FACTORS AFFECTING GROWTH OF THE CONCEPTUS IN LACTATING DAIRY COWS AND NON-LACTATING DAIRY HEIFERS

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by

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

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Presented by Tyler J Stratman

A candidate for the degree of Master of Science

We hereby certify that in our opinion it is worthy of acceptance

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ABBREVIATIONS

A_vol	Amniotic vesicle volume
AI	Artificial Insemination
BCS	Body condition score
BHBA	Beta-hydroxybutyric acid
BS	Blood sample
BW	Body weight
CL	Corpus luteum
CR	Conception rate
CV	Coefficient of variance
DHIA	Dairy Herd Information Association
DIM	Days in milk
DPR	Daughter pregnancy rate
E_vol	Embryo volume
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FFA	Free fatty acid
GH	Growth hormone
GnRH	Gonadotropin releasing hormone
HDR	Heat (estrus) detection rate
IFNτ	Interferon-tau
IGF	Insulin-like growth factor
IGF1	Insulin-like growth factor 1

IM	Intramuscular
IR	Insulin resistance
ISG	Interferon-tau stimulated genes
IVGTT	Intravenous glucose tolerance test
Milk ME	Mature equivalent milk
MINMOD	Minimal model of insulin sensitivity
MHz	Megahertz
NAAB	National Association of Animal Breeders
NEB	Negative energy balance
NEFA	Non-esterified fatty acid
PAG	Pregnancy associated glycoprotein
PD	Pregnancy detection
$PGF_2\alpha$/PGF	Prostaglandin F2-alpha
PMN	Polymorphonuclear cell
PR	21 day pregnancy rate
QUICKI	Quantitative insulin sensitivity check index
RIA	Radioimmunoassay
RQUICKI	Revised quantitative insulin sensitivity check index
TAG	Triacylglycerol
TAI	Timed artificial insemination
US	Ultrasound examination
VFA	Volatile fatty acid

Chapter I

1. LITERATURE REVIEW

1.1. Introduction

The reproductive efficiency of dairy cattle has undergone substantial change in the last 60 years. First service conception rates have declined from 70% in 1951, to 47% in 2013 (Galvão et al., 2013) This decline in reproductive performance coincided with genetic selection for milk production. Using AI, producers improved the genetic quality of their animals by selecting for traits they most prefer. Emphasis was placed on production traits, mainly milk yield, which has a negative correlation with daughter pregnancy rate (DPR) (VanRaden, 2004). Milk production per cow increased from an average of 8636 kg/cow/year in 2004, to 10300 kg/cow/year in 2013 (AgSource, 2013; USDA, 2015). Selection for increased production contributed to the decrease in reproductive efficiency seen in dairy cattle today. A small improvement has occurred recently with first service conception rate in 2014 at an average 55% for Holstein cows (AgSource, 2013) indicating a shift towards improving reproductive function.

Reproductive management of a dairy herd is an important component of overall profitability (Britt, 1985; De Vries, 2006) . The economic value of a pregnancy varies from $200 (Eicker and Fetrow, 2003) in a traditional estrus detection system, to $254-$273 in a system using timed artificial insemination (TAI) (Stevenson, 2001). Reproduction is closely monitored in the progressive dairy herd, and influences other management areas. The reproductive status of a dairy herd can be measured in many ways, but the most common and comprehensive indicator is the 21 day pregnancy rate

(PR). The combination of heat detection, or service rate (HDR) and conception rate (CR) to form the PR allows for a more in-depth analysis of overall reproductive performance. Pregnancy establishment is an important factor, but pregnancy loss is an issue that can cost a producer both time and money.

Although greater production likely contributes to decreased reproductive performance, infertility is a complex issue with many contributing factors (Lucy, 2001). These factors can include poor estrus detection, poor conception rates, anestrus, embryonic loss, disease, or metabolic abnormalities. In high producing dairy cattle, late embryonic or early fetal loss continues at a significant rate until day 56 of gestation (Santos et al., 2004). Embryonic loss is distressing for dairy producers because of the increased cost and time associated with losing an established pregnancy (Whitlock and Maxwell, 2008).

The transition period presents a unique set of challenges to the dairy cow. After parturition, the dairy cow begins lactation, recuperates from calving, and prepares the reproductive system for subsequent pregnancy. Many dairy cows experience a negative energy balance (NEB) during this time period because of an insufficient nutrient intake relative to the energy required for lactation (Grummer, 2007; LeBlanc, 2010). Glucose serves as an energy source for several homeostatic processes. Glucose becomes especially important during the transition period as it is required for the synthesis of milk (Bell, 1995) and is required for the establishment of a subsequent pregnancy (Battaglia and Meschia, 1978).

Glucose metabolism is unique for ruminants because glucose ingested in the diet is converted to volatile fatty acids (VFA) by the rumen microbes (Leek, 2004). One of

the VFA, propionate, undergoes gluconeogenesis in order to produce glucose that can be used by the cow (Aschenbach et al., 2010). Dairy cattle demonstrate differing levels of insulin resistance during lactation; primarily during the transition period (De Koster and Opsomer, 2013). Insulin resistance increases the circulating blood glucose pool because the glucose is not being sequestered into insulin sensitive tissues.

A greater circulating blood glucose pool is advantageous for milk production, but may also be valuable to early embryonic development. Placentomes are not fully formed in the bovine placenta until day 42 of gestation (King et al., 1979) and preceding their formation the gravid uterus relies on facilitated diffusion for glucose supply (Lucy et al., 2012). The research described in this thesis is focused on the associations between glucose and insulin concentrations with embryonic and placental development between days 30 and 50 of gestation in lactating dairy cows and non-lactating heifers.

1.2. Ruminant digestion and metabolism

Ruminants possess unique adaptations of the digestive tract when compared with non-ruminant animals. The most evident being a four compartment stomach comprised of a three compartment forestomach and a single secretory stomach known as the abomasum. The three compartment forestomach includes, in anatomical order, the reticulum, rumen, and omasum. The forestomach is especially important to the ruminant because it is the site of fermentation by rumen microorganisms (Leek, 2004). The microbes digest the beta-1, 4 glucose linkages of cellulose and hemicellulose. This is due to the presence of the enzymes cellulase, hemicellulase, pectin lyase, and fructosanases that are only found in plants and certain microbes. The resulting products of

fermentation are VFA, primarily in the form of acetate, propionate, and butyrate (Leek, 2004). This is markedly different from nonruminant species, where the primary end product of starch digestion is glucose. Because glucose is required for certain metabolic functions, the VFA must be converted back to glucose via a process known as gluconeogenesis (Aschenbach et al., 2010).

The availability of glucose precursors to enter into the gluconeogenesis pathway and the rate of glucose synthesis are the primary rate limiting factors for overall glucose supply. Eighty-five percent of glucose required at the tissue level is produced from VFA (primarily propionate), amino acids, glycerol, and lactate through the process of gluconeogenesis (Fahey Jr. and Berger, 1988; Bell, 1995). The average Holstein cow in the United States produces 10250 kg of milk per lactation, with cows in larger, more intensively managed herds producing an average of 12750 kg per lactation (AgSource, 2013). Seventy two grams of glucose are required for every kg of milk produced, with approximately 60% destined for immediate conversion to lactose (Aschenbach et al., 2010), the primary sugar found in milk (Bell, 1995). This means the average Holstein in the United States requires between 3600 and 7200 g of glucose per day, depending on days in milk or milk production (DIM) (Young, 1977; Aschenbach et al., 2010). Assuming 85% is produced through gluconeogenesis, there is a 500 to1000 g glucose deficiency per day.

Dairy cattle use a number of homeorhetic processes during early lactation to create gluconeogenic substrates for milk production (Aschenbach et al., 2010). Homeorhesis is defined as "coordinated control in metabolism of body tissues necessary to support a physiological state." (Bauman and Currie, 1980). In the instance of

lactation, mobilization of adipose tissue results in non-esterified fatty acid (NEFA) release into the bloodstream (Bauman and Currie, 1980; Bell, 1995; Wathes et al., 2007). The NEFA then travel to the liver to be oxidized directly into energy, partly oxidized into ketone bodies that can be used for energy, or esterified to form triacylglycerol (TAG) (Wathes et al., 2007). Beta-hydroxybutyric acid (BHBA), resulting from fatty acid oxidation, is the most abundant ketone body found in circulation. The BHBA concentration is often used as a means to evaluate metabolic status in postpartum dairy cows (Bell, 1995; Wathes et al., 2007; LeBlanc, 2010; Wathes, 2012). Mobilization of nutrients from tissues within the body to support lactation results in a loss of body condition score (BCS). The loss of condition is an indicator that can be used by producers to monitor the metabolic status of transition cows (Roche et al., 2009).

1.3. Glucose, insulin, and reproduction

Glucose is an important metabolic substrate, and is perhaps most widely thought of as a cellular energy source. Aside from the large requirement needed for lactation (Bell, 1995), glucose plays a vital role in reproductive function. Lactation, and subsequently the available glucose supply, has a significant effect on bovine embryonic growth between days 28 and 42 of pregnancy (Green et al., 2012). For example, non-lactating cows with greater circulating glucose possessed larger embryos in one study (Green et al., 2012). Blood glucose concentrations have also been linked with normal follicular and luteal function in postpartum dairy cows (Nishimoto et al., 2006; Clark et al., 2011), oocyte quality and competence in sheep (Berlinguer et al., 2012), and first

service conception rate (Garverick et al., 2013). Glucose in the postpartum period is important, but is only one part of the metabolic machinery required for lactation.

Glucose is transported within the body mainly through facilitative diffusion or active transport. Facilitative diffusion means glucose uptake is based upon a change in concentration gradient in the circulation versus the target tissue, which allows the glucose to move from the high concentration of circulation to the low concentration of the target tissue. The glucose transporters GLUT 1 and GLUT 3 are the primary means of facilitative diffusion in the body, and act in an insulin independent mechanism. Active transport is the process of actively moving glucose across a cellular membrane via a signaling pathway, most often an insulin dependent mechanism. The primary glucose transporter responsible for actively transporting glucose is GLUT4. It is normally sequestered within intracellular vesicles, but is translocated in response to insulin to the plasma membrane.

Insulin controls blood glucose concentrations, and is equally important when discussing postpartum metabolic requirements. Blood insulin concentrations begin to decrease after parturition as growth hormone (GH) concentrations increase (Sartin et al., 1985). In addition to a decreased concentration of circulating insulin, insulin sensitivity at parturition and in early lactation is decreased (Sano et al., 1993; Bell and Bauman, 1997). A decrease in insulin sensitivity in lactating dairy cattle has also been observed through 100 DIM (Busato et al., 2002). Studies focused on extended lactation demonstrate that an overall increase in insulin sensitivity is seen after 300 DIM, and most notably by 460 DIM (Marett et al., 2015). This observation agrees with other research that has concluded there is a positive correlation between insulin resistance (IR) and high

milk yield (Chagas et al., 2009). The relationship between insulin sensitivity and milk yield is likely due to a heightened need for a large glucose supply to maintain lactation. The udder is an insulin insensitive tissue, and relies on facilitated diffusion and a concentration gradient for glucose uptake (Collier et al., 1984). The udder uses a large majority of the available glucose, which gives rise to a competition for glucose between the mammary gland and other tissues that also rely on facilitated diffusion to receive glucose.

1.4. Indirect measures of insulin sensitivity

Insulin resistance is typically defined as an altered biological response to normal concentrations of insulin most often resulting from either decreased sensitivity or responsiveness (Kahn, 1978; Muniyappa et al., 2008). Direct measurements of insulin sensitivity such as hyperinsulinemic euglycemic glucose clamp or insulin suppression tests are considered to be the most reliable testing methods, but are extremely time consuming and requires the subject to remain in a fasted steady state for an extended period (Muniyappa et al., 2008). These methods, therefore, are often not applicable to large field or epidemiological studies (Holtenius and Holtenius, 2007).

Indirect measures of insulin sensitivity are often based on computer representations or mathematical equations that model the relationship between glucose and insulin in a closed feedback system (Grodsky, 1972; Bergman, 2005). A model, termed the minimal model (MINMOD), was created (Bergman et al., 1979) to take advantage of information gained from serial blood collections after an intravenous glucose tolerance test (IVGTT) to more accurately understand the interactions of glucose

and insulin (Muniyappa et al., 2008; Hahn et al., 2011). The reasoning behind the MINMOD was to account for the assumption that the glucose and insulin interaction would remain closed loop in nature, but the data generated from the IVGTT would illustrate the stimulated response of insulin sensitive tissues (Bergman, 2005). Two pharmacokinetic certainties exist within the MINMOD, and are based upon the assumption that the central blood supply is a "compartment" in which hormone actions are elicited and a remote "compartment" exists that encompasses the peripheral, non-pancreatic tissues (Dhillon and Gill, 2006). The first is that the elevated glucose seen after infusion returns to basal concentration as a result of insulin signaled recruitment of GLUT 4 transporters and acute hyperglycemic recruitment of GLUT 4 transporters (Galante et al., 1995; Bergman, 2005). The second being that the insulin response is slower because it must first diffuse through the first compartment comprised of blood circulation, then to the second remote compartment comprised of peripheral tissues (Bergman, 2005). These two certainties create the base for the two equations that constitute the MINMOD (Bergman, 2005).

$$dG/dt = - [S_G+X(t)] * G$$

Glucose restoration rate = - [glucose effect – insulin effect in remote compartment] * plasma glucose

Equation 1 Minimal Model GLUT 4 transporter clearance of glucose

$$dX/dt = p_2 * l(t) – p_3 * X(t)$$

Increase in remote insulin = fractional rate of insulin appearance in remote compartment * plasma insulin – fractional rate of clearance of insulin from remote compartment * remote insulin

Equation 2 Minimal model glucose diffusion through multiple pharmacokinetic compartments

The unknown parameters in the equation (p_1, p_2, and p_3 and basal plasma glucose) must be attained through rapid blood sampling indicative of an IVGTT (Muniyappa et al., 2008). With these a third equation can be used to determine insulin sensitivity (Bergman, 2005; Muniyappa et al., 2008).

$$S_I = p_3/p_2$$

Equation 3 Minimal Model insulin sensitivity measure

The MINMOD is a valuable tool that allows the quantification of multiple metabolic parameters including insulin sensitivity, rate of glucose clearance in response to hyperglycemia, rate of glucose clearance in response to insulin, and the action of insulin in both the primary compartment and the remote compartment. The ability to identify the unique relationship between glucose, insulin, and differing tissue sensitivities provides a more complete understanding.

1.5. Simple indexes for insulin sensitivity

Previous models, including MINMOD, of insulin sensitivity are based upon results gained from an IVGTT after a fasting period. To counter the need to collect serial blood samples, an index was created to test a single sample of fasted glucose and insulin and is termed the Quantitative Insulin Sensitivity Check Index (QUICKI) (Katz et al., 2000). This index is derived by computing the reciprocal of log glucose and log insulin concentrations (Muniyappa et al., 2008). The results have proven, in several studies, to be reliable, replicable, and accurate (Katz et al., 2000; Mather et al., 2001; Chen et al., 2005) with robust linear correlations to direct methods of insulin sensitivity

measurements (Mather et al., 2001; Rabasa-Lhoret et al., 2003). These qualities have been shown to produce an index that has a strong positive predictive value for development of diabetes in human patients (Hanley et al., 2003). It has been found during the course of several studies that QUICKI is best suited for use in insulin-resistant patients (Muniyappa et al., 2008) but results were less linear and representative when comparing healthy patients (Perseghin et al., 2001). It was found that a revised QUICKI, known as Revised Quantitative Insulin Sensitivity Check Index (RQUICKI), which included a measure of the log of free fatty acid (FFA) concentrations, greatly increased the ability to quantify insulin sensitivity in healthy non-obese human subjects (Perseghin et al., 2001; Rabasa-Lhoret et al., 2003). When RQUIKCI was used to investigate insulin sensitivity in lactating dairy cattle, it was found to not be significantly affected by metabolic adaptions after parturition, unlike the metabolites that constitute the RQUICKI equation (Holtenius and Holtenius, 2007). Multiple studies have elucidated the merit of QUICKI and RQUICKI as a means of determining insulin sensitivity in humans, applicable to both epidemiological and individual trials (Hrebicek et al., 2002; Muniyappa et al., 2008). The use of RQUICKI shows promise in determining insulin sensitivity in dairy cattle, although the relationship of the index across obese and non-obese animals is not well established (Holtenius and Holtenius, 2007).

1.6. Bovine placentation

The bovine placenta is a complex organ that serves a multitude of functions and is the source of a long list of important pregnancy related biomarkers. Ruminants, including the bovine, possess a specialized epitheliochorial placenta, known as a

cotyledonary synepitheliochorial placenta (Peter, 2013). The key feature of the bovine placenta, the placentome, is comprised of the maternal caruncle and the fetal cotyledon, from which the placental name is derived.

An epitheliochorial placenta is comprised of six distinct microscopic levels that separate the maternal and fetal blood supply. These six layers, beginning with the maternal side, are the endometrial capillaries, endometrial interstitium, endometrial epithelium, chorionic epithelium, chorionic interstitium, and chorionic capillaries (Senger, 2005). Between days 20-25 of gestation, the placenta will spread to completely inhabit both uterine horns and will have a translucent appearance. By day 25-30 of gestation the placenta has three distinct, separated macroscopic layers; the chorion, the allantois, and the early amnion. Between days 40-45 of gestation the allantois is closely affiliated with the chorion and separated by a gelatinous layer, but no fusion between the layers is present. By days 50-60 of gestation the allantois and chorion macroscopically appear to be united and distinction of separate layers is difficult (Peter, 2013).

The synepitheliochorial placenta of the bovine has three distinct characteristics that distinguish it from general epitheliochorial placentas. These characteristics are the presence of binucleate giant cells, formation of fetomaternal syncytia, and development of placentomes on the allantochorion surface (Peter, 2013). The binucleate cells and syncytia are formed during early embryo development and placentation before day 20-25 of gestation. Binucleate cells continue to mature and are present throughout gestation, but the hallmark of the gestation period from 30-50 days is the formation of mature placentomes (Senger, 2005; Peter, 2013).

1.7. Placentome formation and function

The cotyledonary placenta's unique characteristic is the presence of placentomes. A placentome is comprised of two parts, the fetal cotyledon and the maternal caruncle. Fetal cotyledons are specialized structures on the outer chorion surface most commonly described as being "button-like" in appearance. In the bovine, small villi of the fetal cotyledon become interdigitated with the corresponding crypts of the maternal caruncle to form the placentome. This forms a convex placentome, opposite of the concave configuration seen in sheep and goats (King et al., 1979; Senger, 2005; Peter, 2013). Histologic examinations performed during the time period of 30-50 days of gestation have provided insight into when placentome formation begins. Before day 30 there is evidence that the maternal caruncular epithelium is in contact with the fetal giant trophoblast cells, but no direct placentome formation has occurred (King et al., 1980). At day 30 of gestation the maternal caruncular epithelium is comprised primarily of cuboidal type cells. At this time point some maternal cells are elongated and multinucleated cells are noted, but are rare (King et al., 1979).

By day 33 the placentomes have become readily detectable microscopically, as they are extended above the uterine surface. The maternal epithelium is comprised of cuboidal cells nearly exclusively, with an occasional multinucleated giant cell. At this stage the fetal villi and maternal crypts are easily discernible microscopically, and interdigitation of the two layers is present in nearly all histological cross sections (King et al., 1979).

At days 36-37 of gestation the placentomes have become macroscopically detectable on the allantochorion surface, beginning to appear first in the immediate

vicinity of the embryo (Peter, 2013). Microscopically, the fetal villi and maternal crypts have become more defined and have progressed deeper into the opposing tissue. Although the villi and crypts have gained depth, there is still no secondary branching of either apparent at this time point (King et al., 1979).

Between days 39-40 of gestation the placentomes have become markedly raised from the allantochorion and uterine surface. The placentomes directly surrounding the embryo are easily distinguishable and appear as darkened oval shaped areas (King et al., 1979; Senger, 2005). The villi of the cotyledons become more complex and secondary branching can be seen microscopically (King et al., 1979). At this point in gestation there are no more than 20 placentomes present on the total surface of the allantochorion (Peter, 2013).

By day 42 of gestation the placentomes have grown in size and complexity. Extensive secondary branching is apparent microscopically in the villi. The maternal epithelium composition has changed from cuboidal cells to a mixture of cuboidal and columnar cells. The fetal cotyledon epithelium is comprised of darker colored, multinucleated columnar cells (King et al., 1979). At this point in gestation, the first placentomes are considered mature and functional (Peter, 2013).

Between days 40-50 of gestation the number of placentomes triples from the approximately 20 observed at 40 days, to an average of 60 total placentomes. This number will continue to increase until approximately day 70 when the mature number of placentomes is reached. The average bovine has 80-90 placentomes total but can have as many as 120 (Peter, 2013).

The placenta plays a key role in the growth of the conceptus through nutrient transport from the maternal circulation to the developing calf. Glucose is the primary substrate required by the conceptus for growth (Battaglia and Meschia, 1978).

Glucose is primarily transported across the uterus and placenta through facilitative diffusion, meaning glucose diffusion is based upon a change in concentration gradient in the maternal circulation in comparison to the gravid uterus and placenta. The glucose transporters GLUT 1 and GLUT 3 are responsible for this action in the uterus (Frolova and Moley, 2011). Active transport of glucose in the placenta and uterus, is controlled via GLUT4 transporters, primarily during later embryonic development. This combination of glucose transporter expression allows glucose to be transported across the membrane in response to a flux in concentration gradient, or during certain time periods, an increase in insulin concentrations.

The expression of GLUT 1 in the placenta and uterus decreases from days 28-42 of gestation. The expression of GLUT 3 in the placenta and the uterus remains similar during this portion of gestation, however, GLUT 4 expression increases between days 28-42 of gestation (Lucy et al., 2012). The expression of GLUT 1 and GLUT 3 is most prominent in placental tissue, but is seen in very small quantities in caruncular tissue or the intercaruncular endometrial tissue. Alternatively, GLUT 4 expression is lesser in placental tissue, but is expressed in greater quantities in the caruncles and intercaruncular endometrium compared with GLUT 1 and GLUT 3 (Green et al., 2012; Lucy et al., 2012).

The pattern and location of glucose transporter expression indicates energy transport is altered as placentome formation begins and is completed. On day 28 of

gestation, preceding placentome formation, facilitated diffusion seems to be the primary method by which glucose enters the uterus and placenta. As the placentomes are beginning to form microscopically on day 35 of gestation, facilitated diffusion transporter GLUT 1 begins to decrease and active transport transporter GLUT 4 begins to increase. At day 42 when the first mature placentomes are present on the placenta, active transport has become much more prevalent in the placentome and facilitated diffusion has decreased in relation to GLUT 1 expression (Lucy et al., 2012). This shift in energy acquisition from diffusion to active transport illustrates the importance of the placentome in appropriating energy for the increased fetal growth seen later in gestation.

The formation of the placentomes between days 30 and 50 of gestation is the key to increasing the surface area of the placenta and establishing communication between the dam and the calf. The increased ability of the placentomes to transport nutrients to the conceptus, especially through insulin mediated glucose acquisition, is fundamental to sustaining fetal growth throughout pregnancy. These changes make this time period an area worthy of continued research concerning the implications on conceptus health, as well as the clinical impacts that have yet to be realized.

1.8. Pregnancy Associated Glycoproteins (PAG)

The placenta produces a number of hormones that have a variety of physiological roles as well as clinical relevance. A group of placental derived proteins, which have received attention in recent years, are the pregnancy-associated glycoproteins or PAGs. PAGs are produced solely by the binucleate cells in the trophectoderm of ruminant ungulates species (Wooding, 1983; Zoli et al., 1992). As the binucleate cells establish

contact with the maternal uterine epithelium, secretory granules containing PAGs are transferred to the maternal circulation via exocytosis (Wooding, 1992). Because PAGs are an exclusive product of the placenta and are present in maternal circulation, development of bovine pregnancy tests are focused on detection of PAGs (Sasser et al., 1986).

There are more than 20 identified bovine PAGs, and their expression during gestation can be either time dependent or continual. The earliest detection of PAGs in maternal circulation can be seen at day 25 of gestation (Green et al., 2000). The detection of PAGs at day 25 of gestation provides an advantage compared with conventional pregnancy detection methods of rectal palpation or ultrasound that are typically performed at day 30 of gestation or later. As gestation progresses the expression of PAGs shifts from a total of 7 PAGs at day 25 to a total of 10 PAGs at day 45 (Green et al., 2000). Many bovine pregnancy tests are designed for early detection, with many commercial kits testing around day 28 of gestation, and thus the efficacy may decrease as gestation progresses and PAG profiles change in the blood (Lawson et al., 2014). Overall the production of a placental specific protein has allowed new technologies to capitalize on this unique aspect and increase the efficiency and productivity of pregnancy diagnosis in the bovine.

1.9. Bovine Embryonic and Fetal Loss

Fertilization rate is the first measure of reproductive success in vivo, but there are several periods of abortion, or conceptus loss, from conception to calving that influence the reproductive success of a dairy herd. When compared with an industry conception

rate of 55% for Holstein cows (AgSource, 2013), the true fertilization rate is much greater. There are reports of the fertilization rate of lactating dairy cows bred via AI reaching highs of 87% (Sartori et al., 2002; Cerri et al., 2009). Conversely, studies have shown fertilization rates of lactating dairy cows to range somewhat largely, from 55.3% in a heat stress environment (Sartori et al., 2002) to 79.5% in an arid desert environment (Ryan et al., 1993), with an average fertilization rate of lactating dairy cows determined to be approximately 76% (Santos et al., 2004). Fertilization rates improved dramatically when cows were housed in cooler climates, or abated from heat stress with rates between 82.4% (Ryan et al., 1993) and 87.8% (Sartori et al., 2002). Non-lactating cows and Holstein heifers, however, consistently show greater fertilization rates, even during heat stress. Fertilization rates as high as 100% have been reported for heifers (Sartori et al., 2002), with dry cows ranging from 66% (Dalton et al., 2001) to 89.9% (Sartori et al., 2002).

