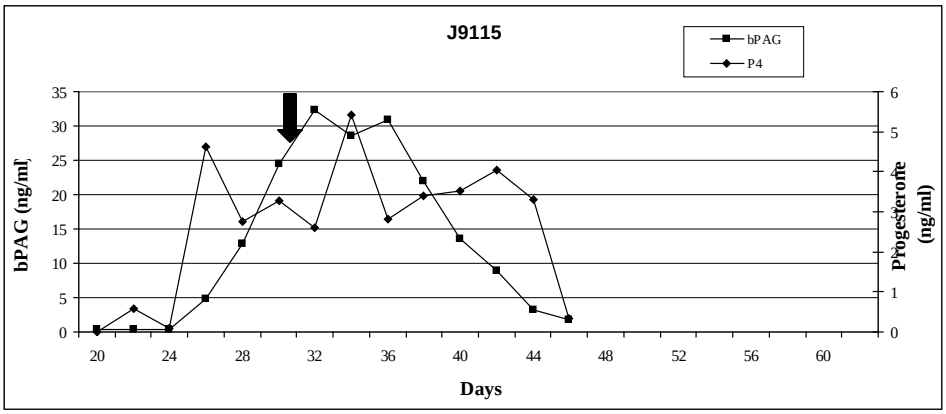


Figure 3.3. Serum concentrations of bovine pregnancy associated glycoproteins (bPAG), in postpartum beef cows (n=40), from three months of gestation to calving (panel A; d 0 = insemination) and serum concentrations of bPAG in six postpartum beef cows in which the last serum sample was collected close to calving (panel B). bPAG were measured with an enzyme linked immunosorbent assay (ELISA; Ab 2 antisera).

A



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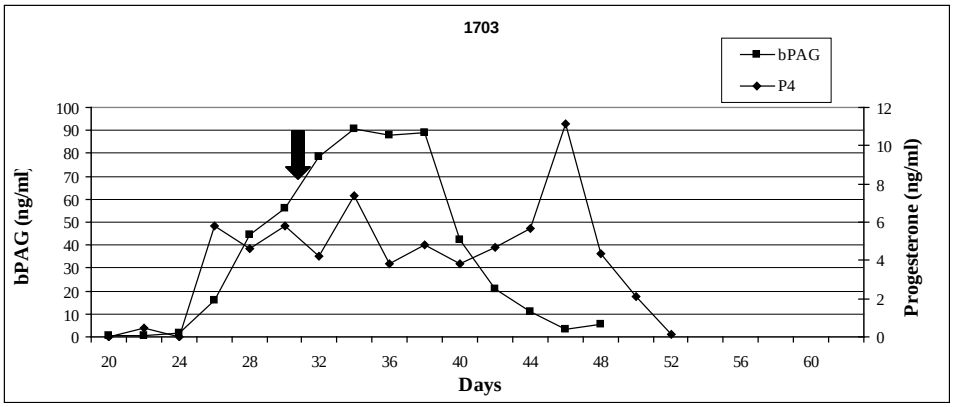

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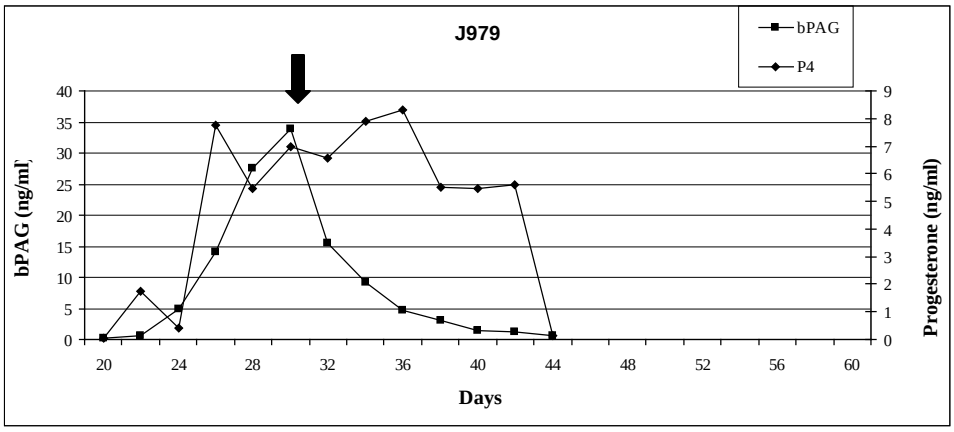


Figure 3.4. Serum concentrations of bovine pregnancy associated glycoproteins (bPAG) and progesterone (P4) in individual postpartum beef cows (panels A, B, and C) that experienced late embryonic/fetal mortality between d 30 and 45 post insemination. The last day that a viable embryo was detected is indicated with an arrow. bPAG concentrations were determined using Ab 1 antisera with an enzyme linked immunosorbent assay (ELISA).

was detected in each of the cows depicted in Figure 3.4, based on the presence of a heart beat was d 30 and the subsequent ultrasonogram for the presence of a viable embryo was on d 45 postinsemination. Serum concentrations of bPAG decreased before the decrease in P4 for all cows in which an embryo died (Figure 3.4). This indicates that the conceptus died before the CL regressed. The timing of the decrease in bPAG relative to the decrease in P4 varied from approximately 8 to 12 d. In panel A of Figure 3.4, bPAG was declining on d 38; whereas, P4 did not decline until d 46. In panel B of Figure 3.4, bPAG was declining on d 40 whereas P4 did not decline until d 48. In panel B of Figure 3.4, bPAG were declining on d 32 whereas P4 did not decline until d 44. Figure 3.5 shows bPAG serum concentrations of the three individual cows from Figure 3.4 graphed against the average bPAG serum concentrations for all cows.

A primary objective of this study was to examine the effect of ovulatory follicle size on serum concentrations of bPAG. There was a definite effect of day ($P < 0.0001$) on serum concentrations of bPAG but no effect of ovulatory follicle size, or ovulatory follicle size by day interaction from d 20 to 60 with either antibody mixture (Figure 3.6). Furthermore, there was an effect of month ($P < 0.0001$) on bPAG but no effect of ovulatory follicle size, or ovulatory follicle size by month interaction from 3 months of gestation to calving (Figure 3.7).

Since uterine environment is influenced by gonadal steroids, the relationship between the preovulatory change in E2 or the postovulatory concentrations of P4 (d 20 to 60) and bPAG was examined. E2 increased from the time of PG (d -2) to insemination (d 0) regardless of ovulatory follicle size (Figure 3.8). For animals that did not show estrus there was no effect of ovulatory follicle size on serum concentrations of E2 on d -2 or 0

