

**Characterization of the Reproductive Tract in Recipients for Bovine Embryo
Transfer: pH and Bacterial Presence**

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Dedication

I would like to dedicate this dissertation to my family; Jennifer, Ty, Kase, and new addition coming spring 2015. Without their love and continued support this degree would not have been possible.

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Abstract

Pregnancy rates of bovine recipients following embryo transfer are key factors in profitability and genetic gains. The uterus is a dynamic organ that is essential for pregnancy; however, no selection criterion for recipient uterine environment exists. At transfer, pH and temperature of the ipsilateral uterine horn at site of embryo deposit and vagina were recorded in experiment one ($n = 120$). Vaginal pH and ipsilateral horn pH were correlated ($r=0.72$, $P < 0.0001$). Vaginal and ipsilateral pH and temperature did not differ between pregnant and non-pregnant animals in experiment one. Measuring uterine environment decreased pregnancy rates (27 versus 55%, $P = 0.03$; pregnant versus non-pregnant, respectively). In experiment two, abattoir collected reproductive tracts ($n = 43$) revealed a correlation between ipsilateral uterine horn and vagina pH ($r = 0.59$, $P < 0.0001$) and temperature ($r = 0.81$, $P < 0.0001$) utilizing the field probe. Logistic regression models in experiment three recipient animals ($n = 136$, vaginal pH and temperature with field probe) predicted pregnancy likelihood based on vaginal pH ($P = 0.15$), vaginal temperature ($P = 0.3$), and rectal temperature ($P = 0.15$). Groups based on these models revealed no impact of vaginal pH ($P = 0.42$) or rectal temperature groups on pregnancy rates ($P = 0.83$ and 0.59 , respectively). Chapter 3 utilized abattoir bovine reproductive tracts ($n = 43$) with an approximate day 7 corpus luteum for analysis of bacteria presence on uterine and vaginal pH. Anaerobic presence impacted pH in ANOVA and regression models ($P = 0.05$ and $P = 0.02$, respectively). Bacterial presence in the ipsilateral horn did not affect ipsilateral pH, and vaginal bacterial growth did not predict ipsilateral horn environment. Thus, vaginal pH was highly correlated to ipsilateral horn pH. Presence of bacteria at site of embryo deposit did not impact ipsilateral horn pH.

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Introduction

World census estimates place the global population of the year 2050 between 9 to 10 billion people (United States Census Bureau 2013), and thus a greater demand for agriculture products will be required with less land and water available. Therefore, the need to improve cattle efficiency through increased fertility and gains in genetic improvements will be demanded. Cattle producers have enjoyed the benefits of the commercial embryo transfer industry for several decades (Seidel *et al.* 1985; Hasler 2001; Betteridge 2003; Seidel and Elsden 2003; Hasler 2006), unfortunately these benefits also come with the frustrations of stagnant improvements in the recovery of viable embryos (Hasler 2001; Hasler 2004; Stroud and Hasler 2006) and the inability to identify suitable recipient animals (McMillan 1998; Stroud and Hasler 2006).

While a great deal of research has focused on the donor animal, semen quality, superovulation protocols and pharmaceuticals (Seidel and Elsden 2003; Hasler 2004; Hasler 2006; Stroud and Hasler 2006), little effort has been directed at optimizing the recipient in terms of uterine environment. This is despite the statistical understanding that the recipient animal is a major source of pregnancy loss in an embryo transfer program (McMillan 1998). Furthermore, the impact of nutrition on the uterine environment has been clearly demonstrated by numerous groups and most notably by Elrod and Butler (1993). Their research showed the impact of dietary crude protein on pH of the uterus and subsequent fertility (Elrod and Butler 1993). In addition, several *in vitro* groups have demonstrated the sensitivity of cultured embryos to changes in pH in multiple species including human, mice, hamster and bovine (Dale *et al.* 1998; Edwards *et al.* 1998; Lane *et al.* 1998; Lane and Bavister 1999; Squirrell *et al.* 2001; Ocon and Hansen 2003; Pool 2004). It has also been documented that a disruption in uterine pH prior to or at standing estrus through the addition of supplemental pharmacological agents leads to altered fertility in artificial insemination programs (Perry and Perry 2008b; Perry and Perry 2008a).

Beyond nutrition and synchronization pharmaceuticals, natural factors may also play a role in the pH of the reproductive tract that may influence fertility. A small amount of literature exists to indicate pH differences in the uterus when differing species of

bacteria are present depending on stage of estrous cycle (Ozenc *et al.* 2010).

However, what is not known is the ideal pH range of the uterus that will allow for early embryonic development at day 7 of the estrous cycle in the bovine when embryos are transferred. Furthermore, is the uterine pH correlated to the vaginal pH to allow this to be utilized as a recipient selection tool to aid the commercial industry? Finally, do varying bacterial loads impact pH of the uterus; and thus preventing recipient animals from establishing a successful pregnancy?

Currently, recipient animals are identified as acceptable solely off the display of estrus and the presence of a corpus luteum on the ovary. These subjective measurements can lead to the embryo being transferred to an animal that possess a uterine environment that is unfavorable for embryonic development. The recipient animal has been clearly defined as the largest contributor to embryo transfer pregnancy success or failure (McMillan 1998). In addition, several current embryo practitioners and company representatives have identified the non-pregnant recipient animal as the biggest cost in an embryo transfer program (Sam Edwards; personal communication) with the number of non-pregnant days being the most crucial (Cary Crow, TransOva; personal communication). Utilizing today's current market prices for slaughter cows and assuming an additional 20% increase in value (Beltrame *et al.* 2007) to identify higher quality recipient animals, would lead to an initial purchase cost of \$1740. In addition, the cost of synchronizing, transferring, and a minimum of 45 days on feed prior to first transfer leads to an estimated \$2056 initial cost. Additionally, the potential cost of the offspring must be considered and could easily be in the thousands of dollars. In contrast to this, several commercial entities exist that offer embryo owners the option to purchase pregnant recipient animals or a live weaned embryo calf without the purchase of the recipient. These options may be a better choice for the embryo owner, but the recipient manager still incurs the above costs and obstacles associated with the recipient animal. Thus, a recipient animal failing to become pregnant represents a very large and real economic burden on the profitability of an embryo transfer program.

The central hypothesis of the current dissertation is that pregnancy status following embryo transfer of recipients is determined by uterine pH (as a reflection of uterine health). Thus, the aim of the current dissertation is to better characterize the

environment of the recipient uterus. The first milestone was to determine if pH values are correlated to any other compartments of the reproductive tract (upper third of uterine horn versus vagina). Secondly, milestone two evaluated the impact that vaginal pH had on the pregnancy rate of recipient animals. Finally, milestone three evaluated the relationship of pH in varying compartments of the reproductive tract with a greater focus on bacterial load. Essentially, the ultimate goal is to gain a better understanding of the uterine environment of recipient cows in order to minimize the negative financial impact these non-pregnant animals have on embryo transfer programs.

Chapter 1. Literature Review

First an understanding of the basic uterine structure and environment must be discussed to gain an appreciation for the normal events that occur in this fascinating organ. Then the impacts of pH on uterine health and fertility can be explored. Ultimately, the interactive effects of pH, nutrition, and bacteria on fertility will be discussed.

Uterine Environment

Basic Uterine Structure

The uterus is a dynamic reproductive organ that is continually in varying states of transition. These periods include prepubertal development, transition to puberty and subsequent change in endocrine status, cyclicity driven by ovarian structures, pregnancy, parturition, and involution. Ultimately, these changes in uterine environment drive the ability of a female to become pregnant. In addition, these changes make it difficult to define what it means for a female to have a “normal” uterine environment. Normalcy is truly a relative term and will vary depending on what time point is being discussed.

It is generally accepted that the uterus is responsible for providing the necessary nutrients and proper environment needed for embryo and fetal development. In fact, this point was first made by Aristotle in the third century BC and again by William Harvey in the 17th century (Gray *et al.* 2001a; Spencer and Bazer 2004). The uterus is comprised of two distinct layers, the endometrium and the myometrium (Gray *et al.* 2001a; Spencer and Bazer 2004). The endometrium consists of the mucosal cells that line the innermost compartment of the uterus separating the musculature from the uterine lumen (Gray *et al.* 2001a; Spencer and Bazer 2004). This is also the layer that is responsible for attachment of the developing embryo during early pregnancy (Guillomot and Guay 1982; Spencer and Bazer 2004). In fact, the hypothesis exists that the attachment of an early embryo to the luminal epithelium permits its elongation (Spencer and Bazer 2004). Furthermore, a vast amount of evidence exists to indicate that without the glandular component of the endometrium, pregnancy will not be possible (Gray *et al.* 2000; Gray *et al.* 2001b; Gray *et al.* 2001c; Gray *et al.* 2002). In contrast, the myometrium consists of smooth muscles and is responsible for providing the uterine contractions needed

during parturition (Gray *et al.* 2001a; Spencer and Bazer 2004). Its role in maintaining a normal luminal environment is limited and it has been proven that estrogen (E₂) and progesterone (P₄) receptors are predominantly located in the cells of the endometrium and not the myometrium (Boos *et al.* 1996).

Uterine Involution

Within the beef industry, multiparous animals are preferred as recipients since questions regarding maternal ability and specifically lactation ability have been answered. Thus in the case of the recipient animal, the “normal” uterine environment typically begins with her ability to undergo uterine involution following parturition. This time point is important due to the fact that a recipient cow’s uterus has a defined period of time to involute before she can receive an embryo. If the uterus fails to fulfill the necessary procedures during involution then subsequent fertility can be impacted. Even during parturition that is characterized as normal, the uterus undergoes varying levels of trauma. In cases of dystocia or retained placentas, the incidence of delayed uterine involution and increased bacteria contamination increase (Kiracofe 1980; Leslie 1983; Sheldon *et al.* 2008b). Sheldon *et al.* (2008b) outlined the required steps for a female following parturition that must occur to prepare herself for a subsequent pregnancy. These events included uterine involution, regeneration of the endometrium, elimination of bacterial contamination of the uterus, and return to ovarian cyclical activity. They further define involution as the physical shrinkage, necrosis and sloughing of caruncles and regeneration of the endometrium. Involution length can vary by species, health status, and endocrine environment of the female (Sheldon *et al.* 2008b). In order for a cow to maintain a 365 day calving interval and assuming a gestation length average of 285 days, a recipient animal has 80 days postpartum to complete involution and become pregnant again. This timeline becomes even shorter when an estimated involution time is subtracted from those 80 days. The time needed from involution (figure 1.1) varies among authors but has been reported to range from 16 to 60 days (reviewed by Kiracofe 1980; Lindell *et al.* 1982; Sheldon *et al.* 2008b). In some cases, recipients may only have one estrous cycle to reestablish pregnancy. Further evidence

exists that fertility increases with each successive estrous cycle following parturition (Thatcher and Wilcox 1973; Thatcher *et al.* 2006).

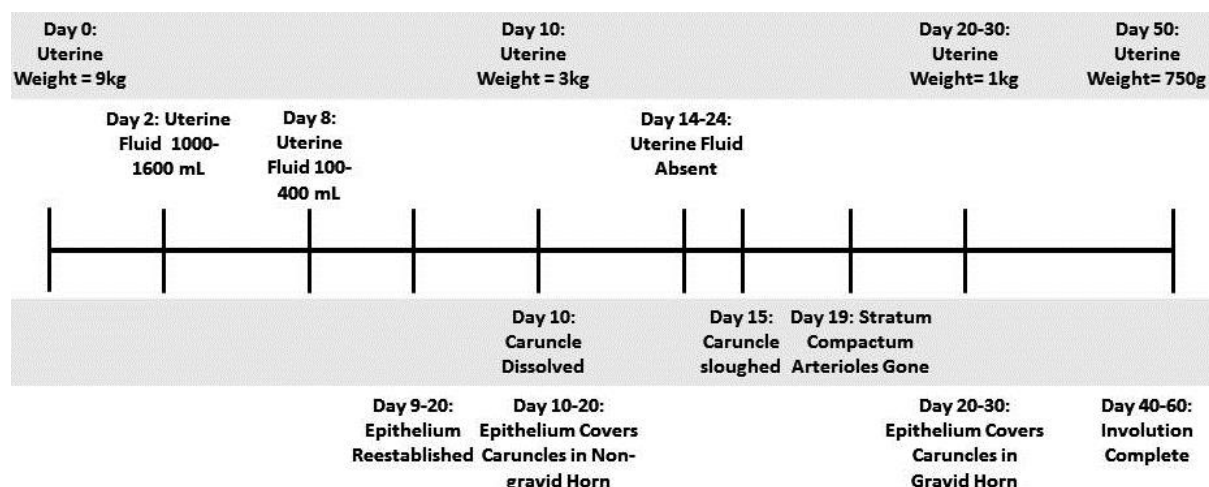


Figure 1.1 Timeline of involution in the cow. Adapted from Kiracofe (1980).

pH Basics

At its simplest definition, pH is the negative logarithmic measure of the hydrogen ions present in a solution and often referred to as the Henderson-Hasselbalch equation (Pool 2004). However, this measurement is based on the mass action law developed over 140 years ago by Peter Waage and Cato Maxmillian Guldberg (Pool 2004). The mass action law states that reactive species A and B can form species C and D while at the same time C and D react back into A and B. This law has been refined over the course of the last century and half to establish a meaningful measurement system to reflect the dissociation of salts and acids in solution, leading to the development of the pH scale. pH is expressed as a numeric value between 1-14 with 7 considered neutral (Pool 2004). What is confusing and of biological importance to remember is that this scale is the negative log of the hydrogen ions present, and thus reflects a fold change in amount rather than a simple numeric change. As an example, simply going from a 7.2 to a 7.4 pH seems minor but in actuality this change represents a 60% change in the amount of hydrogen ions present (Swain 2010b; Swain 2010a).