Conceptus loss is typically divided into three phases; early embryonic, late embryonic, and fetal. The Committee on Bovine Reproductive Nomenclature states the embryonic phase occurs from fertilization to day 42 of gestation, whereas the fetal phase occurs from day 42 of gestation to parturition (Nomenclature, 1972). Early embryonic loss is typically defined in literature as abortion occurring after fertilization and before day 24 of gestation (Ayalon, 1978; Santos et al., 2004; Diskin and Morris, 2008), with late embryonic loss occurring between days 25 and 42 of gestation. The rate of embryonic loss is debated in literature, but it is commonly believed the majority of loss occurs before day 15 of gestation (Ayalon, 1978; Roche et al., 1981). Values associated with embryonic loss vary, with an average value of 23% loss before day 16 of gestation

(Roche et al., 1981). Timelines and reasons for embryonic loss are variable, with lactating dairy cattle representing a unique niche of cattle suffering from prolonged periods of embryonic and fetal loss.

Lactation seems to have a negative effect on embryo quality before day 7 of gestation and a positive correlation with embryonic mortality. Studies have shown high quality embryos only resulted from fertilized oocytes in lactating dairy cows 59% of the time on average, with ranges from 51.5% to 73.5% (Ryan et al., 1993; Sartori et al., 2002; Cerri et al., 2009). This is corroborated when compared with embryos collected from dry cows, for which 82.3% of fertilized oocytes resulted in high quality embryos (Sartori et al., 2002). The addition of heat stress has a compounding negative effect, with only a 33.3% rate of high quality embryos produced from fertilized oocytes in lactating dairy cows and 71.9% in dairy heifers (Sartori et al., 2002). Several factors may contribute to this early conceptus loss, with varying degrees of evidence. Several studies have shown heat stress can increase chromosomal abnormalities, resulting in the improper development and loss of early embryos (Zavy, 1994).

The time period between days 8 and 24 of gestation is important for embryonic survival as maternal recognition of pregnancy, embryo elongation, and attachment are all occurring. Many complex interactions and physiological changes rely on proper signaling and communication to maintain an embryo. It has been estimated that 25- 40% of total embryonic loss occurs between days 8 and 17 of gestation due to interactions between the conceptus and uterine environment with lactation and heat stress influencing total mortality (Thatcher et al., 2001; Berg et al., 2010). In a study conducted with several groups of non-lactating beef heifers, a 7% loss by day 8, 44% loss by day 12, and

34% loss by day 16 was reported (Diskin and Sreenan, 1980). A recent study reports a 5-10% loss between 14 and 18 days of gestation, and an additional 5-10% loss from days 29 to 42 of gestation (BonDurant, 2007). Embryonic survival becomes more likely as gestation progresses, however, risk of loss is still significant, and becomes more worrisome when coupled with high lactation demands.

Late embryonic loss and fetal loss typically occurs at a lesser rate, but is arguably more detrimental to cattle producers owing to the larger loss of investment in both capital and time. Several studies have investigated the occurrence of embryonic loss through day 42 of gestation, however, evidence indicates that in lactating dairy cattle, the rate of late embryonic and early fetal loss is still relevant through day 56 of gestation (Santos et al., 2004). In more than 4800 lactating dairy cattle examined between days 28 and 58 of gestation, it was found the rate of conceptus loss varied between 3.2% and 42.7%, with an average value of 12.8% (Ayalon, 1978; Santos et al., 2004). This is greater when compared with the 10.8% reported for lactating beef cows during the same period (Stevenson et al., 2003). The risk of fetal loss to term is highly variable, but the average is comparable with risk of early fetal loss (Santos et al., 2004).

1.10. Bovine Maternal Recognition of Pregnancy

Conceptus loss has many potential causes, several beyond the scope of this review. Early embryonic losses occur as the embryo moves from the oviduct into the uterus around day 6 of gestation or from days 8-24 of gestation during the period of maternal recognition of pregnancy. Progesterone from the corpus luteum (CL) is important for maintenance of the early pregnancy (Spencer et al., 2007; Robinson et al.,

2008). The importance of progesterone dictates the CL is maintained to allow the bovine pregnancy to sustain. This necessity of CL maintenance underpins the process known as maternal recognition of pregnancy. In cattle and sheep, the secretion of interferon-tau (IFNτ) by the trophoblast of the developing embryo during elongation acts to block pulsatile release of prostaglandin $F_{2\alpha}$ required for luteolysis (Martal et al., 1979; Godkin et al., 1982; Roberts, 1989; Robinson et al., 2006; Robinson et al., 2008). Ginther (1981) showed in a series of experiments that in order for a bovine conceptus to adequately prevent luteolysis, it must be present in the uterine horn ipsilateral to the ovary containing the CL. The need for precise uterine location is due to the local exchange of hormones and other signaling molecules between the ovary and uterine horn via a series of veins and arteries that form a countercurrent exchange (Lukaszewska and Hansel, 1980; Ginther, 1981; Milvae et al., 1996). This local exchange mechanism allows for a small concentration of IFNτ to be effective in signaling maternal recognition of pregnancy by bypassing central circulation.

1.11. Bovine Pregnancy Detection

There are several methods of pregnancy detection employed on progressive dairy operations. These methods range from the most basic such as monitoring cattle for return to estrus, to more complex methods utilizing transrectal ultrasonography or pregnancy associated assays.

Cattle that have been bred, but fail to become pregnant should return to estrus within 21 days, after the completion of the estrous cycle. The observation of a cow or heifer standing to be mounted during this time frame post-insemination is the most basic

method of determining pregnancy status. Observation of return to estrus, however, is not a reliable method of pregnancy detection as it does not prove the presence of a viable pregnancy, but rather the absence of behavioral quiescence.

Transrectal palpation of the reproductive tract is a common, quick, and accurate method of pregnancy detection when performed by a skilled practitioner. The earliest time that consistent, accurate palpation can be performed is approximately 35 days of gestation (Romano et al., 2007). The implementation of transrectal palpation has limitations on a large dairy operation. Accuracy of pregnancy detection requires substantial practice, most notably when performing examinations before 45 days of pregnancy. There is also potential risk of damage to the conceptus involved with this method of pregnancy detection, although others have found no link between palpation and conceptus loss (Ball and Carroll, 1963; Abbitt et al., 1978; Romano et al., 2007).

Transrectal ultrasonography of the reproductive tract is considered by many to be the gold standard of pregnancy detection. The benefits of ultrasonography compared with palpation are evident in the information available to the practitioner. The conceptus can be visualized and a heartbeat can be confirmed, ovarian activity can be monitored, uterine health can be examined, and fetal sex can be determined all with the use of ultrasonography (Fricke, 2002; DesCoteaux et al., 2009). This amount of information is hugely advantageous, but the fact that transrectal ultrasonography can be used as early as 26 days of gestation for accurate pregnancy detection, shortens the days open for a cow or heifer that needs to be rebred is even more advantageous (Romano et al., 2006).

The detection of PAGs in the maternal circulation has proven to be another reliable method of pregnancy detection. As previously discussed, the presence of PAGs

is pregnancy specific and is first detectable at day 25 of gestation (Green et al., 2000). A radioimmunoassay (RIA) was developed in the 1980s (Sasser et al., 1986) to detect PAG in blood. With time a more sensitive enzyme-linked immunosorbent assay (ELISA) was developed that allowed for the accurate detection of PAGs earlier in gestation (Green et al., 2005). This ELISA has become increasingly sensitive with alterations and studies have now shown PAGs can be detected in milk samples at quantities high enough to diagnose pregnancy comparable to PAG detection in blood (Leblanc, 2013; Gajewski et al., 2014; Lawson et al., 2014). These assays have become very simple, and IDEXX now offers the ELISA in a format marketed towards on farm diagnosis for producers with little laboratory skill. The advent of an assay that can accurately diagnose pregnancy using a milk sample in a short period of time offers a viable option for producers with limited access to trained practitioners or a small population of animals to test.

1.12. Ultrasonographic Determination of Conceptus Growth

The merits of ultrasonography for pregnancy detection have been covered previously, but another benefit of ultrasonography is the ability to determine gestational age based on conceptus size. In dairy herds utilizing AI predominantly, determining the age of a fetus is not a common concern because day of last service is known. In beef or dairy herds utilizing natural mating, however, the accurate estimation of gestational age is an invaluable tool (Fricke and Lamb, 2005; Poock and Wilson, 2011). Several studies have gathered information concerning conceptus growth at different stages of gestation post-slaughter (Eley et al., 1978; Riding et al., 2008; Green et al., 2012) such as fluid volume, conceptus weight, membrane weight, and fluid weight among other things.

There is also evidence lactation affects the size and weight of the developing embryo between days 28-42 of gestation. Embryos from lactating dairy cattle were smaller than embryos collected from dry dairy cattle at the same gestational time points (Green et al., 2012). The collection of the conceptus and fetal fluids and membranes is the gold standard for accurate determination of gestational age, but has obvious limitations for implementation outside of research.

A more appropriate model for field implementation is utilizing conceptus measurements gathered via transrectal or transabdominal ultrasonography. Early studies from O.J. Ginther's lab show developmental benchmarks such as formation of limbs, hooves, and ribs are fairly uniform in heifers (Curran et al., 1986) (Table 2). A series of experiments performed by W. Kahn also illustrate a large number of anatomical landmarks can be used to determine advanced fetal age, including eye size to tibia or humerus length (Kahn, 1989; Kähn, 1990).

These experiments show the feasibility of determining gestational age via ultrasonography, however, the speed of detection when used by practitioners is also important to consider. With this consideration in mind, the most common measurements used now are crown-rump length for embryos between 20-55 days of gestation (Jones and Beal, 2003; Lamb and Fricke, 2004; Poock and Wilson, 2011) (Table 1). Head circumference, head length, or orbital width is used for fetuses after 55 days of gestation and up to approximately 110 days of gestation, because the fetus is typically too large to accurately measure the entire body length (Jones and Beal, 2003; Chavatte-Palmer et al., 2006; Poock and Wilson, 2011). Using these measuring parameters, practitioners can accurately determine gestational age within 4.5 days when using crown rump

measurements and 6.9 days when using head measurements (Jones and Beal, 2003; Poock and Wilson, 2011), providing a valuable tool to producers and practitioners while also offering a less invasive procedure for collecting research data concerning the developing conceptus.

Table 1.1 Gestational Age Determined by Crown-Rump Length

Gestational Age (day)	Crown-Rump Length (mm)	Reference
25	5.0 - 7.0	(DesCoteaux et al., 2009)
<30	9.0	(Poock and Wilson, 2011)
30	8.0 - 12.0	(DesCoteaux et al., 2009)
35	10.0	(Poock and Wilson, 2011)
35	13.89	(Chavatte-Palmer et al., 2006)
35	13.0 - 17.0	(DesCoteaux et al., 2009)
40	20.0	(Poock and Wilson, 2011)
40	17.0 - 24.0	(DesCoteaux et al., 2009)
45	30.0	(Poock and Wilson, 2011)
45	23.0 - 26.0	(DesCoteaux et al., 2009)
50	40.0	(Poock and Wilson, 2011)
50	34.48	(Chavatte-Palmer et al., 2006)
55	50.0	(Poock and Wilson, 2011)
50	35.0 - 45.0	(DesCoteaux et al., 2009)

Table 1.2 Embryonic Organ Development Timeline

Gestational Age (day)	Anatomical Landmark	Reference
29.1	Spinal Cord	(Curran et al., 1986)
29.1	Forelimbs	(Curran et al., 1986)
29.5	Amnion	(Curran et al., 1986)
31.2	Hindlimbs	(Curran et al., 1986)
40	Eye	(Kähn, 1990)
40	Optic lense	(Curran et al., 1986)
40	Fetal movement	(DesCoteaux et al., 2009)
44.6	Split Hooves	(Curran et al., 1986)
52.8	Ribs	(Curran et al., 1986)

1.13. Fetal Sexing

Another valuable tool that is available via ultrasound examination is the determination of the sex of the fetus. The process and implantation of fetal sexing using transrectal ultrasonography was first described in cattle and horses (Curran and Ginther, 1989; Curran et al., 1989; Curran and Ginther, 1991). The visualization of the genital tubercle is first possible at approximately 53 days of gestation (Curran et al., 1989), however, final migration of the tubercle is completed between days 55-57 of gestation (Curran et al., 1989) making the time period of 57-70 days of gestation optimal for field use (Curran and Ginther, 1991). Multiple studies have shown that with appropriate training, the degree of accuracy associated with fetal sexing can be as high as 83-100% (Curran et al., 1989; Curran and Ginther, 1991; Lamb and Fricke, 2004; Fricke and Lamb, 2005). The ability to determine the sex of the fetus has economic benefits for dairy and beef producers. This information can often be used to make management or sales decisions, or fulfill sales contracts requiring offspring of certain sex (Fricke and Lamb, 2005)

1.14. Summary

Reproductive efficiency is an important asset to a dairy producer that holds large economic impact on the outcome of the operation. Considerable effort is devoted to producing pregnancies in dairy cattle, and thus the loss of a pregnancy, especially in the later portion of embryonic development, has many negative implications. The relationship between the metabolism and insulin sensitivity of lactating dairy cattle and the ability to produce and retain a pregnancy is an area of study that has the potential to

realize an index to predict pregnancy loss or embryonic growth. Increased understanding of conceptus loss and the growth of an embryo or fetus in lactating animals could lead to potential intervention or early identification. The goal of such an index would be preventing economic loss or greater days open for cattle producers.

As an initial attempt to better understand why embryos die, we performed two studies where we measured how fast the conceptus grew. The overall hypothesis was that the embryo was responding to specific characteristics of the cow. For example, milk production or body condition score. It was also possible that circulating metabolites affected the pregnancy. If these effects were large enough then it could lead to embryonic loss if the conceptus grows too slowly to reach the critical stage of placentation when embryonic loss is much less.

Chapter II

2. FACTORS AFFECTING EMBRYONIC GROWTH BETWEEN 33 AND 45 DAYS AFTER ARTIFICIAL INSEMINATION

2.1. Introduction

The reproductive efficiency of dairy cattle has undergone substantial change in the last 60 years. First service conception rates have declined from 70% in 1951, to 47% in 2013 (Galvão et al., 2013). Emphasis has been placed on production traits, mainly milk yield, which has a negative correlation with daughter pregnancy rate (DPR) (VanRaden, 2004). Milk production per cow has increased from an average of 8636 kg/cow/year in 2004, to 10300 kg/cow/year in 2013 (AgSource, 2013; USDA, 2015). While increased production likely contributes to decreased reproductive performance, infertility is a complex issue with many contributing factors (Lucy, 2001). These factors include transition cow disease, metabolic abnormalities, anovulation, poor estrus detection, poor conception rates, and embryonic loss.

Given the importance of pregnancy to the economic viability of a dairy there is focus on understanding the multiple factors that contribute to the establishment of pregnancy as well as pregnancy loss. Dairy cows undergo homeorhetic mechanisms during the transition to lactation. Endocrine and metabolic changes associated with homeorhesis cause immunosuppression, most notably during the first 30 d of lactation. This is thought to be a caused by suppressed PMN (polymorphonuclear cells; neutrophils) function (Graugnard et al., 2012; LeBlanc, 2012; Wathes, 2012; Ingvartsen and Moyes, 2013). The first 30 days of lactation are important for reproductive health

because the uterine epithelium recovers from the previous pregnancy, uterine involution occurs, and PMN infiltration removes debris and harmful organisms (Gilbert, 2011; LeBlanc, 2012; Wathes, 2012; Lucy et al., 2014). Impaired immune function is a factor in the development of transition diseases such as metritis and endometritis. These uterine diseases are risk factors for embryonic loss (Santos et al., 2009). In their recent work, Machado et al. (2015) and Ribeiro (2013) found that cows with metritis were 2.16 times more likely to undergo embryonic loss. The presence of PMN in the uterus may give insight into the uterine health of the postpartum cow, and subsequent reproductive performance including embryonic loss.

Glucose is important during the postpartum period because glucose is used for the synthesis of milk (Bell, 1995) and is also required for growth of the conceptus while the cow is lactating (Battaglia and Meschia, 1978). After pregnancy establishment, dairy cattle must maintain a pregnancy while simultaneously partitioning nutrients to sustain lactation. It is possible that low glucose in postpartum cows affects the growth of the pregnancy (Lucy et al., 2014). A faster growing pregnancy associated with greater glucose and other metabolite concentrations may prevent embryonic loss.

Several studies have investigated the occurrence of embryonic loss through 56 of gestation (reviewed by Santos et al., 2004). In over 4800 lactating dairy cattle examined between days 28 and 58 of gestation, it was found that the rate of loss varied between 3.2% and 42.7% in dairy herds, with an average value of 12.8% (Ayalon, 1978; Santos et al., 2004). Loss in dairy cows was greater than the 10.8% reported for lactating beef cows during the same period (Stevenson et al., 2003).

Although embryonic loss is a relatively common occurrence on dairies, we do not fully understand the factors that lead to embryonic loss. Green et al. (2012) studied cows that were either lactating or not lactating postpartum. They found that lactating cows had smaller embryos. Other studies have also shown an association between decreasing bovine PAG between d 30 and 45 of gestation and late embryonic/early fetal loss in beef cattle by d 56 of gestation (Pohler et al., 2013). The data from Pohler et al. (2103) also suggest that embryos with lower PAG concentrations are more likely to undergo embryonic loss. Likewise the data from Green et al. (2012) suggest that lactation leads to smaller embryos, perhaps suggesting a link between a small embryo in lactating cows and embryonic loss.

We designed a study to examine the effect of common cow level factors on the development of the conceptus between days 33 and 45 of gestation (period of late embryonic loss). We tested production related factors as well as circulating hormones and metabolite concentrations. We also tested the cows for subclinical endometritis using a cytobrush examination. The hypothesis was that typical production measures, hormone and metabolite concentrations, and uterine health would impact the rate of growth of the conceptus between d 33 and 45 of gestation. Slower growth would be associated with embryonic loss. This could help explain why lactating dairy cows undergo embryonic loss.

2.2. Materials and Methods

2.2.1. Estrus Synchronization and Pregnancy Diagnosis

One hundred and eight lactating dairy cows (100 Holstein and 8 Guernsey) from the University of Missouri Foremost Research Center were used. Animals were enrolled in the PreSynch OvSynch56 protocol beginning at approximately 42 d postpartum (Figure 1). The PreSynch protocol consisted of an injection of $PGF_{2\alpha}$ (5 mL Lutalyse; 25mg dinoprost tromethamine, IM; Zoetis Inc., Florham Park, NJ) with a second $PGF_{2\alpha}$ injection 14 d later. The OvSynch56 protocol began 14 d after PreSynch completion with an injection of GnRH (2 mL Factrel; 100 µg gonadorelin, IM; Zoetis Inc., Florham Park, NJ), followed 7 d later by a $PGF_{2\alpha}$ injection, followed 56 h later by a GnRH injection, and completed with timed artificial insemination 16 h after. Cows were examined by using transrectal ultrasonography 32 d after AI for pregnancy diagnosis. Pregnant cows were defined as the presence of an embryo with a visible heartbeat. Animals that were diagnosed as non-pregnant at examination were re-enrolled in the OvSynch56 protocol on the same day as diagnosis. Animals that failed to become pregnant to first or second AI were removed from the study (Figure 2.1).

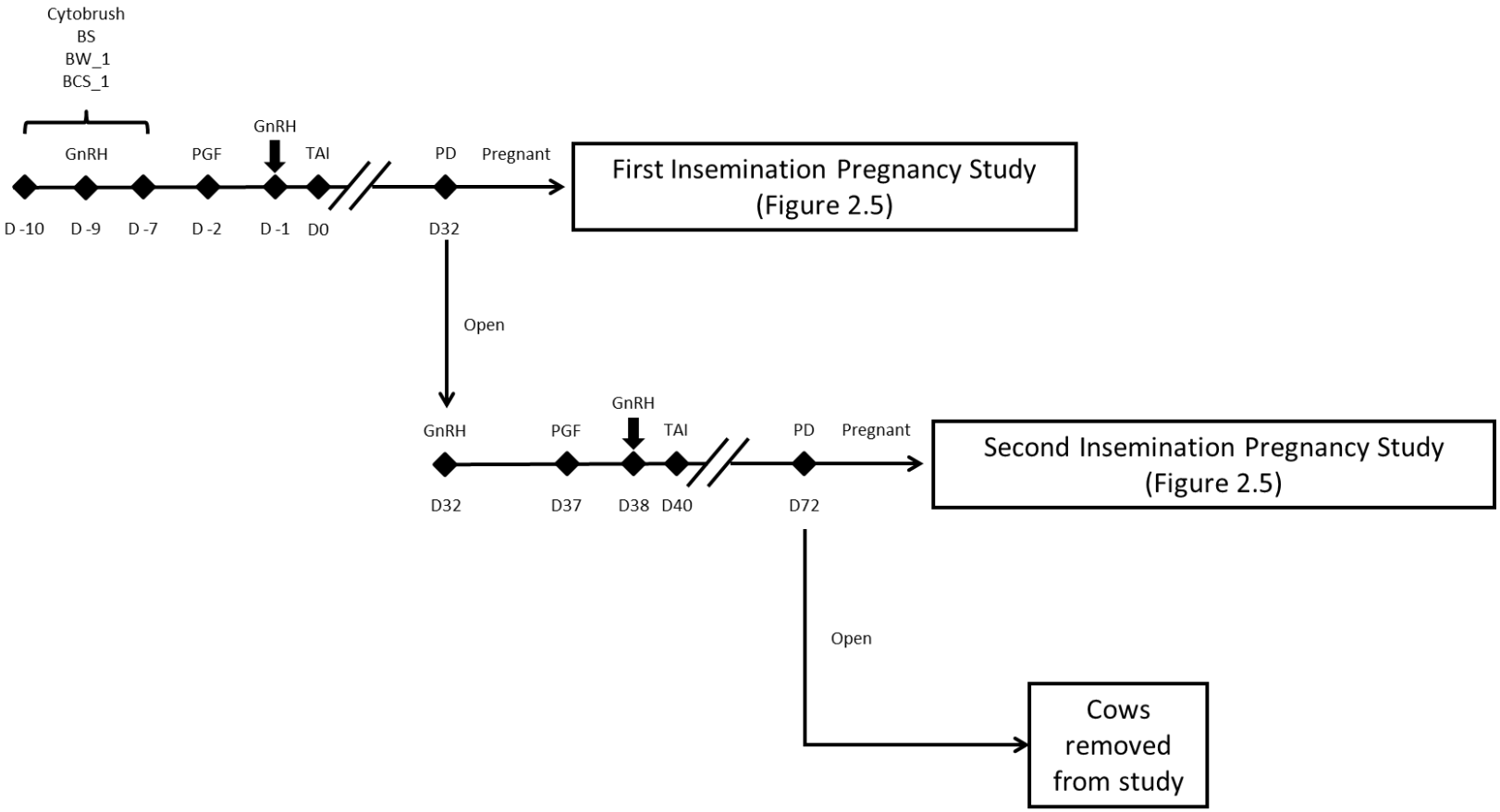

Figure 2.1 Timeline for cytobrush examination and OvSynch synchronization for cows that became pregnant to first AI, second AI, or were removed from the study for failure to become pregnant. US = ultrasound, BS = blood sample, BW_1 = first of two body weight measurements, BCS_1 = first of two body condition score measurements, PD = pregnancy detection.

2.2.2. Endometrial Cytology

An endometrial sample was collected for cytological examination by using a cytobrush (Figure 2.2; Cytobrush Plus cell collector, Cooper Surgical, Inc., Trumball, CT) 1±2 days after the first GnRH injection of the initial OvSynch56 protocol approximately 70 d postpartum (Figure 2.1). The cytobrush was modified for use, with the handle cut to approximately 7 cm in length. The cytobrush was then screwed onto a 65 cm x 0.063 mm stainless steel rod with a threaded end, and placed inside a 44.45 cm bovine insemination gun (Continental Plastic Corp., Delavan, WI). The insemination gun was placed inside a plastic sheath (Continental Plastic Corp., Delavan, WI) to protect the cytobrush while passing through the cervix, and finally placed inside a plastic sleeve (Continental Plastic Corp., Delavan, WI) to protect the sheath while passing through the vagina. The vulva was cleaned with paper towels and the insemination gun was advanced through the vagina to the cervix. The plastic sleeve was then punctured to allow the insemination gun and plastic sheath to pass through the cervix. Once in the uterus, near the horn bifurcation, the cytobrush was advanced out of the plastic sheath to make contact with the uterine wall. Cytological samples were collected by moving the cytobrush in a clockwise manner while in contact with the endometrium. The cytobrush was then retracted inside the sheath and removed from the animal. Slides for cytology were immediately prepared by rolling the cytobrush onto clean glass microscope slides and fixed with ctyofixative (CytoPrep Fixative, Fischer Scientific Co., Pittsburgh, PA). Slides were stained with a modified Wright-Geimsa protocol (HEMA-3 stain series, Fischer Scientific Co., Kalamazoo, MI) within 4 hours of collection.