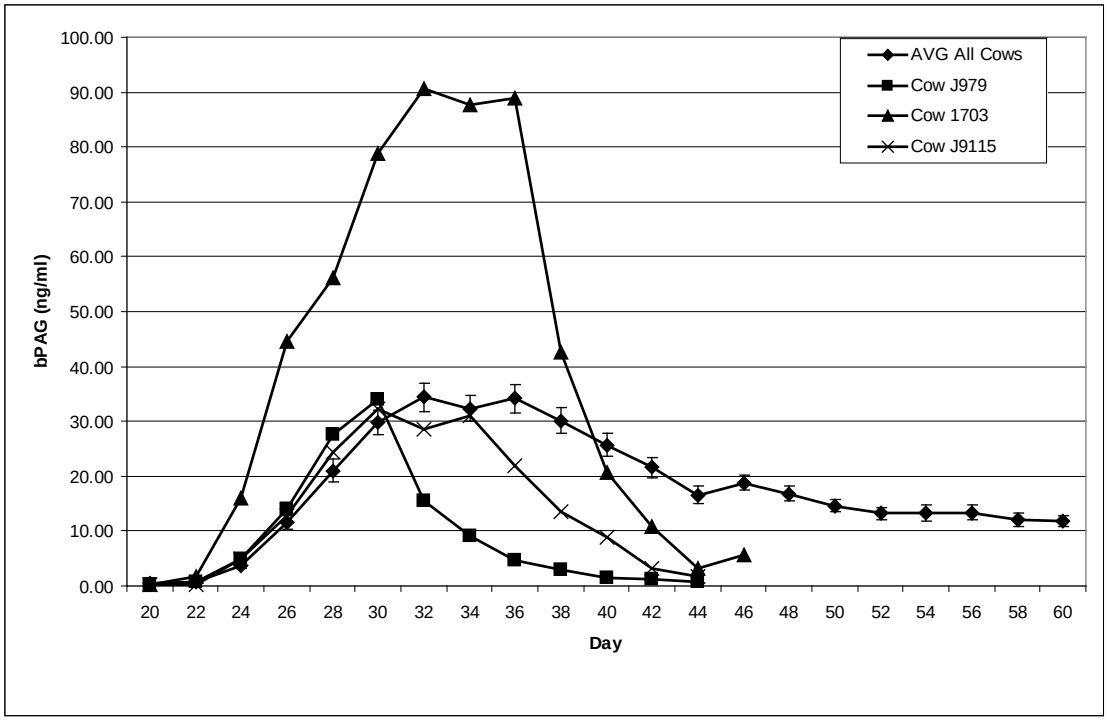
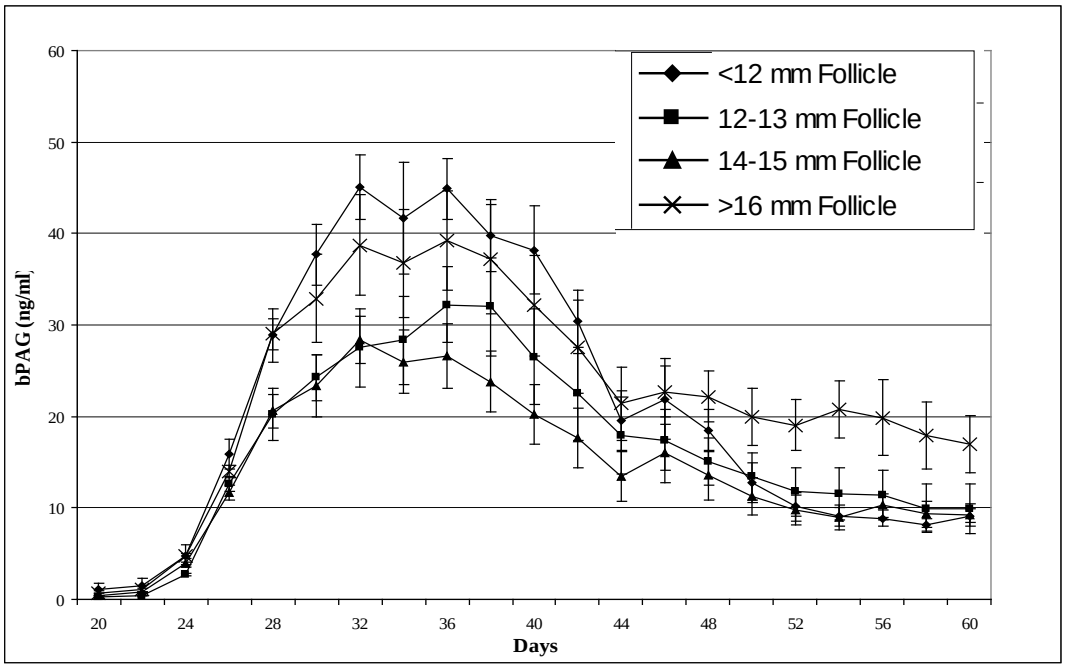


Figure 3.5. Serum concentrations of bovine pregnancy associated glycoproteins (bPAG) in three individual postpartum beef cows that experienced late embryonic/fetal mortality between d 30 and 45 post insemination compared with mean serum concentrations of bPAG for all cows that maintained a pregnancy. bPAG concentrations were determined using Ab 1 antisera with an enzyme linked immunosorbent assay (ELISA).

A



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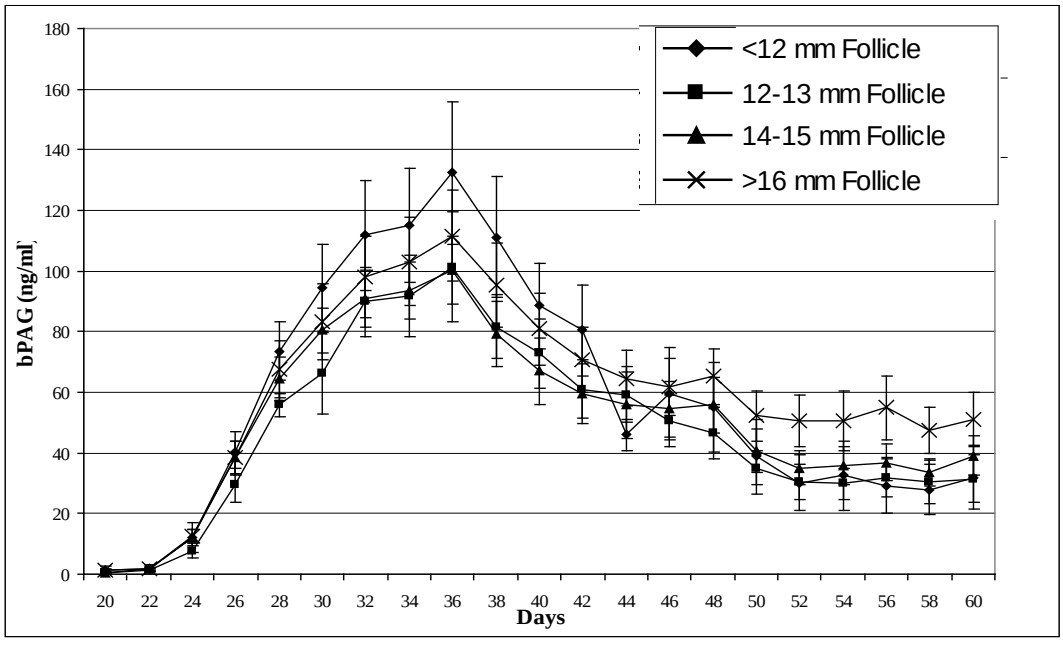


Figure 3.6 Effect of ovulatory follicle size on serum concentrations of bovine pregnancy associated glycoproteins (bPAG) as detected with antibody 1 (panel A; Ab 1 antisera; n = 44 [ovulatory follicle size, P = 0.77; day, P < 0.0001; ovulatory follicle size by day, P = 0.08]) or antibody 2 (panel B; Ab 2 antisera; n = 44 [ovulatory follicle size, P = 0.66; d, P < 0.0001; ovulatory follicle size by day, P = 0.05]) in an enzyme linked immunosorbent assay (ELISA) from d 20 to 60 of gestation (d 0 = insemination).

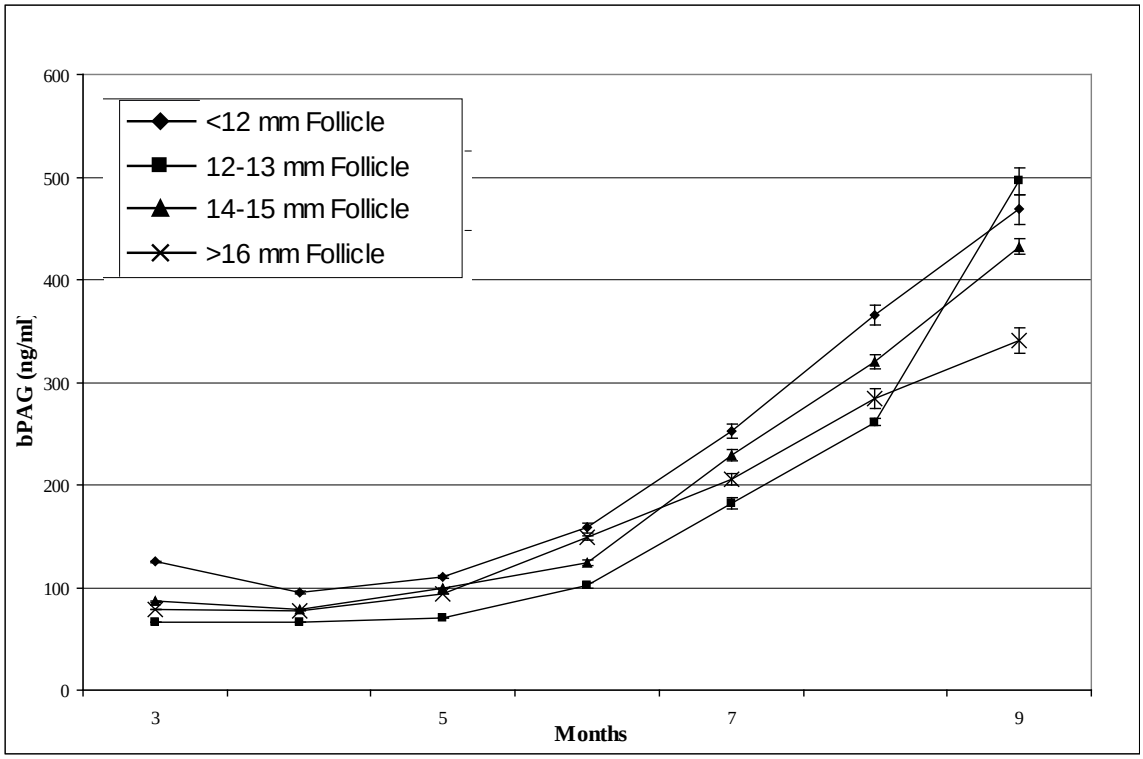


Figure 3.7. Effect of ovulatory follicle size (ovulatory follicle size, P = 0.56; month, P < 0.0001; ovulatory follicle size by month, P = 0.23) on serum concentrations of bovine pregnancy associated glycoproteins (bPAG) as detected with Ab 2 antisera (n = 40) in an enzyme linked immunosorbent assay (ELISA) from three months of gestation to calving.