As can be seen from the mass action law, it is of extreme importance to realize that pH is a dynamic state that is ever changing based on the environment (Pool 2004;

Swain 2010b; Swain 2010a). In particular, the amount of carbon dioxide (CO₂) and bicarbonate has a large impact on the pH of a solution (Pool 2004; Swain 2010b; Swain 2010a). The dynamics of pH should be thought of in 3 stages equilibration, set point, and stabilization (Swain 2010b; Swain 2010a). The equilibration stage is the interaction of gaseous CO₂ dissolving in solution to carbonic acid and reaching equilibrium with the dissolved bicarbonate. Once equilibrium has been established so has the set point or maximum equilibrium between the two compounds. The stabilization stage is actually oscillations of dissociation and re-association of the compounds in an attempt to maintain the set point. While the amount of dissolved carbonic acid and bicarbonate are major effectors of pH, these two compounds can also be influenced by temperature and proteins present in the solution (Swain 2010b; Swain 2010a). Increased temperatures will lead to more acidic solutions or an increase in the amount of dissolved carbonic acid.

It is widely known and accepted that pH of blood and the general system is 7.4 and has historically been used as the benchmark for homeostasis. However, from a reproductive setting this is not necessarily the case, especially for the uterine environment and the developing embryo (Pool 2004; Swain 2010b; Swain 2010a). Efforts have been made in the past to establish the pH and ion concentrations of the reproductive tract of multiple species (Nichol *et al.* 1997; Hugentobler *et al.* 2004; Hugentobler *et al.* 2007), but this task has proved challenging. Thus the majority of what we currently know about the needs of the developing oocyte and early embryo are derived from *in vitro* culture systems with the aim of producing viable embryos for subsequent transfer. This information holds great importance for both farm animals and humans as well. It is also important to realize that the ability to survive varying pH environments may be species specific. This fact is highlighted by review information published by Pool (2004) and these specific differences will be highlighted and discussed later. First, the basic cellular tools available to the embryo need to be discussed.

Extracellular pH (pHe) impacts Intracellular pH (pHi)

Extensive work across multiple species has been performed to establish the impacts of pHe on pHi (Zhao *et al.* 1995; Dale *et al.* 1998; Edwards *et al.* 1998; Lane *et al.* 1998; Lane *et al.* 1999; Lane and Bavister 1999; Phillips *et al.* 2000; Squirrell *et al.* 2001; Erdogan *et al.* 2005; FitzHarris and Baltz 2006; FitzHarris *et al.* 2007; FitzHarris and Baltz 2009). From these efforts, it was determined that the embryo is able to adapt to varying pH levels but that an optimal range may exist in a species specific manner. In fact, a range of pH levels exist that embryos may survive in and can be lower than the 7.4 benchmark as the system wide neutral point.

Regardless of species, the general behavior of cells pHi to pHe is the same. Initially, there is a quick response of pHi to follow the pHe level to a specific “trigger point” (Swain 2010b; Swain 2010a). At this point, the cell possess three mechanisms that allow for pHi adjustments to be made (Swain 2010b; Swain 2010a). A single mechanism appears to function in alkaline environments while two mechanisms function to correct acidic pHi and have different trigger points. The bicarbonate/chlorine exchanger ($\text{HCO}_3^-/\text{Cl}^-$ exchanger) works in alkaline environments, while the sodium dependent, bicarbonate/chlorine exchanger (Na^+ , $\text{HCO}_3^-/\text{Cl}^-$ exchanger) and sodium/hydrogen antiporter (Na^+/H^+ antiporter) function to correct acidic pHi (Swain 2010b; Swain 2010a). Furthermore, these mechanisms vary among species (Zhao *et al.* 1995; Lane *et al.* 1998; Lane and Bavister 1999; Phillips *et al.* 2000) and reviewed by Fitzharris and Baltz (2009). It has also been demonstrated that 8-16 cells bovine embryos do possess these mechanisms to combat acidosis efficiently (Lane and Bavister 1999), while similar stage embryos do not respond as well to alkaline environments as evidenced by an incomplete recovery of treated versus control embryos (Lane and Bavister 1999). When challenged with a mild alkaline environment above the resting point of 7.2 caused 8-16 cell bovine embryos to arrest development. Conversely, similar stage embryos challenged in an acidic environment continued development.

Impact of pH on cellular components

The cell's ability to correct extreme levels of pH_i determines its ability to develop and maintain viability. Even though early embryos of varying species have the ability to withstand a wide range of pH environments, this does not ensure that their developmental competence is the same as those that develop at a pH closer to that species neutral set point (Swain 2010b; Swain 2010a). Extreme pH_i levels have negative consequences on intracellular functions and organization. Many of these allow the embryo to develop properly (reviewed by Sun and Schatten 2006). Of importance to the cell is the disruption of normal mitochondria migration during varying stages of early development (reviewed by Sun and Schatten 2006). The mitochondria are important for energy production in early embryos (reviewed by Ramalho-Santos *et al.* 2009) and abnormal distribution of mitochondria during early embryonic development can prevent cell division and lead to increased cell fragmentation and altered growth (reviewed by Ramalho-Santos *et al.* 2009). Furthermore, mitochondria function in the production of reactive oxygen species; this, may influence apoptosis induction under stress conditions (reviewed by Ramalho-Santos *et al.* 2009). In brief, if an embryo is sufficiently stressed due to altered pH environments, apoptosis may be induced and lead to decreased fertility. Research performed in 2-cell hamster embryos indicated that altered pH (both acidic and alkaline) disrupted the migrations of mitochondria (Squirrell *et al.* 2001) and that returning cultured embryos to control media following a short exposure to increased or decreased pH levels allowed these embryos to develop to levels near control embryos. Similarly, studies in the mouse model found glycolytic activity was reduced when a non-metabolizable weak acid was added to culture medium of unfertilized zygotes (Edwards *et al.* 1998), but activity was not reduced in the morula stage embryos (Edwards *et al.* 1998). This research would indicate that a disruption in mitochondrial function had occurred (reviewed by Ramalho-Santos *et al.* 2009).

In addition, microfilament organization has been disrupted due to exposure to both acidic and alkaline environments (Squirrell *et al.* 2001). Microfilaments serve multiple important roles in the developing embryo (reviewed by Sun and Schatten 2006). A specific example is the role in chromosome separation during cell division

(reviewed by Sun and Schatten 2006). Abnormalities in chromosome separation have been shown to lead to decreased embryonic survival (Hansen 2002).

Impact of pH on Bovine Fertility

Given the understanding of pH impacts on cells, does pH have a similar impact on bovine and other farm animals? A great deal of effort has evaluated this question over recent decades (Elrod and Butler 1993; Elrod *et al.* 1993; Butler *et al.* 1996; McEvoy *et al.* 1997; Ocon and Hansen 2003; Hammon *et al.* 2005; Rhoads *et al.* 2006; Karen *et al.* 2011). The majority of these publications indicate that alterations that result in an acidic uterine environment during the luteal phase result in decreased fertility (Elrod and Butler 1993; Elrod *et al.* 1993; Butler *et al.* 1996; McEvoy *et al.* 1997; Rhoads *et al.* 2004; Rhoads *et al.* 2006). The acidic environment is induced by feeding excessively high levels of protein in the diet (Elrod and Butler 1993; Elrod *et al.* 1993; Butler *et al.* 1996; Rhoads *et al.* 2004; Rhoads *et al.* 2006), which is often required to maximize livestock production. Efforts from Elrod and Butler (1993) highlighted this fact and in a manner that coincides with an important time point for embryo transfer practitioners. This study fed heifers protein levels to be either at maintenance or in excess of daily requirements. In doing so, they altered the plasma urea nitrogen levels of the heifers and decreased the uterine pH on day 7 of the estrous cycle. Furthermore, the pH levels of both the low and high groups were reduced at estrus but the high group's pH remained low on day 7 indicating that the excess protein altered the uterine environment. In addition, work from the same lab (Elrod *et al.* 1993) determined that this effect is unique to the uterus and not seen in other systems.

This information led to the further investigations to confirm the specific metabolites of protein metabolism that may cause these changes. One such effort infused levels of urea directly into circulation to match levels observed in the previous efforts (Rhoads *et al.* 2004). In doing so, they were able to obtain similar pH readings previously mentioned by Elrod and Butler (1993) indicating that the excess urea in circulation leads to the acidic uterine pH. In addition, this group indicated that individual cows show varying degrees of response to urea infusions. This highlights the additional need for characterization of recipient animals for use in embryo transfer programs.

Additionally, Hammon and co-workers (2005) examined the concentration of urea present in the uterus on day 0 and 7 of the estrous cycle. From their efforts, cows with a high plasma urea nitrogen level (indicative of excess crude protein) had higher concentrations of urea in uterine flushing's compared to cows with low plasma urea nitrogen levels. These findings are similar to that of Jordan and co-workers (1983) in that higher plasma urea levels lead to increased luminal concentrations in the uterus.

However, the question remains, does pH impact the embryo or secretory function of the uterus? Thus research has been performed to determine the negative effects upon embryo development (McEvoy *et al.* 1997; Ocon and Hansen 2003) or uterine secretions important for embryo survival (Butler 1998; Butler 2005; Hugentobler *et al.* 2007) as influenced by increased urea and alter pH levels.

The curious aspect of these results is that the negative impacts on fertility are seen at day 7 of the estrous cycle when a compacted morula or blastocyst is present in the uterus (Elrod and Butler 1993; Butler *et al.* 1996). In other species, the negative effects of altered pH appear to be more problematic for maturing oocytes and early stage/pre-compaction embryos (Lane *et al.* 1998; Squirrell *et al.* 2001). A similar result was also reported in bovine embryos (Ocon and Hansen 2003), but at the same time later stage bovine embryos were not challenged in this experiment (Ocon and Hansen 2003). Perhaps this indicates altered secretory functions of the uterus rather than embryonic cell disruption.

In fact, great effort has been put forth evaluating the secretory patterns of the bovine oviduct and uterus (Jordan *et al.* 1983; Butler 1998; Kenny *et al.* 2002; Hugentobler *et al.* 2004; Butler 2005; Hugentobler *et al.* 2007). It appears that the secretion pattern for specific ions varies between the oviduct and uterus and some are related to stage of the estrous cycle (Hugentobler *et al.* 2007). Several of these ions are important for embryonic development and early maturation (Hugentobler *et al.* 2007). However, of strict importance is the realization that PGF_{2α} levels are increased from endometrial cells in response to elevated plasma urea nitrogen levels *in vitro* culture (reviewed by Butler 1998). The culture of endometrial cells *in vitro* directly reflect the response of these cells to elevated urea independent of other factors in the whole animal unit. It has been clearly demonstrated that increased PGF_{2α} have a negative

impact on the developing embryo (Scenna *et al.* 2008b). This would provide a potential link for decreased fertility seen on day 7 of the estrous cycle and increased plasma urea levels (Elrod and Butler 1993).

Bacteria and Endocrine interaction delay involution and fertility

Bacteria

Bacterial infections of the uterus play an important role in decreasing fertility by inhibiting the functions of the uterus along with interfering with normal functioning of the hypothalamus-pituitary-ovary axis needed for cycling and fertility (Sheldon and Dobson 2004b). A great deal of historic effort has been applied to the cattle species as they appear to be more susceptible to postpartum uterine infections than other livestock species (reviewed by Sheldon and Dobson 2004b). Unfortunately for cattle, it is estimated that approximately 40% of dairy cows experience clinical uterine infections within 1 week postpartum (Sheldon *et al.* 2009). This figure is potentially on the conservative low side since it does not include subclinical cows or beef cattle that are not as frequently examined postpartum as dairy cattle. Another estimate by Griffin *et al.* (1974a) estimates that 90% of uteri have bacterial invasion within the first 10 days postpartum. The ability of a female to become pregnant then relies on her ability to clear these infections and will be discussed in a later section.