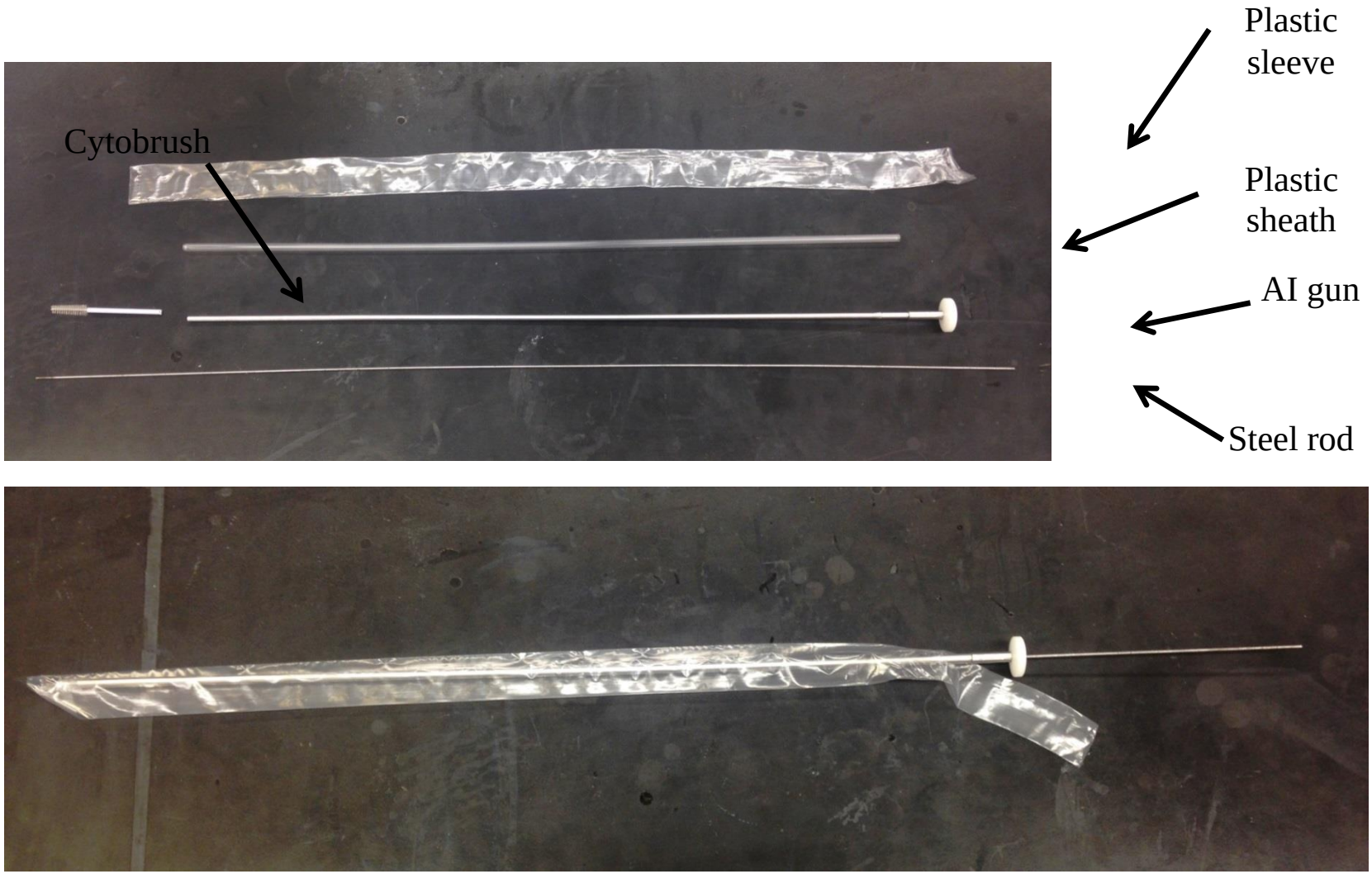

Figure 2.2 Cytobrush apparatus including stainless steel rod inside insemination gun covered by plastic sheath and plastic sleeve.

2.2.3. Cytology Slide Examination

Slides were examined at two different times for the presence of polymorphonuclear cells (PMN; neutrophils) (Figure 2.3). If neutrophils were found then a minimum of 200 cells were counted at 400X magnification (Leica Microsystems Inc., Buffalo Grove, IL) to determine the number of neutrophils as a percentage of total cells present.

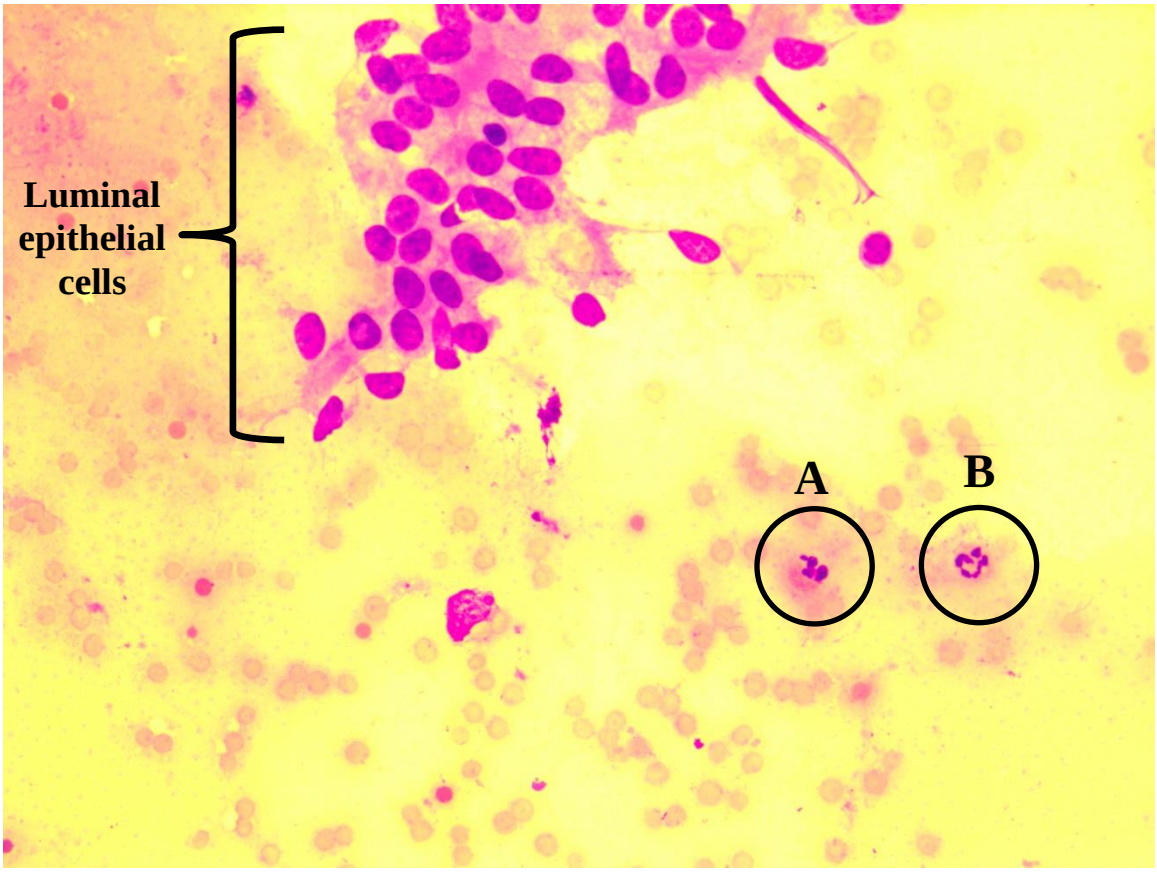

Figure 2.3 Polymorphonuclear cells (neutrophil; A, B) and luminal epithelial cells. The %PMN was the percentage of neutrophils relative to luminal epithelial cells.

2.2.4. Clinical endometritis examination

A vaginal examination was performed using a device constructed to be similar to the Metricheck device (Pleticha et al., 2009) (Figure 2.4). The examination was performed immediately preceding the cytobrush examination. The vulva was cleansed, and the Metricheck was advanced to the cranial aspect of the vagina, then retracted caudally out of the animal. Material covering the apparatus was scored 0 to 3 (0 = translucent mucus, 1 = mucus containing flecks of white purulent material, 2 = less than half the apparatus covered in white purulent material, and 3 = more than half the apparatus covered in purulent material or any blood present) following the guidelines from Pleticha et al.,(2009).

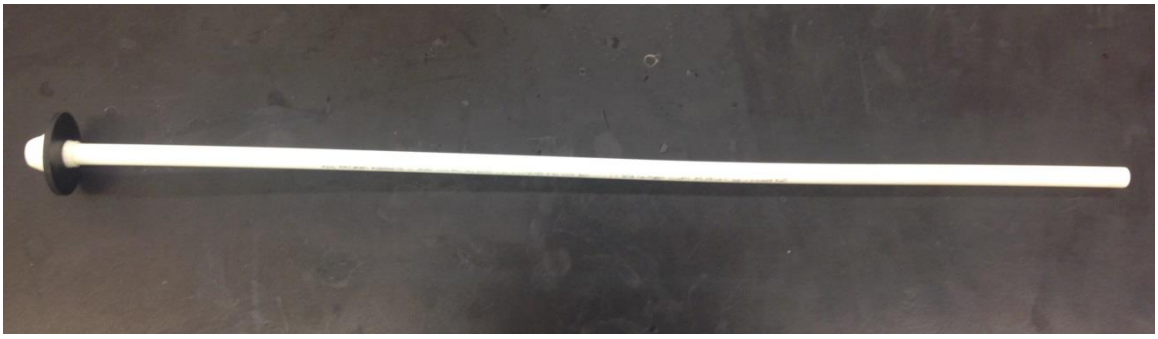

Figure 2.4 Device constructed to be similar to the Metricheck device. A black plastic washer (4 cm diameter) was fitted onto a plastic handle (50 cm in length).

2.2.5.Measurement of the conceptus using ultrasound

Pregnancy diagnosis was performed by a veterinarian using a Sonosite Edge equipped with a variable MHz linear probe (SonoSite Inc., Bothell, WA) 32 d after AI. The location of the embryo (right or left horn) was recorded. All cows pregnant with a single conceptus (n=56; 122 ± 18 DIM) were examined by transrectal ultrasonography on d 33, 35, 38, 40, 42, and 45 of pregnancy using an Aloka 900 ultrasound with a 7.5 MHz transducer (Hitachi Aloka Medical Ltd., Wallingford, CT) (Figure 2.5). Cows pregnant with twins were not included because there were too few in number (n=5). Length (l) and width (w) of the embryo and amniotic vesicle were measured. The volume for the embryo (E_vol) and amniotic vesicle (A_vol) was calculated as an ellipsoid [volume= $4/3*\pi*(0.5*l)*(0.5*w)*(0.5*w)$]. Fetal sex was determined via transrectal ultrasonography between days 60 and 80 of gestation and confirmed at birth. Calf birth weight was recorded using an electronic livestock scale (Tru-Test Inc., Mineral Wells, TX).

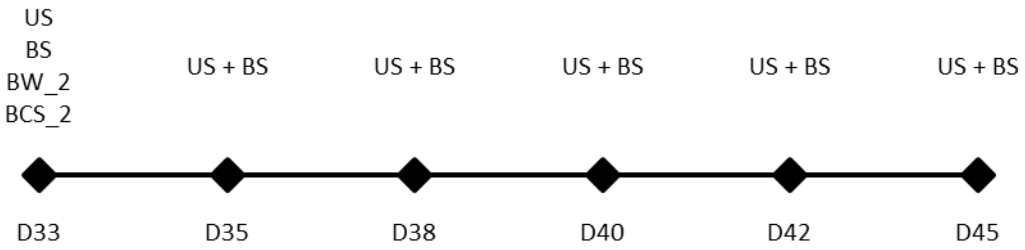

Figure 2.5 Timeline for blood sample collection and ultrasound examination of the embryo and amniotic vesicle on days 33, 35, 38, 40, 42, and 45 of gestation for cows that became pregnant to first or second AI. US = ultrasound, BS = blood sample, BW_2 = second of two body weight measurements, BCS_2 = second of two body condition score measurements.

2.2.6. Blood Collection and Hormone/Metabolite Analysis

Immediately before each ultrasound exam, 10 mL of whole blood was collected from either the coccygeal vein or artery into a tube containing EDTA (10 mL Monoject 16mm x 100mm blood collection tubes, Covidien, Minneapolis, MN) and immediately placed on ice for transport back to the laboratory. Centrifugation of whole blood (1500 x *g* for 15 min) was performed to separate plasma. Plasma was removed and stored at -20°C in polypropylene tubes until hormone and metabolite analysis.

2.2.7. Plasma metabolites

Plasma glucose concentrations were determined enzymatically with the glucose oxidase method (Pointe Scientific Inc., Canton, MI). A standard curve was created using a glucose standard. The standard curve points were 0, 25, 50, 100, 200, and 400 mg/dL. Absorbance was quantified using an ELx808 absorbance reader at 500 nm (BioTek Instruments Inc., Winooski, VT). A predictive equation based on linear regression was created from the standard curve. The equation was used to estimate the glucose concentrations in the samples.

Plasma NEFA concentrations were determined using a NEFA C kit (Wako Diagnostics, Richmond, VA). A standard curve was created using a NEFA standard. The standard curve points were 0, 250, 500, 1000, 2000, and 4000 μEq/L. Colorimetric development was quantified using an ELx808 absorbance reader at 550 nm (BioTek Instruments Inc., Winooski, VT). A predictive equation based on linear regression was created from the standard curve. The equation was used to estimate the NEFA concentrations in the samples.

2.2.8. Plasma hormones

Plasma GH and plasma IGF1 were analyzed by validated RIA (Rhoads et al., 2008). Samples were analyzed in a single assay with the intraassay coefficients of variation (CV) for plasma GH and IGF1 at 7.85% and 8.64%, respectively. Plasma progesterone concentrations were analyzed by validated RIA (Kirby et al., 1997). Samples were analyzed in four assays. The interassay CV for low control (0.29 ng/mL) was 28.13%; for the medium control (5.55 ng/mL) was 6.08%, and for the high control (10.79 ng/mL) was 11.83%. The intrassay CVs for RIA 1, 2, 3, and 4 were 10.37%, 10.52%, 14.82%, and 12.59%, respectively. Plasma insulin concentrations were analyzed by bovine insulin ELISA assay (Alpco Diagnostics, Salem, NH).

2.2.9. Body condition scoring and body weight

All cows were scored for BCS [1 (thin) to 5 (obese)] at cytobrush examination and again on d 33 of gestation (pregnant cows) by two technicians and scores were averaged. Body weight was also measured at cytobrush for all cows and again at d 33 of gestation (pregnant cows) using an electronic livestock scale (Tru-Test Inc., Mineral Wells, TX). The first BCS, the second BCS, the difference in BCS (second minus the first), and the average BCS was included in the statistical analyses. Likewise, the first BW, the second BW, the difference in BW, and the average BW was included in the statistical analyses.

2.2.10. Milk production

Milk production records were obtained from official DHIA test records. Milk_1 was the period around the cytobrush examination. The DHIA test before and DHIA test after the cytobrush examination were averaged for milk_1. Milk_2 was the period around the ultrasound examinations. The DHIA test before and the DHIA test after the series of ultrasound examinations were averaged for milk_2. Milk_diff was defined as milk_2 minus milk_1. Milk_mean was defined as the average of milk_1 and milk_2.

2.2.11. Parity

For the purposes of statistical analyses, parity ≥2 was defined as second parity and first parity cows were analyzed as first parity.

2.2.12. Statistical Analyses-Day of cytobrush

The data collected on the day of cytobrush examination were analyzed for the effects of breed, parity, and status (pregnant or open). The dependent variables tested were plasma concentrations of hormone and metabolites, BCS, BW, %PMN in the cytobrush cytology, and milk production.

2.2.13. Statistical Analyses- Embryo measurement using ultrasound

Embryo measurements were made on days 33, 35, 38, 40, 42, and 45 of pregnancy. The measurements were length and width of the embryo and the amniotic vesicle. Embryo measurements were used to calculate volumes based on the assumption that the embryo was an ellipsoid. The volume of an ellipsoid is $4/3\pi abc$. In this

experiment, a=1/2 embryo length and b and c=1/2 embryo width. The abbreviations used were embryo length (EL), embryo width (EW), embryo volume (E_vol), amniotic vesicle length (AL), amniotic vesicle width (AW), and amniotic vesicle volume (A_vol). The measurements were subject to second order quadratic regression to output regression coefficients for each cow and each measurement (Figure 2.6). Second order regression was selected because preliminary analysis revealed that the third order term was not significant when introduced into the statistical model. The regression equation, therefore, for each cow consisted of EL, EW, E_vol, AL, AW, and A_vol each with an intercept (b_0) and coefficient for the linear (b_1) and quadratic (b_2) component of the polynomial where $y=b_0+b_1*day+b_2*day^2$.

The b_0, b_1, and b_2 terms were analyzed by using a GLMSELECT procedure in SAS. GLMSELECT was used because it can accept both class and continuous variables in the model. The stepwise procedure in the GLM SELECT was used to identify significant effects in the model. Cutoffs for stay and entry were set at P = 0.1. A flow chart describing the analysis of regression coefficients is shown (Figure 2.7).

The effects introduced into the model are listed in Table 2.1 and are fully described in Table 2.2. In most cases, the observations were made once per cow (for example, BCS1, BCS2, etc.). For hormonal data, however, there were measurements made at each ultrasound examination. Preliminary analyses showed a significant effect of cow but not day. A PROC SUMMARY statement, therefore, was used to create an average for each cow across all days. These data were merged with other cow data to create a file with one line of data per cow. These data were then merged with the coefficients for each of EL, EW, E_vol, AL, AW, and A_vol. The GLMSELECT

procedure was run individually for EL, EW, E_vol, AL, AW, and A_vol and each coefficient was tested (b_0, b_1, and b_2) (Figure 2.7).

Once the coefficients were analyzed, a repeated measures analysis was performed. A flow chart for repeated measures analysis is shown (Figure 2.8). Repeated measures is different from the previous analysis because there are multiple measures for each cow (repeated measure of day). For repeated measures, a second order regression was fitted for each of EL, EW, E_vol, AL, AW, and A_vol and a predicted measurement (based on fitted curve) was outputted for each day (Figure 2.6). This was done so that minor day to day variability could be removed hence reducing noise. The r^2 for the fitted values and the actual values was >0.9.

Each of EL, EW, E_vol, AL, AW, and A_vol were then subjected to a repeated measures analysis in PROC MIXED. Most of the variables found in Table 2.1 were not significant so a minimal model was tested. This minimal model included likely variables that could affect growth of the embryo.

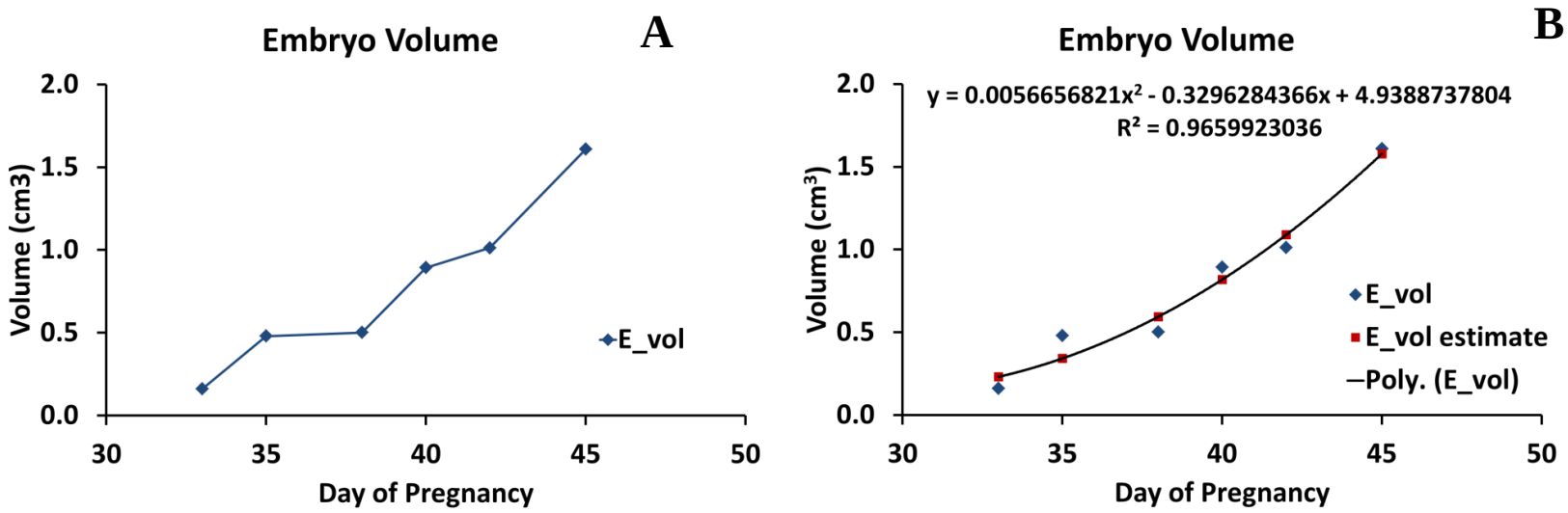

Figure 2.6 Raw data for E_vol between d 33 and 45 of gestation (A) and second order polynomial regression fitted to the same data (B). Predicted measurements (E_vol estimate) for each day were outputted based on the fitted curve.

Table 2.1 Complete list of effects analyzed for influence on embryonic and amniotic vesicle size including descriptive statistics.

Effect	N	Mean	Std Dev	Min.	Max.
Continuous					
NEFA, µEq/L	56	65.41	61.23	31.25	348.54
Glucose, mg/dL	56	66.26	5.46	51.11	82.34
GH, ng/mL	56	5.62	1.92	2.35	13.39
IGF1, ng/mL	56	85.94	20.72	50.02	131.47
Insulin, µU/L	55	0.310	0.077	0.203	0.704
Progesterone, ng/mL	56	8.60	2.26	4.71	13.47
BCS_1	56	3.11	0.25	2.38	4.00
BCS_2	55	3.22	0.31	2.50	4.38
Change in BCS	55	0.11	0.20	-0.38	0.50
Mean BCS	56	3.16	0.27	2.44	4.19
BW_1, kg	55	590.40	65.58	495.45	819.09
BW_2, kg	54	614.01	64.85	503.64	820.91
Change in BW, kg	53	23.57	25.37	-34.55	78.18
Mean BW	56	1322.21	140.84	1099.0	1804.0
Month at ultrasound	56	1.98	0.84	1.0	3.0
DIM at day 32	56	122.23	17.54	100.00	164.00
Birth Weight, kg	44	40.54	4.48	30.45	50.00
Milk_1, kg/day	56	35.78	8.12	21.36	58.18
Milk_2, kg/day	56	35.61	6.71	20.91	54.55
Milk_diff, kg/day	56	-0.06	4.65	-18.64	10.91
Milk_mean, kg/day	56	35.68	7.10	20.91	50.91

Milk ME	56	24151.27	3690.26	16548.0	32166.0
Uterine PMN %	56	1.57	3.49	0	19.00
Metricheck	56	0.89	0.93	0	3
Discrete					
Breed	56	-	-	-	-
Sire	56	-	-	-	-
Parity	56	-	-	-	-
Insemination Number	56	-	-	-	-
Sex	56	-	-	-	-
Horn	56	-	-	-	-

Table 2.2 Explanation of independent variables that were used in the analysis of conceptus growth.

Continuous variables – hormone and metabolites	
Plasma NEFA	Averaged over the six time collection days (d 33, 35, 38, 40, 42, and 45).
Plasma glucose	Averaged over the six time collection days (d 33, 35, 38, 40, 42, and 45).
Plasma insulin	Averaged over the six time collection days (d 33, 35, 38, 40, 42, and 45).
Plasma IGF1	Averaged over the six time collection days (d 33, 35, 38, 40, 42, and 45).
Plasma GH	Averaged over the six time collection days (d 33, 35, 38, 40, 42, and 45).
Plasma progesterone (P4)	Averaged over the six time collection days (d 33, 35, 38, 40, 42, and 45).
Continuous variables – production	
Milk_ME	Mature milk lactation equivalent. Average of DHIA records at the time of cytobrush exam and ultrasound.
Milk_1	Daily milk production at the time of the cytobrush exam. Average of month DHIA test data before and after cytobrush exam.
Milk_2	Daily milk production at the time of the ultrasound exams. Average of month DHIA test data before and after ultrasound exams.
Milk_diff	Milk_2 minus Milk_1
Milk_mean	Average of Milk_1 and Milk_2
BCS_1	Body condition score at the time of cytobrush exam (average of two different evaluators).
BCS_2	Body condition score at the time of ultrasound exam (average of two different evaluators).

BCS_diff	BCS_2 minus BCS_1
BCS_mean	Average of BCS_1 and BCS_2
BW_1	Body weight at the time of cytobrush exam.
BW_2	Body weight at the time of ultrasound exam.
BW_diff	BW_2 minus BW_1
BW_mean	Average of BW_1 and BW_2
Continuous – other	
DIM	Days in milk on d 32 of pregnancy
BW_calf	Birth weight of calf
PMN%	Percentage of neutrophils in cytological analysis of cytobrush exam.
Discrete	
Insemination number	Pregnancies were from either a first or second insemination postpartum
Sire	NAAB registration number of bull used at each insemination
Month	Month (January, February, March, or April) during which the ultrasound exam was conducted
Breed	Holstein or Guernsey
Parity	Defined as either first or ≥2 parity
Metricheck score	0 to 3 based on the amount of purulent material or blood present
Location	Pregnancy detected in the right or left horn
Sex	Sex of calf (male or female)

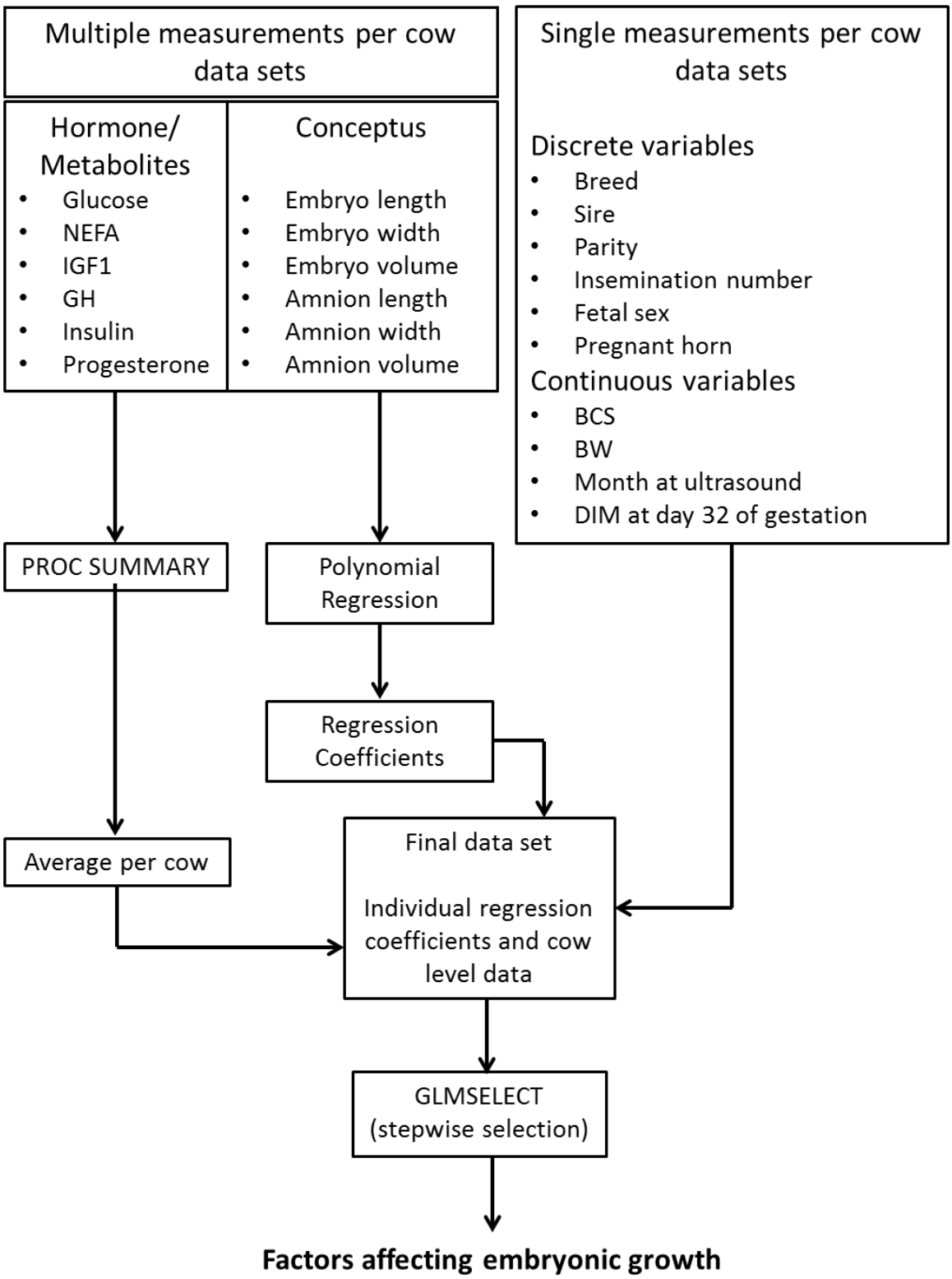

Figure 2.7 Statistical analyses flow chart. Polynomial regression coefficients of each of EL, EW, E_vol, AL, AW, and A_vol were analyzed.