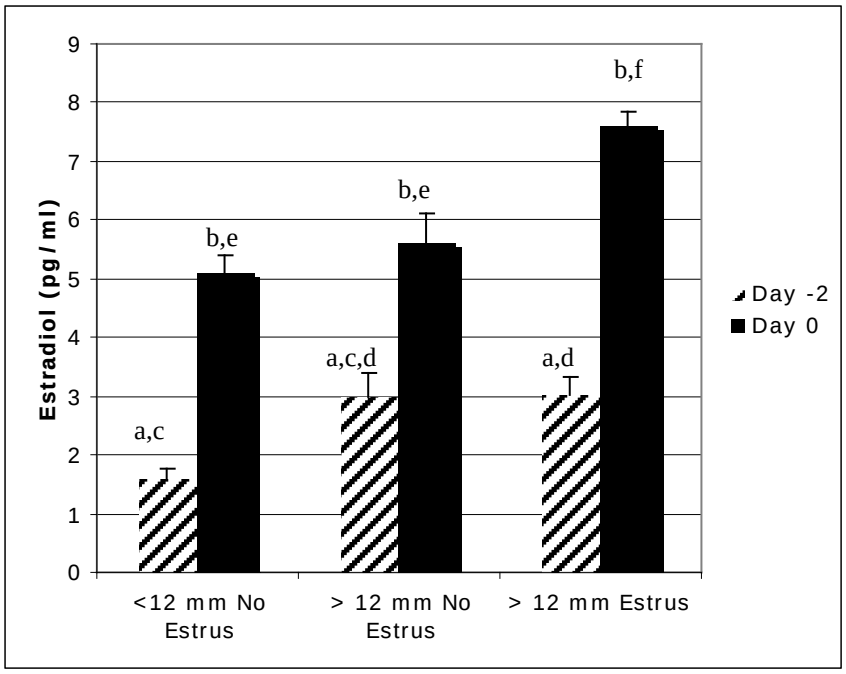


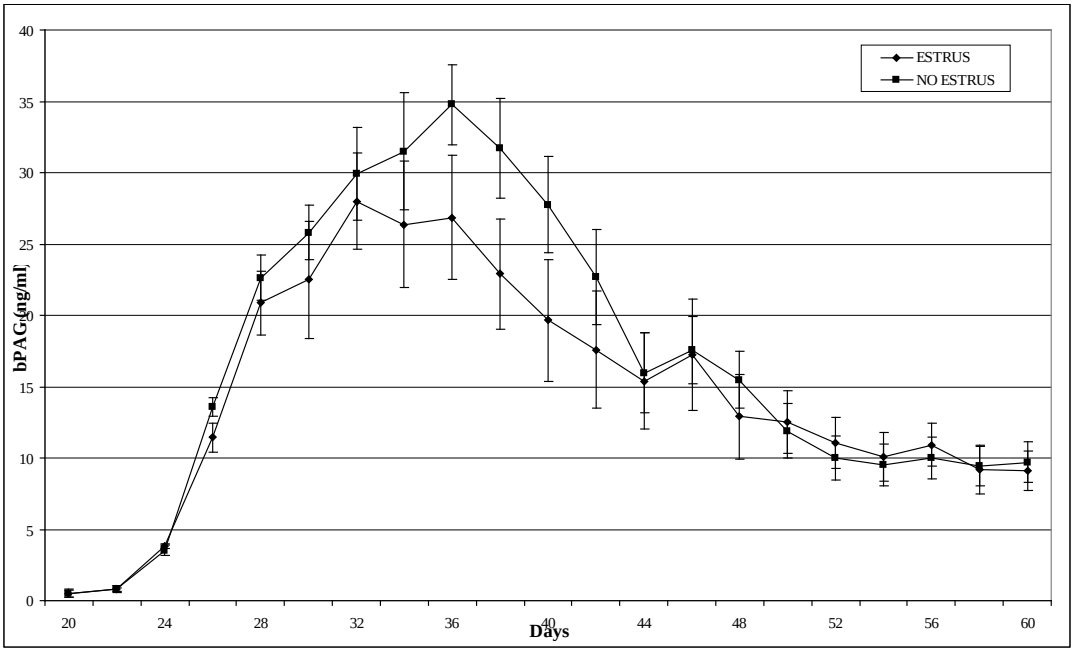
Figure 3.8. Effect of expression of estrus (estrus vs. no estrus) and ovulatory follicle size (< 12 mm - no estrus, n = 5; $\geq$ 12 mm - no estrus, n = 12; $\geq$ 12 mm - estrus, n = 30) on mean (± SEM) serum concentrations of estradiol (E2) on d -2 (PG injection) and 0 (GnRH 2 and insemination). Mean (± SEM) E2 on d -2 and 0 within treatment groups having different superscripts differ ($^{ab}P < 0.001$). Mean (± SEM) E2 on d -2 among treatment groups having different superscripts differ ($^{cd}P < 0.05$). Mean (± SEM) E2 on d 0 among treatment groups having different superscripts differ ($^{ef}P < 0.001$).

(Figure 3.8). Animals that showed estrus had higher serum concentrations of E2 at insemination than the animals that did not show estrus (Figure 3.8). Whether or not the animals showed estrus on d 0 did not effect the serum concentrations of bPAG from d 20 to 60 (Figure 3.9) or from three months of gestation to calving (Figure 3.10). There was no relationship between the change in E2 from d -2 to 0 and the release of bPAG (area under the curve; $r^2 = 0.01$; Figure 3.11). There was an effect of day ($P < 0.0001$) on P4 concentrations from d 20 to 60 but no effect of ovulatory follicle size or ovulatory follicle size by day interaction (Figure 3.12). There was no relationship between mean P4 and the release of bPAG (area under the curve; $r^2 = 0.05$; Figure 3.13).

DISCUSSION

Pregancy associated glycoproteins are expressed in binucleated trophoblast cells of the placenta and exocytosis of granules toward the maternal capillary beds allows trophectoderm cell products (e.g. PAG) to enter the maternal blood supply upon placentation (Wooding, 1983). Detection of circulating concentrations of bPAG has been used to diagnose pregnancy in a variety of ruminant species including cattle, sheep, goats, buffalo, bison, moose, and elk (Sousa et al., 2006). The half life of bPAG is an important factor affecting the efficacy of bPAG for pregnancy diagnosis. For example, PSPB is a bPAG with a long half life (≈ 8 d; Sasser et al., 1986) that has made it difficult to accurately detect pregnancy in cows that have a relatively short interval from calving to conception. The half life of other bPAG family members are reported to be 1.7 to 4.3 d during early gestation (Green et al., 2005; Busch, 2005). Bovine PAG immunoreactivity

A



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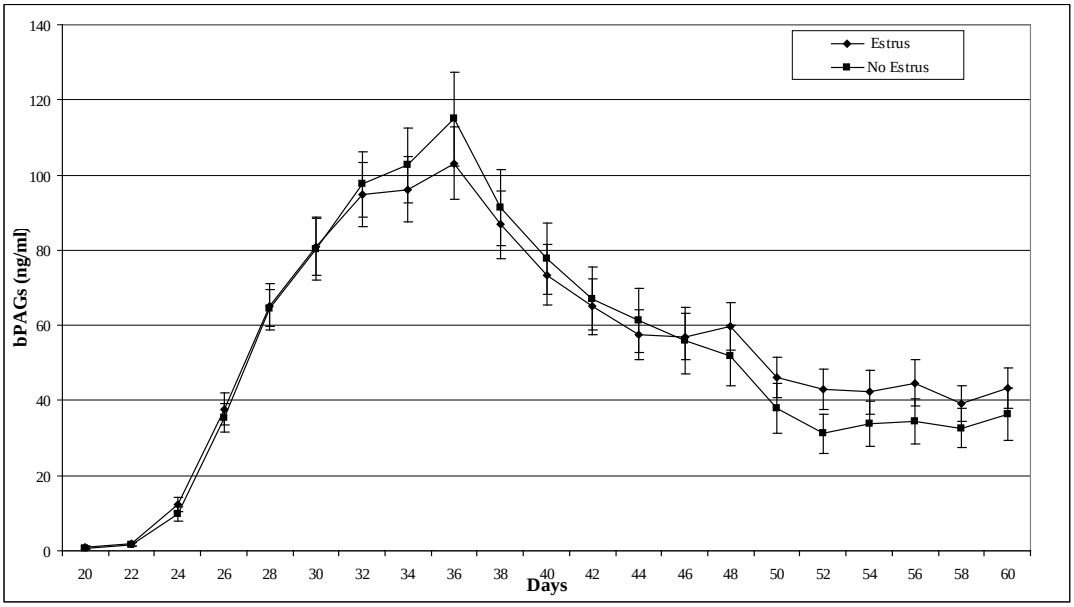