The pattern of species that infects the uteri of cattle is non-specific (Griffin *et al.* 1974a; Griffin *et al.* 1974b; Sheldon *et al.* 2002; McDougall 2005) and appears to be more reflective of species present in the environment associated with that animal location. However, the most commonly isolated species are *Arcanobacterium pyogenes*, *Escherichia coli*, and *Fusobacterium necrophorum* (Griffin *et al.* 1974a; Griffin *et al.* 1974b; Sheldon *et al.* 2002; McDougall 2005). All of which are known environmental pathogens and are also typically associated with infections with severe lesions and pus (Sheldon *et al.* 2002).

Definition of Bacterial Infections

For the producer and practitioner, communicating clearly as to the level of bacterial infection often proves to be a challenge. In recent years, greater effort has been made to develop a class system (table 1.1) to determine the level uterine tissue involvement and severity of infection (Sheldon *et al.* 2006). This has aided in the development of treatment strategies for combating infection and will be discussed in more detail shortly. These cattle are more problematic to diagnosis and typically lead to an increased culling rate (Sheldon *et al.* 2006).

Table 1.1 Uterine infection classification and their associated definitions (Sheldon et al. 2006).

Class	Clinical Definition
Puerperal Metritis	Acute systemic illness due to uterine infections. Defined by fetid red-brown watery uterine discharge and pyrexia within the first 10 days postpartum. Typically associated with cows with abnormal parturition.
Clinical Endometritis	Presence of purulent (>50% pus) or mucopurulent (50% pus/50% mucus) uterine exudate in the vagina 21 days postpartum and without systemic signs
Subclinical Endometritis	Endometrial inflammation determined by cytology in the absence of purulent fluid in the vagina
Pyometra	Accumulation of purulent or mucopurulent fluid within the uterine lumen causing distention and associated with an active <i>corpus luteum</i> and a closed cervix

Role of Eicosanoids and Progesterone Following Parturition

PGF_{2α}* and *PGE₂

Following parturition and uterine involution, PGF_{2α} plays a permissive role for resuming estrous cyclicity (Lindell *et al.* 1982; Kindahl *et al.* 1992). In cattle that are free of bacterial infection, PGF_{2α} is a normal component of the estrous cycle causing lysis of the *corpus luteum* (CL) to remove negative effects of progesterone for subsequent ovulation to occur (figure 1.2).

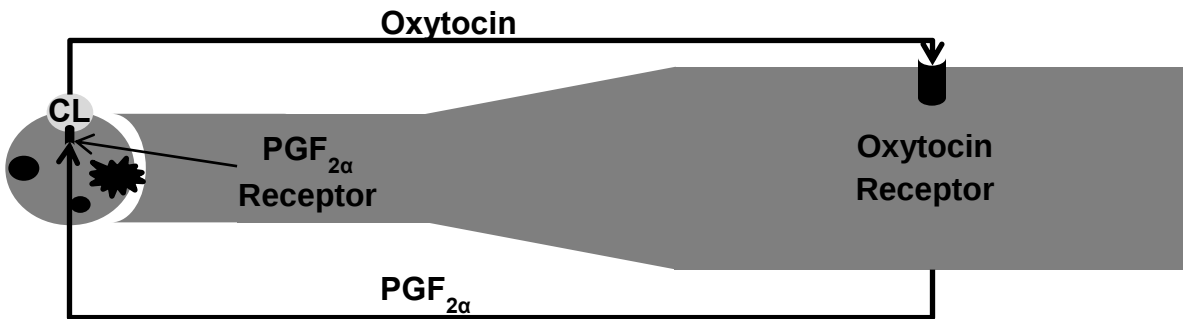


Figure 1.2 Luteolytic feedback loop between the uterine epithelium production of PGF_{2α} and CL derived oxytocin.

Bacterial infections have been shown to have varying and contradicting effects on PGF_{2α}. These impacts range from a reduction of PGF_{2α} release or shorter duration of release (Bekana *et al.* 1996; Lewis 2003) to an increase level of PGF_{2α} metabolites indicating a greater level of release (Lindell *et al.* 1982; Regassa and Noakes 1999). A decrease in production level or time may lead to negative consequences on involution. It is accepted that PGF_{2α} causes smooth muscle contraction along with its known luteolytic functions. These contractions are important for the involution process. In addition, PGF_{2α} functions as part of the immune system leading to inflammation of tissues that works to recruit other immune cells to the site of injury. It is possible that during an infection, PGF_{2α} functions in an immune role, rather than in a reproductive role. To this end, PGF_{2α} would not cause luteolysis of the *corpus luteum* (CL) resulting in prolonged life of the CL leading to further and more severe infection. Efforts in sheep and cattle (Lindell *et al.* 1982; Regassa and Noakes 1999) have indicated an increased level of PGFM, a metabolite of PGF_{2α}, from ewes with bacterial infection of the uterus during involution. This evidence would support the role of PGF_{2α} as an immune modulator rather than facilitating restoration of the estrous cycle.

Indeed, this immune modulatory role of PGF_{2α} is supported by a several additional authors in both sheep and cattle species. Furthermore, the broad class of eicosanoids that PGF_{2α} belongs to have varying degrees of immune functioning and can be regulated by disease state. Of the eicosanoids, PGF_{2α} and prostaglandin E₂ (PGE₂) appear to have the largest impact on uterine health status and play antagonistic roles to each other.

From the literature it appears that a relationship does exist for increased levels of $\text{PGF}_{2\alpha}$ metabolites in the blood supply when uterine infections are present. To test this hypothesis, numerous laboratories have evaluated the level of circulating 13,14-dihydro-15-keto-prostaglandin $\text{F}_{2\alpha}$ (PGFM) during natural or induced uterine disease states (Del Vecchio *et al.* 1992; Seals *et al.* 2002; Lewis 2003; Kaneko and Kawakami 2009). These authors all indicated that PGFM concentrations are elevated when uterine infections are present. These increased levels of PGFM support two hypothesis of $\text{PGF}_{2\alpha}$ role during the postpartum period. First, $\text{PGF}_{2\alpha}$ has a positive impact on the chemotaxis and activity of neutrophils (Hoedemaker *et al.* 1992a; Hoedemaker *et al.* 1992c) but only during the follicular phase of the estrous cycle (Del Vecchio *et al.* 1992; Ramadan *et al.* 1997). In fact, it has been well understood since the 1950s that immune function is decreased during the luteal phase (Black *et al.* 1953; Rowson *et al.* 1953) and will be discussed more shortly. Secondly, the increased levels of PGFM validate an increase in $\text{PGF}_{2\alpha}$ during an infectious state but the interestrus interval is increased compared to healthy animals (Del Vecchio *et al.* 1992). This finding is in contrast to the “normal” role of $\text{PGF}_{2\alpha}$ to cause luteolysis of the *corpus luteum* (CL) to allow for ovulation to occur. These findings further illustrate that $\text{PGF}_{2\alpha}$ is being released in a more tonic fashion rather than a pulsatile release that is needed for luteolysis CL (Kaneko and Kawakami 2009).

In contrast to $\text{PGF}_{2\alpha}$, an additional subset of literature exists investigating the role of PGE_2 during uterine infections. In general, PGE_2 has a luteotrophic effect on the CL along with anti-inflammatory immune roles (Slama *et al.* 1991). Furthermore, PGE_2 works to reduce the natural phagocytic and bactericidal properties of neutrophils (Slama *et al.* 1991; Hoedemaker *et al.* 1992b; Roper *et al.* 1994). It is also curious to realize that bacterial strains may impact the level of production of both PGE_2 and $\text{PGF}_{2\alpha}$. This ability would indicate that these strains have the ability to control uterine environment to allow for increase survivability and increased infection. This ability works in agreement that infections are more severe and immune functions are decreased during the luteal phase (Black *et al.* 1953; Rowson *et al.* 1953). Perhaps, the bacteria are driving the production of PGE_2 in an effort to extend the life of the CL in order to promote their survival.

However, this ability also appears to be specific to certain strains of bacteria rather than a general fact. For example, *Escherichia coli* (*E. coli*) infections appear to switch production of uterine endometrial cells from PGF_{2α} to PGE₂ (Herath *et al.* 2009). This is of importance because *E. coli* is a commonly found environmental pathogen that can play a predisposing role for further uterine infections (Herath *et al.* 2009). This suggestion becomes possible when combined with the luteotrophic and anti-immune functions of PGE₂ previously described. Thus, it would appear that *E. coli* can direct the uterine environment to promote bacterial survival and infection. In contrast, *Arcanobacterium pyogenes* (*A. pyogenes*) infections result in increased levels of PGFM in circulation (Kaneko and Kawakami 2009). This would indicate that the immune response to *A. pyogenes* would be increased in an effort to remove the infection. However, as mentioned previously, the release of PGF_{2α} appears to be a tonic rather than a pulsatile release. In the research performed by Kaneko and Kawakami (2009), a small percentage of cows ovulated a first wave dominate follicle following intrauterine infusion of *A. pyogenes* which led to increased levels of PGFM. However, more cows did not ovulate in response to this increased level indicating that the released PGF_{2α} is serving an immune function rather than a luteolytic role. In fact Kaneko and Kawakami (2009) actually reported a reverse in endocrine profiles of the cows that ovulated. They reported that cows that ovulated actually had higher levels of PGE₂ metabolites. This may be indicative of a threshold PGF_{2α}/ PGE₂ ratio rather than an absolute role of PGE₂.

Progesterone (P₄)

The negative effects of P₄ on uterine health and uterine infections have been explored since the early 1950's (Black *et al.* 1953; Rowson *et al.* 1953). These two groups compared the immune responses of the uterus during the follicular and luteal phases of the estrous cycle in response to sterile semen (Black *et al.* 1953) and bacterial-infected semen (Rowson *et al.* 1953). Rowson and co-workers (1953) were concerned that embryo transfer techniques might lead to an increased uterine infection since embryos were transferred on day 7 of the luteal phase. To further characterize the endocrine hormones that would elicit increased infections, they ovariectomized cows and then supplemented cows with estrogen or estrogen and progesterone combined

followed by insemination with infected semen on day 7 of the luteal phase. The cows supplemented with estrogen alone did not develop clinical infections while the cows supplemented with the combination of estrogen and progesterone developed clinical infections. This early work has led to further efforts to characterize effects of progesterone on the immune functions of the uterus. More recent efforts by Lewis (2003) utilizing similar techniques in ewes that were ovariectomized and supplemented with progesterone found that both neutrophils and lymphocyte migration and production appeared to be decreased during high progesterone states. Their assumptions are based on the indirect relationship of these two immune cells in the circulation during low progesterone states. They assume that during low progesterone conditions, neutrophils and lymphocytes are moving toward the site of infections and would not be in the circulation. However, a direct measure of the neutrophils in the uterus was not performed and thus highlights a weakness in this particular research. Thus, it appears that the immune response of the uterus is under the control of $\text{PGF}_{2\alpha}$, PGE_2 , and P_4 . In addition, bacteria can play a role to manipulate the conditions needed for optimal growth and infection, creating a dynamic interrelationship between the endocrine and immune systems.

Follicular Dynamics are impacted by Uterine Bacterial Infections

Beyond the local uterine environment, bacterial infections can affect the endocrine system to alter the follicular dynamics both at the ovarian level and at the control center of the hypothalamus/pituitary glands. The recruitment, growth, selection, and ovulation of follicles containing an oocyte are a well-orchestrated interaction between endocrine components of the hypothalamus-pituitary-ovary axis (reviewed by Diskin *et al.* 2003; Arrais and Dib 2006). It is generally accepted that gonadotropin releasing hormone (GnRH) from the hypothalamus is secreted into the portal system of the pituitary stalk and is transported to the anterior pituitary to cause the secretion and release of either luteinizing hormone (LH) or follicle stimulating hormone (FSH) (reviewed by Diskin *et al.* 2003). Secretion of FSH acts in an endocrine fashion at the ovarian level to cause a cohort of follicles to be recruited for growth (reviewed by Diskin *et al.* 2003). Next, LH is responsible for the continued growth of the follicles until a

dominate follicle is selected and estrogen production increases to a threshold level causing a pulsatile release of LH resulting in ovulation (reviewed by Diskin *et al.* 2003). The determining factor for the secretion of either LH or FSH is dependent upon the negative or positive feedback of estrogen from the dominant follicle at the ovarian level. Disruptions in this feedback system could lead to delayed or perturbed ovulation rates following parturition if bacterial infections are present (Sheldon *et al.* 2002).

Efforts performed by Slama and co-workers (1991) during a broad study of PGE₂ effects in postpartum cattle found that intrauterine infusions of PGE₂ lead to increased uterine infections and decreased follicular activity. In particular, treated cows had fewer numbers of smaller follicles than the control group. However, a difference did not exist for diameter of the largest follicle or in ovulation between the two groups. This can possibly be explained by the fact that these cattle were early postpartum and had not resumed normal estrous cyclicity. Taken in this context, the decreased number of growing follicles becomes indicative of decreased follicular functioning if bacterial infections are present. In contrast, an additional source evaluated the effect of *E. coli* upon the preovulatory surge of LH in cattle (Peter *et al.* 1989). This group infused *E. coli* endotoxin into the uterus of cows and followed the growth and ovulation via ultrasonography. They report that all of the control cows ovulated during the time periods expected while the treated cows developed follicular cysts that persisted for 7 to 21 days. Regardless, both experiments indicate that bacterial infections disrupt the normal follicular patterns of cattle and other research exists to support this as well (Battaglia *et al.* 1999; Battaglia *et al.* 2000; Williams *et al.* 2001; Sheldon *et al.* 2002; Herath *et al.* 2007). However, what is not understood from these previously discussed groups are the exact mechanisms that are impaired.