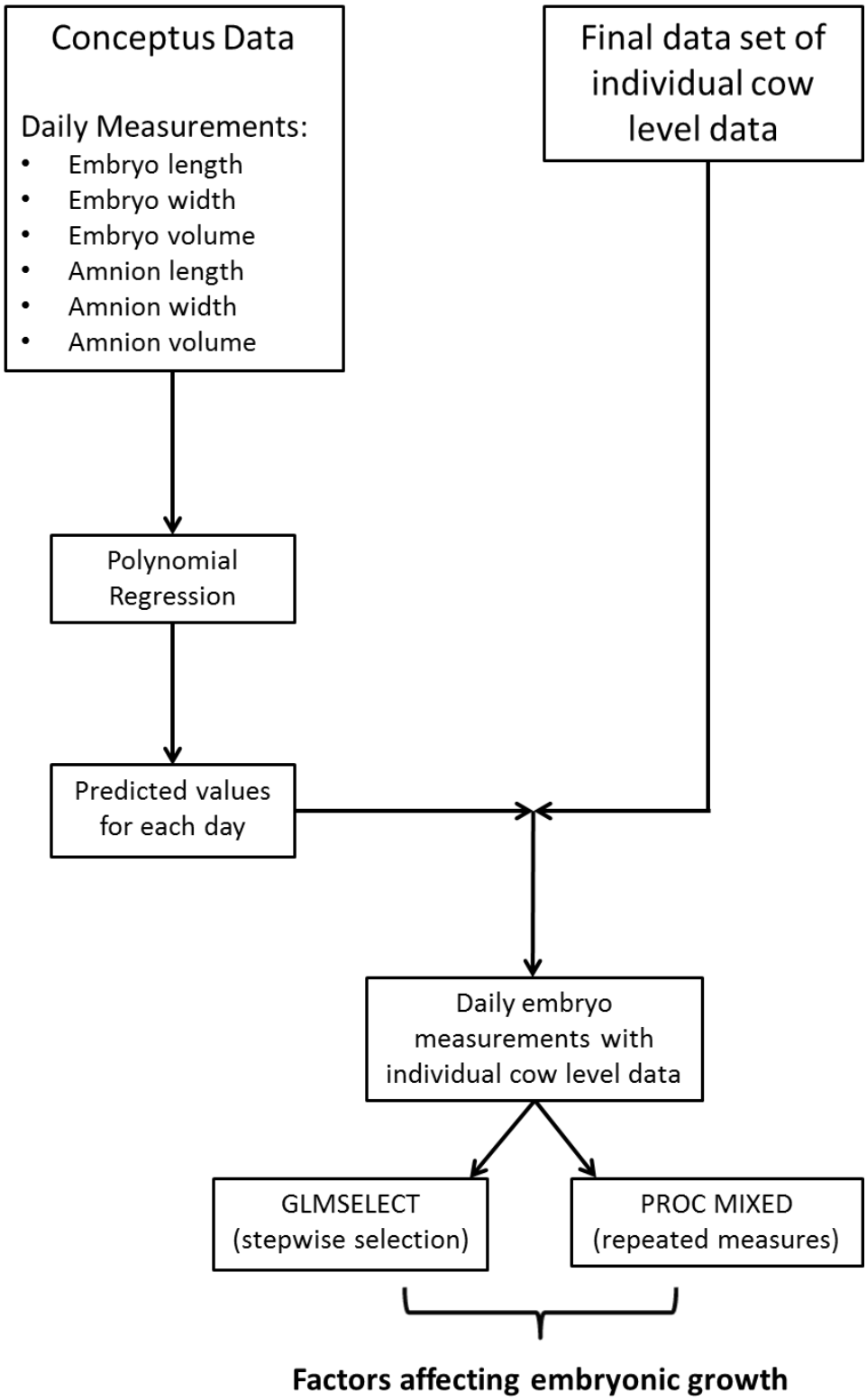

Figure 2.8 Second statistical analyses flow chart. Daily predicted values for EL, EW, E_vol, AL, AW, and A_vol were analyzed using GLMSELECT and PROC MIXED for repeated measures.

2.3. Results

2.3.1. Cows at cytobrush exam

There were 58 first parity and 48 ≥ 2 parity cows at the cytobrush exam. Of these, 65 became pregnant to 1 or 2 inseminations and 41 did not become pregnant. There was an effect of status (pregnant versus open) for BW (P <0.008) and % PMN (P <0.029). Cows that did not become pregnant weighed less and had a greater % PMN at the cytobrush exam (Table 2.3). Parity affected IGF1 (P <0.001) and BW (P <0.001). As expected first parity cows had greater IGF1 concentrations and lesser BW at the cytobrush exam (Table 2.3). There was a status by parity interaction for progesterone concentrations because first parity open cows had lesser progesterone concentrations than first pregnant parity cows (Table 2.3). The opposite was true for second or greater parity cows because open cows had greater progesterone concentrations than pregnant cows (Table 2.3). There was a status by parity interaction for GH concentrations and BCS. First parity cows that did not become pregnant had greater GH concentrations, and decreased BCS (Table 2.3). There was an effect of status because open cows produced less milk. (P <0.036) and parity because first parity cows produced less milk (P <0.001) (Table 2.3).

2.3.2. Cows at day 33 of gestation

There were 39 first parity and 27 ≥ 2 parity cows at day 33 of gestation. There was an effect of parity for BCS_2 (P <0.001) and BW_2 (P<0.055) determined on day 33 of gestation. First parity cows had greater BCS but lesser body weight (Table 2.4). Parity affected the change in BCS (P <0.001) between cytobrush exam and day 33 of

gestation. First parity cows experienced a greater gain in BCS compared with second or greater parity cows (Table 2.4).

Table 2.3 Concentrations of plasma hormones and metabolites, body condition score (BCS) (1 to 5 scale, thin to obese), body weight (BW), and percentage of uterine polymorphonuclear cells (PMN; neutrophils) on the day of cytobrush exam for cows that were either first or second or greater parity, or pregnant or open after two inseminations.

	Parity = 1		Parity ≥ 2		P value		
	Open	Pregnant	Open	Pregnant	Status	Parity	S*P
Number of cows	20	37	21	27			
Glucose, mg/dL	65.9 ± 1.4	65.1 ± 1.0	65.5 ± 1.3	63.8 ± 1.2	NS	NS	NS
NEFA, µEq/L	89.9 ± 25.0	110.2 ± 17.7	56.7 ± 24.4	79.1 ± 21.5	NS	NS	NS
Progesterone, ng/mL	3.4 ± 0.7	6.4 ± 0.5	5.9 ± 0.7	3.4 ± 0.6	NS	NS	0.001
GH, ng/mL	11.8 ± 1.4	6.7 ± 1.0	7.6 ± 1.3	8.5 ± 1.2	0.090	NS	0.016
IGF1, ng/mL	72.7 ± 4.2	84.3 ± 3.0	65.0 ± 4.1	63.8 ± 3.7	NS	0.001	0.092
BCS	2.96 ± 0.06	3.18 ± 0.04	3.10 ± 0.06	3.04 ± 0.05	NS	NS	0.012
BW, kg	513 ± 13	558 ± 9	642 ± 13	659 ± 11	0.008	0.001	NS
% PMN	4.8 ± 2.0	2.1 ± 1.4	5.8 ± 1.9	0.5 ± 1.7	0.029	NS	NS
Milk, kg/day	28.8 ± 1.6	31.5 ± 1.1	40.0 ± 1.5	43.4 ± 1.4	0.036	0.001	NS

[1] NS = Not significant, P > 0.15

Table 2.4 Body condition score at day 33 of gestation (1 to 5 scale, thin to obese), change in body condition score from day of cytobrush to day 33 of gestation, body weight at day 33 of gestation, and change in body weight from day of cytobrush to day 33 of gestation for cows that were either first or second parity.

	Parity		P value
	1	≥ 2	
Number of cows	39	27	
BCS_2	3.32 ± 0.04	3.08 ± 0.05	0.001
Change in BCS	0.14 ± 0.03	0.04 ± 0.04	0.055
BW_2, kg	586 ± 8	675 ± 10	< 0.001
Change in BW, kg	61.3 ± 13.2	30.4 ± 15.9	0.139

[1] NS = Not significant, P > 0.15

2.3.3. Embryo and amnion measurements using ultrasound

The parameters (b_0, b_1, and b_2) for the fitted curves for EL, EW, E_vol, AL, AW, and A_vol were analyzed for all of the effects listed in Table 2.1. A GLMSELECT procedure was used. The results from the GLMSELECT are shown in Table 2.5. Very few of the analyzed variables had any effect on the parameters that defined the individual curves for each cow. Significant effects of month (EW, E_vol, AW), breed (EW), and IGF1 (EW) were detected (Table 2.5). None of the variables affect EL, AL, or A_vol.

In a second round of statistical analyses, the effects were analyzed using GLMSELECT with a repeated measures model. The individual data points for EL, EW, E_vol, AL, AW, and A_vol were fitted and a predicted value for each day was outputted. These outputted data were analyzed for all effects listed in Table 2.1. The results are shown in Table 2.6. Again, very few of the analyzed variables were significant. The effects of day and cow were typically significant (as expected). The significant effect of day reflected the growth in the conceptus over time. The significant effect of cow indicated that the conceptus differed in size for different cows. We noted an effect of month*day for EL, EW, and E_vol. An effect of month was also found when the coefficients were analyzed (Table 2.5). In terms of hormone concentrations, there was an effect of insulin*day on EL and EW. Other effects on the conceptus were less consistent across measurements (BW_1, BCS_2, breed, insemination).

Table 2.5 Results of the GLMSELECT procedure where the most appropriate model for parameter coefficients was selected by a stepwise procedure.

Curve	Parameter	Significant model effects at P <0.05
EL	b_0	None
	b_1	None
	b_2	None
EW	b_0	Breed (P <0.008), Month (P <0.012), IGF1 (P <0.04)
	b_1	Breed (P <0.009), Month (P <0.007), IGF1 (P <0.04)
	b_2	Breed (P <0.006), Month (P <0.006), IGF1 (P <0.04)
E_vol	b_0	Month (P <0.001)
	b_1	Month (P <0.001)
	b_2	Month (P <0.001)
AL	b_0	None
	b_1	None
	b_2	None
AW	b_0	Month (P <0.037)
	b_1	Month (P <0.039)
	b_2	Month (P <0.05)
A_vol	b_0	None
	b_1	None
	b_2	None

Table 2.6 Output of GLMSELECT with repeated measures

Dependent Variable	Significant model effects at P <0.05
EL	Cow (P <0.001), Day (P <0.001), Month*Day (P <0.002), BW_1*Day (P <0.002), BCS_2*Day (P <0.014), Insulin*Day (P <0.035)
EW	Cow (P <0.001), Month*Day (P <0.001), Breed*Day (P <0.023), Insulin*Day (P <0.030)
E_vol	Cow (P <0.001), Day (P <0.001), Month*Day (P <0.001), Insemination*Day (P <0.035)
AL	Cow (P <0.001), Day (P <0.001), BW_1*Day (P <0.004)
AW	Cow (P <0.001), Day (P <0.001)
A_vol	Cow (P <0.001), Day (P <0.001)

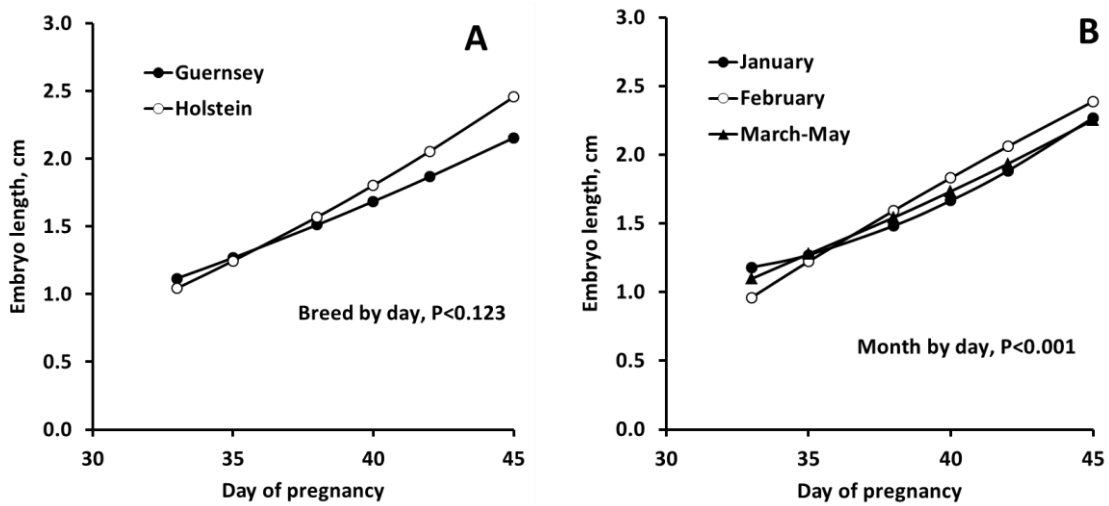

Figure 2.9 Embryo length (day 33-45 of gestation) for cows that were either Guernsey or Holstein breed (A) or were pregnant in January, February, or March to May (B).

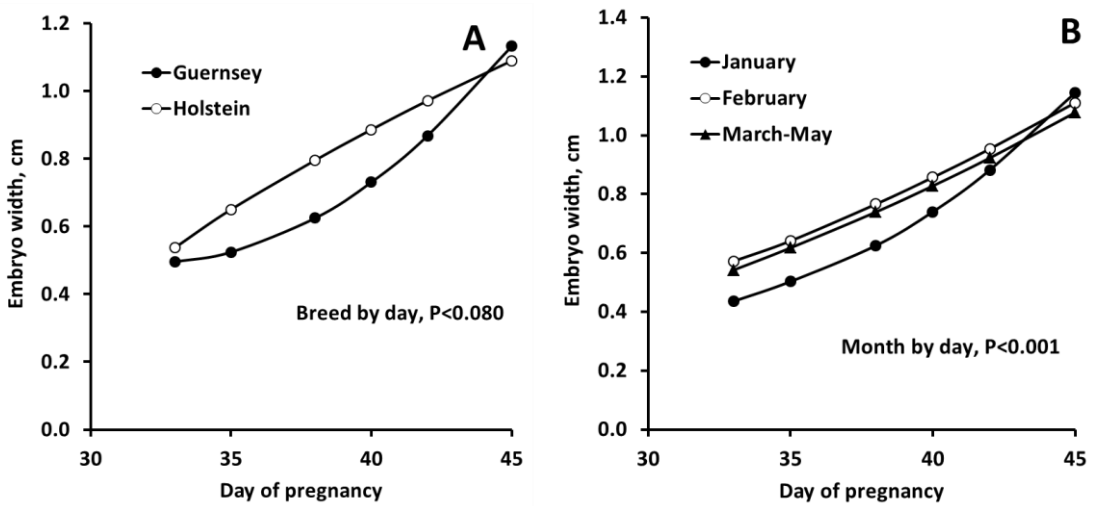

Figure 2.10 Embryo width (day 33-45 of gestation) for cows that were either Guernsey or Holstein breed (A) or were pregnant in January, February, or March to May (B).

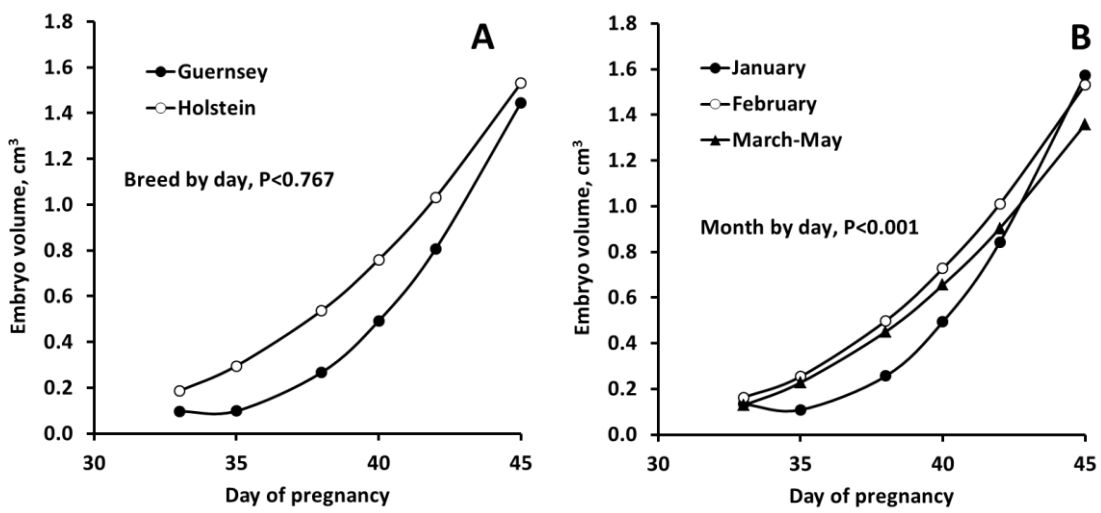

Figure 2.11 Embryo ellipse volume (day 33-45 of gestation) for cows that were either Guernsey or Holstein breed (A) or were pregnant in January, February, or March to May (B).

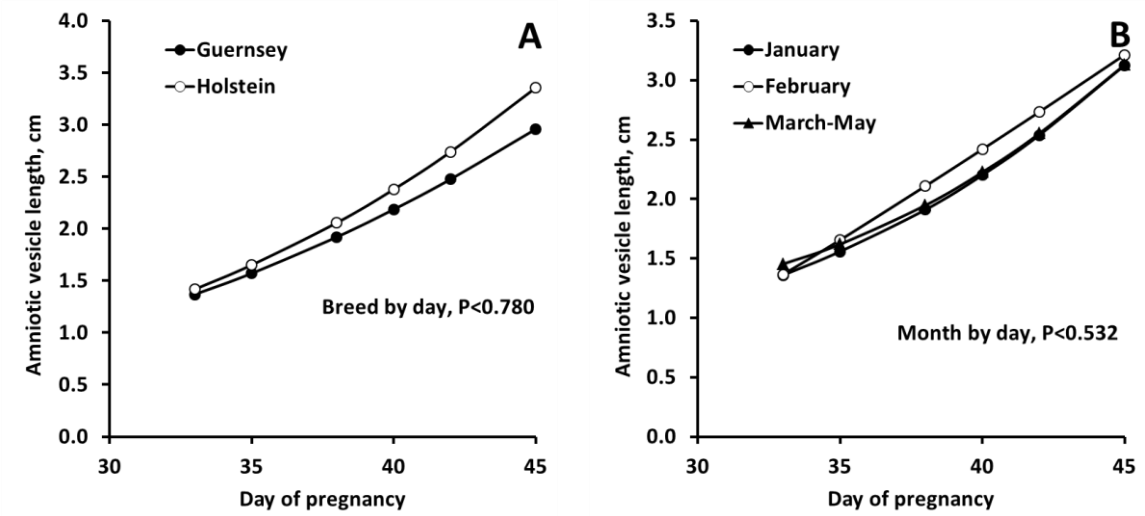

Figure 2.12 Amniotic vesicle length (day 33-45 of gestation) for cows that were either Guernsey or Holstein breed (A) or were pregnant in January, February, or March to May (B).

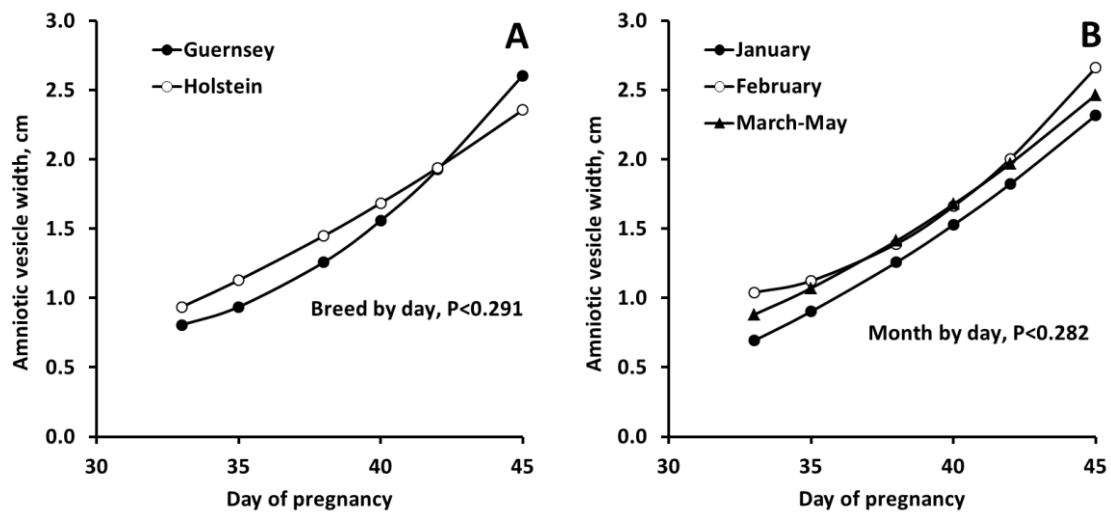

Figure 2.13 Amniotic vesicle width (day 33-45 of gestation) for cows that were either Guernsey or Holstein breed (A) or were pregnant in January, February, or March to May (B).

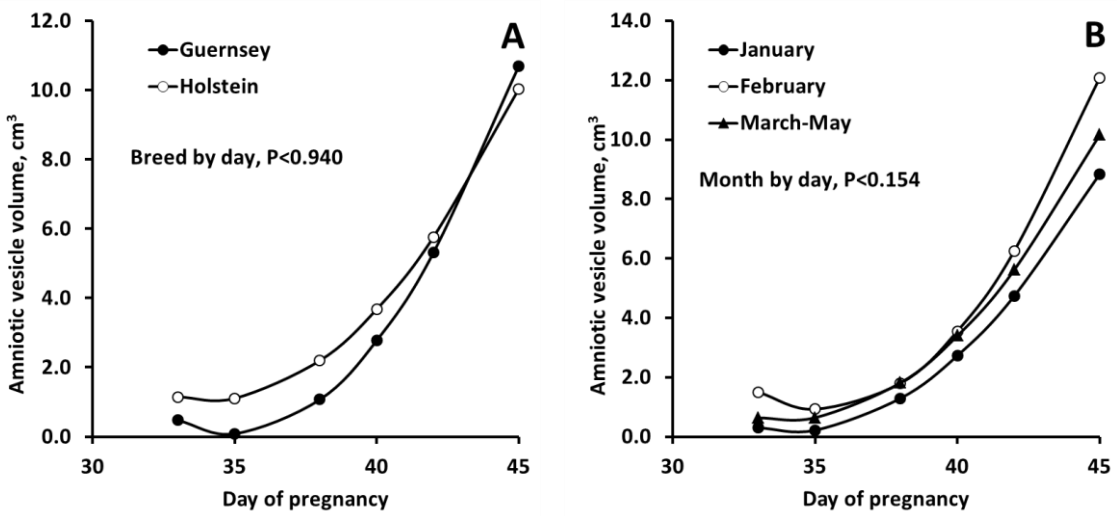

Figure 2.14 Amniotic vesicle ellipse volume (day 33-45 of gestation) for cows that were either Guernsey or Holstein breed (A) or were pregnant in January, February, or March to May (B).

2.3.4. PROC MIXED conceptus measurements

The GLMSELECT procedure is used to identify likely components of a statistical model. The GLMSELECT analyses suggested effects of month, breed, BW_1, IGF1, and insulin. These effects were followed up by using a repeated measures analysis in PROC MIXED. In these analyses cow was nested in breed*month and was fitted as a random variable. Day was a repeated discrete variable. Breed and month were discrete. BW_1, IGF1, and insulin were continuous. The results of the model are shown in Table 2.7. The effects of month, day, and breed or their interactions with day were often significant. There also appeared to be effects of BW_1, IGF1, and insulin on the EL (interaction with day).