Figure 3.9. Effect of expression of estrus (estrus (n=27) vs. no estrus (n=17) on d 0 (insemination) on serum concentrations of bovine pregnancy associated glycoproteins (bPAG) from d 20 to 60 of gestation (d 0 = insemination). Serum concentrations of bPAG were determined with antibody 1 (panel A; Ab 1 antisera; n = 44 [estrus*ovulatory follicle size, P = 0.28; day, P < 0.0001; estrus*ovulatory follicle size by day, P = 0.03]) or antibody 2 (panel B; Ab 2 antisera; n = 44 [estrus*ovulatory follicle size, P = 0.65; day, P < 0.0001; estrus*ovulatory follicle size by day, P = 0.5]) in an enzyme linked immunosorbent assay (ELISA).

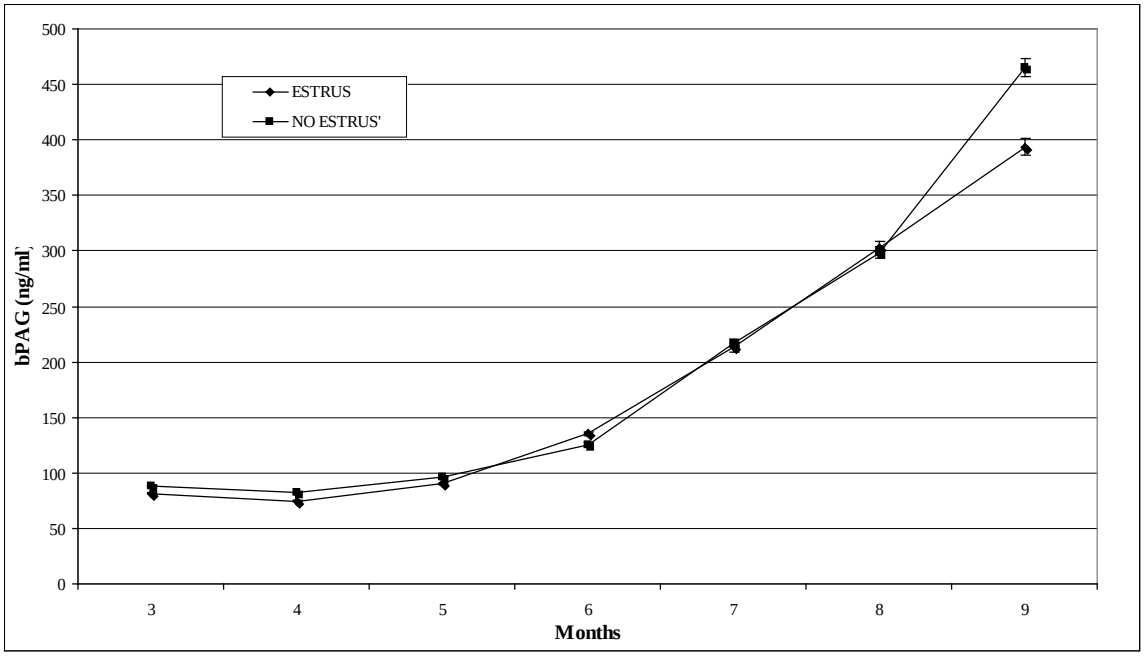


Figure 3.10. Effect of expression of estrus ([estrus; n = 25, vs. no estrus; n = 15] estrus*ovulatory follicle size, P = 0.47; month, P < 0.0001; estrus*ovulatory follicle size by month, P = 0.73) on d 0 (insemination) on serum concentrations of bovine pregnancy associated glycoproteins (bPAG) from three months of gestation to calving. Serum concentrations of bPAG were determined with Ab 2 antisera in an enzyme linked immunosorbent assay (ELISA).

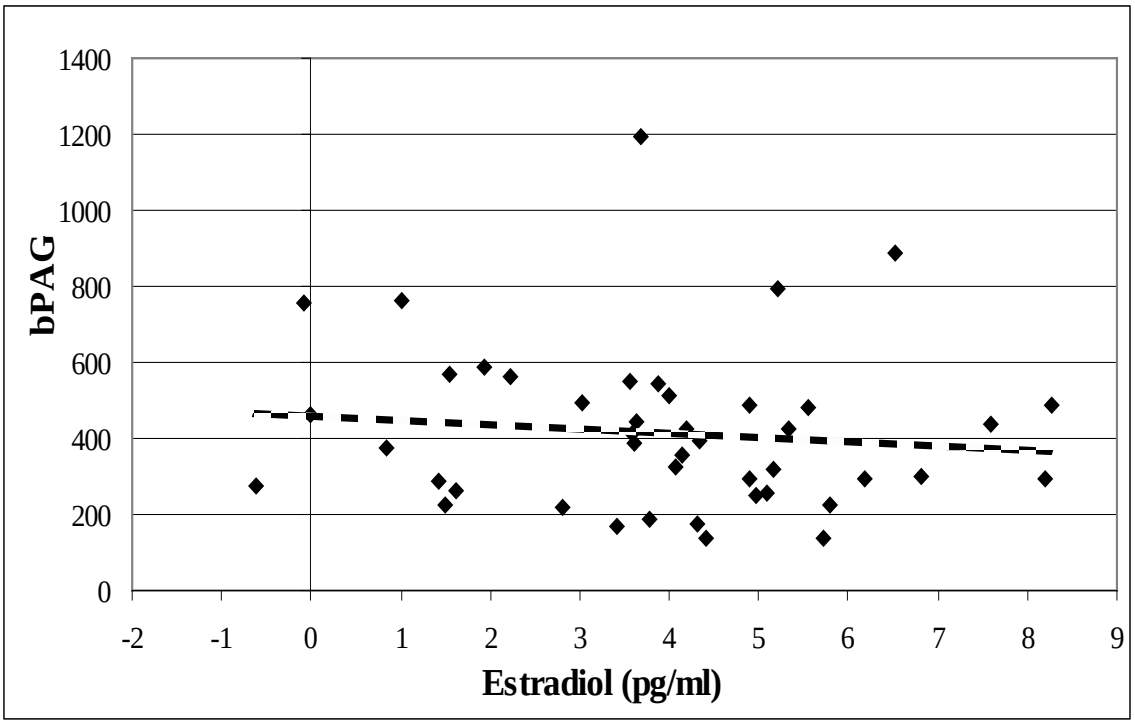
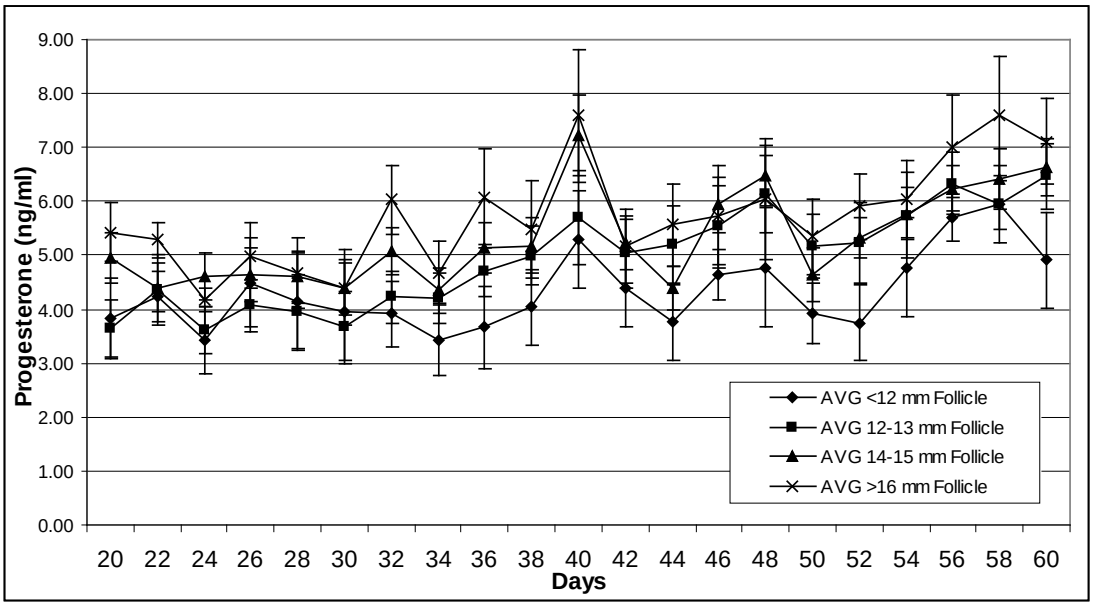


Figure 3.11. Regression of bovine pregnancy associated glycoproteins (bPAG; area under the curve) on change in estradiol (E2) from d -2 to 0 (d -2 = injection of PGF; d 0 = insemination [$r^2 = 0.01$]). bPAG were measured with an enzyme linked immunosorbent assay (ELISA; Ab 2 antisera).