A subset of relatively more recent research has been conducted in an aim to determine at what level the bacterial infections interrupt follicular dynamics (Battaglia *et al.* 1999; Battaglia *et al.* 2000; Williams *et al.* 2001; Sheldon *et al.* 2002; Herath *et al.* 2007). Work performed by Battaglia and co-workers (2000) illustrated that endotoxin has multiple effects on the normal follicular process in ewes. Following exposure to endotoxin, the estradiol increase is delayed leading to a delayed LH surge. This surge was not completely abolished but rather was delayed compared to control ewes and

ovulations still occurred just in a delayed fashion. In addition, follicular dynamics seemed to be restored during the following cycle as all ewes ovulated normally. The treated ewes had a longer interestrus interval compared to the controls, but this is possibly a product of a delayed ovulation from the previous cycle. In contrast to this, research performed by the same group a year earlier (Battaglia *et al.* 1999) caused both a delay and abolished LH surge depending on timing of endotoxin infusion. These efforts attempted to identify specific time points in the LH surge induction that endotoxin elicited the greatest effect. First, ewes were challenged for 30 hours to mimic the entire processing time needed to produce an LH surge and results are similar to that of the previously described experiment (Battaglia *et al.* 2000). Both studies confirm the LH surge was delayed but not blocked in ewes (Battaglia *et al.* 1999; Battaglia *et al.* 2000). Secondly, estradiol supplementation was provided to ovariectomized ewes for 14 hours followed by an endotoxin challenge. This experiment determined if an “initial” reading time exists in which elevated estradiol is absolutely crucial for LH surge initiation and then estradiol is not required later during the surge induction. These efforts were unable to delay or block the LH surge, indicating that after 14 hours of elevated estradiol, an LH surge will occur regardless of infection (Battaglia *et al.* 1999). Finally, an effort was made to evaluate the effect of endotoxin and estradiol supplemented together during the early 14 hour period to assess the importance of the elevated estradiol during an “initial” reading time point. This procedure blocked the LH surge in 62% (5 of 8 treated) ewes, and indicates a required time point for increased estradiol on the LH surge. This requirement was further validated in a repeated sampling period in the same literature (Battaglia *et al.* 1999). However, the question still remains, does endotoxin disrupt hypothalamic sensitivity to estrogen to produce GnRH, is the pituitary sensitivity to GnRH impaired, or is the decreased amount of estradiol of follicular origin the cause of the impaired LH surges?

From a previous effort, Williams and co-workers (2001) noticed circumstantially that GnRH pulses were not impacted by endotoxin while the LH surge was. This led them to a targeted effort to evaluate GnRH specifically. Their first experiment showed the normally expected relationship of GnRH pulse leading to a LH surge in control animals; while endotoxin treated ewes had a reduced LH pulse while GnRH pulse was

similar to controls. Furthermore, in the presence of endotoxin, LH pulses were absent or reduced even when additional exogenous GnRH was supplemented. Endotoxin directly affects the sensitivity of the pituitary to respond to GnRH provided that sufficient estradiol is present to generate a GnRH pulse (Battaglia *et al.* 1999; Battaglia *et al.* 2000) . So it would appear that the effects of bacterial infection play a role at the pituitary level. In addition, it appears that it may play a role at the ovarian level if altered follicular growth leads to reduced estradiol production.

In fact, research has been performed targeting the effect of bacterial infection on the local ovarian environment. In contrast to the previously described works (Peter *et al.* 1989; Slama *et al.* 1991), Sheldon *et al.* (2002) reported a decrease in the size of the dominant follicle of cows with a high bacterial score on day 7 postpartum. This in turn would lead to a decreased level of estradiol in circulation. Indeed, cows with a high bacteria score had reduced circulating estradiol levels on days 15 and 16 compared to cows with low bacteria scores.

Finally, Herath and co-workers (2007) identified the specific cells of the follicle that were impacted by *E. coli* infections. Within the theca and granulosa cells of the follicle, a two-cell theory has been established for the production of estradiol (Fortune and Quirk 1988). Briefly, the theca cells are responsible for the production of androgens, while the granulosa cells express aromatase activity and can convert androgens to estradiol. Thus, it is important to look at the effects of bacterial endotoxins on both cell lines. To this end, Herath *et al.* (2007) determined that *E. coli* infections did not affect theca cell numbers or production of androgens. However, mRNA for aromatase production in the granulosa cells was reduced and would lead to a decrease in estradiol production by these cells. At the same time, granulosa cell numbers and LH and FSH receptor mRNA were not impacted. It is important to note also that lipopolysaccharide (LPS) levels, that indicate *E. coli* infections, are elevated in follicular fluid (Herath *et al.* 2007). This confirms that components of bacterial infections are small enough to be transported into the follicle and stimulate the reported detrimental effects.

Treatment Strategies for Uterine Bacterial Infections

Antibiotics and Prostaglandin F_{2α}

It has been commonly recognized that uterine infections has a negative impact on reproductive performance and has been further illustrated by the previous discussion. In fact, it has been reported that cows with subclinical endometritis had a 70% increase in pregnancy rate when treated with a PGF_{2α} analog (Kasimanickam *et al.* 2005) and further highlights the impact of uterine infections on reproductive performance. The unfortunate aspect of uterine infection is agreement on treatment procedures for resolution. A great deal of research exist evaluating varying treatment options under differing conditions (Smith *et al.* 1998; Tenhagen and Heuwieser 1999; Drillich *et al.* 2001; LeBlanc *et al.* 2002; Sheldon and Dobson 2004a; Kasimanickam *et al.* 2005; Goshen and Shpigel 2006; Drillich *et al.* 2007; Galvão *et al.* 2009; Kaufmann *et al.* 2010; Brick *et al.* 2012). Though this is a small subset of the vast amount of research available, they still highlight the competing information that has been documented on treatment efficacy. One consistent point that appears is that animals that are left untreated have a greater rate of pregnancy failure than treated animals. Thus, the argument can be made that it is more important to treat regardless of protocol than it is to leave untreated. In addition, stage of the estrous cycle may dictate the protocol used. In general, research that compared PGF_{2α} analogs to antibiotic treatments showed no difference in infection resolution (LeBlanc *et al.* 2002; Kasimanickam *et al.* 2005; Kaufmann *et al.* 2010). However, if a CL is present it is recommended to use a PGF_{2α} or PGF_{2α} analog treatment protocol to remove the CL and aid in uterine clearance (Lewis 1997a; Sheldon and Dobson 2004a). This technique would agree with the above literature review, in that the CL, through progesterone, is inhibiting the natural immune response and that removal will aid in uterine infection clearance.

Literature Summary

Evidence does exist for altered pH to impact fertility in the bovine at day 7 of the estrous cycle. The effect may be mediated by increased $\text{PGF}_{2\alpha}$ in response to acidic pH, direct effects of pH on the developing embryo, or a combination of both. In addition, the acidic uterine pH is a result of increased plasma urea nitrogen in the circulation. This increase is due to increased levels of crude protein in the diet above what is required by the animal. These altered pH environments have been shown to impact the development of *in vitro* oocytes and embryos in multiple species. However, limited work has been performed on the bovine recipient animal to assess pH of the uterus on day 7 of the estrous cycle at the exact time and location of transfer.

Furthermore, it has also been shown that bacterial challenges can affect fertility of cattle. Specifically, bacterial infections alter normal physiological events, proper functioning of the immune system, and endocrine aspects of the hypothalamus-pituitary-ovary axis. Realizing that a majority of cattle experience infections postpartum allows for speculation that these factors may combine with altered pH environments to effect fertility of the bovine recipient animal. Limited work exists in regards to recipient information (Rhoads *et al.* 2006) and to date it appears no effort has been made to measure pH of the bovine uterus at the time and location of embryo transfer. Other models have used plasma urea nitrogen levels as their assessment of altered uterine environments. Thus, a need exists to characterize the pH, bacterial, and the tandem effects of these two factors on the environment of specific locations of the bovine reproductive tract.

Chapter 2. Characterization and Association of Uterine Horn and Vaginal pH and Temperature Relationships with Pregnancy Rates in Bovine Recipient Animals

Abstract

Pregnancy rates following embryo transfer are a key factor in the profitability and improved genetic gains in today's cattle industry. The recipient animal accounts for the majority of this pregnancy failure due to suboptimal selection criteria based solely on expression of estrual behavior and presence of a corpus luteum seven days following estrus. Thus, the need for additional selection tools to better identify recipient animals is needed. Two experiments characterized uterine pH and temperature in association with vaginal pH and temperature, while a third determined vaginal pH and temperature effects on pregnancy rate. Bovine recipients in experiment one ($n = 120$) indicated that a strong vaginal to uterine pH association ($r = 0.72$, $P < 0.0001$) and a strong vaginal to uterine temperature association ($r = 0.63$, $P < 0.0001$) existed. However, vaginal and ipsilateral uterine horn pH values did not differ in cows that were pregnant versus non-pregnant ($P = 0.37$ and 0.98 , respectively). Similarly, vaginal, ipsilateral uterine horn, and rectal temperatures were not different in pregnant versus non-pregnant animals ($P = 0.58$, 0.93 , and 0.91 , respectively). Techniques to measure uterine environment decreased pregnancy rates (27 versus 55%, $P = 0.03$; pregnant versus non-pregnant, respectively). Experiment two, utilizing abattoir collected reproductive tracts ($n = 43$) further validated the presence of a uterine to vaginal association for pH ($r = 0.59$, $P < 0.0001$) and temperature ($r = 0.81$, $P < 0.0001$). Logistic regression models in experiment three ($n = 136$ recipient animals) predicted the likelihood of pregnancy based on vaginal pH ($P = 0.15$), vaginal temperature ($P = 0.3$), and rectal temperature ($P = 0.15$). From these models, groups were established and effects on pregnancy rates were analyzed. No difference was seen between acidic, neutral, or alkaline pH groups on recipient pregnancy rates ($P = 0.42$). Similarly, no effects were seen for high, middle, or low vaginal and rectal temperature range groups for pregnancy rates. Data for experiments one and two were combined to assess impacts related to location of embryo deposit, transfer score (reflection of transfer difficulty), and time spent in recipients to transfer embryo and collect data. Embryos deposited more cranially in the uterus were more likely to establish pregnancy ($P = 0.08$). The lower degree of difficulty in transfers (score 1) also tended to establish more pregnancies ($P = 0.07$). In conjunction with these, the shorter amount of time spent transferring embryos and

collecting data increased pregnancy rates ($P = 0.03$). Thus, associations are present between vaginal and ipsilateral uterine horn pH and temperature. Traditionally accepted variables of influence (transfer difficulty, location of embryo deposit in uterine horn, and time in cow) continue to impact pregnancy success.

Introduction

The goal of any beef operation is to maximize production while controlling costs to improve profitability. With the development of a commercial embryo transfer program in the late 1980's, new technologies have become increasingly available to the beef producer (Betteridge 2003); however, the efficiency of these techniques have remained unchanged (Hasler 2006). Specifically, the largest source of variability related to embryo transfer (ET) is pregnancy establishment in the bovine recipient animal (McMillan 1998; Stroud and Hasler 2006). Across the literature, recipient pregnancy rates after ET are quite variable and range from 34 to 72 percent (Spell *et al.* 2001a; Sartori *et al.* 2006; Vasconcelos *et al.* 2006). Currently, the method for identifying an acceptable recipient is limited to display of estrus and presence of a corpus luteum on day 7 of the estrous cycle (Spell *et al.* 2001b). Little effort has been applied to develop tools to improve recipient identification and highlights an area of needed efforts.

It has been well characterized that increasing levels of crude protein (CP) in bovine diets has an inverse relationship with pregnancy rates in artificial inseminations mating systems (Elrod and Butler 1993; Butler 1998; Kane *et al.* 2004). These diets are positively associated with increased levels of plasma urea nitrogen (PUN) in circulation (Elrod and Butler 1993). Within and specific to the uterus only, increased PUN levels lead to an acidic pH environment (Elrod and Butler 1993; Elrod *et al.* 1993). Specific to bovine embryo transfer is the knowledge that this effect of pH fluctuations is most notably seen on day 7 of the estrous cycle and coincides with timing of embryo transfers. Normally, the pH of the uterus shifts from a neutral to a slightly acidic pH at estrus with a return to near neutral by day 7 of the estrous cycle (Elrod and Butler 1993; Perry and Perry 2008b; Perry and Perry 2008a; Ozenc *et al.* 2010). However, in the case of cattle fed excess levels of CP, this return to neutral is absent causing the uterus to remain more acidic on day 7 compared to control animals (Elrod and Butler 1993;

Butler 1998). The acidic uterine environment lead to a 20% reduction in artificial insemination pregnancy rates (Elrod *et al.* 1993).