In a first series of graphs EL, EW, E_vol, AL, AW, and A_vol for Holstein versus Guernsey and January, February, and March to May are shown (Figures 2.9 to 2.14). Specific tests of significance can be found in Table 2.7. The depicted relationship is not always significant. In some cases, the graph is shown for completeness of interpretation. For the embryo, the general pattern that was observed was for a smaller embryo in Guernsey cows and a smaller embryo in cows that were pregnant in January. Embryos were typically smaller on days 35 to 40 and converged by day 45. For the amniotic vesicle (Figures 2.12 to 2.14), the data were generally not significant but followed a similar trend when compared with the embryo.

Interactions with continuous variables were then plotted (Figures 2.15 to 2.17). In this analysis the slope of the effect at each individual day was calculated from parameter estimates given by PROC MIXED (solution included in the model statement). Once the slope was calculated then it was multiplied by ±2 standard deviations (Table 2.1) to

calculate a deviation in embryo or amniotic vesicle size on each day. The deviated values (plus two SD and minus two SD) represent 95% of the population on each side of the average.

The data for EL, EW, and E_vol are shown in Figures 2.15 to 2.17. Data were not plotted for amniotic vesicle because they were generally not significant. In terms of EL, cows with low IGF1 had a flatter growth trajectory (Figure 2.15A). For insulin, cows with low insulin had faster embryo growth but more linear trajectory. Likewise, the growth of low BW_1 cows was faster but more linear.

Although there appeared to be differences in growth curves for EW and E_vol (Figures 2.16 and 2.17), these data were not significant except for an effect of insulin on EW and E_vol. The effects of insulin were main effects meaning that the interaction with day was not found. Cows with greater insulin had thinner embryos (Figure 2.16B) and had lesser embryonic volume (Figure 2.17B)

Table 2.7 Results of the PROC MIXED analyses of conceptus measurements with a statistical model that included breed, month, day, BW_1, IGF1, and insulin.

Model Effect	Df	EL	EW	E_vol	AL	AW	A_vol
Breed	1	NS	0.061	0.043	NS	NS	NS
Month	2	NS	0.003	0.012	NS	0.005	0.004
Day	5	0.001	NS	0.007	0.001	NS	NS
BW_1	1	NS	0.03	0.022	NS	0.04	0.08
IGF1	1	NS	NS	NS	NS	NS	NS
Insulin	1	0.022	0.047	0.021	0.08	NS	NS
Breed*Day	5	NS	0.08	NS	NS	NS	NS
Month*Day	10	0.001	0.001	0.001	NS	NS	NS
BW_1*Day	5	0.026	NS	NS	0.03	NS	NS
IGF1*Day	5	0.01	NS	NS	NS	NS	NS
Insulin*Day	5	0.001	NS	NS	NS	NS	NS

[1] NS = Not significant, P > 0.15

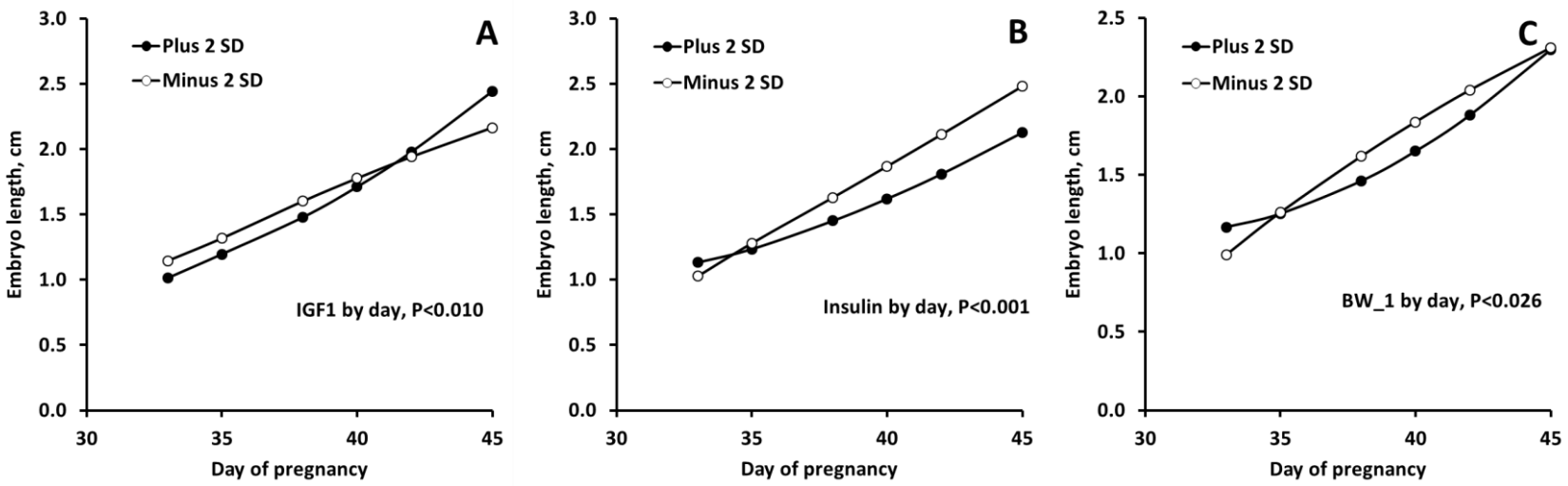

Figure 2.15 Embryo length (day 33-45 of gestation) for cows that were two standard deviations above or below the population mean IGF1 concentration (A) or were two standard deviations above or below the population mean insulin concentration (B), or were two standard deviations above or below the population mean BW at cytobrush examination (C).

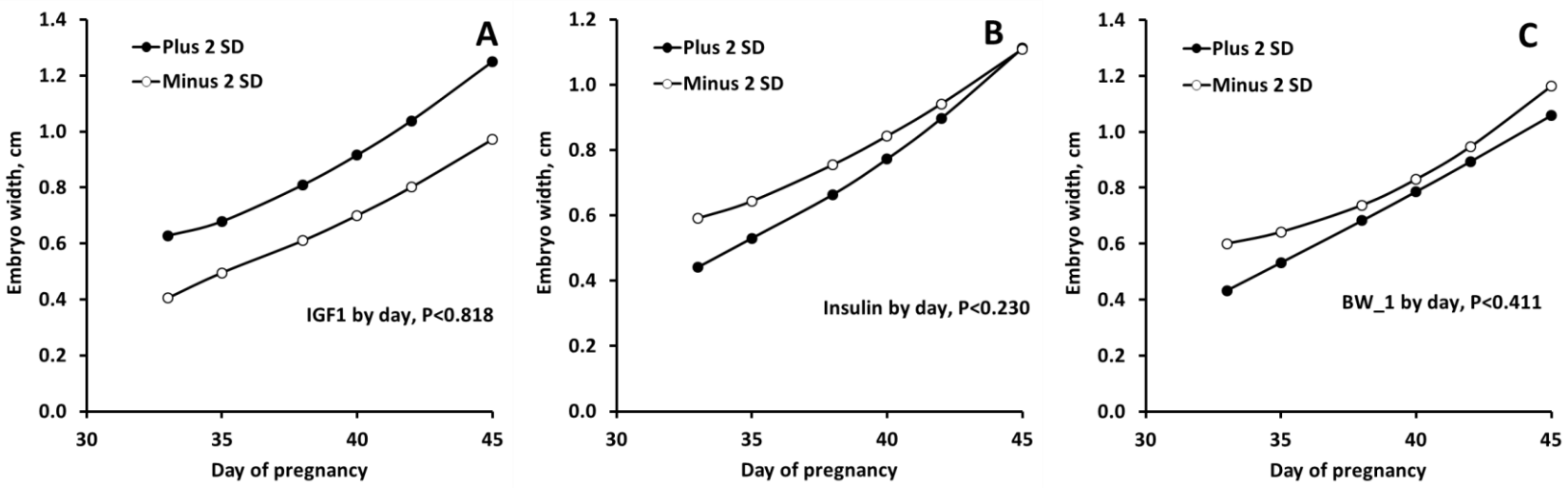

Figure 2.16 Embryo width (day 33-45 of gestation) for cows that were two standard deviations above or below the population mean IGF1 concentration (A) or were two standard deviations above or below the population mean insulin concentration (B), or were two standard deviations above or below the population mean BW at cytobrush examination (C).

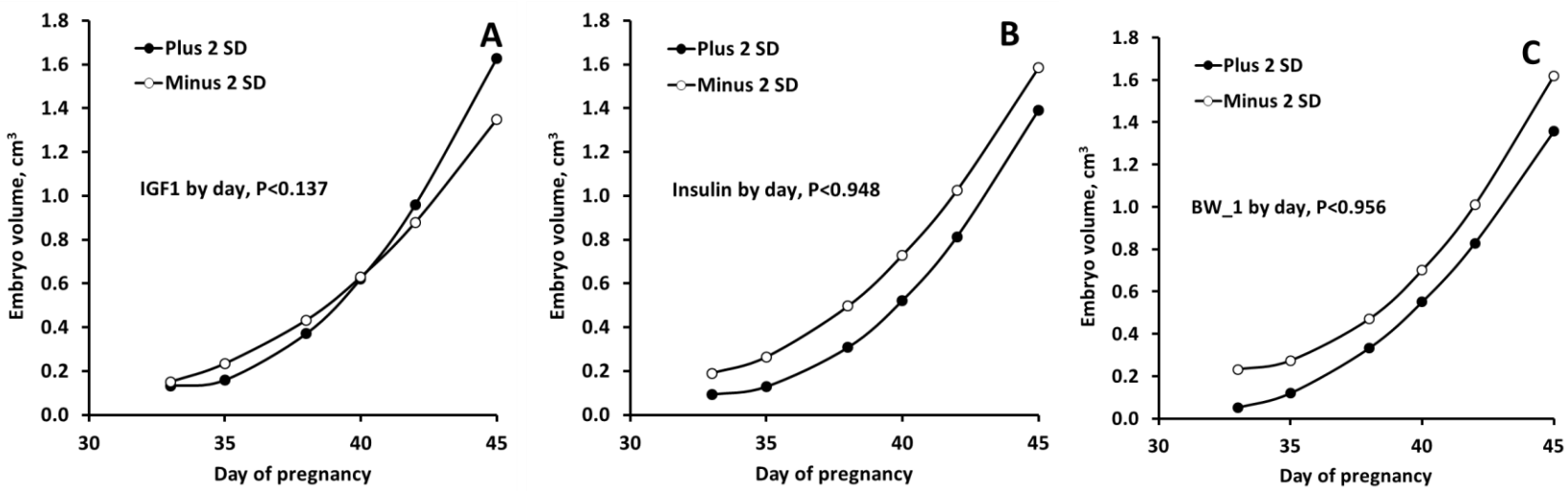

Figure 2.17 Embryo ellipse volume (day 33-45 of gestation) for cows that were two standard deviations above or below the population mean IGF1 concentration (A) or were two standard deviations above or below the population mean insulin concentration (B), or were two standard deviations above or below the population mean BW at cytobrush examination (C).

2.4. Discussion

We examined relationships between hormones, metabolites, and common production parameters and the growth of the conceptus (embryo and amniotic vesicle). Surprisingly, we were unable to identify a relationship with most of the cow level measures that were included in the statistical model (Table 3.1). The only consistent effect that we observed was month of the ultrasound exam, breed of cow, a modest effect of circulating insulin and IGF1 concentrations, and BW_1. The cows that were examined in January had embryos that grew more slowly initially (Figures 2.9, 2.10, and 2.11). The embryo growth rates caught up later (after day 45 of gestation). Guernsey cows had smaller embryos than Holstein cows. IGF1 appeared to be associated with a larger embryo but low insulin and lower BW_1 were associated with a larger embryo.

The measurements that were made on each cow were selected to characterize the metabolic state of the animal. For example, we examined BCS and milk production at two times as well as changes across time. We also selected hormones (GH, IGF1, and insulin) and metabolites (glucose and NEFA) known to be important to metabolism. Progesterone which is typically associated with embryo growth (Wiltbank et al., 2014) was tested. With the exception of insulin, IGF1, and BW_1, none of the measures were found to be associated with how the embryo grew over time during the period that we studied (d 33 to 45 of gestation).

Perhaps some of these results should not have been unexpected. In their extensive review of embryonic loss, Santos et al. (2004) was unable to find evidence for a relationship between milk production and embryo loss. Our data would indicate that milk production does not affect how fast the embryo grows and, therefore, may not affect

embryo loss. Santos et al. (2004) did report effects of parity and BCS at AI on embryonic loss but we did not find a large effect of these factors. Our hypothesis was that cow-level factors could affect how fast the embryo grows and, therefore, impact the potential for embryonic loss. It is very possible that the growth of the embryo and the potential for embryonic loss are not related.

As stated above, month had a large effect on the growth of the embryo. January cows had slower growing embryos. January cows were also almost exclusively pregnant to first insemination. Thus, there was clearly some confounding in the data. Both insemination number and month were included in the model so presumably month was the overriding effect. We are unsure why the embryos were slower growing in January. Perhaps photoperiod or ambient temperature can interact with the growth of the embryo. We are aware of data that suggests an effect of ambient temperature but this is associated with heat stress rather than cold stress. Heat stressed cows calve lower body weight calves, perhaps because blood is shunted to the periphery (vasodilation) (Collier et al., 1982). We are unaware of photoperiod effects on growth of the conceptus. When we examined our hormone and metabolite data, month was not significant. There was nothing obvious that differed for cows pregnant in January compared with other cows.

In addition to month, we also found a consistent effect of breed (Guernsey cows had smaller embryos compared to Holstein cows). We are not aware of additional data in the literature showing differences in breeds during this period of gestation.

There were effects of metabolic hormones. For example, IGF1 appeared to improve embryo growth particularly late (around day 45 of gestation). Both insulin and BW_1 appeared to be inhibitory toward the growth of the embryo. In general, greater

IGF1 is associated with improved health and energy balance (Wathes, 2012). So, this could suggest a faster growing embryo in cows with greater IGF1. We typically think of an association between IGF1 and insulin (positive, more insulin equals more IGF1) so it was surprising that the cows with greater insulin had smaller embryos. Perhaps this means that there is a threshold above which insulin redirects nutrients away from the pregnancy and toward the liver and muscle for glucose storage as glycogen. Indeed, pregnancy generally antagonizes insulin function evidenced by gestational diabetes where the placenta produces signals that block the release and action of insulin (Buchanan and Xiang, 2005). It may be that we are observing the antagonism between insulin and embryo growth in our study.

Embryonic loss is associated with uterine health. In general, cows with metritis or other uterine disease are likely to have embryonic loss (Santos et al., 2009; Wathes, 2012). We attempted to examine whether the growth of the embryo was greater or lesser in cows with significant %PMN before pregnancy. What was found was that cows that did not get pregnant had a greater %PMN compared with pregnant cows. The percentage, therefore, was associated with infertility. We did not find, however, that the %PMN in cows that became pregnant determined how fast the embryo grew. There was a threshold of %PMN for pregnancy but of the cows that became pregnant this measurement was not important to the growth of the embryo. We did note that one of the two cows that lost an embryo during the study (cow # 2704) had a Metricheck score of 3 and 1.5% PMN in the uterus. The cow did not conceive to the first insemination, but eventually lost the embryo after the second insemination.

Although we failed to find relationships with the measurements, we did discover unique features of embryonic growth. For example, different patterns were found for how embryos grew from days 33 to 45 of gestation (Figure 2.18). We found that some embryos grew at a near linear rate with little fluctuation in growth rate. Another subset of embryos, however, displayed slower initial growth before day 40 of gestation with rapid daily growth after day 40 of gestation. Interestingly, embryos growing in both pattern types were nearly the same size by day 45 of gestation. This timeline suggests that as placentome formation becomes established, some slower growing embryos undergo rapid growth to become similar to the linear growing embryos. Perhaps these two phases represent a period where the embryo is primarily dependent on histotrophic mechanisms and a period when the embryo is dependent on nutrients crossing through the placenta.

We examined two cows from this study which experienced embryonic loss during the period of 33 to 45 days of gestation. We found that embryo measurements were typically within two standard deviations of the average length, width, and volume found in literature in the days preceding embryonic death. After death the embryo measurements decreased and were subsequently smaller than the published average (Figure 2.19). It did not appear that a small embryo was responsible for embryonic loss.

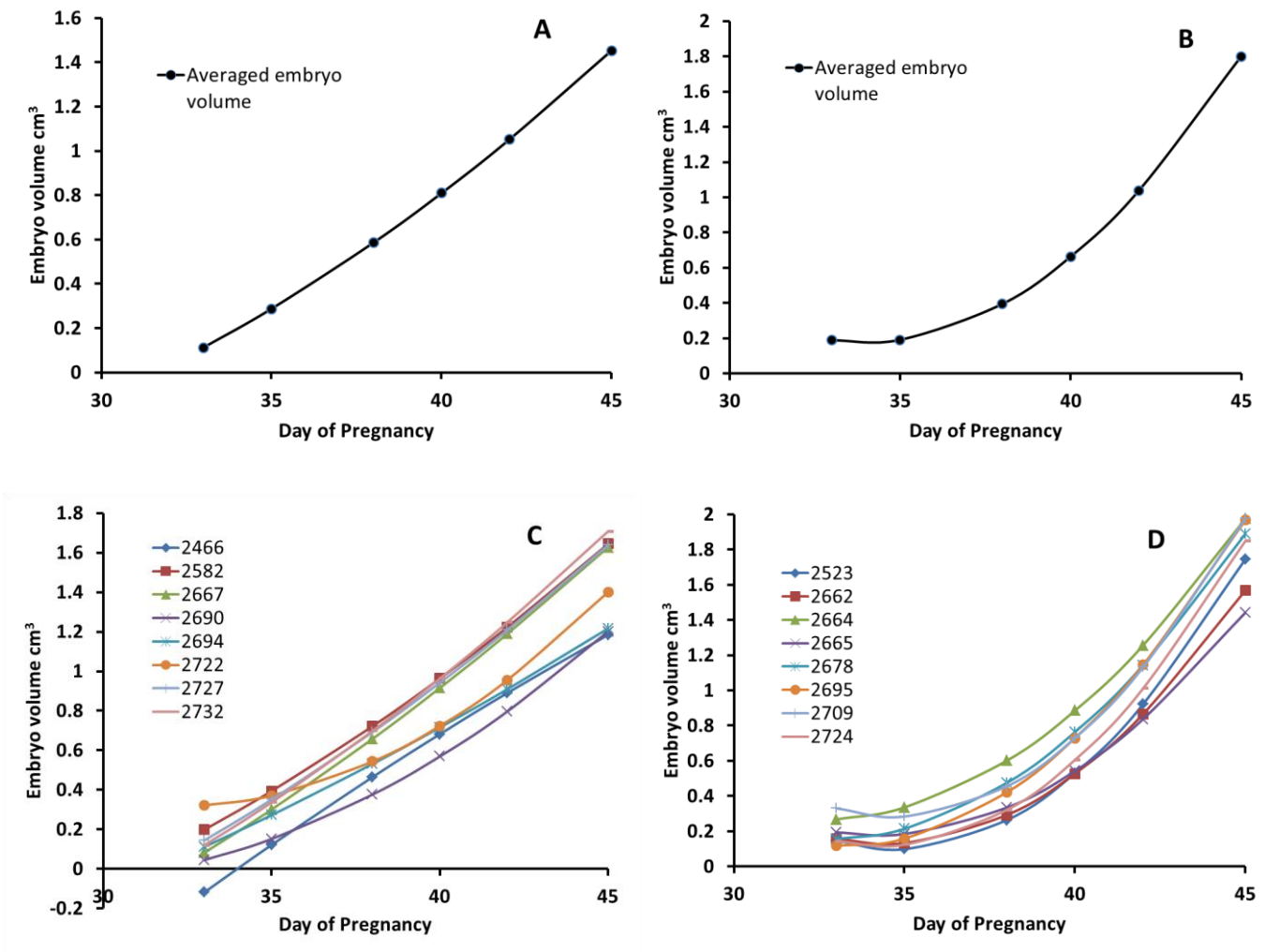

Figure 2.18 Different rates of growth embryonic growth between days 32 and 45 of gestation. Some embryos grow at a more linear rate (A) and some embryos experience a period of slower initial growth, but end at a similar size (B).

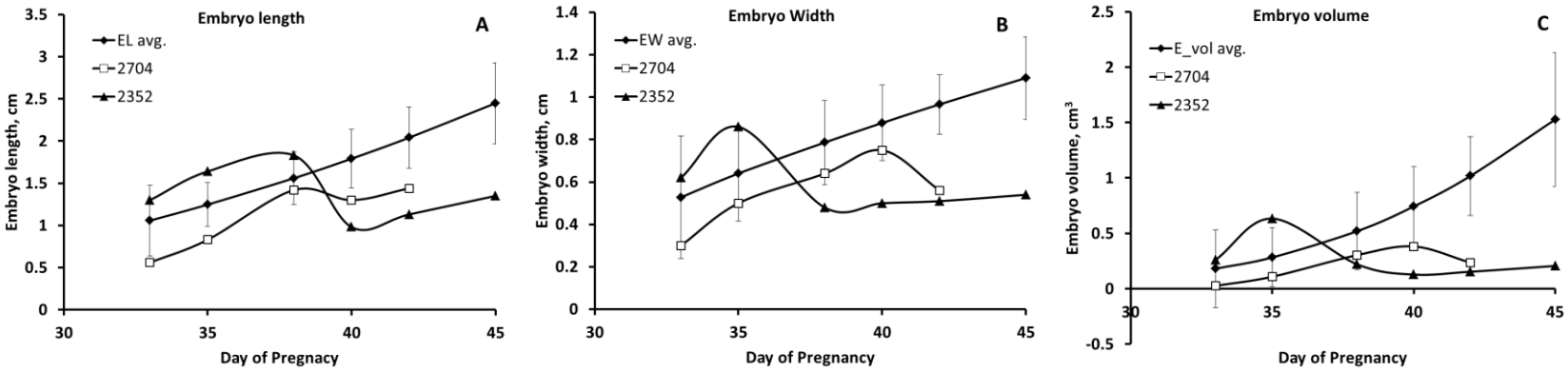

Figure 2.19 Average rate of growth (within two standard deviations) for embryo length (A), embryo width (B), and embryo ellipsoid volume (C) compared with two cows that underwent embryonic loss.

2.5. Conclusion

In conclusion, we examined a large number of cow level factors for their effects on growth of the embryo from day 33 to 45 of gestation. These cow-level factors were largely not significant. There were effects of month and breed and small effects of circulating IGF1 and insulin concentrations. One interpretation of these data is that the metabolism of the cow has a relatively small effect on the growth of the conceptus (both embryo and amniotic vesicle). There were clearly differences between cows in the manner in which embryos grew (Figure 2.18) but we do not know what controls development in faster compared with slower growing embryos.

Chapter III

3. EMBRYONIC GROWTH IN LACTATING COWS AND NON-LACTATING HEIFERS FROM DAY 33 TO 45 OF GESTATION

3.1. Introduction

In our first study, Chapter II, we found a relationship of breed, month, BW_1, insulin, and IGF1 on the development of the conceptus. We did not find that other indicators of the metabolism of the cow had a major effect on the growth of the conceptus. To follow up on these initial observations further, we designed a study to examine non-lactating heifers and lactating cows. These study groups were chosen because we could test potential effects of milk production in an extreme case (non-lactating virgin animal compared with lactating animal).

We also knew that a single measurement of glucose or insulin perhaps did not fully characterize the insulin status of the cow. To explore this possibility, we included a glucose tolerance test in this second year study. The objective was to measure growth of the conceptus in heifers compared with lactating cows and to perform a glucose tolerance test on the same animals under the same conditions. The hypothesis was that lactating cows and non-lactating heifers would differ in terms of growth of the conceptus (embryo and amniotic vesicle) and that indices of insulin sensitivity would differ and could be related to embryonic growth as well.

3.2. Materials and Methods

3.2.1. Estrus Synchronization and Pregnancy Diagnosis

Fifty lactating dairy cows (42 Holstein and 8 Guernsey) from the University of Missouri Foremost Research Center were used. Animals were enrolled in the PreSynch OvSynch56 protocol beginning approximately 37 days postpartum (Figure 3.1). The PreSynch protocol consisted of an injection of $PGF_{2\alpha}$ (5 mL Lutalyse; 25mg dinoprost tromethamine, IM; Zoetis Inc., Florham Park, NJ) with a second $PGF_{2\alpha}$ injection 14 d later. The OvSynch56 protocol began 14 d after PreSynch completion with an injection of GnRH (2 mL Factrel; 100 µg gonadorelin, IM; Zoetis Inc., Florham Park, NJ), followed 7 d later by a $PGF_{2\alpha}$ injection, followed 56 h later by a GnRH injection, and completed with timed artificial insemination 16 h after.

The non-lactating heifers (37 Holstein and 3 Guernsey) were also from the University of Missouri Foremost Research Center. Heifers were moved to the breeding lot when they reached a specific target body weight. Heifers were injected with $PGF_{2\alpha}$ (5 mL Lutalyse; 25mg dinoprost tromethamine, IM; Zoetis Inc., Florham Park, NJ) when they moved to the breeding lot. Estrus detection was performed for 7 days following injection using estrus detection aids (Estrotect patches; MAI Animal Health, Elmwood, WI) and AI performed 12 hours after detection of estrus. A second $PGF_{2\alpha}$ injection was administered to any heifers not in estrus by 7 days.

Cows and heifers were examined by using transrectal ultrasonography 32 d after AI for pregnancy diagnosis. Pregnancy was defined as the presence of an embryo with a visible heartbeat. Cows that were diagnosed as non-pregnant at examination were re-enrolled in the OvSynch56 protocol on the same day as diagnosis. Heifers were re-inseminated at return to estrus. Animals that failed to become pregnant to first or second AI were removed from the study (Figure 3.1).