A



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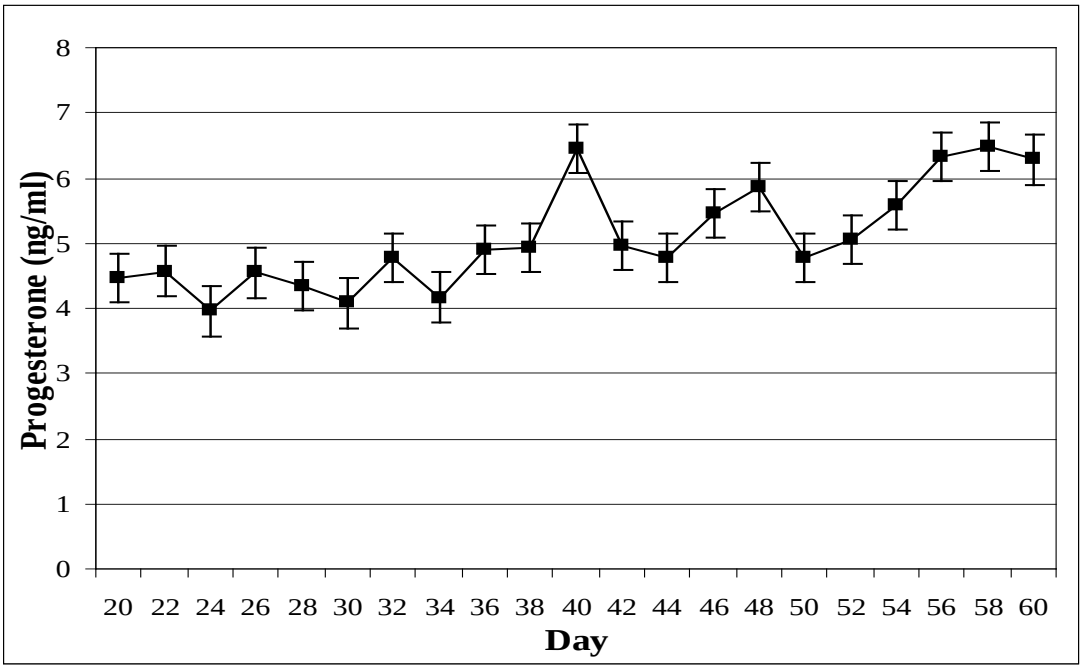

Figure 3.12. Effect of ovulatory follicle size (panel A; n = 44 [ovulatory follicle size, P = 0.57; day, P < 0.0001; ovulatory follicle size by day, P = 0.44]) and time (panel B) on serum concentrations of progesterone (P4) from d 20 to 60 of gestation (d 0 = insemination).

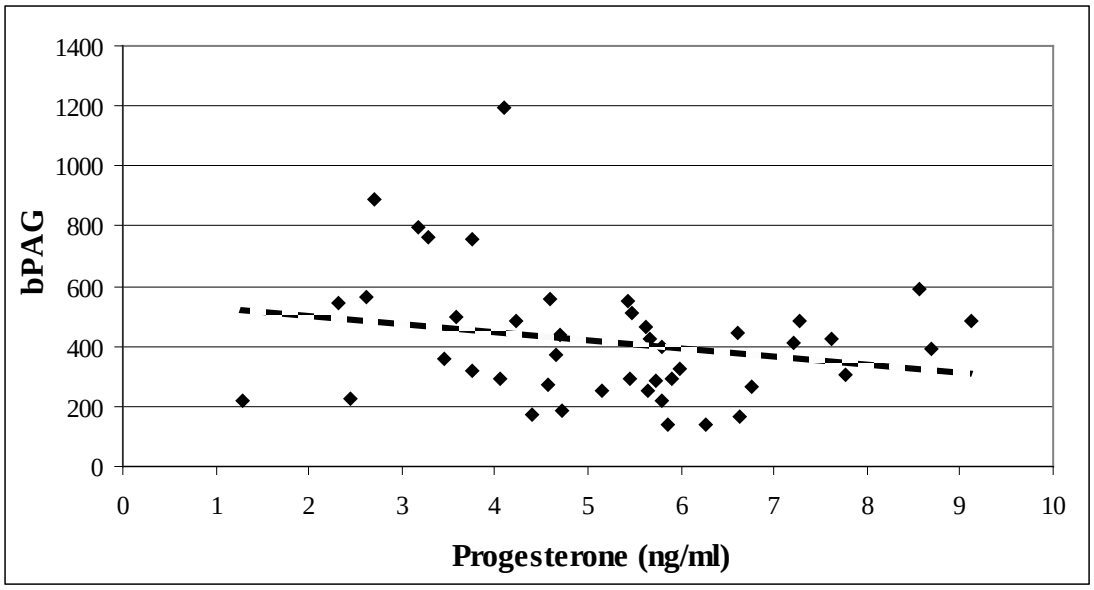

Figure 3.13. Regression of bovine pregnancy associated glycoproteins (bPAG; area under the curve) on mean serum concentrations of progesterone (P4; d 20 to 60 of gestation; d 0 = insemination [$r^2 = 0.05$]). bPAG were measured with an enzyme linked immunosorbent assay (ELISA; Ab 2 antisera).

increased rapidly between d 24 and 28 post insemination. Circulating PAG declined briefly around d 60 and then increased steadily throughout the remainder of gestation until a few weeks prior to parturition. Near calving, serum concentrations of bPAG rose rapidly, and peaked during the week prior to calving (Green et al., 2005). In the current study, circulating concentrations of bPAG were increased above baseline by d 24 and there was a dramatic increase in bPAG from about 6 months of gestation until calving. The increase in bPAG during the last trimester may have been due to the release of sequestered bPAG from tissue or accessory sources of bPAG (Green et al., 2005). This increase may also reflect the relatively rapid increase in placental size during the last trimester. As reported by others there was significant animal to animal variation in circulating concentrations of bPAG; however, the cause of this variation is currently unknown.

Pregnancy stage, breed, parity, body weight of the dam, sex of the fetus and birth weight of the fetus have all been shown to affect bPAG concentration during gestation (Lobago et al., 2009). In addition, first calf heifers, dams with low body weight, dams that carried a female fetus, and dams that delivered a calf having a lower birth weight had higher serum bPAG concentrations than their respective counterparts (Lobago et al., 2009). Dams with a low BCS have also been shown to have higher serum concentrations of bPAG (Sousa et al., 2003). Furthermore, it has been shown that the stage of gestation and fetal number influence serum bPAG concentration (Patel et al., 1997). As a cow progresses through gestation serum bPAG concentration increase and cows carrying twins have higher serum concentrations of bPAG than cows carrying singletons (Patel et al., 1997).

Although the role of bPAG throughout gestation, remains unclear, a couple of hypothesis have been proposed. First, local immunomodulation may be necessary in early pregnancy to reduce the maternal immune response and establish or maintain the histioincompatible feto-maternal unit (Dosogne et al., 1999). Also placental blood flow is exponentially increased towards parturition and could account for some of the increase in bPAG concentrations (Green et al., 2005). This could cause an increased extraction of secretory products from the trophoblast by maternal circulation. (Dosogne et al., 1999) This is supportive of the theory that bPAG play a role in the initiation of parturition through remodeling of the placental attachment or in the placental release mechanism (Zoli et al., 1992). Szafranska and others (2007) have suggested that PAG may be luteoprotective chorionic-origin signals during implantation and placentation as they are able to interact with gonadotropin receptors of luteal phase animals.