Impacts of altered pH environments have been clearly demonstrated through *in vitro* culture models in multiple species (Dale *et al.* 1998; Edwards *et al.* 1998; Lane *et al.* 1998; Lane *et al.* 1999; Lane and Bavister 1999; Phillips *et al.* 2000). These efforts indicate that negative effects of altered extracellular pH directly impact the developmental ability of oocytes and embryos. Influences on cellular organization of organelles and cytoplasmic structure have been suggested as the base for this developmental disruption (Ramalho-Santos *et al.* 2009). In addition, it has been hypothesized that altered pH levels can interfere with the secretion and direction of secretion of prostaglandin F_{2α} (PGF_{2α}) from uterine endometrial cells (Gilbert *et al.* 1996). Endometrial cells produced and secreted increased amounts of PGF_{2α} in a uterine luminal direction (Gilbert *et al.* 1996; Butler 1998). Increased PGF_{2α} would then pose direct negative effects on the embryo (Scenna *et al.* 2008a; Scenna *et al.* 2008b). Together, these two lines of evidence would indicate direct negative effects of altered pH for embryonic survival in an *in vivo* environment exist.

The majority of past efforts have focused on the impacts of acidic uterine environments in natural mating and artificial insemination programs (Elrod and Butler 1993; Butler *et al.* 1996; Butler 1998; Perry and Perry 2008b; Perry and Perry 2008a). Furthermore, considering that undefined factors allow some embryo transfer recipients to outperform others (Stroud and Hasler 2006) prompts the additional need to characterize these animals. Past efforts only utilized circulating plasma urea nitrogen concentrations as an indirect indicator of altered uterine pH levels. Given the absence of direct characterization of recipient uteri, emphasizes the need to directly characterize the uterine pH environment on day 7 of the estrous cycle. To our knowledge, no work exists that measured uterine pH at the site and time of embryo transfer in the bovine recipient. Thus, the hypothesis was developed that altered uterine pH values on day 7 of the estrous cycle will impact the pregnancy success of bovine recipient animals. Multiple experiments were conducted to assess this impact. First, a pH measurement system was developed to allow pH reading to be collected at the site of embryo transfer along with other compartments of the reproductive tract to assess relationships between

compartments. Pregnancy success was compromised during experiment one as a result of our techniques. Therefore, study two explored the relationship of uterine pH and temperature using the same probe in both locations. Based on these findings the third experiment assessed the impact of vaginal pH on pregnancy in bovine recipients.

Material and Methods

Development of pH measurement system

The first step to evaluate the current hypothesis was to develop a pH measurement system able to capture uterine pH and temperature data at the site and time of embryo deposit. This system utilized an esophageal pH probe (MicroElectrodes Inc., Bedford, NH, USA), a reference pH probe (Fisher Scientific, Suwanee, GA, USA), and a thermistor (Measurement Specialties, Shrewsbury, MA, USA), to measure and record pH and temperature of the reproductive tract, respectively. Collectively, these instruments will be referred to as the uterine probe. The uterine pH probe was housed in a retrofitted embryo transfer sheath (PETS, Inc., Canton, TX, USA) for protection during the embryo transfer process. The metal tip of the sheath was removed and a small hole was drilled into the tip to allow for the pH probe to be inserted. The pH probe was held in place by the size of the cable inside the sheath and with a small amount of epoxy in the end of the sheath opposite the metal tip to prevent backing out (figure 2.1). This allowed the cable to flex along with the sheath and the attached embryo transfer gun to prevent probe damage and accidental removal. The thermistor utilized one of the pre-fabricated holes for embryo deposit located in the metal tip of the sheath (figure 2.1). The thermistor was passed through one of these holes and attached using heat conductive epoxy to decrease the amount of time needed to record a temperature reading. The wire and cables of the thermistor were held in place similarly to the pH probe (figure 2.1). The uterine probe was fitted to a standard embryo transfer gun to allow for data collection at the site and time of embryo deposit. The esophageal probe is a single cell measurement pH probe only. Therefore, a reference probe inserted into the vagina is required to complete the system. Values for pH and temperature were visualized on an Orion 3 Star portable pH meter (Thermo Scientific, Beverly, MA, USA).

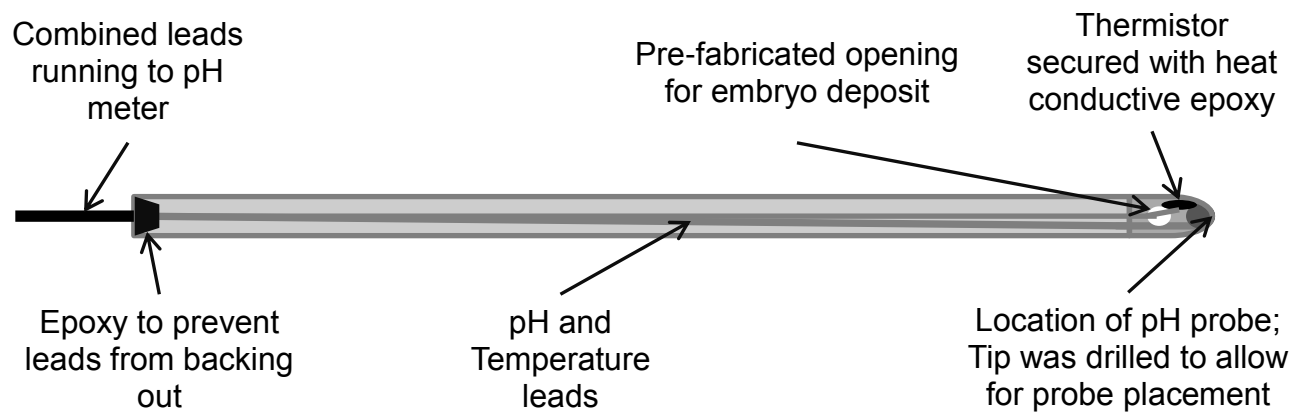


Figure 2.1 Schematic of uterine pH probe in retrofitted embryo transfer sheath.

This study was performed at three University of Tennessee research and education centers across the Tennessee. All procedures were performed in accordance with the approval of the University of Tennessee IACUC. Experiments 1 and 3 were performed in concurrent succession over a three year period.

Experiment 1: Determination of uterine to vaginal relationships with uterine probe

The first experiment (n=120) was performed to determine pH and temperature correlations between the uterine horn ipsilateral to the corpus luteum and the vagina. In addition, impacts of data collection technique, pH values, and temperature values on recipient pregnancy rate were also evaluated. Recipient cows (3-10 years of age; Angus and Angus crossbred, Gelbvieh and Gelbvieh crossbred; body condition score of 5 to 8 on a 1 to 9 scale (Richards *et al.* 1986)) from three research centers (East Tennessee Research and Education Center, Knoxville TN; Plateau Research and Education Center, Crossville TN; Highland Rim Research and Education Center, Springfield TN) were synchronized using an Eazi-Breed CIDR (1.38 grams progesterone, Zoetis, Florham Park, NJ, USA) vaginal insert for 8 days. A single injection of prostaglandin (Lutalyse, 25 mg dinoprost tromethamine, Zoetis, USA) was administered one day prior to insert removal. Detection of estrus (visual observation 2x daily; estrus alert patches (Estroject, Rockway Inc., Spring Valley, WI, USA); or Heat Watch system (CowChips, Manalapan, NJ, USA) occurred for 84 hours post implant removal with embryo transfer occurring approximately 7 days after estrus was displayed.

At time of embryo transfer, recipient cows were restrained and administered a 3mL epidural of lidocaine (LidoJect, 2% lidocaine/mL, Henry Shein Animal Health, Dublin, OH, USA) prior to manipulation. The external vulva, rectum, tail, and surrounding area were cleaned with a mild disinfectant diluted in water. Recipient's ovaries were scanned with an Aloka 900 (Hitachi-Aloka Medial, Wallingford, CT, USA) ultrasound with a 7.5 MHz linear transducer for presence of a corpus luteum (CL). The location of the CL (side; left or right ovary), size, and presence of cavity were documented to allow for luteal tissue volume estimation. Recipients possessing a normal CL and not associated with follicular or luteal cysts received an embryo. Depending on availability, fresh and frozen embryos were transferred (by experience technicians) using standard nonsurgical techniques via embryo transfer gun that had been fitted with the uterine probe (as discussed previously). Once at the site of transfer, the reference pH probe (Fisher Scientific, Suwanee, GA, USA) was inserted into the vagina of the cow to complete the circuit to allow a pH to be recorded.

Initially, the embryo transfer (ET) gun and associated probes were inserted and the pH reading was allowed to stabilize for data collection. Following the first several transfers, this technique was adjusted to record a pH reading every 15 seconds for 1 minute and a temperature reading at 1 minute (figure 2.2). This adjustment was in response to an increased amount of time required for a stable pH reading. Typically, pH is a measurement of hydrogen ions in a solution (Pool 2004) and the uterine environment possess very little liquid or solution (Hugentobler *et al.* 2004; Hugentobler *et al.* 2007). Therefore, the 15 second intervals were developed to decrease the amount of time spent in each recipient. Following embryo deposition, the uterine probe was retracted to the vagina and a similar technique was performed to capture vaginal pH reading and temperature in the caudal region of the vagina (near the vaginal fornix, figure 2.2) with the uterine probe. Between cows, the probe was rinsed with de-ionized water and maintained in a standard solution of 7 pH. Finally, a rectal temperature and blood sample were collected on each recipient prior to exiting the chute. Blood samples were maintained on ice or under refrigeration and centrifuged allowing for serum storage for progesterone analysis. Each day, a subset of recipients did not have pH measurements taken to serve as a positive control to test the impact of extra

manipulation on pregnancy status. Elapsed time to complete transfer and collect data from recipient animals ranged from 2 to 30 minutes.

The uterine probe and Orion 3 Star portable pH meter (Thermo Scientific) were calibrated beginning, during, and end of each transfer day in known standards (Fisher Scientific) of 4, 7, and 10 pH (uterine probe intra- and inter- coefficients of variations were 1.2% and 3%, respectively).

Values of pH from each of the 4 time points (15, 30, 45, and 60 seconds) were pooled and averaged for each location within the reproductive tract. Uterine and vaginal temperature readings were recorded in Celsius while rectal temperature (GLA M525/550 Hi Performance Digital Thermometer, GLA Agriculture Electronics, San Luis Obispo, CA, USA) was recorded in Fahrenheit and converted to Celsius for analysis.

Pregnancy diagnosis was performed approximately 30 days post transfer using an Aloka 900 (Hitachi-Aloka Medial) ultrasound with a 7.5 MHz linear transducer. Pregnancy diagnosis was subsequently confirmed with a second pregnancy diagnosis at approximately 180 days of gestation and final confirmation based on calving date.

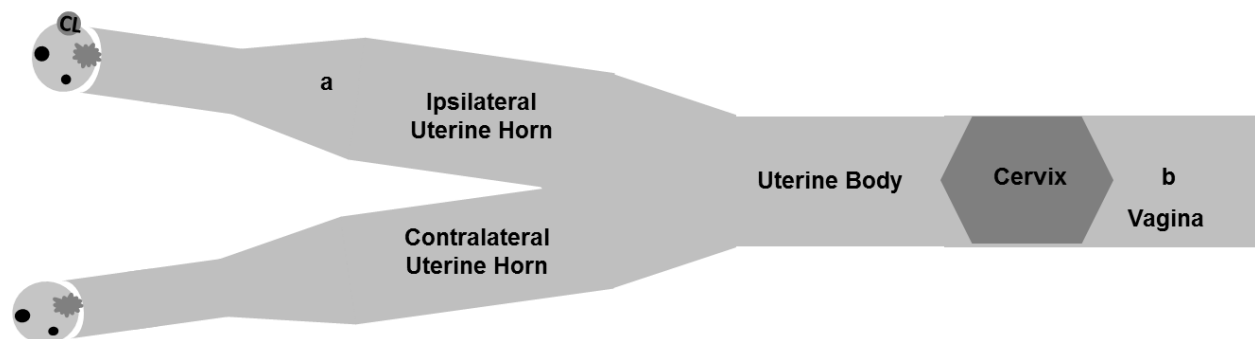


Figure 2.2 Diagram of locations within the reproductive tract where pH and temperature measurements were recorded. Location A and B corresponds to the approximate locations of pH and temperature measurements within the ipsilateral uterine horn and cranial vagina, respectively.