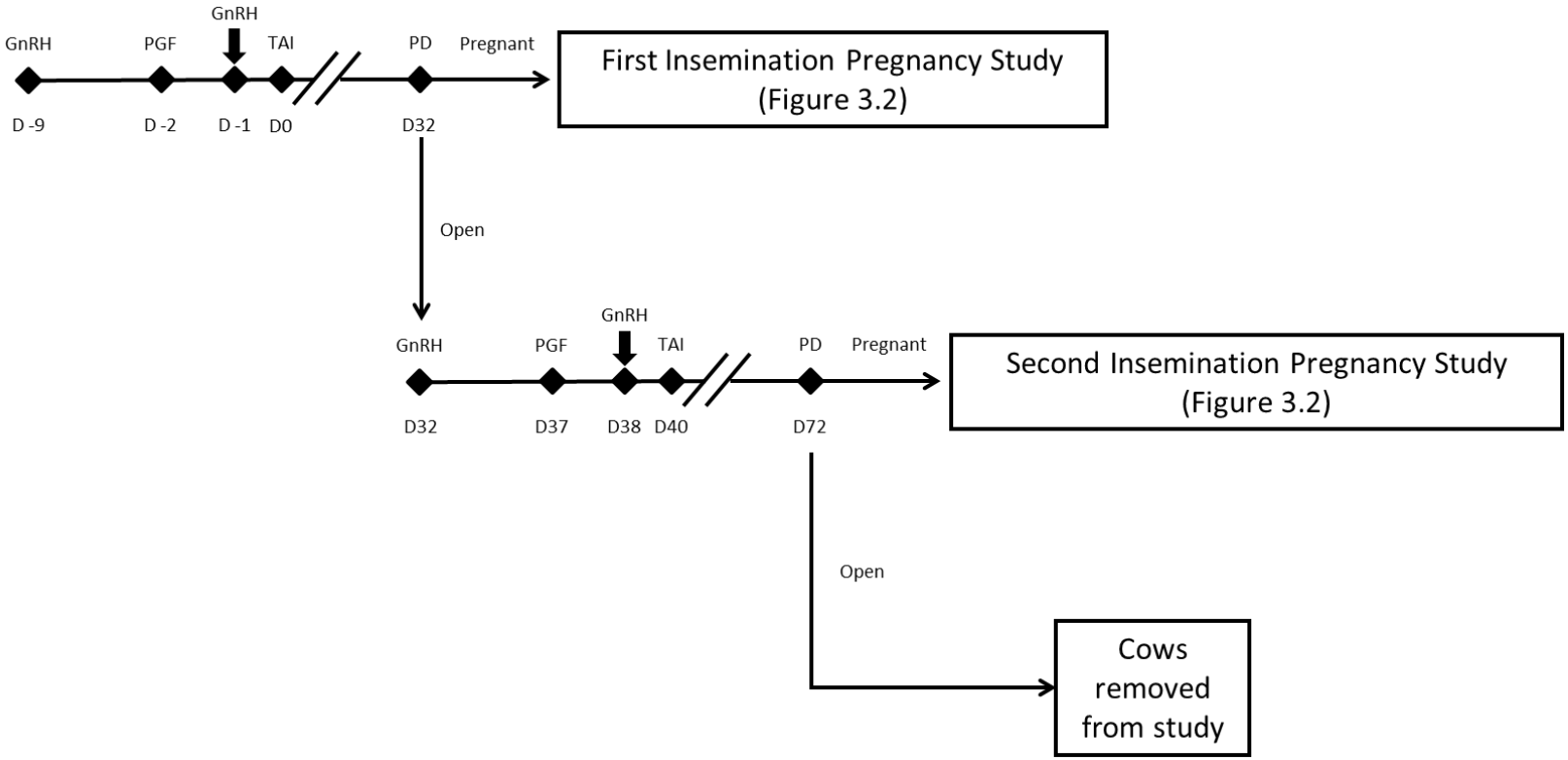

Figure 3.1 Protocols for OvSynch synchronization for cows which became pregnant to first insemination, second insemination, or were removed from the study for failure to become pregnant.

3.2.2. Ultrasonographic Evaluation of Embryo Growth

Pregnancy diagnosis was performed by a veterinarian using a Sonosite Edge equipped with a variable MHz linear probe (SonoSite Inc., Bothell, WA) 32 d after AI. The location of the embryo (right or left horn) was recorded. All cows (n=50; 115 ± 15 DIM) and heifers (n=40) pregnant with a single conceptus were examined by transrectal ultrasonography on d 33, 35, 38, 40, 42, and 45 of pregnancy using an Aloka 900 ultrasound with a 7.5 MHz transducer (Hitachi Aloka Medical Ltd., Wallingford, CT) (Figure 3.2). Cows pregnant with twins were not included because they were too few in number (n=4). Length (l) and width (w) of the embryo and amniotic vesicle were measured. The volume for the embryo (E_vol) and amniotic vesicle (A_vol) was calculated as an ellipsoid [volume= $4/3*\pi*(0.5*l)*(0.5*w)*(0.5*w)$]. Fetal sex was determined via transrectal ultrasonography between days 60 and 80 of gestation and confirmed at birth.

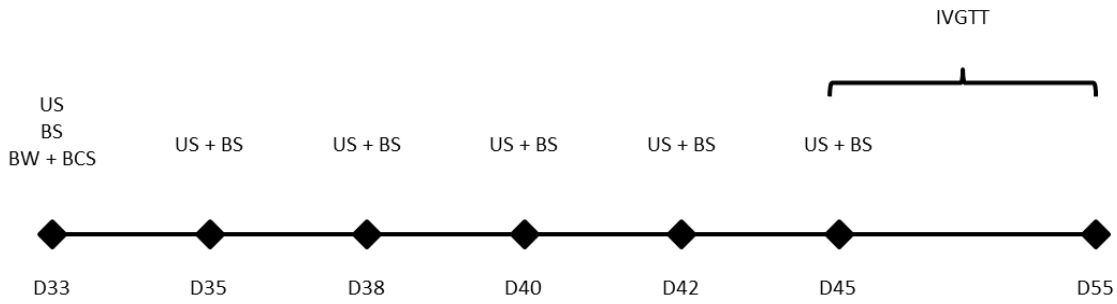

Figure 3.2 Protocol for blood sample collection and ultrasound examination of the embryo and amniotic vesicle on days 33, 35, 38, 40, 42, and 45 of gestation for cows and heifers that became pregnant to first or second insemination. An intravenous glucose tolerance test (IVGTT) was performed after the ultrasound exams were completed. BW = body weight measurement, BCS = body condition score measurement.

3.2.3. Blood Collection and Hormone/Metabolite Analysis

Immediately before each ultrasound exam, 10 mL of whole blood was collected from either the coccygeal vein or artery into a tube containing EDTA (10 mL Monoject 16mm x 100mm blood collection tubes, Covidien, Minneapolis, MN) and immediately placed on ice for transport back to the laboratory. Centrifugation of whole blood (1500 x *g* for 15 min) was performed to separate plasma. Plasma was removed and stored at -20°C in polypropylene tubes until hormone and metabolite analysis.

Plasma glucose concentrations were determined enzymatically with the glucose oxidase method (Pointe Scientific Inc., Canton, MI). A standard curve was created using a glucose standard. The standard curve points were 0, 25, 50, 100, 200, and 400 mg/dL. Absorbance was quantified using an ELx808 absorbance reader at 500 nm (BioTek Instruments Inc., Winooski, VT). A predictive equation based on linear regression was created from the standard curve. The equation was used to estimate the glucose concentrations in the samples.

Plasma insulin concentrations were analyzed for samples corresponding to time points 0, 12, and 40 minutes from the intravenous glucose tolerance test. Sample concentrations were determined by bovine insulin ELISA assay (Mercodia AB, Uppsala, Sweden). A standard curve was created using an insulin standard. The standard curve points were 0, 0.05, 0.15, 0.5, 1.5, and 3 ng/mL. Samples above the standard curve were diluted with zero calibrator and re-ran.

3.2.4. Body Condition Scoring and Body Weight

All cows were scored for BCS [1 (thin) to 5 (obese)] at d 33 of gestation by two technicians and scores were averaged. Body weight was also determined at d 33 of gestation and again at IVGTT using an electronic livestock scale (Tru-Test Inc., Mineral Wells, TX). The average BCS was included in the statistical analyses as BCS_2 to be consistent with the terms in Chapter II. The first BW and second BW were analyzed and included in the statistical analyses as BW_2 to be consistent with the terms from Chapter II.

3.2.5. Intravenous Glucose Tolerance Test

Fifty-nine lactating dairy cows (50 Holsteins and 9 Guernseys) and 40 non-lactating dairy heifers (37 Holstein and 3 Guernsey) between 45-55 days of gestation were used. Animals were moved from free-stall or lot housing to tie-stall housing at approximately 0600 h and feed was withheld until the end of the IVGTT. The right or left jugular vein was catheterized using a 14g x 133mm catheter (MILCATH, Mila International Inc., Erlanger, KY). Catheters were sutured and super-glued to the skin to insure retention. The entire apparatus was flushed with 12 mL heparinized saline (10 units/mL) and capped (INT Stopper, Braun Medical Inc., Bethlehem, PA). The animals were allowed to acclimate to the tie-stalls until approximately 1630 h, when they were milked and extension sets were attached to prepare for IVGTT. The catheter was fitted with a large bore T-port extension set (15 cm, 0.6 mL priming volume; Braun Medical Inc., Bethlehem, PA), to facilitate rapid dextrose infusion, with a small bore extension set (91 cm, 1.2 mL priming volume; ICU Medical Inc., San Clementine, CA) attached to the T-port to facilitate blood collection. The entire apparatus was again primed with 12 mL

heparinized saline (10 units/mL). Three mL of blood was collected through the apparatus and discarded before 20 mL of blood was collected into two tubes containing EDTA (10 mL Monoject 16mm x 100mm blood collection tubes, Covidien, Minneapolis, MN) at time points of -2, 0, 2, 4, 6, 8, 10, 12, 15, 18, 20, 23, 26, 30, 35, 40, 50, 60, and 90 minutes for all animals. Immediately following blood collection, the apparatus was flushed with 5 mL of heparinized saline (10 units/mL). Dextrose (VetOne Dextrose 50% injection, MWI Veterinary Supply, Boise, ID) was administered via large bore extension set immediately following blood collection at time point 0 min, at a dosage of 300 mg/kg, with infusion completed in < 2 minutes. Immediately following dextrose administration the entire collection apparatus was flushed with 10 mL heparinized saline (10 units/mL). Blood tubes were immediately transferred to 4°C and allowed to sit for approximately 30 minutes before centrifugation of whole blood (1,500 x *g* for 15 min) was performed to separate plasma. Plasma was removed and stored at -20°C in polypropylene tubes until hormone and metabolite analysis.

3.2.6. Milk production

Milk production records were obtained from daily parlor milk weight records. Milk_2 was the period around the ultrasound examinations. The daily milk weights during the series of ultrasound examinations were averaged for milk_2.

3.2.7. Parity

For the purposes of statistical analyses, parity ≥2 was defined as second parity and first parity cows were analyzed as first parity.

3.2.8. Statistical Analyses- Embryo measurement using ultrasound

The approach to the statistical analysis was the same manner as Chapter II. The primary difference being that we did not include as many cow level effects as previous. A list of tested effects is presented for both heifers (Table 3.1) and cows (Table 3.2). A description of the tested effects is presented in Table 3.3. Polynomial regressions for individual cows were fitted and regression coefficients were outputted for EL, EW, E_vol, AL, AW, and A_vol. The regression coefficients were analyzed for cow-level factors. The cow-level factors included data from the glucose tolerance test (glucose and insulin concentrations from 0, 12, and 40 minutes).

In a second series of analyses, predicated values for each day were outputted and analyzed with a repeated measures approach. Cow-level factors including data from the glucose tolerance test were included in the model. In each case, the initial analyses were done with a GLMSELECT procedure and the second set of analyses were done with PROC MIXED (repeated measures model). The PROC MIXED employed a reduced model that was based on the results of the GLMSELECT procedure.

In addition to the analyses of the conceptus, we performed an analysis of the complete glucose profile during the glucose tolerance test. We examined the effects of breed (Holstein or Guernsey), type (cow or heifer), time, and interactions. Time was the repeated variable and animal (type*breed) was fitted as random.

Table 3.1 Complete list of effects analyzed for influence on embryonic and amniotic vesicle size and type of analysis performed for heifers.

Effect	N	Mean	Std Dev	Min.	Max.
Continuous					
Glucose, mg/dL	40	75.62	4.16	68.92	83.74
Gluc_0, mg/dL	38	87.85	9.39	71.84	124.45
Gluc_12, mg/dL	38	236.91	20.77	183.65	281.96
Gluc_40, mg/dL	38	148.37	23.63	99.40	211.95
GDiff_12, mg/dL	38	149.06	18.56	103.18	188.88
GDiff_40, mg/dL	38	60.52	20.29	15.13	101.06
GDiff_12_40, mg/dL	38	176.39	21.24	132.92	238.38
Ins_0, ng/mL	38	0.53	0.28	0.03	1.64
Ins_12, ng/mL	38	6.04	1.87	2.19	10.14
Ins_40, ng/mL	38	3.19	1.19	1.05	5.51
Ins_diff_12, ng/mL	38	5.50	1.82	2.16	9.76
Ins_diff_40, ng/mL	38	2.66	1.04	0.91	4.73
Ins_diff_12_40, ng/mL	38	2.85	1.66	-0.81	6.91
BCS_2	40	3.69	0.26	3.25	4.38
BW_2, kg	40	432.16	40.06	342.27	543.64
Age, months	40	15.15	1.27	12.00	18.00
Discrete					
Breed	40	-	-	-	-
Sire	40	-	-	-	-
Month at ultrasound	40	-	-	1	3

Insemination Number	40	-	-	-	-
Sex	40	-	-	-	-
Horn	40	-	-	-	-

Table 3.2 Complete list of effects analyzed for influence on embryonic and amniotic vesicle size and type of analysis performed for cows.

Effect	N	Mean	Std Dev	Min.	Max.
Continuous					
Glucose, mg/dL	50	62.97	5.04	46.48	72.17
Gluc_0, mg/dL	46	70.76	9.28	49.41	96.81
Gluc_12, mg/dL	46	225.51	26.45	163.88	278.31
Gluc_40, mg/dL	46	139.90	23.39	84.94	187.35
GDiff_12, mg/dL	46	154.76	23.05	111.45	207.24
GDiff_40, mg/dL	46	69.15	20.47	27.91	117.45
GDiff_12_40, mg/dL	46	156.37	20.56	119.56	222.70
Ins_0, ng/mL	46	0.19	0.08	0.07	0.43
Ins_12, ng/mL	46	1.86	1.38	0.55	9.47
Ins_40, ng/mL	46	0.88	0.50	0.35	2.64
Ins_diff_12, ng/mL	46	1.67	1.37	0.44	9.36
Ins_diff_40, ng/mL	46	0.69	0.48	0.20	2.50
Ins_diff_12_40, ng/mL	46	0.98	1.03	0.02	6.86
BCS_2	50	3.25	0.25	2.63	3.88
BW_2, kg	50	624.97	79.84	392.27	836.59
Milk_2, kg/day	50	82.38	19.73	33.66	120.13
DIM at day 32	50	114.56	14.94	100.00	153.00
Discrete					
Breed	50	-	-	-	-
Sire	50	-	-	-	-
Parity	50	-	-	-	-

Month at ultrasound	50	-	-	1	3
Insemination Number	50	-	-	-	-
Sex	50	-	-	-	-
Horn	50	-	-	-	-

Table 3.3 Explanation of independent variables that were used in the analysis of conceptus growth

Continuous variables – hormone and metabolites	
Plasma glucose	Averaged over the six time collection days (d 33, 35, 38, 40, 42, and 45).
Continuous Variables-glucose tolerance test	
Gluc_0	Glucose concentration at time 0 minutes of glucose tolerance test
Gluc_12	Glucose concentration at time 12 minutes of glucose tolerance test
Gluc_40	Glucose concentration at time 40 minutes of glucose tolerance test
Gluc_diff_12	Gluc_12 minus Gluc_0
Gluc_diff_40	Gluc_40 minus Gluc_0
Gluc_diff_12_40	Gluc_12 minus Gluc_40
Ins_0	Insulin concentration at time 0 minutes of glucose tolerance test
Ins_12	Insulin concentration at time 12 minutes of glucose tolerance test
Ins_40	Insulin concentration at time 40 minutes of glucose tolerance test
Ins_diff_12	Ins_12 minus Ins_0
Ins_diff_40	Ins_40 minus Ins_0
Ins_diff_12_40	Ins_12 minus Ins_40
Continuous variables – production	
Milk_2 (cows only)	Daily milk production at the time of the ultrasound exams. Average of month DHIA test data before and after ultrasound exams.
BCS_2	Body condition score at the time of ultrasound exam (average of two different

	evaluators).
BW_2	Body weight at the time of ultrasound exam.
Continuous – other	
DIM (cows only)	Days in milk on d 32 of pregnancy
Age (heifers only)	Age in months
Discrete	
Insemination number	Pregnancies were from either a first or second insemination postpartum
Sire	NAAB registration number of bull used at each insemination
Month	Month (January, February, March, or April) during which the ultrasound exam was conducted
Parity (cows only)	Defined as either first or ≥2 parity
Location	Pregnancy detected in the right or left horn
Breed	Holstein or Guernsey
Sex	Sex of calf (male or female)

3.3. Results

3.3.1. Glucose tolerance test results

Glucose concentrations across the entire time are shown (Figure 3.3). There were significant effects of time ($P < 0.001$), breed*time ($P < 0.002$), and type*time ($P < 0.012$). There was also a highly significant breed*time*type interaction ($P < 0.001$). The three way interaction appeared to be explained by a relatively low glucose response in the Guernsey cows (Figure 3.3). Other categories appeared similar in terms of glucose clearance.

The results of the glucose tolerance test were analyzed at time points 0, 12, and 40 minutes when insulin was measured (Table 3.4). There were clearly effects of type and breed*type throughout. At time 0, heifers had more glucose than cows and Holsteins had more glucose than Guernseys. Insulin at time 0 followed the same trend for heifers compared with cows but insulin was less in Guernseys. After glucose infusion, Holstein cows and heifers and Guernsey heifers were largely similar for glucose but Guernsey cows were less. Insulin release was greater in heifers and also greater in Guernsey cows compared with Holstein cows at both 12 minutes and 40 minutes. When changes in insulin or glucose were calculated for 12 minutes to 40 minutes, there were effects of type for both insulin (Ins_diff_12_40; $P < 0.012$) and glucose (Gluc_diff_12_40; $P < 0.001$). The drop in glucose was greater in heifers compared with cows. The decrease in plasma insulin was also greater in heifers compared with cows.

3.3.2. Embryo and amnion measurements using ultrasound

The parameters (b_0, b_1, and b_2) for the fitted curves for EL, EW, E_vol, AL, AW, and A_vol were analyzed for all of the effects listed in Tables 3.1 and 3.2. A GLMSELECT procedure was used. The results from the GLMSELECT are shown in Tables 3.6 (heifers) and 3.7 (cows). Very few of the analyzed variables had any effect on the parameters that defined the individual curves for each animal. Significant effects of month (EL, EW, E_vol, AL, and A_vol), age (EL), and BW_2 (AL) were detected for heifers (Table 3.6). No other effects were significant for measures of the embryo. With respect to the amniotic vesicle, there were additional effects of sire, ins_0, and ins_diff_12_40 but these were relatively weak ($P < 0.05$). For cows, the ins_0 affected EL, E_vol, and AL. Other effects on the cow conceptus were associated with parity, sex, gluc_diff_12, and BCS_2. In general these effects were weakly significant ($P < 0.05$).

In a second round of statistical analyses, the effects were analyzed using GLMSELECT with a repeated measures model. The individual data points for EL, EW, E_vol, AL, AW, and A_vol were fitted and a predicted value for each day was outputted. These outputted data were analyzed for all effects listed in Tables 3.1 and 3.2. The results are shown in Table 3.8 (heifers) and 3.9 (cows). Again, very few of the analyzed variables were significant.

For heifers (Table 3.8), there were effects of "cow" (meaning heifer) and day on most measures of the conceptus. The significant effect of cow indicates that heifers differed for growth of the conceptus individually. Day indicates that the conceptus grew over time. Day*month was also significant which agreed with the analysis of coefficients. There were additional significant effects but these were generally $P < 0.05$ and did not achieve a high level of significance.

For cows (Table 3.9), there were again the expected effects of cow and day. There were also effects of ins_0*day which agrees with the findings of the coefficient analysis. Another effect that was highly significant was insemination number. There were some effects of the glucose tolerance test, but these were generally not highly significant ($P < 0.05$).

3.3.3. PROC MIXED conceptus measurements

The GLMSELECT analyses for this chapter suggested effects of day, month, and breed. Additionally, we tested for the effects of type (cow or heifer). These effects were followed up by using a repeated measures analysis in PROC MIXED. In these analyses cow was nested in breed*type*month and was fitted as random. Day was a repeated discrete variable. The results of the model are shown in Table 3.10. The effects of month, day, breed, and type or their interactions with day were often significant.

In a first series of graphs EL, EW, E_vol, AL, AW, and A_vol for heifer versus cows, Holstein versus Guernsey, and January, February, and March to May are shown (Figures 3.7 to 3.9). For the embryo, the general pattern that was observed was for a smaller embryo in Guernsey cows and a smaller embryo in cows that were pregnant in January. Heifers did appear to have larger embryos at d 33 of gestation, but then became smaller than cows as pregnancy progressed. For the amniotic vesicle (Figures 3.10 to 3.12), the data were generally not significant but followed a similar trend when compared with the graphs depicting growth of the embryo.

Final models were constructed for heifers and cows separately. For heifers the model included effects of month, day, and ins_0 (Table 3.11). A similar model was fitted for cows (Table 3.12) except insemination number in place of month was fitted.

For heifers, the effects of day and month*day were highly significant for EL, EW, and E_vol (Table 3.11). The effect of ins_0 was only significant for EW. For cows, the effects of day and insemination number by day were highly significant (Table 3.12). Ins_0 by day was significant for EL and E_vol.

Interactions with continuous variables were plotted. In this analysis the slope of the effect at each individual day was calculated from parameter estimates given by PROC MIXED (solution included in the model statement). Once the slope was calculated then it was multiplied by ±2 standard deviations to calculate a deviation in embryo or amniotic vesicle size on each day. The deviated values (plus two SD and minus two SD) represent 95% of the population on each side of the average.

The data for EL, EW, and E_vol in heifers are shown in Figures 3.10 to 3.12, and the cow data are shown in Figures 3.13 to 3.15. Data were not plotted for amniotic vesicle because they were generally not significant. The analysis of embryonic growth in heifers generally showed large statistically significant effects of month. These can be seen in the plots of the individual month (Figures 3.10 to 3.12, B). The interaction of month appears to be caused by similar sizes on d 33 and 45 with smaller sizes for January embryos on d 38 and 40. Insulin interactions with day were generally not significant for heifers. There was a significant effect of insulin on heifer EW. For EW, greater insulin was associated with a wider embryo (greater EW; Figure 3.14). This difference was detected early (before d 40).

The analyses of embryo growth in cows demonstrated an effect of insemination number of EL and E_vol (insem*day P <0.01 for each). This interaction appeared to be caused by larger embryos for second insemination cows after d 40. There were effects of ins_0 on the embryo as well (significant for EL and E_vol). For ins_0, cows at plus two standard deviations had larger embryos; particularly at d 45.

Table 3.4 Results from a fasted 90 minute intravenous glucose tolerance test using Guernsey and Holstein animals consisting of lactating cows and non-lactating heifers. See Table 3.2 for definitions.

	Guernsey				Holstein						
	Cow (n=8)		Heifer (n=3)		Cow (n=42)		Heifer (n=37)		P-Value		
	lsmean	SEM	lsmean	SEM	lsmean	SEM	lsmean	SEM	Breed	Type	Breed*Type
Glucose	65.68	1.59	81.37	2.60	62.45	0.69	75.16	0.74	0.004	0.001	NS
Gluc_0	61.63	3.39	84.75	5.17	72.39	1.43	88.11	1.51	0.033	0.001	NS
Gluc_12	192.32	8.27	239.64	12.64	231.47	3.50	236.68	3.70	0.026	0.002	0.001
Gluc_40	108.72	8.16	149.21	12.46	145.50	3.46	148.30	3.65	0.025	0.007	0.019
Gdiff_12	130.69	7.53	154.89	11.50	159.08	3.19	148.57	3.37	NS	NS	0.019
Gdiff_40	47.08	7.32	64.46	11.18	73.11	3.10	60.18	3.27	NS	NS	0.035
Gdiff_1240	145.24	7.87	175.18	12.02	158.36	3.33	176.49	3.52	NS	0.002	NS
Ins_0	0.11	0.07	0.21	0.11	0.20	0.03	0.56	0.03	0.002	0.001	0.060
Ins_12	2.76	0.60	4.54	0.91	1.70	0.25	6.17	0.27	NS	0.001	0.022
Ins_40	1.09	0.33	2.43	0.51	0.84	0.14	3.25	0.15	NS	0.001	0.096
Idiff_12	2.64	0.59	4.33	0.90	1.49	0.25	5.61	0.26	NS	0.001	0.036

Idiff_40	0.98	0.30	2.22	0.45	0.64	0.13	2.69	0.13	NS	0.001	NS
Idiff_1240	1.66	0.51	2.10	0.77	0.86	0.21	2.91	0.23	NS	0.012	NS
BCS_2	3.31	0.09	3.50	0.15	3.24	0.04	3.70	0.04	NS	0.001	NS
BW_2	555.17	21.48	373.64	35.08	638.26	9.37	436.90	9.99	0.001	0.001	NS

NS = Not significant, P > 0.15

Table 3.5 Results of the mixed model analysis of glucose concentration during intravenous glucose tolerance test for Guernsey and Holstein cows and heifers.