Ovulatory follicle size at the second GnRH injection (CO-Synch protocol) affects pregnancy rates in beef cattle. GnRH-induced ovulation of follicles > 12 mm had higher pregnancy rates compared to cows induced to ovulate follicles < 12 mm (Lamb et al., 2001). Furthermore, cows (Perry et al., 2005) and heifers (Perry et al., 2007) that were induced to ovulate a small dominant follicle had decreased pregnancy rates in comparison to those that ovulated a large dominant follicle following CO-Synch. However, cows that showed estrus and spontaneously ovulated small dominant follicles had pregnancy rates comparable to cows that were induced to ovulate a large follicle. In the current experiment, cows that ovulated a 12 to 13 mm follicle had lower pregnancy rates (50%) than cows that ovulated a 14 to 15 mm follicle (86%).

Perry et al, (2005), reported an increased incidence of late embryonic/fetal mortality from d 27 to 68 following GnRH-induced ovulation (CO-Synch protocol) of small ovulatory follicles with the greatest loss occurring from d 25 to 39. The increased incidence of late embryonic/fetal mortality may be associated with inadequate oocyte competence (Lonergan et al., 2003), reduced P4 production from the CL (Diskin and Morris, 2008), inadequate uterine environment (Barnes, 2000), or inadequate placental function (Facciotti et al., 2009). In the present study, late embryonic/fetal loss occurred around the time of embryo uterine attachment (d 21 to 42; Peters, 1996) as has been reported by others (Vasconcelos et al., 1999; Cartmill et al., 2001; Moreira et al., 2001; Perry et al., 2005). An important objective of this study was to monitor bPAG as a marker of conceptus viability. In the present study, circulating concentrations of bPAG decreased before circulating concentrations of P4 in cows that lost an embryo. This observation supports the concept that embryonic death (no heartbeat) and reduced placental function (decreased production of bPAG) occur before luteolysis. A similar relationship between embryo viability at 30 d of gestation and circulating concentrations of bPAG was reported by Busch (2005).

Identification of a marker of conceptus viability in the maternal circulation would be helpful in investigating mechanisms by which the physiological maturity of an ovulatory follicle might affect the incidence of late embryonic/fetal mortality. As an initial step, we chose to examine the relationship between ovulatory follicle size and bPAG in postpartum beef cows. Busch (2005) reported that GnRH-induced ovulation of large follicles resulted in increased circulating concentrations of bPAG from d 24 to 60 post AI compared to GnRH-induced ovulation of small follicles. Interestingly,

circulating concentrations of bPAG in cows in which ovulation of a large follicle was induced by GnRH or occurred spontaneously were not different. However, the preceding observations were not supported by the data presented. There was no effect of ovulatory follicle size or ovulatory follicle size by time interaction. In an attempt to reconcile the discrepancy between the study by Busch (2005) and the current study, a subset of serum samples from cows ovulating large or small follicles from each study were run in the same PAG ELISA. The results were identical to the original findings (Figure B.1); therefore, a difference in assay conditions could not account for the discrepancy between the study by Busch (2005) and the current study.

An increase in preovulatory concentrations of estradiol are reported to be required for the establishment of pregnancy (Moore, 1985). Animals that exhibited standing estrus within 24 h of TAI had greater serum concentrations of E2 and greater pregnancy success compared to animals that did not exhibit estrus (Perry et al., 2005, 2007; Perry and Perry, 2008a). Similarly, in the current study, cows that showed estrus had increased serum concentrations of E2 at GnRH 2 and had a higher pregnancy rate on d 30 (79%) than cows that did not show estrus (55%). Preovulatory concentrations of E2 may impact the establishment and maintenance of pregnancy via several mechanisms: uterine pH (Perry and Perry, 2008a), preparation of granulosa cells for luteinization (McNatty et al., 1979), and(or) preparation of the endometrium for pregnancy (Zelinski et al., 1980).

Estradiol may impact sperm transport by altering the uterine environment around the time of fertilization (Perry and Perry 2008a). Perry and Perry (2008b) showed a decreased uterine pH at the second GnRH injection in cows that had elevated concentrations of E2. An increase in sperm longevity, presumably due to decreased

uterine pH, might increase fertility of cows that have elevated serum E2 concentrations (Acott and Carr, 1984; Goltz et al., 1988; Jones and Bavister, 2000).

Estradiol may have a role in the preparation of follicular cells for luteal formation and function. The ability of luteinized human granulosa cells to secrete P4 was increased when cells were collected from follicles that had increased follicular fluid concentrations of E2 compared to the granulosa cells collected from follicles with a lower concentration of E2 (McNatty et al., 1979). Also, the secretion of P4 in ewes given an aromatase inhibitor before induced ovulation was delayed (Benoit et al., 1992). With this in mind, cows that exhibited standing estrus and had higher serum E2 concentrations during the two d prior to insemination may have attained a concentration of E2 necessary to adequately prepare the follicular cells for luteinization regardless of follicular size and induced an adequate number of uterine P4 receptors, as discussed previously. E2 has been associated with the induction of endometrial progesterone receptors (Zelinski et al., 1980). However, in the current study, there was no effect of the change in E2 between d -2 and 0 on the secretion of bPAG.

The rate of increase in serum concentrations of P4 during luteal formation and development affects the establishment of pregnancy (Beltman et al., 2009). Previous studies noted that pregnant cows have greater serum concentrations of P4 shortly after insemination (d 6 to 10) than non-pregnant cows (Mann et al., 1999; Perry et al., 2005; Busch et al., 2008). Furthermore, cows that had an earlier rise in P4 had embryos that were further developed than cows that had a delayed rise in P4 (Mann and Lamming, 2001). These data indicate that exposure to an earlier rise in P4 may contribute to the establishment of pregnancy.

In the current study, there was no effect of ovulatory follicle size on mean serum concentrations of P4 in pregnant cows from d 20 to 60, which is in agreement with Busch et al. (2008). There was also no relationship between serum concentrations of P4 and bPAG (Figure 3.13; Figure D.1). Reduced P4 production after GnRH-induced ovulation of small follicles was not observed from d 20 to 60 in cows that conceived. Since reduced serum concentrations of P4 do not contribute to late embryonic/fetal mortality in postpartum cows in which small dominant follicles were induced to ovulate with GnRH (Perry et. al.; 2005 and current study), it is possible that inadequate induction of endometrial P4 receptors by E2 played a role. Perry et al.(2005) reported that serum concentrations of E2 were lower at GnRH-induced ovulation of small compared to large dominant follicles and E2 was reported to induce endometrial P4 receptors (Zelinski et. al.; 1980).

In summary, no difference in serum concentrations of bPAG or serum P4 levels was found between the different GnRH-induced follicle groups. Cows that exhibited standing estrus had higher serum E2 concentrations prior to insemination and were more likely to become pregnant after insemination.

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APPENDIX A.1

Enzyme-linked Immunosorbent Assay for Bovine Pregnancy-Associated Glycoproteins Protocol

Serum concentrations of bovine pregnancy associated glycoproteins (bPAG) were determined by enzyme-linked immunosorbent assay (ELISA) as described below. The following procedure was developed and validated in cattle by Green et al., (2005).

The ELISA employed a mixture of monoclonal antibodies {(Ab 1 = A6 and J2 [antibodies identify bPAG 4,6,7,16 and 20]; Ab 2 = A6, J2, and L4 [antibodies identify bPAG 4,6,7,16, 20 and 21])} to trap bPAG in a 96-well ELISA plate and an anti-PAG polyclonal rabbit antiserum to bind to the immobilized bPAG. The entire complex was detected by using an alkaline phosphatase-conjugate anti-rabbit antibody. Sheep anti-mouse Fc acts as a substrate for the monoclonal antibody mixture to attach to. A blocking solution is then added to coat the wells of the plate. The monoclonal mixture is then added to attach to the sheep anti-mouse substrate. Cow sera is then added, bPAG antigen attach to the antibodies in the monoclonal mixture. Unbound antigen is washed away and anti-PAG polyclonal IgG is added which attaches to the bPAG antigen. AP-conjugated goat anti-rabbit antibody is then added to the wells. Para-nitrophenyl phosphate (PNPP) attaches to the AP-conjugation on the antibody. The PNPP undergoes a colorimetric change. This color is detected by the plate reader and the absorbance is measured.