Experiment 2: Validation of uterine to vaginal relationships with field probe

The objective of experiment two was to validate uterine to vaginal relationship using abattoir collected bovine reproductive tracts ($n = 43$). Data collection for experiment two was performed in conjunction with a companion experiment (Chapter 3). However, only the pH and temperature values are reported in this experiment. Tracts were recovered approximately 15 to 45 minutes post sacrifice and identified using visual evaluation for pregnancy status and presences of an approximately day 7 corpus luteum (CL) using a method similar to Ireland and co-workers (1980). Furthermore, tracts were selected on a gross normal appearance to avoid potential cases of clinical metritis. The side, size, color, and external presence of a cavity were documented for each CL. Following characterization of CLs, pH and temperature readings were collected in three compartments of the reproductive tract (upper third of the contr- and ipsilateral horns in relation to the CL and vagina, figure 2.3). Each location was first swabbed with an iodine scrub solution and allowed to briefly dry. Then a scalpel blade was used in a stabbing motion to pierce the tract in each location. The scalpel was then rotated and incision was made using an “in to out” motion. The incision was held open with a large set of tweezers allowing pH and temperature readings to be collected. A 2 cell pH probe (Thermo Scientific, Beverly, MA, USA) and Orion 3 Star portable pH meter (Thermo Scientific) were used to capture pH readings. This probe was termed the “field probe” since it would serve as a model for future field applications. A pH reading was recorded every 15 seconds for one minute and a temperature reading recorded at the end of one minute. Between measurement collections the probe was rinsed with de-ionized water and maintained in pH buffer of 7 (Fisher Scientific). Prior to use each day, the probe and meter were calibrated in known standards of 4, 7, and 10 pH (Fisher Scientific) and the calibration slope was recorded (inter- coefficients of variations were 1.2%).

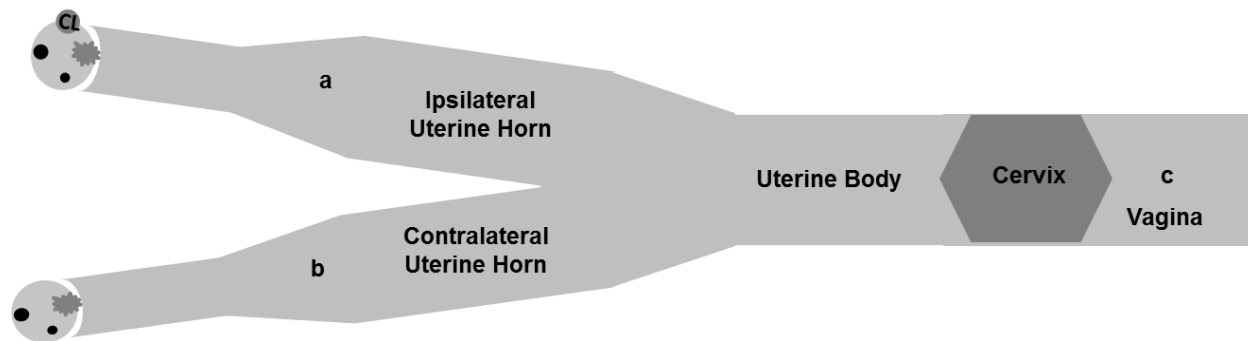


Figure 2.3 Diagram of locations within the reproductive tract where pH and temperature measurements were recorded. Location A, B, and C corresponds to the approximate locations of pH and temperature measurements within the ipsilateral uterine horn, contralateral uterine horn, and cranial vagina, respectively.

Experiment 3: The relationship of vaginal pH to pregnancy status of recipients

Recipient animals (n = 136) in experiment three were handled similarly to those in experiment one. The recipient pool (3-10 years of age; Angus and Angus crossbred, Gelbvieh and Gelbvieh crossbred, Holstein; body condition score of 5 to 8 on a 1 to 9 scale for beef (Richards *et al.* 1986) and 1 to 5 scale for dairy (Edmonson *et al.* 1989)) were from three research centers (East Tennessee Research and Education Center, Knoxville TN; Plateau Research and Education Center, Crossville TN; Highland Rim Research and Education Center, Springfield TN) over a one and one half year period. Recipients were synchronized using an Eazi-Breed CIDR (1.38 grams progesterone, Zoetis, Florham Park, NJ, USA) vaginal insert for 8 days. A single injection of prostaglandin (Lutalyse, 25 mg dinoprost tromethamine, Zoetis, USA) was administered one day prior to insert removal. Detection of estrus (visual observation 2x daily; estrus alert patches (Estroject, Rockway Inc., Spring Valley, WI, USA); or Heat Watch system (CowChips, Manalapan, NJ, USA) occurred for 84 hours post implant removal with embryo transfer occurring approximately 7 days after estrus was displayed.

At time of embryo transfer, recipient cows were restrained and administered a 3mL epidural of lidocaine (LidoJect, 2% lidocaine/mL, Henry Shein Animal Health, Dublin, OH, USA) prior to manipulation. The external vulva, rectum, tail, and

surrounding area were cleaned with a mild disinfectant diluted in water. Recipient's ovaries were scanned with an Aloka 900 (Hitachi-Aloka Medial, Wallingford, CT, USA) ultrasound with a 7.5 MHz linear transducer for presence of a corpus luteum (CL). The location of the CL (side; left or right ovary), size, and presence of cavity were documented to allow for luteal tissue volume estimation. Recipients possessing a normal CL and not associated with follicular or luteal cysts received an embryo. Depending on availability, fresh and frozen embryos were transferred (by experience technicians) using standard nonsurgical techniques. Following transfer, the field probe (Thermo Scientific) was inserted approximately 6-8 inches into the vagina to capture pH (every 15 seconds for 1 minute) and temperature (at 1 minute) observations (figure 2.2). Between cows, the probe was rinsed with de-ionized water and maintained in a standard solution of 7 pH. The field probe and Orion 3 Star portable pH meter (Thermo Scientific) were calibrated (beginning, during, and end of each transfer day) in known standards (Fisher Scientific) of 4, 7, and 10 pH (field probe intra- and inter- coefficients of variations were 0.7% and 1.3%, respectively).

Finally, a rectal temperature and blood sample were collected on each recipient prior to exiting the chute. Blood samples were maintained on ice or under refrigeration and centrifuged allowing for serum storage for progesterone analysis. Each day, a subset of recipients did not have pH measurements taken to serve as a positive control to test the impact of extra manipulation on pregnancy status. Elapsed time to complete transfer and collect data from recipient animals ranged from 2 to 30 minutes. Pregnancy status based on the amount of time needed to complete transfers and record data was evaluated. Recipients from both experiments 1 and 3 were combined to determine effects of excess manipulation on pregnancy status. As a result, groups were established as those cows that required 14 to 25 (high, n=48), 10 to 13 (middle, n=61), and 6 to 9 minutes (low, n=33).

Values of pH from each of the 4 time points (15, 30, 45, and 60 seconds) were pooled and averaged for each location within the reproductive tract. Uterine and vaginal temperature readings were recorded in Celsius while rectal temperature (GLA M525/550 Hi Performance Digital Thermometer, GLA Agriculture Electronics, San Luis Obispo, CA, USA) was recorded in Fahrenheit and converted to Celsius for analysis.

Animals were confirmed pregnant at day 30 post transfer by ultrasonography and verified based on calving dates.

Progesterone Assay

For experiments one and three, radioimmunoassays for progesterone (intra- and inter- coefficients of variations were 2.56% and 8.56%, respectively) were performed with Coat-A-Count progesterone kits (Siemens, Washington, D.C., USA). Recipients with progesterone levels below 2ng/ml at time of embryo transfer were removed from analysis (n= 14). In addition, luteal volume was estimated using techniques previously described (Lüttgenau *et al.* 2011), recipients with an estimated luteal volume below zero were discarded from analysis (n= 2).

Statistical Analysis

Experiment 1: Determination of uterine to vaginal relationships with uterine probe

All statistical analysis was performed using SAS 9.4 (SAS Institute, Cary, NC, USA). Extreme outliers for pH (below 4.7 and above 10.0 uterine pH; below 6.02 and above 10.7 vaginal pH), time in recipient (below 5 and above 26 minutes), and progesterone concentration (below 1.9 ng/mL) were removed from the data set. For experiment one, the relationship between the ipsilateral uterine horn and the vagina was assessed using mixed model ANOVA (GLIMMIX with an ilink back transformation). Similarly, the effects of ipsilateral uterine horn and vaginal pH and temperature effects on recipient pregnancy rates were assessed with mixed model ANOVA (GLIMMIX with an ilink back transformation). GLIMMIX is required due to the binomial distribution of pregnancy data and the ilink back transformation returns least squares means estimates back to percent pregnant estimates. In addition GLIMMIX can also be used to evaluate normally distributed data and was thus used for all ANOVA tests in experiment one. Multiple ANOVA models were evaluated utilizing a “put and take” method of variable selection. A mixed model ANOVA was performed evaluating ipsilateral uterine horn and vaginal pH and temperature as the dependent variables with pregnancy status as a fixed effect. This provided mean separation and average mean pH and temperature values of each location. Following this, pH and temperature value groups

were categorized using the PROC Univariate function. From this pH and temperature groups were segregated based on quartiles produced by PROC Univariate (upper 25%, high; middle 50%, mid; bottom 25%, low). From this an additional mixed model ANOVA was performed with pregnancy status as the dependent variable and pH and temperature as fixed effects.

The additional mixed model ANOVA evaluating the effect of additional manipulation of pregnancy status was performed. This model contained pregnancy status as the dependent variable with the “treatment group” variable as a fixed effect. This model essentially evaluated the positive control influence on pregnancy rates. All mixed model ANOVA results are presented as least squares means plus standard error of the means.

Following ANOVA modeling, the linear relationships of ipsilateral uterine horn environment to the vaginal environment was performed with PROC CORR producing simple correlations. Correlations results are presents as the correlation r value with corresponding p values.

Experiment 2: Validation of uterine to vaginal relationships with field probe

Extreme outliers for progesterone concentration (below 1.0 ng/mL) were removed from the data set. Correlation analysis was performed using Pearson correlation in SAS 9.4 (SAS Institute, Cary, NC) and presented as the correlation r value with resulting p values. The following variable combinations were analyzed for correlations: ipsilateral horn pH and temperature, contralateral horn pH and temperature, and vagina pH and temperature.

Experiment 3: The relationship of vaginal pH to pregnancy status of recipients

Extreme outliers for pH (below 4.7 and above 10.0 uterine pH; below 6.02 and above 10.7 vaginal pH), time in recipient (below 5 and above 26 minutes), and progesterone concentration (below 1.9 ng/mL) were removed from the data set. For experiment three, the effect of vaginal pH and temperature effects on recipient pregnancy rates were assed with mixed model ANOVA (GLIMMIX with an ilink back transformation). GLIMMIX was required due to the binomial distribution of pregnancy

data and the ilink back transformation returns least squares means estimates back to percent pregnant estimates. In addition GLIMMIX can also be used to evaluate normally distributed data and was thus used for all ANOVA tests in experiment three. Multiple ANOVA models were evaluated utilizing a “put and take” method of variable selection. A mixed model ANOVA was performed evaluating vaginal pH and temperature as the dependent variables with pregnancy status as a fixed effect. The same procedure was utilized for rectal temperature. This provided mean separation and average mean values of each location.

Next, logistic regression utilizing pregnancy status as the dependent variable and multiple variables as the fixed effects was performed. Similar to ANOVA models, logistic regression is used to analyze binomially distributed data such as pregnancy status. Results are presented as slope estimates (untransformed) plus standard error of significant variables. A quadratic polynomial was performed and used to identify optimal ranges for vaginal pH, vaginal temperature, and rectal temperature. This produced pH value groups as acidic (pH 6.89 and below, $n = 19$), neutral (pH 6.9 to 7.39, $n = 54$), and alkaline (pH 7.40 and above, $n = 24$). Three groups for vaginal temperature were low (34°C and below), mid ($34\text{-}35^{\circ}\text{C}$), and high (36°C and above). Similar groups for rectal temperature were established as low (38.4°C and below), mid ($38.5\text{-}39.0^{\circ}\text{C}$), and high (39.1°C and above). Based on the established groups, mixed model ANOVA analysis was performed with pregnancy status as the dependent variable and vaginal pH, vaginal temperature, and rectal temperature as fixed effects.

Finally, from the combined data sets of experiments one and three, multiple variables were evaluated for their influence on pregnancy status as the dependent variable in mixed model ANOVAs. These variables included: embryo donor, sire, fresh or frozen transfer type, embryo stage and quality, tract position/size score (Young *et al.* 2010), transfer location (cranial third, middle third, or caudal third of the uterine horn ipsilateral to the ovary with a CL), transfer score (range of 1 to 3 with 1 being excellent and 3 poor based on difficulty of reaching transfer site and location within the uterine horn), corpus luteum quality (1 to 3 reflecting a good, average, poor quality respectively, modified from Spell *et al.* (2001b)), luteal volume, progesterone concentration, transfer technician, transfer date, and time in cow. Groups were established for the time in cow

variable using PROC Univariate to separate groups based on quartiles as discussed previously (14 to 25 (high, $n=48$), 10 to 13 (middle, $n=61$), and 6 to 9 minutes (low, $n=33$)).