Effect	DF	P-value
Breed	1	0.0033
Type	1	0.0001
Breed*Type	1	0.004
Time	18	<.0001
Breed*Time	18	0.0021
Type*Time	18	0.0118
Breed*Type*Time	18	<.0001

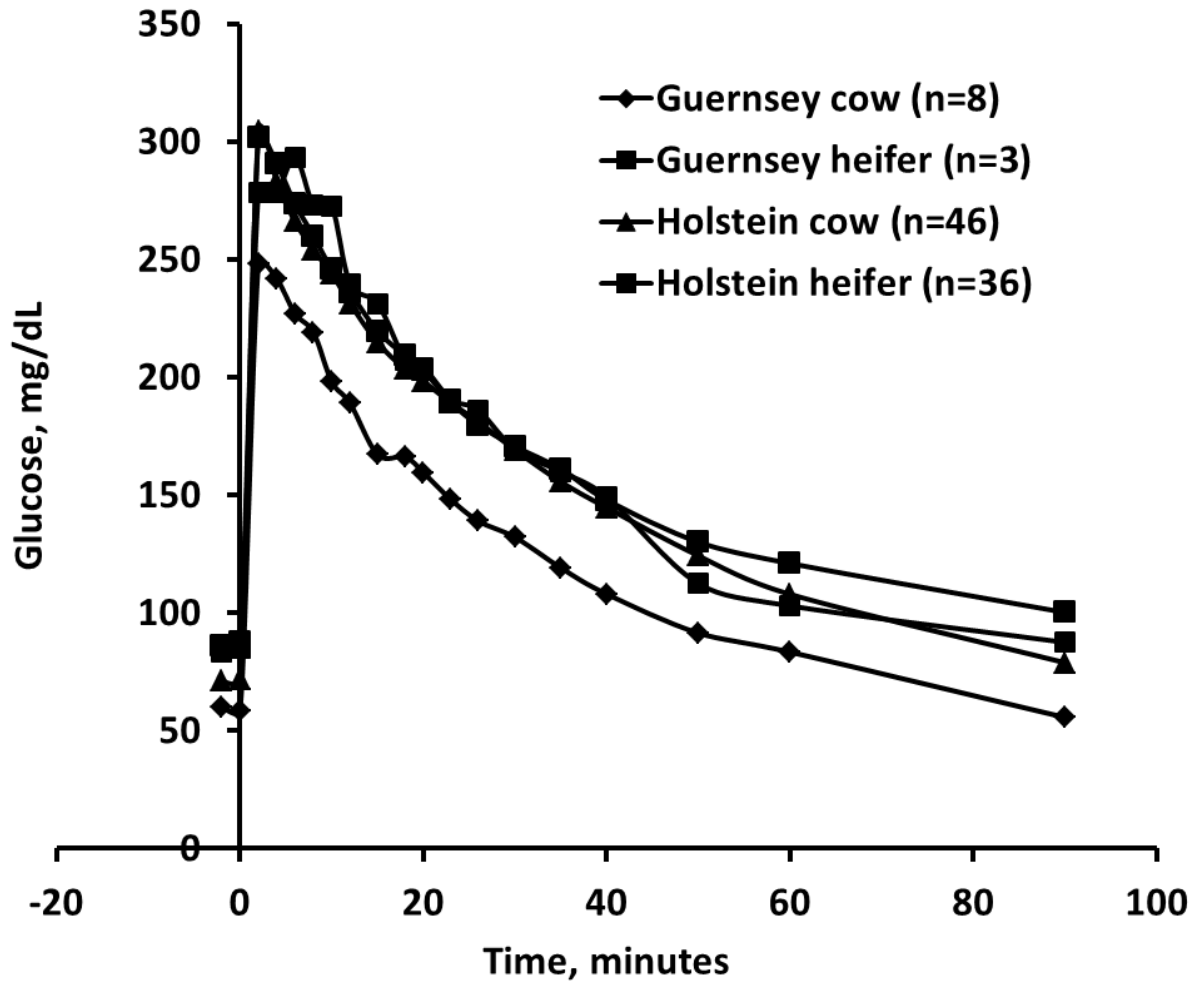

Figure 3.3 Graph of glucose concentrations during course of intravenous glucose tolerance test for Guernsey and Holstein cows and heifers.

Table 3.6 Results of the GLMSELECT procedure where the most appropriate model for coefficients was selected by a stepwise procedure for heifers.

Curve	Parameter	Significant model effects at P <0.05
EL	b_0	Month (P <0.001), Age (P <0.014)
	b_1	Month (P <0.001), Age (P <0.011)
	b_2	Month (P <0.001), Age (P <0.009)
EW	b_0	Month (P <0.004)
	b_1	Month (P <0.003)
	b_2	Month (P <0.002)
E_vol	b_0	Month (P <0.001)
	b_1	Month (P <0.001)
	b_2	Month (P <0.001)
AL	b_0	Month (P <0.003), Sire (P <0.047), BW_2 (P <0.008), Ins_0 (P <0.025)
	b_1	Month (P <0.013), Sire (P <0.050), BW_2 (P <0.010), Ins_0 (P <0.024)
	b_2	Month (P <0.004), BW_2 (P <0.013), Ins_0 (P <0.025)
AW	b_0	Ins_diff12_40 (P <0.033)
	b_1	Ins_diff12_40 (P <0.028)
	b_2	Ins_diff12_40 (P <0.024)
A_vol	b_0	Month (P <0.011), Ins_diff12_40 (P <0.051)
	b_1	Month (P <0.001), Ins_diff12_40 (P <0.048)
	b_2	Month (P <0.013), Ins_diff12_40 (P <0.046)

Table 3.7 Results of the GLMSELECT procedure where the most appropriate model for coefficients was selected by a stepwise procedure for cows.

Curve	Parameter	Significant model effects at P <0.05
EL	b_0	Ins_0 (P <0.005)
	b_1	Ins_0 (P <0.006)
	b_2	Ins_0 (P <0.006)
EW	b_0	None
	b_1	None
	b_2	None
E_vol	b_0	Ins_0 (P <0.040)
	b_1	Ins_0 (P <0.038)
	b_2	Ins_0 (P <0.036)
AL	b_0	Ins_0 (P <0.015), Sex (P <0.039)
	b_1	Gluc_diff12 (P <0.039), Ins_0 (P <0.017), Parity (P <0.047)
	b_2	Gluc_diff12 (P <0.037), Ins_0 (P <0.020), Parity (P <0.044)
AW	b_0	None
	b_1	BCS_2 (P <0.050)
	b_2	BCS_2 (P <0.049)
A_vol	b_0	None
	b_1	None
	b_2	None

Table 3.8 Output of GLMSELECT with repeated measures for heifers

Dependent Variable	Significant model effects at P <0.05
EL	Cow (P <0.001), Day (P <0.001), Month*Day (P <0.001), Age*Day (P <0.001), Ins_0*Day (P <0.017), Ins_40*Day (P <0.049)
EW	Cow (P <0.001), Month*Day (P <0.008), Gluc_diff_12*Day (P <0.001), Glucose*Day (P <0.001)
E_vol	Cow (P <0.001), Day (P <0.001), Month*Day (P <0.002), Gluc_diff_12*Day (P <0.003), Glucose*Day (P <0.010)
AL	Cow (P <0.001), Day (P <0.001), Month*Day (P <0.013)
AW	Cow (P <0.001), Day (P <0.001), Ins_diff_12_40*Day (P <0.002), Glucose*Day (P <0.041)
A_vol	Cow (P <0.002), Day (P <0.001), Ins_diff_12_40*Day (P <0.002)

Table 3.9 Output of GLMSELECT with repeated measures for cows

Dependent Variable	Significant model effects at P <0.05
EL	Cow (P <0.001), Day (P <0.001), Insemination*Day (P <0.007), Ins_0*Day (P <0.008)
EW	Cow (P <0.001), Day (P <0.001), Breed*Day (P <0.040), Month*Day (P <0.020), Gluc_0*Day (P <0.020)
E_vol	Cow (P <0.001), Day (P <0.001), Insemination*Day (P <0.003) Ins_0*Day (P <0.010)
AL	Cow (P <0.001), Day (P <0.001), Gluc_diff_12*Day (P <0.010), Ins_0*Day (P <0.044), Parity*Day (P <0.020)
AW	Cow (P <0.001), Day (P <0.001), Insemination*Day (P <0.002), Gluc_diff_40*Day (P <0.028)
A_vol	Cow (P <0.002), Day (P <0.001), Insemination*Day (P <0.002), Parity*Day (P <0.001), Month*Day (P <0.029), Gluc_diff_12_40*Day (P <0.021), Ins_0 (P <0.022)

Table 3.10 Results for tests of significance using a mixed model analysis for Year 2 (cows and heifers combined; n = 90). Type = heifer versus cow.

Model Effect	Df	EL	EW	E_vol	AL	AW	A_vol
Day	5	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
Type	1	NS	NS	NS	NS	NS	NS
Month	2	0.0006	NS	NS	NS	NS	NS
Breed	1	0.0003	0.0613	0.0081	0.0035	0.0165	0.013
Day*Type	5	0.0003	0.0141	0.0001	NS	0.0564	NS
Day*Month	10	0.0003	0.0002	0.0001	0.0014	NS	NS
Day*Breed	5	NS	NS	NS	NS	NS	0.0454
Day*Type*Month	12	NS	NS	0.0553	NS	NS	NS

[1] NS = Not significant, P > 0.15

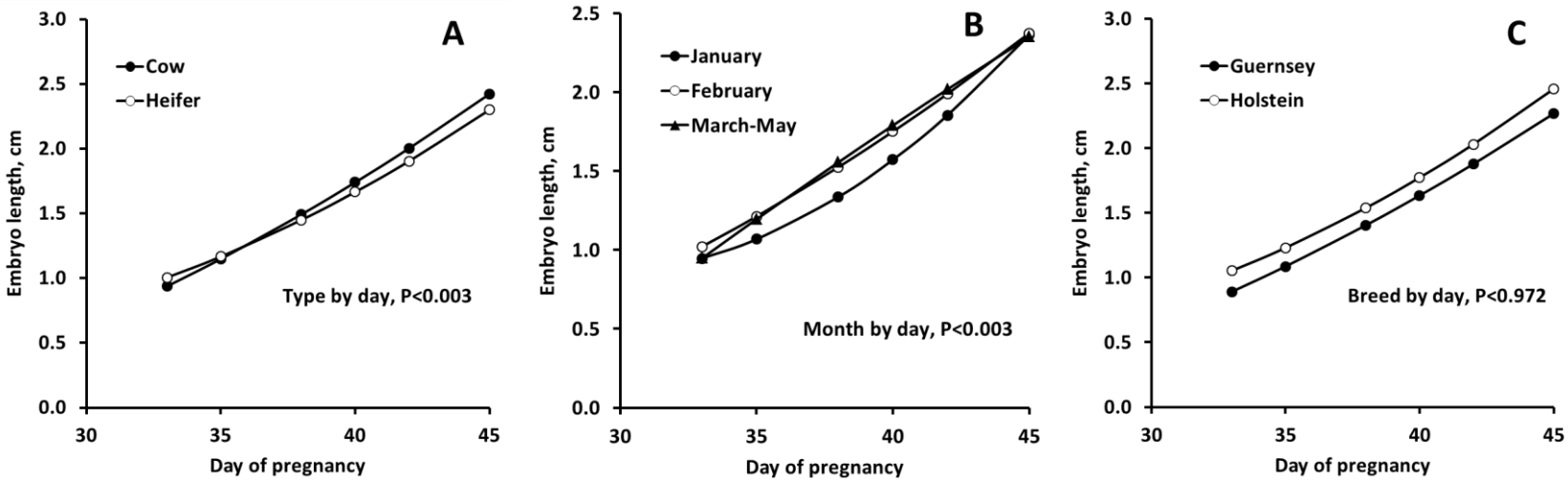

Figure 3.4 Embryo length (day 33-45 of gestation) for cows versus heifers (A), that were pregnant during the month of January, February, or March to May (B), or were either Guernsey or Holstein breed (C).

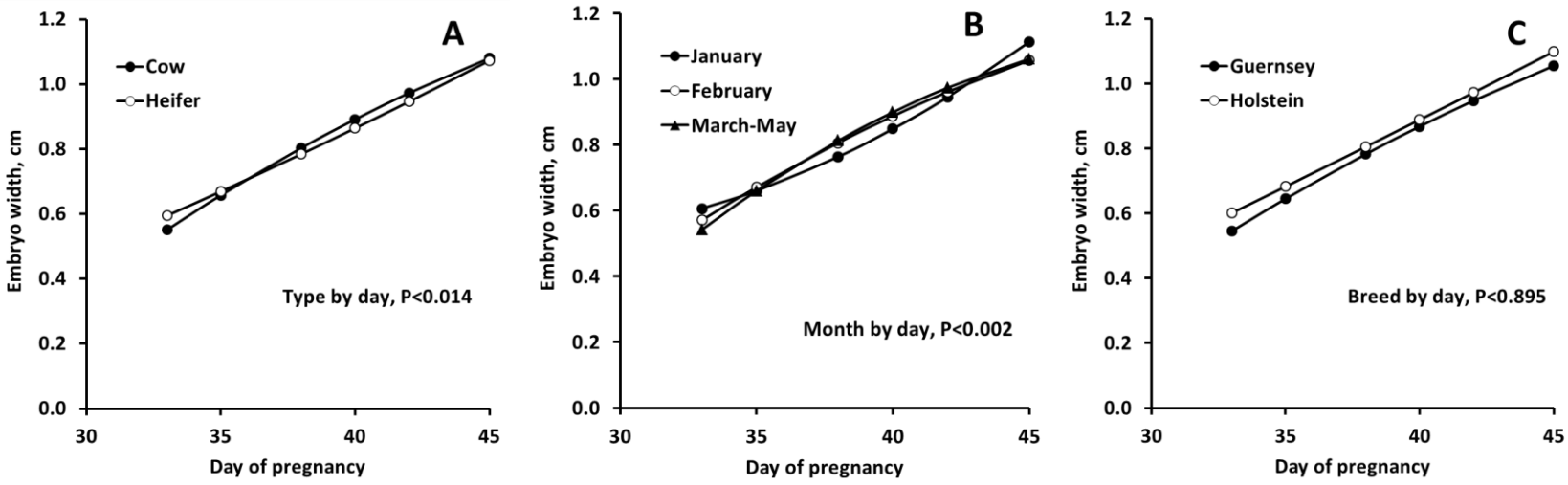

Figure 3.5 Embryo width (day 33-45 of gestation) for cows versus heifers (A), that were pregnant during the month of January, February, or March to May (B), or were either Guernsey or Holstein breed (C).

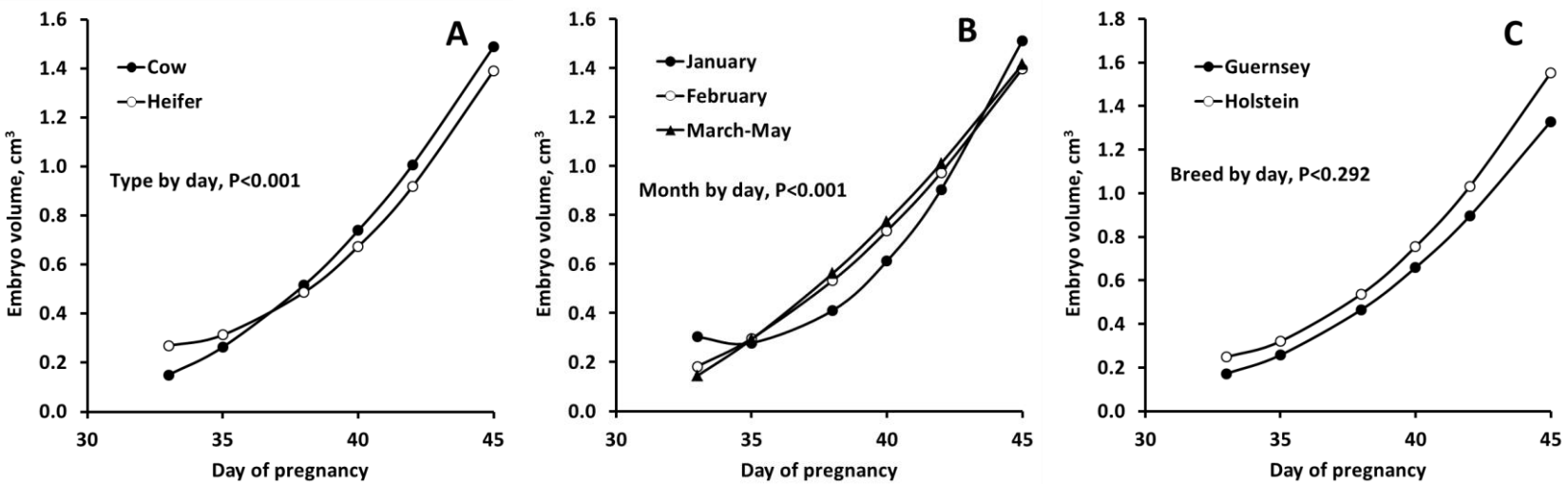

Figure 3.6 Embryo ellipse volume (day 33-45 of gestation) for cows versus heifers (A), that were pregnant during the month of January, February, or March to May (B), or were either Guernsey or Holstein breed (C).

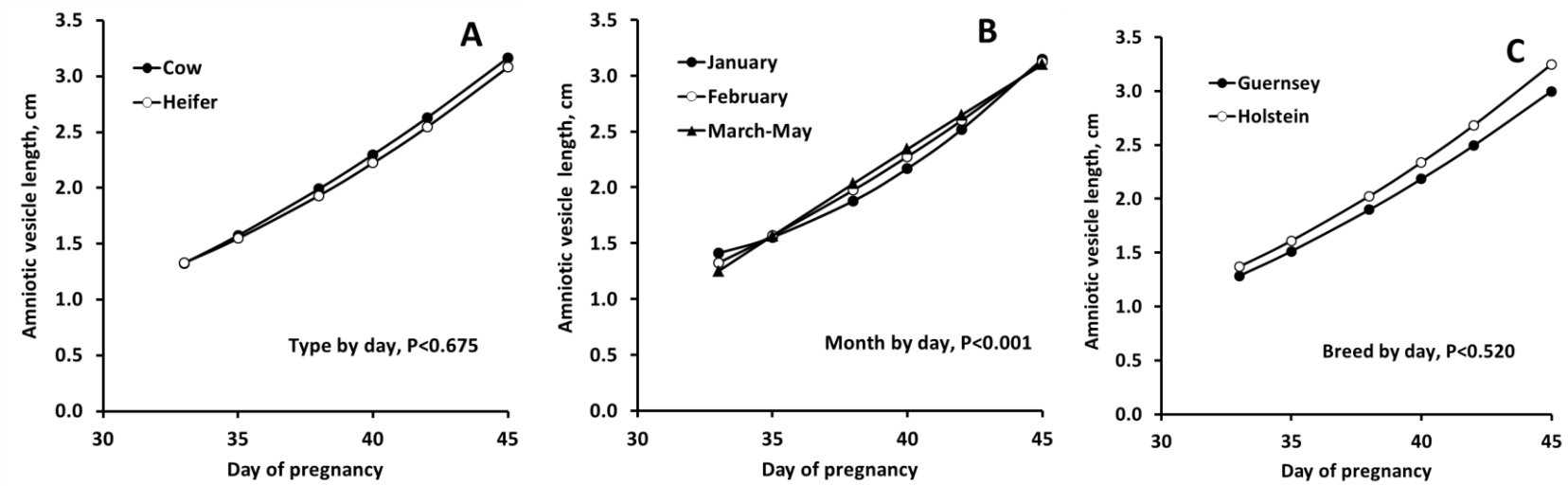

Figure 3.7 Amniotic vesicle length (day 33-45 of gestation) for cows versus heifers (A), that were pregnant during the month of January, February, or March to May (B), or were either Guernsey or Holstein breed (C).

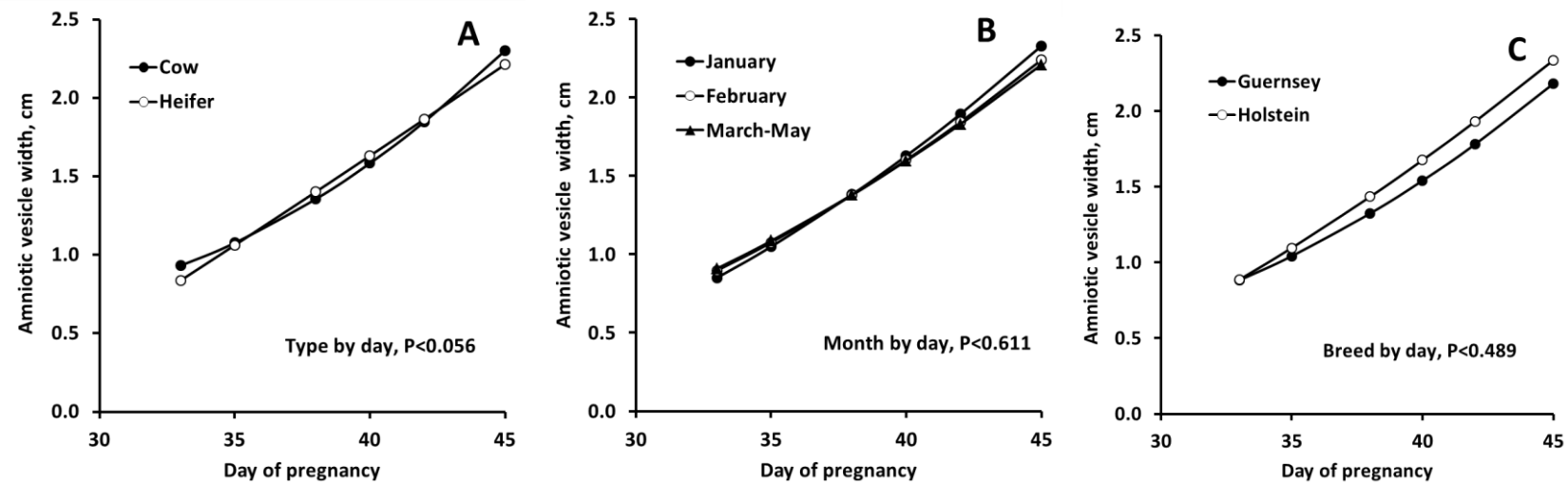

Figure 3.8 Amniotic vesicle width (day 33-45 of gestation) for cows versus heifers (A), that were pregnant during the month of January, February, or March to May (B), or were either Guernsey or Holstein breed (C).

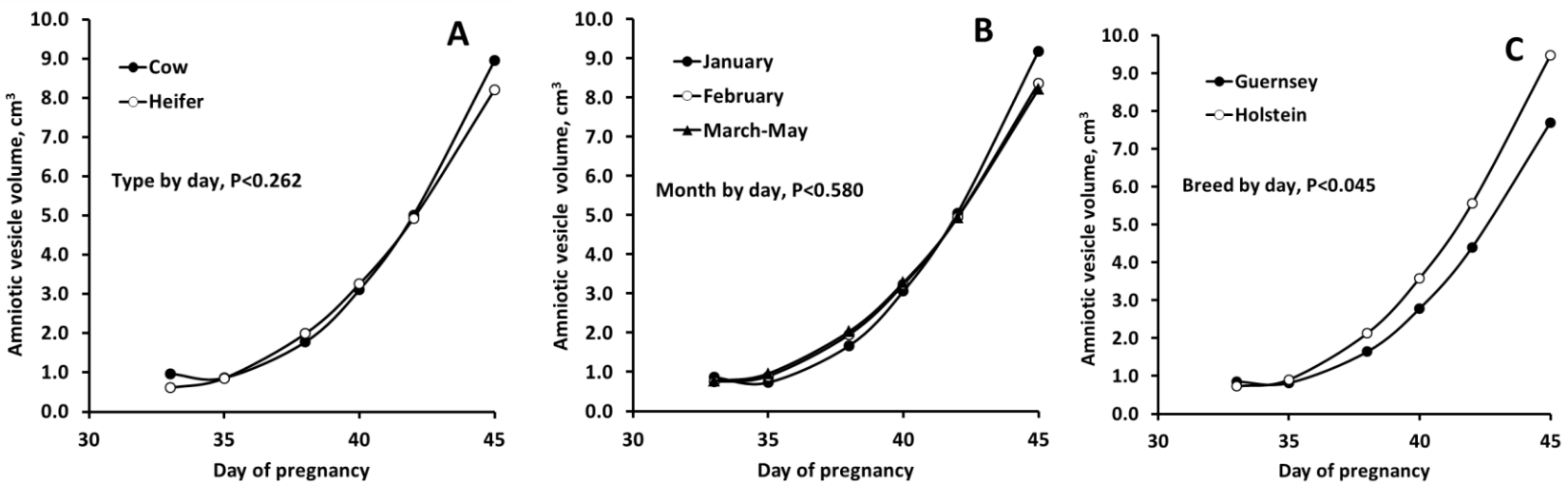

Figure 3.9 Amniotic vesicle ellipse volume (day 33-45 of gestation) for cows versus heifers (A), that were pregnant during the month of January, February, or March to May (B), or were either Guernsey or Holstein breed (C).

Table 3.11 Results of the final MIXED model analysis for heifers

		P-Value		
Effect	DF	EL	EW	E_Vol
Day	5	<0.001	<0.001	<0.001
Month	2	NS	NS	NS
Ins_0	1	NS	0.011	NS
Month*Day	10	<0.001	<0.001	<0.001
Ins_0*day	5	NS	0.062	NS

[1] NS = Not significant, P > 0.15

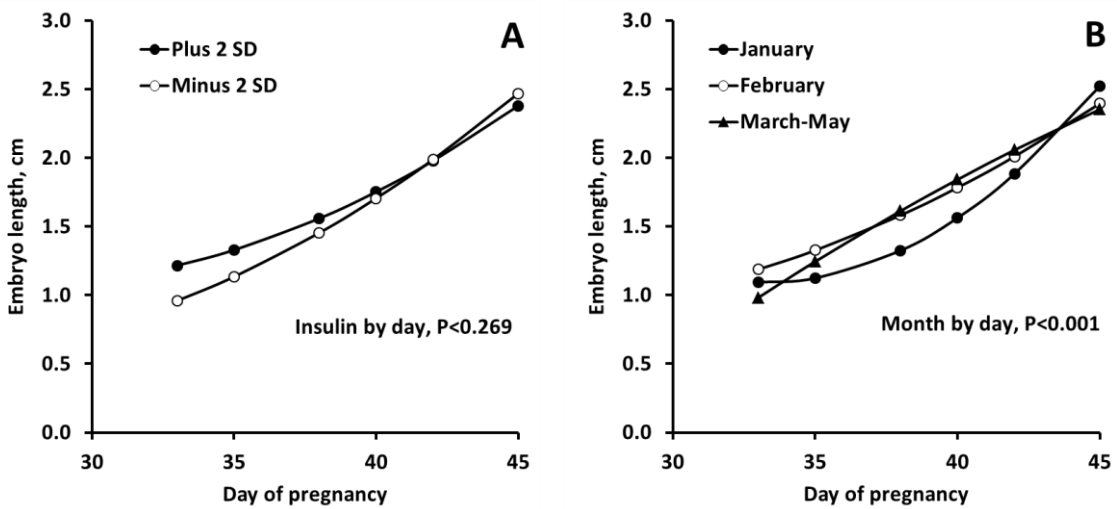

Figure 3.10 Embryo length (day 33-45 of gestation) for heifers that were two standard deviations above or below the population mean insulin concentration (A) or were pregnant during January, February, or March to May (B).