The trapping monoclonal antibodies were oriented in the wells by the use of 1μg/well (stock = 10 mg/ml) of sheep anti-mouse Fc (Jackson ImmunoResearch, West Grove, PA) that had been incubated in the wells in the presence of 0.1 M sodium

bicarbonate, pH 9.5 overnight at 4°C. The anti-mouse antibody was removed by inverting the plates and tapping them on a clean paper towel. The wells were filled with blocking solution (1.5% nonfat dry milk[NFDM]) and incubated at room temperature for 1 h. The blocking solution was removed, and 100 μL of a monoclonal mixture (500 ng/mL each of monoclonal antibodies A6 and J2 [Ab 1] or A6, J2 and L4 [Ab 2], diluted in TBST) was added to each well and incubated at room temperature for 1 h. The antibody solution was removed, the wells washed once with 0.15 M NaCl, 0.05% Tween-20 with a 96 well plate washer on the 5 costar setting (ELx450, BioTek, Winooski, VT), and 50 μL of TBST was added to well A to F to keep them moist. Next, 100 μL of either bovine sera from pregnant animals or serially diluted bPAG standards (in non-pregnant heifer serum; total volume = 150 mL) was added to duplicate wells. Non-pregnant heifer serum alone was included as a blank. The plates were incubated overnight at 4°C on a rocker. The following day the plates were washed twice on the costar 5 setting of an ELx450 Microplate Washer, and 100 μL of a 15 μg/mL anti-PAG polyclonal immunoglobulin (IgG) was added to each well at room temperature for 1 h. The plate was washed once on the costar 5 setting, and 100 μL of AP-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA; diluted 1:2000) added to the wells (room temperature, 20 to 30 min). The plate was washed twice on the costar 5 setting, and 100 μL of 1 mg/mL PNPP (Sigma, St. Louis, MO) was added to each well. After a 30 minute incubation period an EL 808 plate reader (BioTek, Winooski, VT) was used to measure the absorbance at 405 nm in the wells. A standard curve was included on every ELISA plate and was generated by non-linear regression of a LOG (ng bPAG) versus

Absorbance plot by using Graphpad Prism software (version 3.02 for Windows, San Diego, CA; www.graphpad.com).

APPENDIX A.2

Bovine Pregnancy-Associated Glycoproteins Enzyme-linked Immunosorbent Assay

Procedure

I. Reagents

- **Sheep Anti-Mouse Fc:** 1μg per well (10 mg/ml; add 0.1 ml per well) of sheep anti-mouse Fc (Jackson ImmunoResearch, West Grove, PA)
- **Blocking Solution:** 1.5% nonfat dry milk (NFDM) in 0.1M sodium bicarbonate buffer (pH 9.5)
- **Monoclonal Mixture:** 500 ng/mL of each of monoclonal antibodies A6 and J2 (Ab 1) or A6, J2 and L4 (Ab 2), diluted in TBST/1.5% NFDM
    - Mix 30 ml TBS and 20 ml 1.5% NFDM
- **TBST:** Combine 0.2 M Tris and 1.5 M NaCl. Dilute the mix in 1 liter of distilled H_2O Tween 20
- **Anti-PAG Polyclonal IgG:** 15 μg/mL solution
    - Add 100 μl per well in TBST/1.5% NFDM
- **AP-Conjugated Goat Anti-Rabbit IgG:** Jackson ImmunoResearch, West Grove, PA; diluted 1:2000 in AP Buffer
- **AP Buffer:** Combine 12.11 g Tris pH 9.5, 5.84 g NaCl, 1.02 g $MgCl_2$. Dilute the mix in 1 liter of distilled H_2O
- **Para-Nitrophenyl Phosphate:** 1 mg/mL PNPP; Sigma, St. Louis, MO in AP buffer

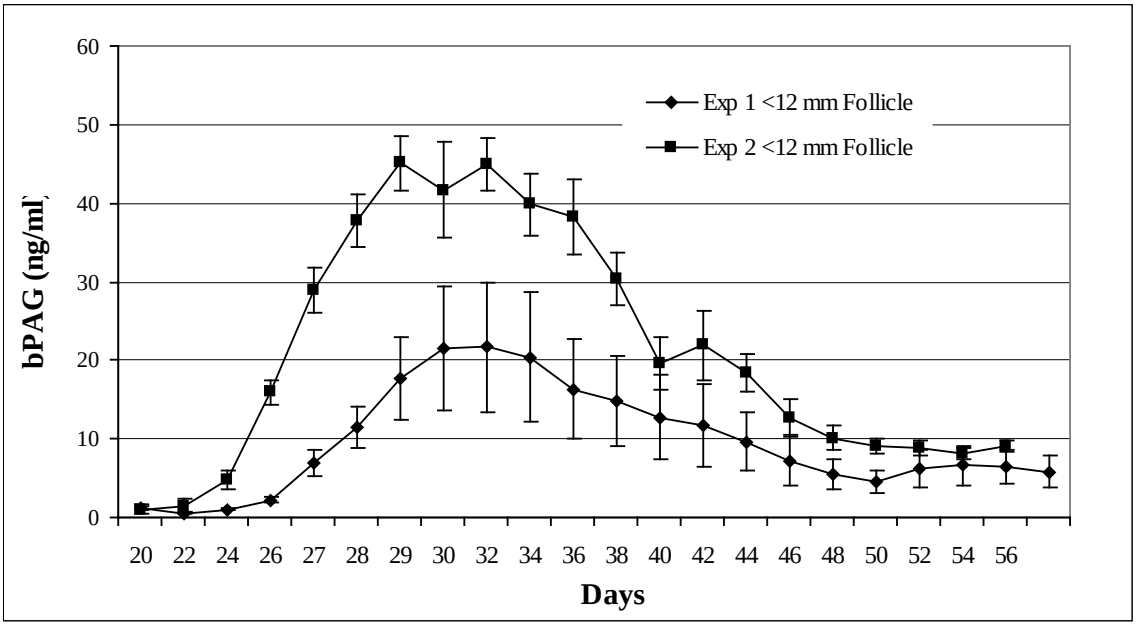
II. Procedure

1. Pipette 100 μL of sheep anti-mouse Fc into each well of a 96 well plate
2. Incubate plates overnight at 4°C
3. Remove anti-mouse antibody by inverting the plates and tapping them on a clean paper towel
4. Pipette 200 μL of blocking solution into each well of the plate
5. Incubate plates at room temperature for 1 h
6. Remove blocking solution by inverting the plates and tapping them on a clean paper towel
7. Pipette 100 μL of monoclonal mixture into each well of the plate
8. Incubate plates at room temperature for 1 h
9. Remove monoclonal mixture by inverting the plates and tapping them on a clean paper towel
10. Wash plates one time with 0.15 M NaCl, 0.05% Tween-20 with a 96 well plate washer on the 5 costar setting
11. Pipette 50 μL of TBST into each well of the plate (rows A to F)
12. Pipette 100 μL of non-pregnant heifer serum into rows G to H
13. Pipette 100 μL of bovine sera into wells of rows A to F in duplicate of the plate.

14. Pipette serially diluted bPAG standards (TBST/NFDM) into rows G to H of the plate
15. Incubate plates overnight at 4°C on a rocker
16. Remove sera and TBST by inverting the plates and tapping them on a clean paper towel
17. Wash plates twice with 0.15 M NaCl, 0.05% Tween-20 with a 96 well plate washer on the 5 costar setting. Turn the plate between wash cycles
18. Pipette 100 μL of anti-PAG polyclonal IgG into each well of the plate
19. Incubate plates at room temperature for 1 h
20. Remove anti-PAG polyclonal IgG by inverting the plates and tapping them on a clean paper towel
21. Wash plates one time with 0.15 M NaCl, 0.05% Tween-20 with a 96 well plate washer on the 5 costar setting
22. Pipette 100 μL of AP-conjugated goat anti-rabbit IgG into each well of the plate
23. Incubate plates at room temperature for 30 min
24. Remove AP-conjugated goat anti-rabbit IgG by inverting the plates and tapping them on a clean paper towel
25. Wash plates twice with 0.15 M NaCl, 0.05% Tween-20 with a 96 well plate washer on the 5 costar setting. Turn the plate between wash cycles.
26. Pipette 100 μL of para-nitrophenyl phosphate into each well of the plate
27. Incubate plates at room temperature for 30 min
28. Use plate reader to measure absorbance at 405 nm in the wells
29. Use Graphpad Prism software to plot LOG (ng bPAG) versus absorbance. Rows G to H of the plate are the standard curve of each plate.