Results

Experiment 1: Determination of uterine to vaginal relationships with uterine probe

A strong positive correlation between vaginal pH and uterine horn pH was discovered ($P > 0.0001$, table 2.1). Uterine horn temperature was strongly and positively correlated to vaginal temperature ($P > 0.0001$, table 2.1). A positive correlation did exist for corpus luteum volume and progesterone concentration ($r = 0.26$, $P = 0.0025$, data not shown).

Vagina and ipsilateral uterine horn pH means did not differ between cows that established a pregnancy versus those that did not establish pregnancy ($P = 0.37$ and 0.98 , respectively; figure 2.4). Similarly, vagina and ipsilateral horn temperature means did not differ between cows that established a pregnancy versus those that did not establish pregnancy ($P = 0.58$ and 0.93 , respectively; figure 2.5). Rectal temperature did not affect pregnancy status of recipient animals ($P = 0.91$, figure 2.5). When vagina and ipsilateral horn pH values were separated into groups based on quartiles, no effect was found on pregnancy rates ($P = 0.45$ and 0.77 , respectively; figure 2.6). Vagina, ipsilateral, and rectal temperature groups were also not different ($P = 0.50$, 0.86 , and 0.60 , respectively; figure 2.7).

The impact of measuring ipsilateral uterine horn pH on pregnancy status was evaluated through the assessment of positive controls. This model revealed that measuring ipsilateral horn pH significantly decreased pregnancy rates of recipients compared to those that did not have ipsilateral uterine horn pH measured ($P = 0.03$, figure 2.8).

Table 2.1 Correlation and associated *p* values of selected pH and temperature values between compartments of the bovine reproductive tract

Uterine Horn Probe	Vagina pH (n = 66)	
	Correlation	<i>P</i> Value
Ipsilateral Uterine Horn pH	0.72	<0.0001
Vagina temperature (n = 66)		
	Correlation	<i>P</i> Value
Ipsilateral Uterine Horn Temperature	0.63	<0.0001

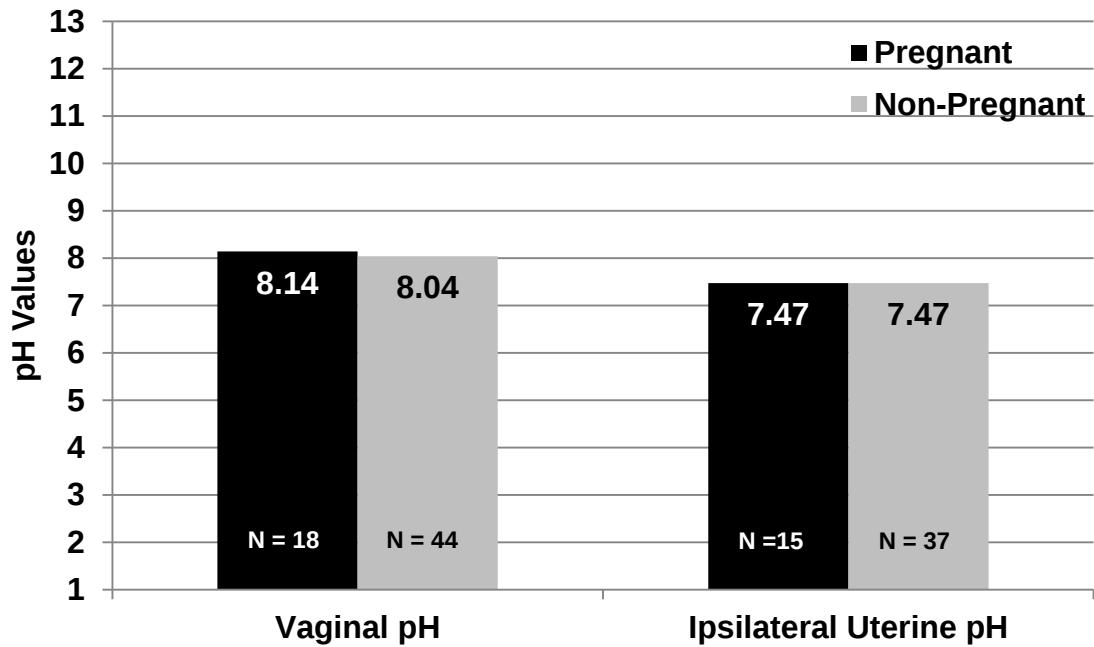


Figure 2.4 pH values of the vagina and ipsilateral uterine horn for pregnant and non-pregnant recipient animals. Bars within groups did not differ ($P = 0.37$ and 0.98 , vagina and ipsilateral uterine horn, respectively). Pooled SEM = 0.08 and 0.07 , vagina and ipsilateral uterine horn, respectively.

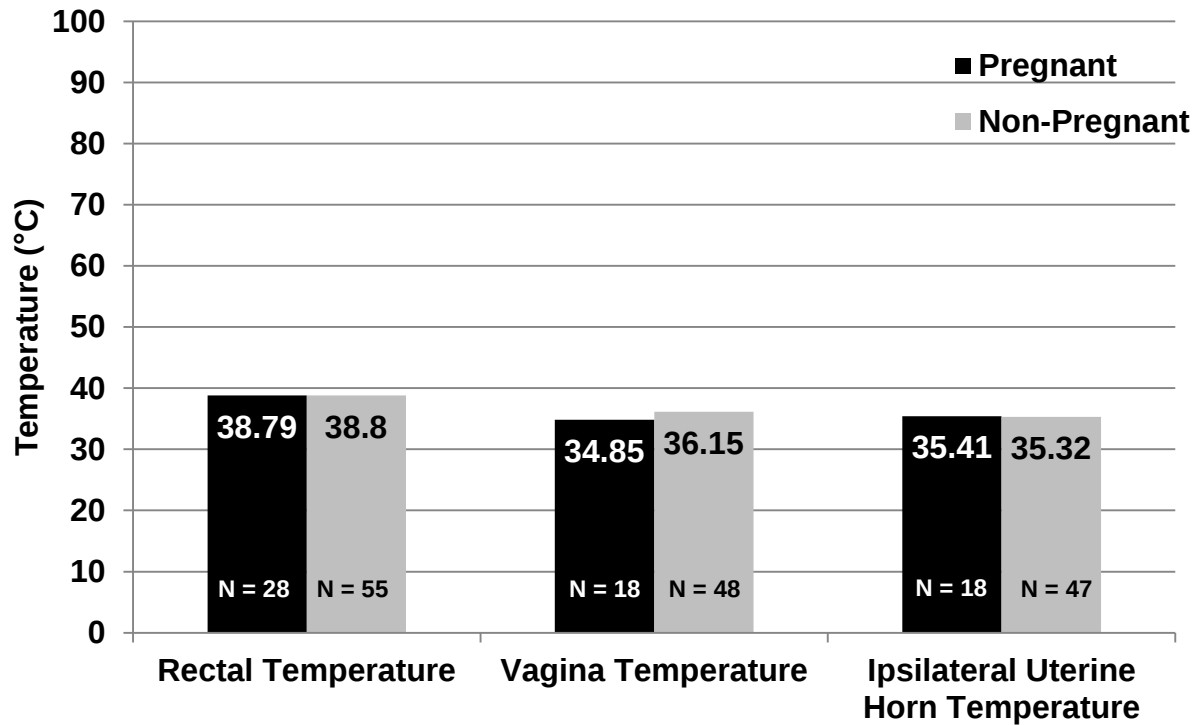


Figure 2.5 Temperature values of the rectum, vagina, and ipsilateral uterine horn for pregnant and non-pregnant recipient animals. Bars within groups did not differ ($P = 0.91$, 0.58 , and 0.93 , rectum, vagina, and ipsilateral uterine horn, respectively). Pooled SEM = 0.06 , 1.60 , and 0.63 , rectum, vagina, and ipsilateral uterine horn, respectively.

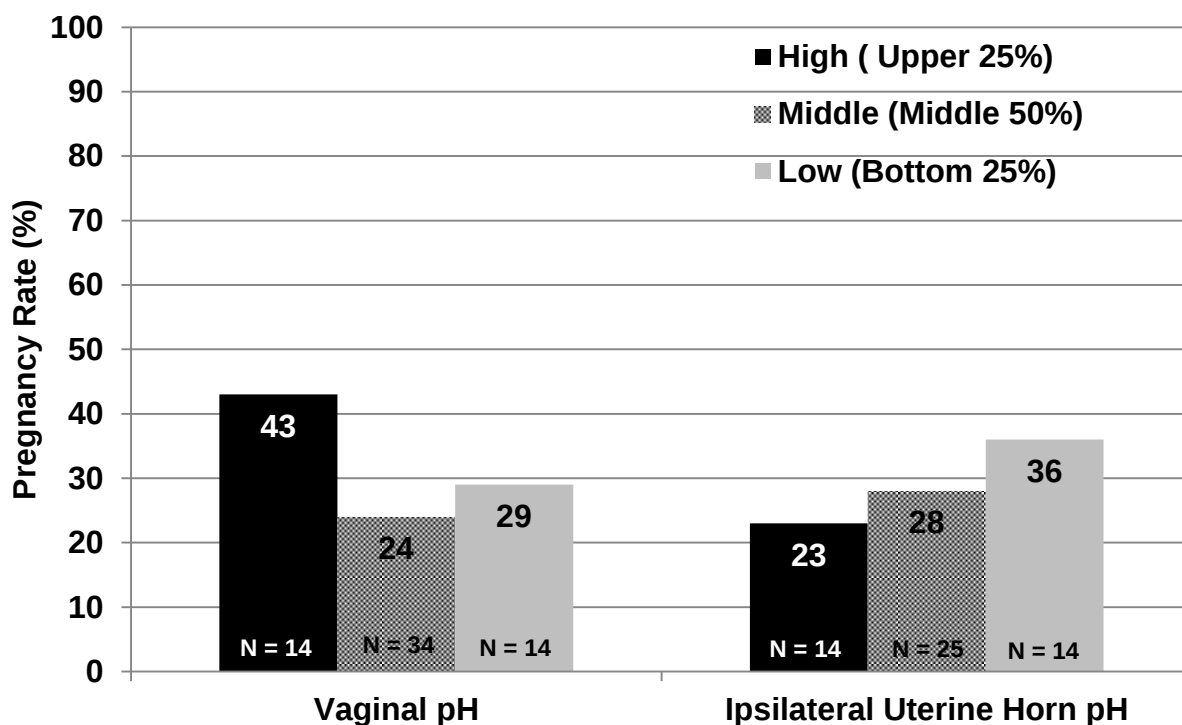


Figure 2.6 Pregnancy rates of recipients based on pH of the vagina and ipsilateral uterine horn pH values separated into groups based on data distribution into the upper, middle, and lower quartiles. For vagina pH high = 8.29 and higher, middle = 7.78 to 8.28, and low = 7.77 and lower; ipsilateral uterine horn pH high = 7.70 and higher, middle = 7.21 to 7.69, and low = 7.20 and lower. Bars within groups did not differ ($P = 0.45$ and 0.77 , vagina and ipsilateral uterine horn, respectively). Pooled SEM = 0.11 and 0.13, vagina, and ipsilateral uterine horn, respectively.

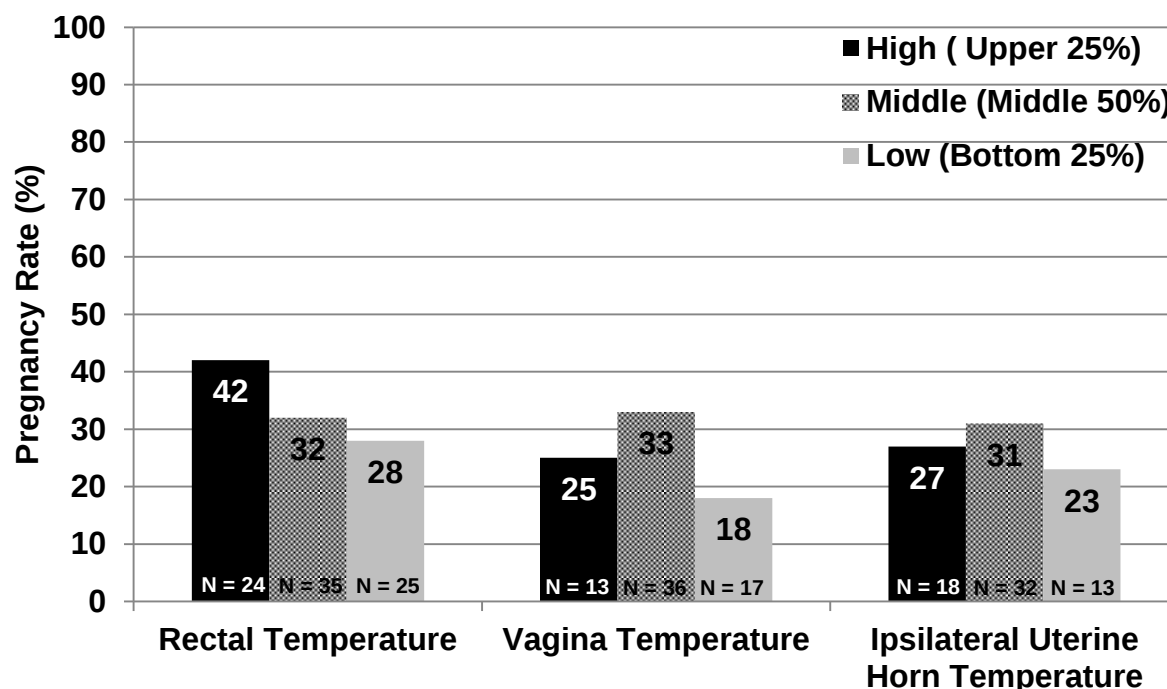


Figure 2.7 Pregnancy rates of recipients based on temperature of the rectum, vagina, and ipsilateral uterine horn with temperature values separated into groups based on data distribution into the upper, middle, and lower quartiles. For rectal temperature high = 39.17 and higher, middle = 38.68 to 39.16, and low = 38.67 and lower; vagina temperature high = 37.97 and higher, middle = 31.61 to 37.96, and low = 31.60 and lower; ipsilateral uterine horn temperature high = 38.28 and higher, middle = 31.81 to 38.27, and low = 31.80 and lower. Bars within groups did not differ ($P = 0.60, 0.50, \text{ and } 0.86$, rectum, vagina, and ipsilateral uterine horn, respectively). Pooled SEM = 0.09, 0.1 and 0.1, rectum, vagina, and ipsilateral uterine horn, respectively.