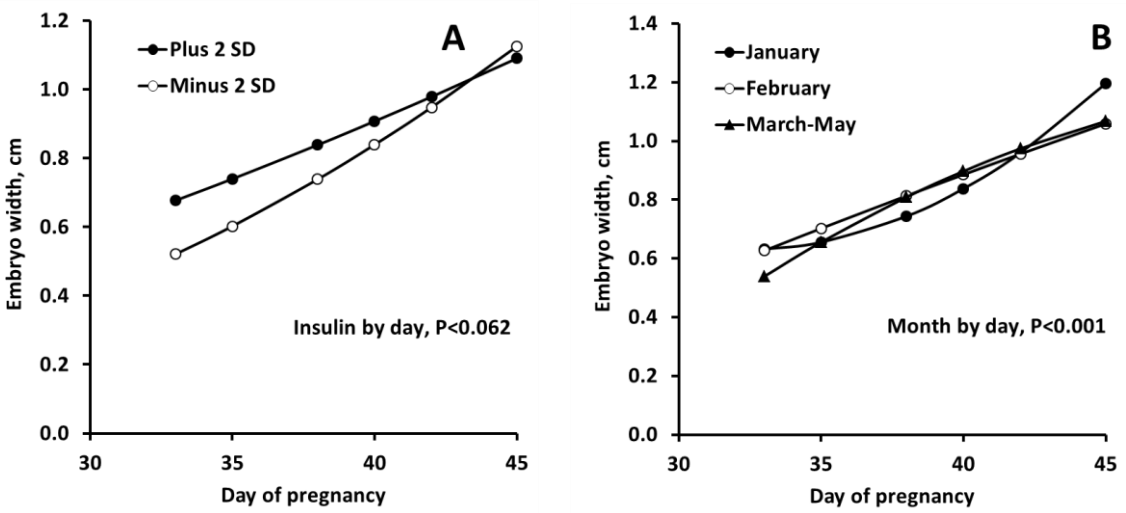

Figure 3.11 Embryo width (day 33-45 of gestation) for heifers that were two standard deviations above or below the population mean insulin concentration (A) or were pregnant during January, February, or March to May (B).

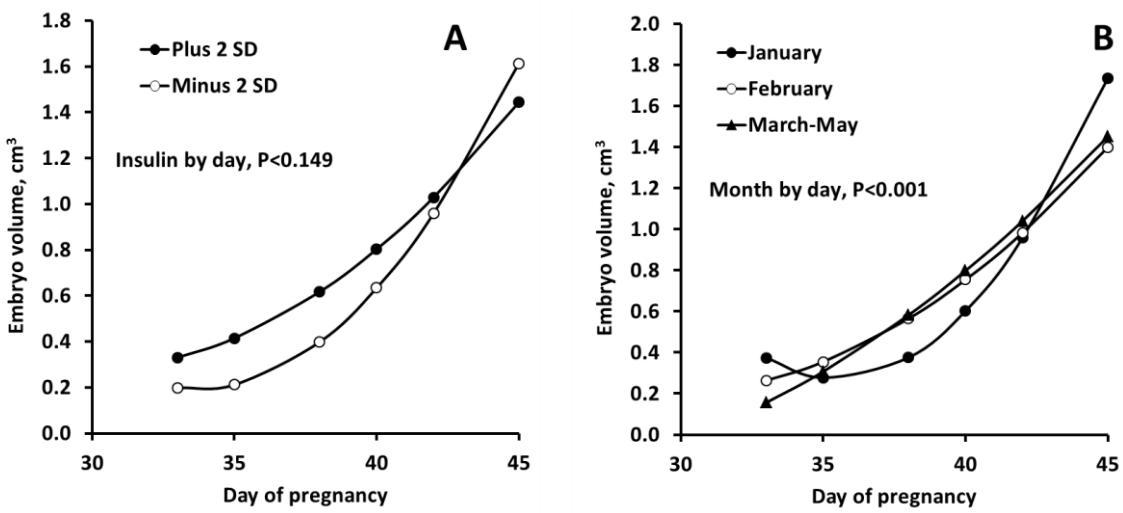

Figure 3.12 Embryo ellipsoid volume (day 33-45 of gestation) for heifers that were two standard deviations above or below the population mean insulin concentration (A) or were pregnant during January, February, or March to May (B).

Table 3.12 Results of the final MIXED model analysis for cows

		P-Value		
Effect	DF	EL	EW	E_Vol
Day	5	<.0001	<.0001	<.0001
Insemination number	1	0.0956	NS	0.0605
Ins_0	1	NS	NS	NS
Insem*Day	5	0.0028	NS	0.0024
Ins_0*day	5	0.0004	NS	0.0021

[1] NS = Not significant, P > 0.15

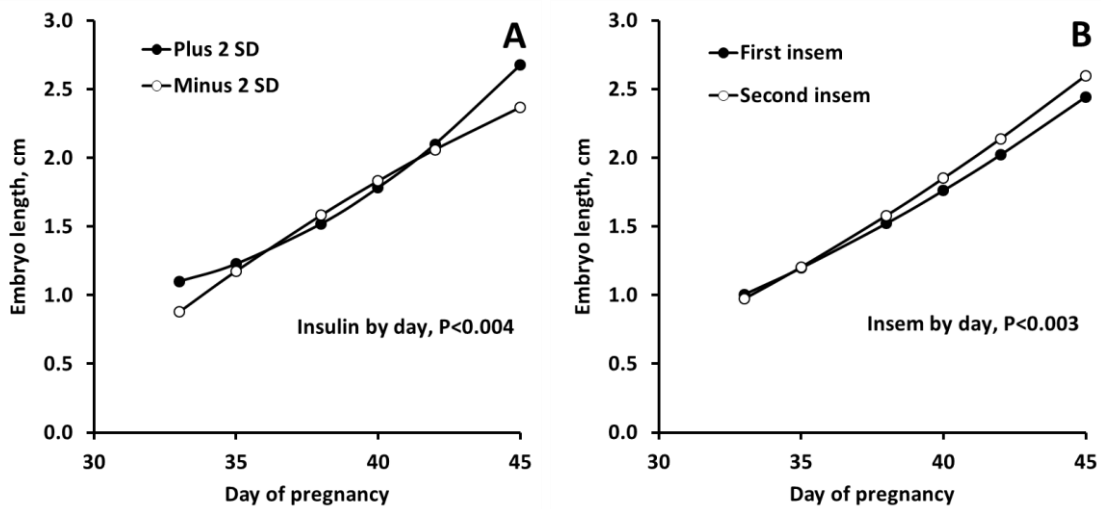

Figure 3.13 Embryo length (day 33-45 of gestation) for cows that were two standard deviations above or below the population mean insulin concentration (A) or were pregnant to either first or second insemination (B).

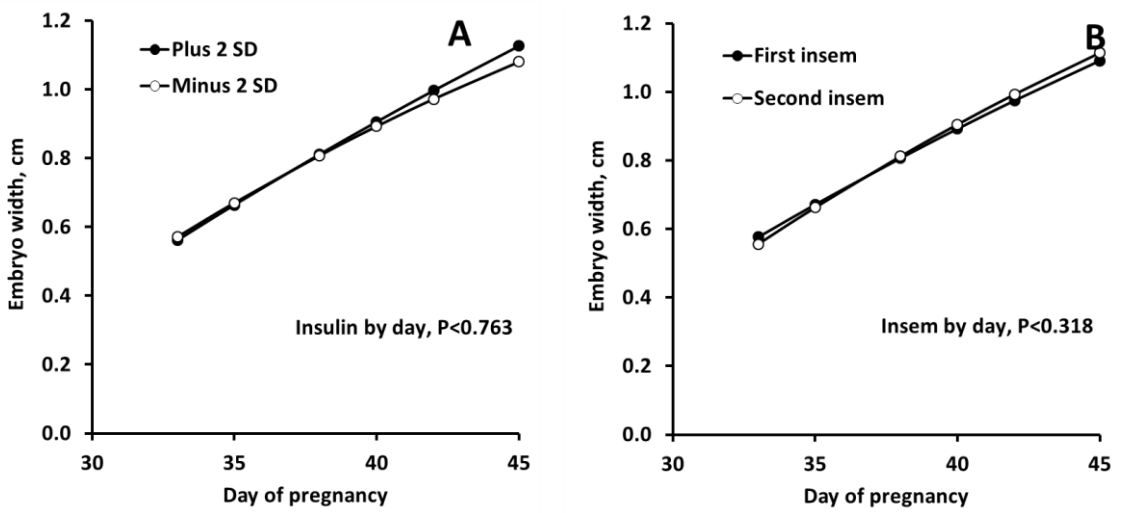

Figure 3.14 Embryo width (day 33-45 of gestation) for cows that were two standard deviations above or below the population mean insulin concentration (A) or were pregnant to either first or second insemination (B).

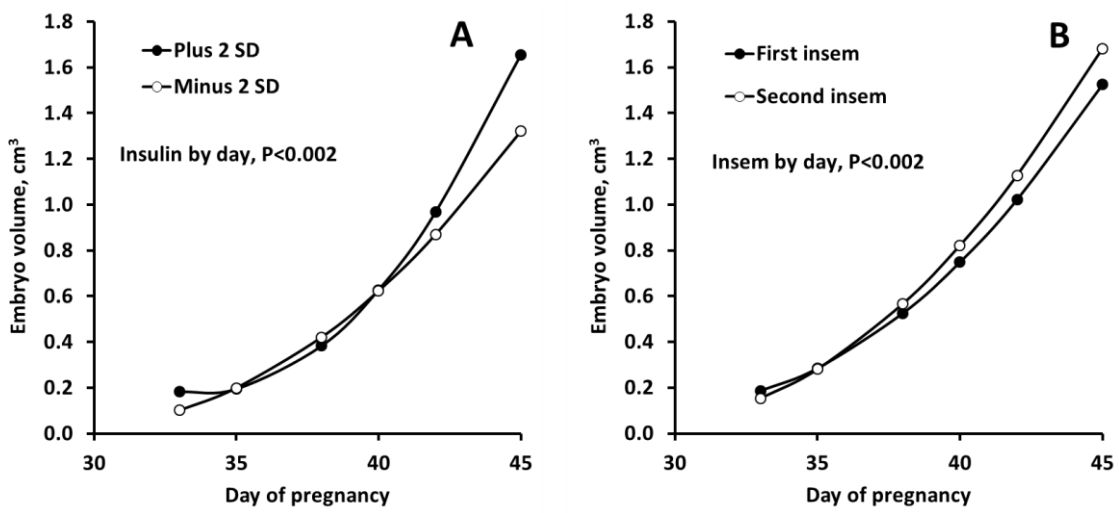

Figure 3.15 Embryo ellipsoid volume (day 33-45 of gestation) for cows that were two standard deviations above or below the population mean insulin concentration (A) or were pregnant to either first or second insemination (B).

3.4. Discussion

We examined cows and heifers for growth of the conceptus. As with chapter II, we found major effects of breed, month, or insemination number in both heifers and cows but small effects of insulin or other metabolic measures. We did observe a large difference in the growth of the conceptus between heifers and cows.

The effects of month (January animals had slower growing embryos) was also seen in Year 1 (Chapter II). In this chapter, month was significant for heifers and insemination number was significant for cows. We are assuming that insemination number is a proxy for month in cows. As mentioned in Chapter II, we are not aware of published effects of winter season on the growth of the embryo. This could be explained by photoperiod or perhaps ambient temperature. Heat stress decreases the growth of the embryo so there is precedence for an effect of ambient temperature on the embryo. Photoperiod is known to affect IGF1 (longer days is associated with greater IGF1 concentrations) so perhaps there is an interaction there as well (Dahl et al., 2012). We did see a positive relationship with IGF1 and growth of the embryo in Chapter II.

We also observed that the Guernsey embryos were smaller. This may be true in both cows and heifers and agrees with results from Chapter II. We found that heifers had smaller embryos than cows. The comparison between heifers and cows is imperfect because cows are lactating and heifers are not, but also because heifers are virgins and cows are not. Thus we do not know if the differences can be explained by a virgin uterus or by the lactational status of the animal. Most of the differences for heifers and cows were found for the embryo (not the amniotic vesicle).

One interesting finding for the heifers was that they appear to have larger embryos on d 33 of gestation. Perhaps this is associated with greater insulin concentrations in heifers versus cows. We wonder if they may indicate biological differences in heifers compared with cows during the early period of embryonic growth. In their analyses of ISG expression, Green et al. (2010) found that ISG expression was greater in heifers compared with cows. They hypothesized a larger embryo in heifers. Our data perhaps suggest the same thing (larger embryo early) (Figure 3.6 A)

The results of the glucose tolerance test demonstrated the unique metabolic differences between a lactating and non-lactating animal. Surprisingly, the glucose profile after glucose infusion was largely similar (with the exception of Guernsey cows). Thus, the clearance of glucose differed minimally between the groups. The only exception to this was the Guernsey cows. For some reason Guernsey cows began with lower resting glucose levels and did not achieve the same maximum concentration compared with the other groups (Figure 3.3).

In terms of insulin release, the cows released much less insulin than the heifers. Thus, in a lactating cow, clearance of glucose is primarily achieved by non-insulin dependent mechanisms. Uptake of glucose by the mammary gland is the most likely non-insulin dependent mechanism. A heifer seems to be more dependent on insulin to lower blood glucose concentrations. Regardless of the mechanism, the decrease in blood glucose that was achieved was largely the same.

We performed glucose tolerance tests because we believed we could identify aspects of insulin sensitivity that could explain how fast the embryo grew. Surprisingly, for the cows we found very few IVGTT parameters that explained how fast the embryo

grew. As with Chapter II, basal insulin (Ins_0) concentrations seemed to explain aspects of embryo growth. Somewhat different from Chapter II, we found that insulin was associated with larger embryos, particularly on day 45 of gestation.

There is considerable work to be done in terms of understanding exactly how insulin and glucose can affect embryonic growth. The fact that both years showed an effect, but the effect was not entirely the same indicates that we do not understand other mitigating factors.

Heifers appeared to have larger embryos early if they had greater insulin concentrations (Figure 3.10). This would suggest a role for insulin during the early period of embryonic growth. Both heifers and cows had effects other than insulin. For example, insemination number in cows and month in heifers. These two variables are confounded. Essentially all January cows were pregnant to first insemination. So, it makes sense that first insemination cows had smaller embryos because most were cows pregnant in January. Heifers were similar to cows because January heifers also had smaller embryos.

There were three cows and three heifers that lost their fetuses between d 45 and approximately d 70 (date of fetal sex determination). The data for these animals are plotted and compared with the averages for E_vol and A_vol for all heifers and cows (Figures 3.16). In terms of E_vol, two embryos were clearly retarded in growth (2958 and 2982). Other embryos were not largely different from average E_vol, although, there were two that were considerably larger with a flatter trajectory. When A_vol was examined, 5 of 6 were below the average. These cows may have been in the early stages of embryonic loss and the amnion was expanding slower from d 33 to 45.

In conclusion, we tested heifers and cows for growth of the conceptus. The conclusions from this trial were largely the same compared with Year 1. Holsteins had larger embryos compared with Guernseys and animals pregnant in January had smaller embryos. Heifers and cows differed in terms of glucose tolerance test response because heifers released more insulin. The release of insulin, however, did not explain the differences in embryonic growth. There were effects of insulin but these effects were relatively small, as can be found in Chapter II. Thus, there must be other mitigating factors that could control the growth of the embryo. It did not appear that the effects of lactation or metabolism alone were large enough to cause embryonic loss through a mechanism involving reduced embryo size.

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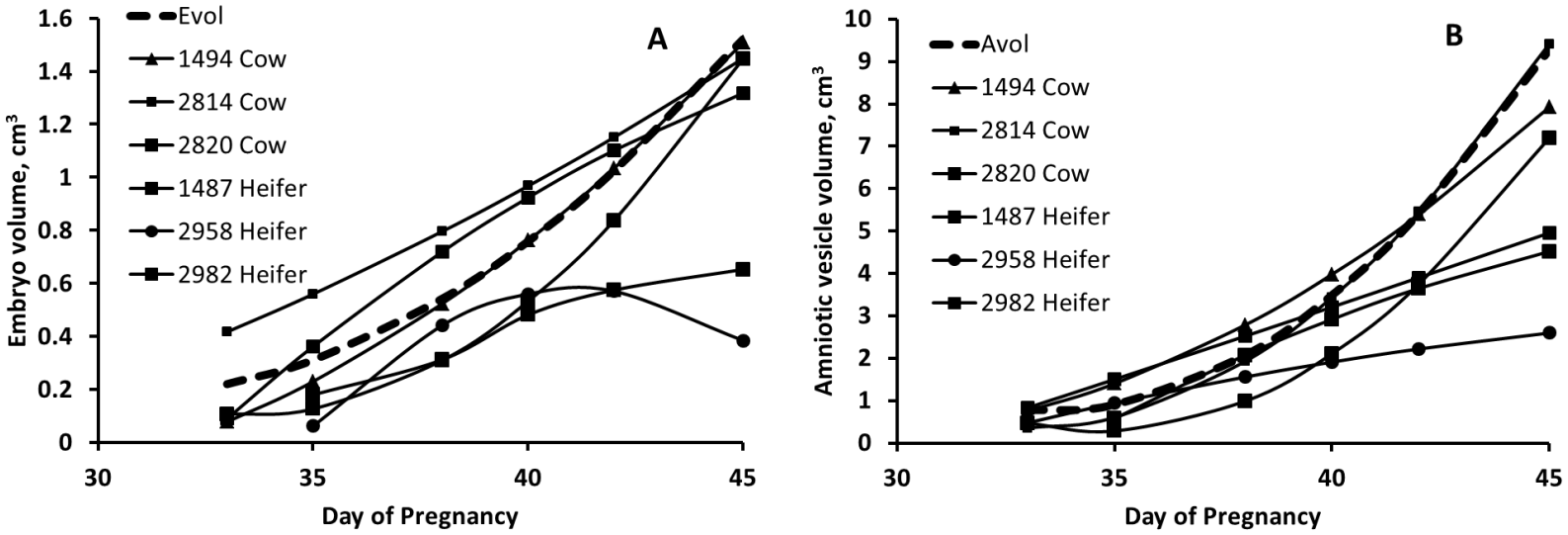

Figure 3.16 Embryo volume (A) and amniotic vesicle volume (B) from d 33 to 45 for three cows and three heifers that experienced fetal loss between d 45 and approximately d 70 of gestation.

Chapter IV

4. SUMMARY AND CONCLUSIONS

The reproductive efficiency of a dairy operation is a pivotal factor in overall profitability. One area of particular frustration for producers has always been embryonic and fetal mortality. Research from Pohler et al. (2013) has shown a relationship between late embryonic mortality and circulating PAG concentrations. A hypothesis which emerged was that a smaller placenta could be responsible for the decreased PAG concentration. The goal of the research presented in this thesis was to explore the relationship between multiple cow-level factors and late embryonic growth rate (33 to 45 days after AI) in lactating cows and non-lactating heifers. Our hypothesis was that factors such as milk production, fetal sex, glucose concentration, or horn (side of pregnancy) could influence embryonic growth, perhaps resulting in embryos that were challenged to grow and died as a result.

Over one hundred pregnancies from a variety of animals were examined during these experiments. It quickly became obvious that healthy embryos grew at different rates and trajectories (Figure 2.18). The breed of cow or heifer influenced the size of the embryo (Guernsey cows and heifers had smaller embryos than Holsteins) consistently. Month of pregnancy was also a factor that continually influenced embryo size (cows and heifers pregnant in January had smaller embryos compared with February or March to May). There were small effects of IGF1 and insulin concentrations on embryo size with differing relationships seen from year 1 to year 2 (Figure 4.1). These findings have led us to believe that variation in the rate of embryonic growth is a normal phenomenon and can

be seen in the data represented. Natural variation of embryonic growth does not seem to influence embryonic survival, and does not support our original hypothesis.

If the rate of embryonic growth does not influence embryonic mortality, further exploration and research must be completed to fully understand what factors are important. Our research shows that many factors including progesterone, glucose, milk production, fetal sex, and horn (side of pregnancy) do not have an effect on the rate of embryonic growth (Figure 4.1). Other researchers have proposed alternate hypotheses for factors influencing embryonic loss. Cows in herds using timed AI protocols have increased embryonic loss compared with herds inseminating after observed estrus (Lucy, 2001). Perhaps the repeated stimulation by artificial hormone treatments influences the development of the oocyte in an adverse manner. An influence of sire was reported by Lopez-Gatius et al. (2002), but the effect was only seen for one sire during a single trial so could potentially be an isolated finding. The findings that are more robust and repeatable include the health status of the cow shortly after calving and continuing into lactation.

Clinical disease has a direct influence on the ability of an embryo to survive. Chebel et al. (2004) reported that cows with clinical mastitis were 2.8 times more likely to experience embryonic loss. Similarly Lopez-Gatius (1996) showed that cows experiencing a retained placenta or pyometra were 1.8 and 2.6 times more likely to lose an embryo. These data together seem to point toward embryonic loss being caused by health factors or a suboptimal uterine environment. We feel that further research exploring the relationship between uterine environment shortly postpartum and subsequent embryo growth could potentially explain some embryonic loss.

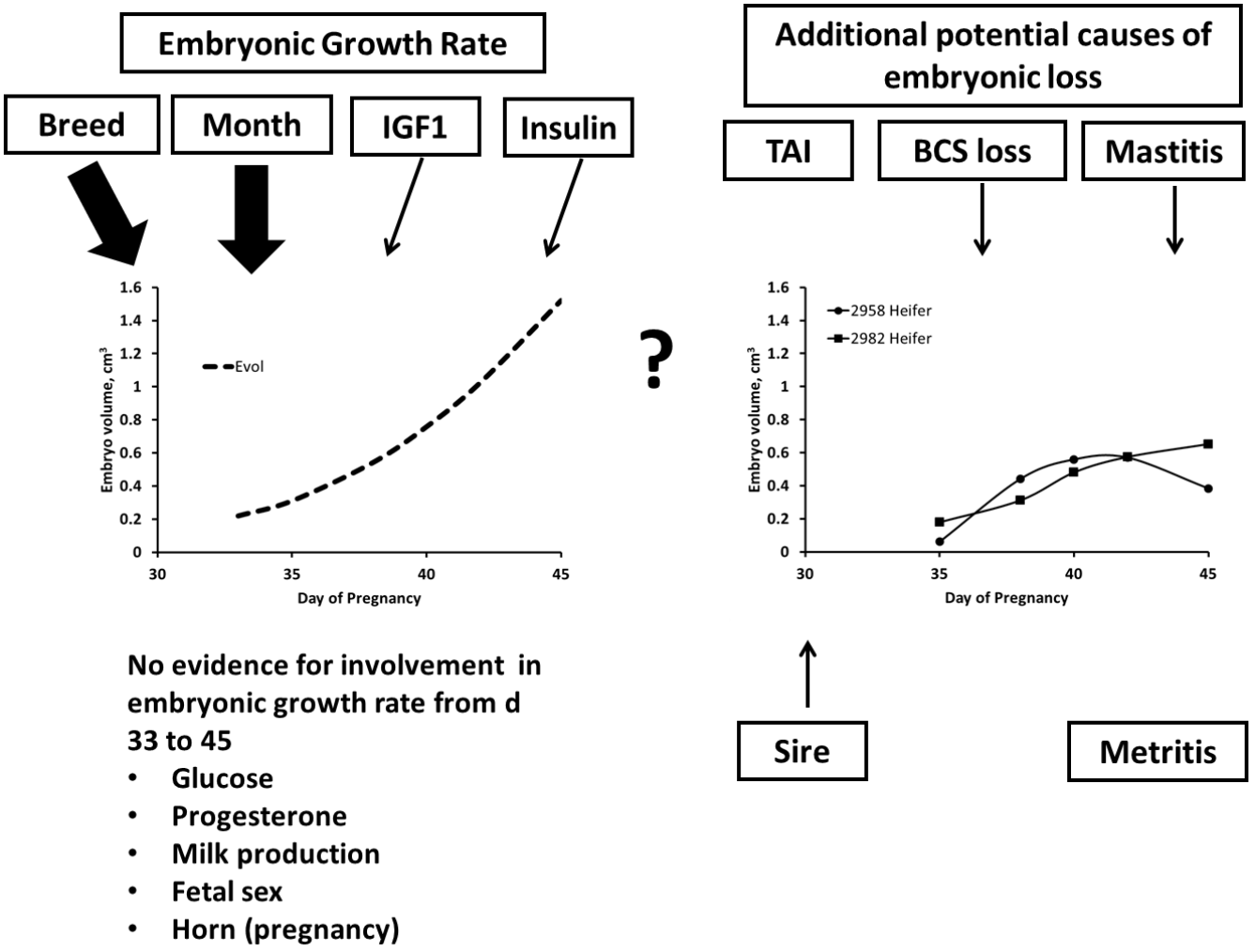

Figure 4.1 Factors influencing embryonic growth compared with potential causes of increased embryonic and fetal mortality.

LITERATURE CITED

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