APPENDIX B.1

A



B

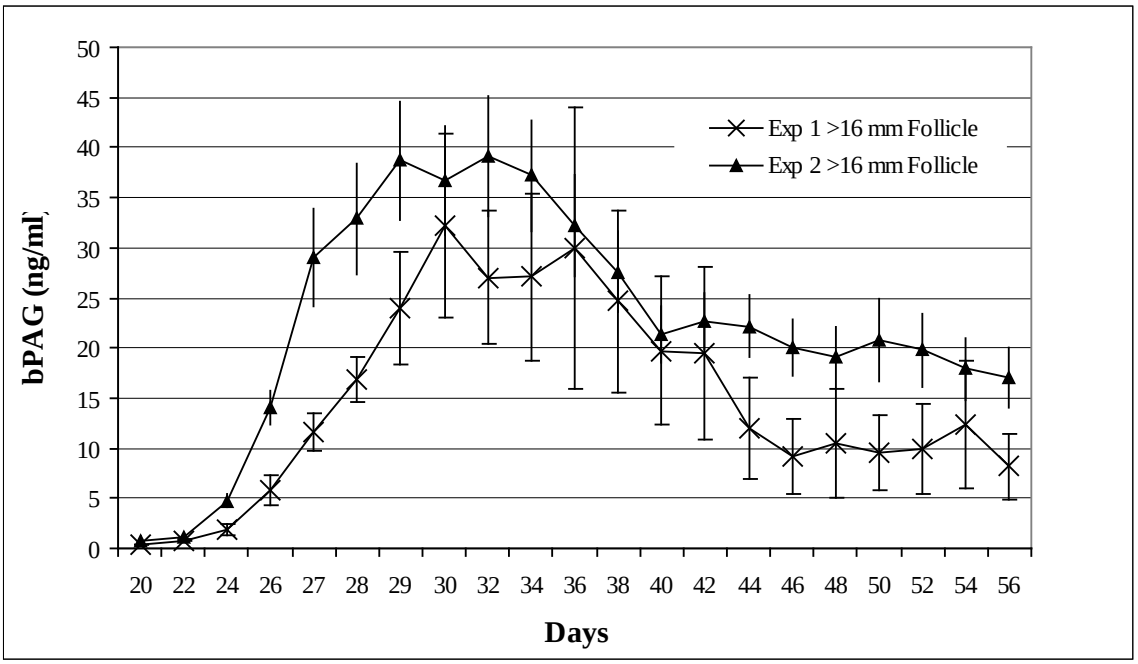


Figure B.1. Effect of ovulatory follicle size (panel A = < 12 mm, Exp 1 n = 5, Exp 2 n = 5; panel B = > 16 mm, Exp 1 n = 5, Exp 2 n = 14) on serum concentrations of bovine pregnancy associated glycoproteins (bPAG) from d 20 to 60 (d 0= insemination) for experiment 1 (Ab 2) and experiment 2 (Ab 1). All samples were assayed by using an enzyme linked immunosorbent assay (ELISA).

APPENDIX C.1

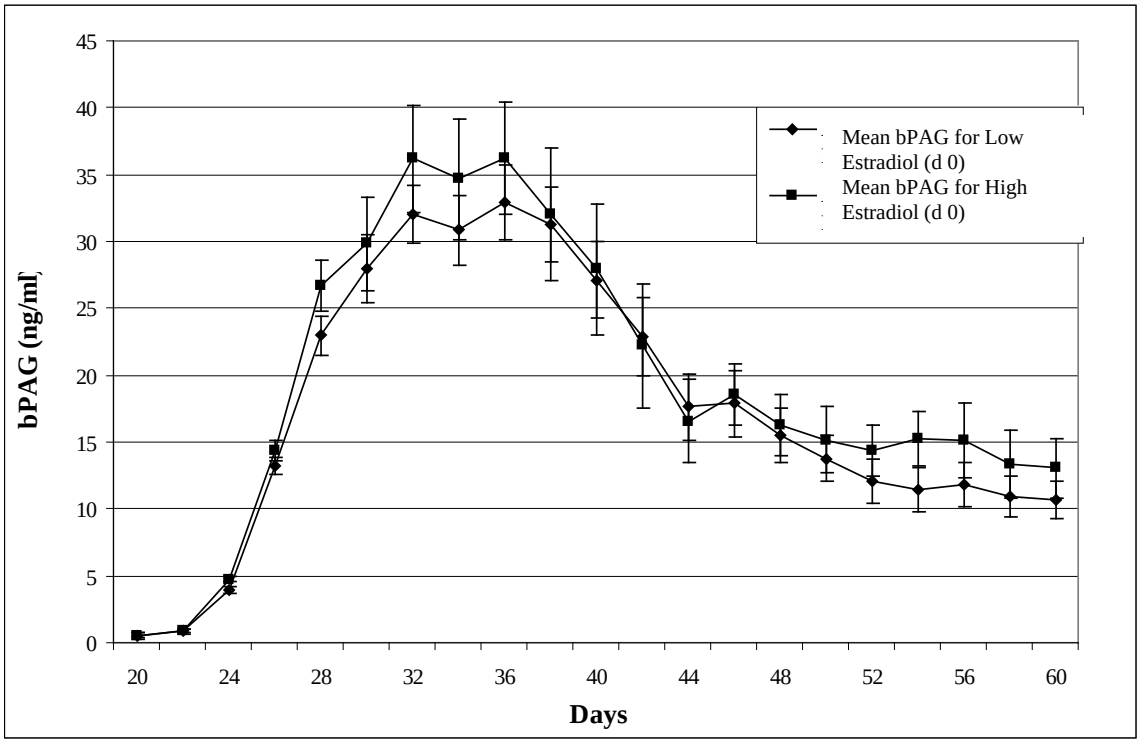


Figure C.1. Comparison of serum concentrations of bovine pregnancy associated glycoproteins (bPAG) in cows with low (n = 24) or high (n = 23) serum concentrations of estradiol (E2) on d 0 (insemination). Bovine PAG were measured with an enzyme linked immunosorbent assay (ELISA; Ab 1 antisera).

APPENDIX D.1

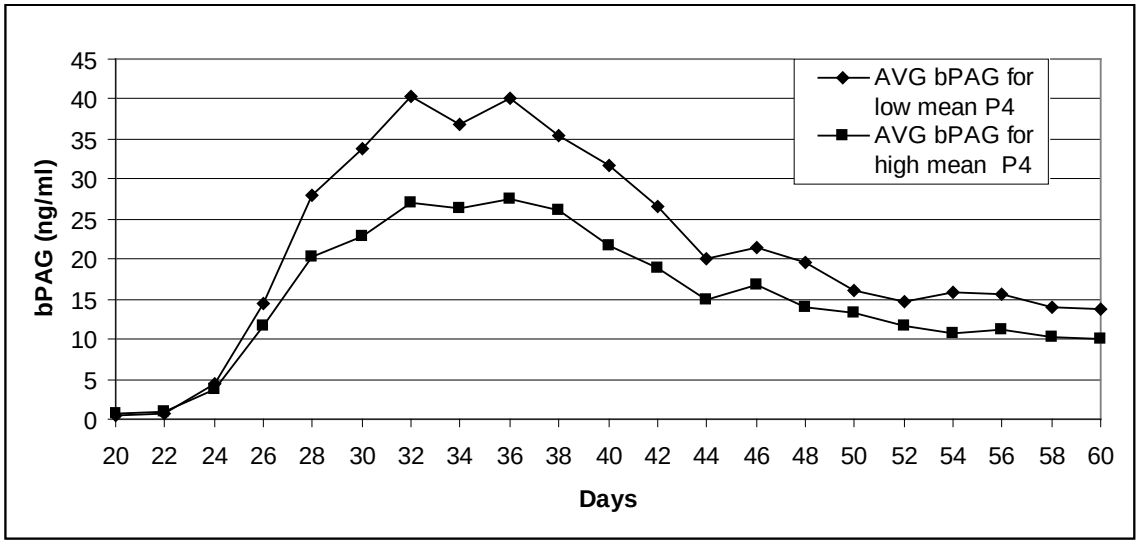


Figure D.1. Comparison of serum concentrations of bovine pregnancy associated glycoproteins (bPAG) in cows with low (n = 22) or high (n = 22) serum concentrations of progesterone (P4) from d 20 to 60 (d 0 = insemination). Bovine PAG were measured with an enzyme linked immunosorbent assay (ELISA; Ab 1 antisera).

VITA

Cia Johnson, formerly Cia Scott, was raised in Humansville, Missouri by her mother Tracy Mason and dad, Tylin Kent Mason. She has two brothers, Kylin (age 21) and Ethan (age 16). Although Cia was raised within the city limits of the rural town and she spent a great deal of time on her grandparents' farms. Cia participated in 4-H and FFA, showed horses, raised beef cattle, and engaged in speaking and judging contests. Cia's family also raised hogs for family use and market rabbits. Cia began her college career at the University of Missouri-Columbia in the fall of 2002 as an Animal Science major. She was accepted into the college as a Pre-Veterinary Medical Scholar giving her automatic acceptance to veterinary college pending meeting the requirements of the program. Cia graduated with a B.S. in Animal Science *cum laude* in the summer of 2005. She was accepted into both veterinary college and graduate school for fall 2005. Cia began her dual degree program of veterinary medicine and M.S. Animal Science, in the fall of 2005 shortly after marrying her long-time boyfriend, Robert Johnson. Cia graduated from the veterinary college in May 2009 and started her career as the Assistant Director of Animal Welfare at the American Veterinary Medical Association while completing her thesis.