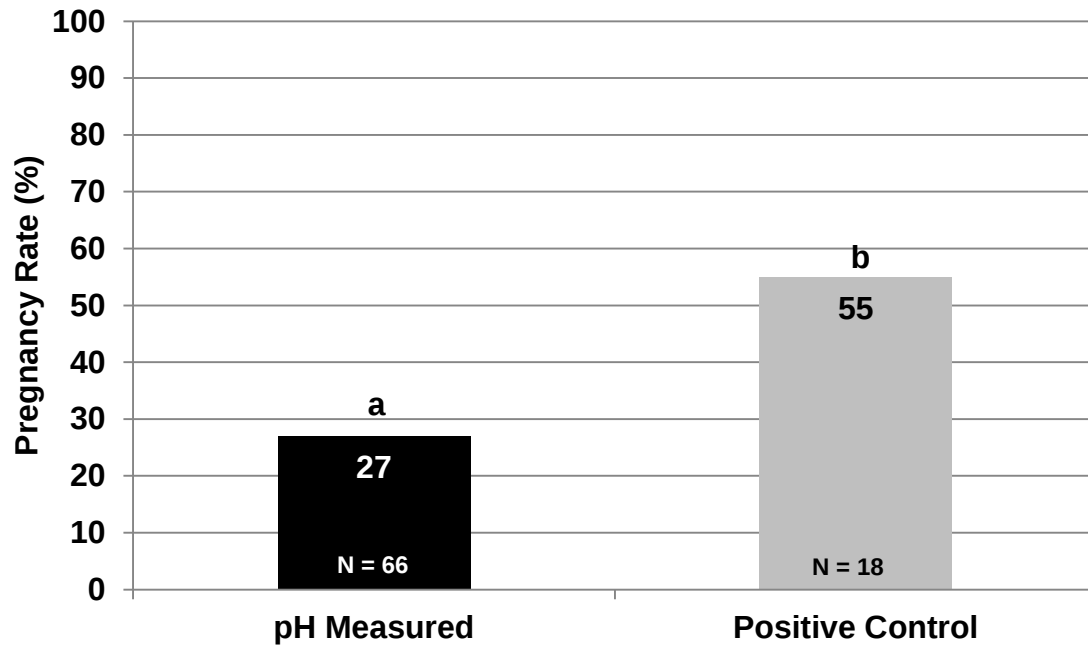


Figure 2.8 Pregnancy rate (%) of recipient animals in which reproductive tract pH measurements were taken compared to recipients that did not have pH measurements recorded (positive controls). Bars within treatments with different superscripts differed (^{a,b} $P = 0.03$). Pooled SEM = 0.06.

Experiment 2: Validation of uterine to vaginal relationships with field probe

Vagina pH values differed from ipsilateral uterine horn and contralateral uterine horn pH means ($P < 0.0001$, table 2.2). Specifically, the vaginal pH was higher (more alkaline direction) compared to the pH of both horns. However, the pH means between the ipsilateral and contralateral horn did not differ. Conversely, temperature means did not differ across tract locations ($P = 0.28$, table 2.2).

Correlations analysis revealed that ipsilateral uterine horn pH was correlated to contralateral uterine horn pH and that both horns were correlated to vagina pH utilizing the field probe (table 2.2). Similarly, ipsilateral uterine horn temperature was correlated to contralateral uterine horn temperature and both horns were correlated to vagina temperature (table 2.2). However, no temperature of any compartment was correlated to any compartmental pH.

Experiment 3: The relationship of vaginal pH to pregnancy status of recipients

Vaginal pH measurements (measured with the field probe) did not differ between recipients that established a pregnancy versus those that did not establish a pregnancy ($P = 0.88$, figure 2.9). Similarly, vaginal temperature and rectal temperature did not differ between recipients that established pregnancy versus those that did not ($P = 0.81$ and 0.60 , respectively, figure 2.10). However, to further explore impacts of vaginal pH, vaginal temperature, and rectal temperature on recipient pregnancy rates, logistic regression modeling was performed, revealing an optimal range of vaginal pH for improved pregnancy probability ($P = 0.15$, figure 2.11). An optimal range for increased pregnancy probability based on vaginal temperature and rectal temperature was also identified ($P = 0.3$ and 0.15 , figure 2.12 and figure 2.13, respectively). Based on the vaginal pH regression model, three pH categories for vaginal pH was established and analyzed utilizing mixed models, revealing no significant differences between groups ($P = 0.42$, figure 2.14). Again, based on these regression models, three temperature categories were established and analyzed with mixed models for effects on recipient pregnancy status. Neither vaginal temperature, nor rectal temperature impacted pregnancy rates of recipients within temperature groups ($P = 0.83$ and 0.59 , figure 2.15, respectively).

Table 2.2 Results of pH and temperature correlation analysis and means vagina, ipsilateral uterine horn, and contralateral uterine horn. Correlation *r* values and associated *p* values of pH and temperature within compartments of the bovine reproductive tract. Locations means are presented as the mean average in that location $\pm$ standard error of the mean (SEM).

Tract Location	Vagina pH	Ipsilateral pH	Contralateral pH	Vagina temperature	Contralateral temperature	Ipsilateral temperature
	(<i>P</i> value)	(<i>P</i> value)	(<i>P</i> value)	(<i>P</i> value)	(<i>P</i> value)	(<i>P</i> value)
Ipsilateral pH correlation	0.59 (0.001)	-	0.63 (<0.0001)	-	-	-
Ipsilateral temperature correlation	-0.04 (0.8)	-0.3 (0.17)	-0.03 (0.86)	0.81 (<0.0001)	0.81 (<0.0001)	-
Contralateral temperature correlation	0.11 (0.51)	-0.38 (0.82)	0.02 (0.87)	1.00 (<0.0001)	-	-
Vagina temperature correlation	0.11 (0.51)	-0.04 (0.82)	0.02 (0.87)	-	-	-
pH & temperature means	6.72 $\pm$ 0.05 ^A	6.35 $\pm$ 0.05 ^A	6.40 $\pm$ 0.05 ^B	28.25 $\pm$ 0.27 ^C	28.25 $\pm$ 0.27 ^C	28.15 $\pm$ 0.27 ^C

Values within pH & temperature means row with different letters differed (^{A,B} $P < 0.0001$; ^C $P = 0.28$)

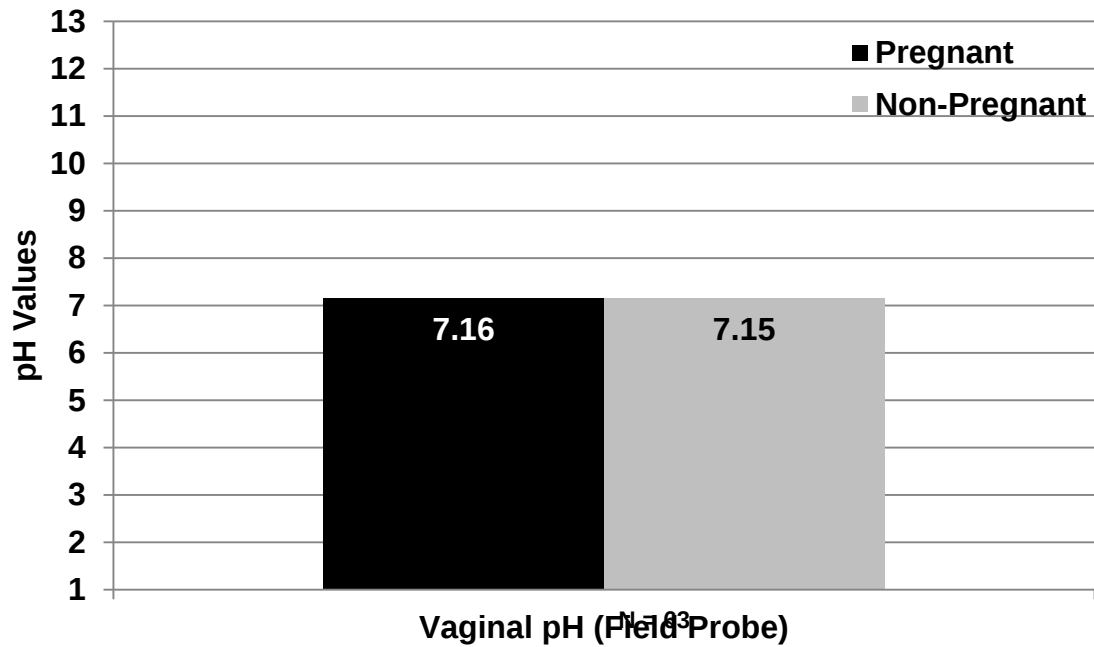


Figure 2.9 pH values of the vagina (measured with field probe) for pregnant and non-pregnant recipient animals. Bars did not differ ($P = 0.88$). Pooled SEM = 0.04.

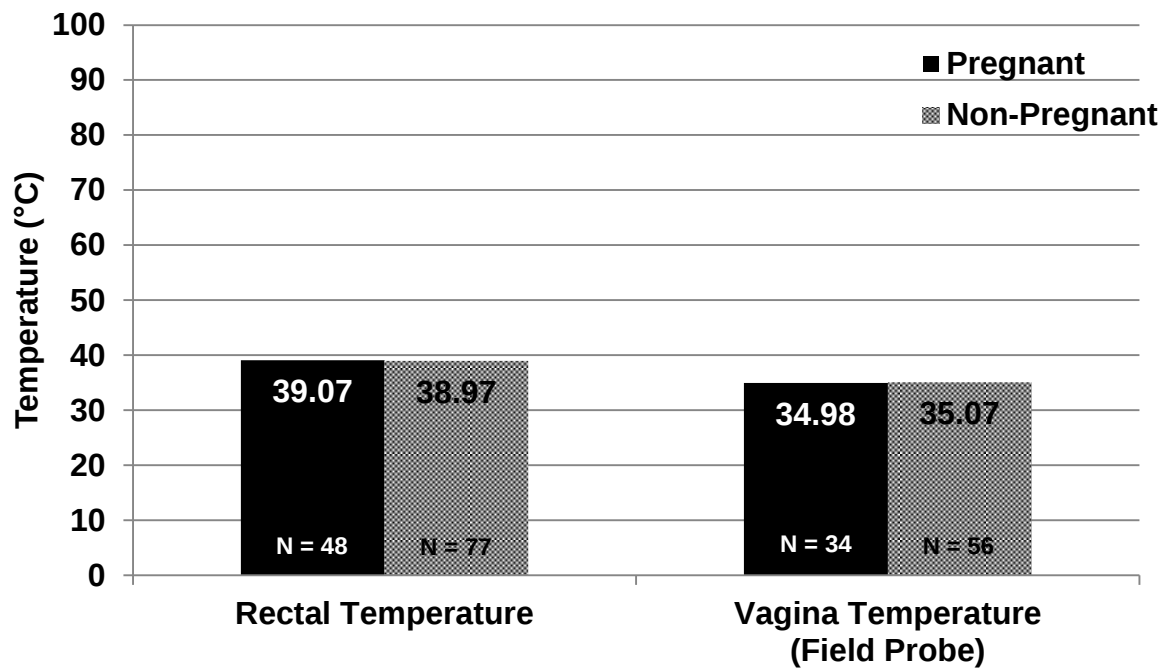


Figure 2.10 Temperature values of the rectum and vagina for pregnant and non-pregnant recipient animals. Bars within groups did not differ ($P = 0.60$ and 0.81 , rectal and vagina temperature respectively). Pooled SEM = 0.13 and 0.24 , respectively.

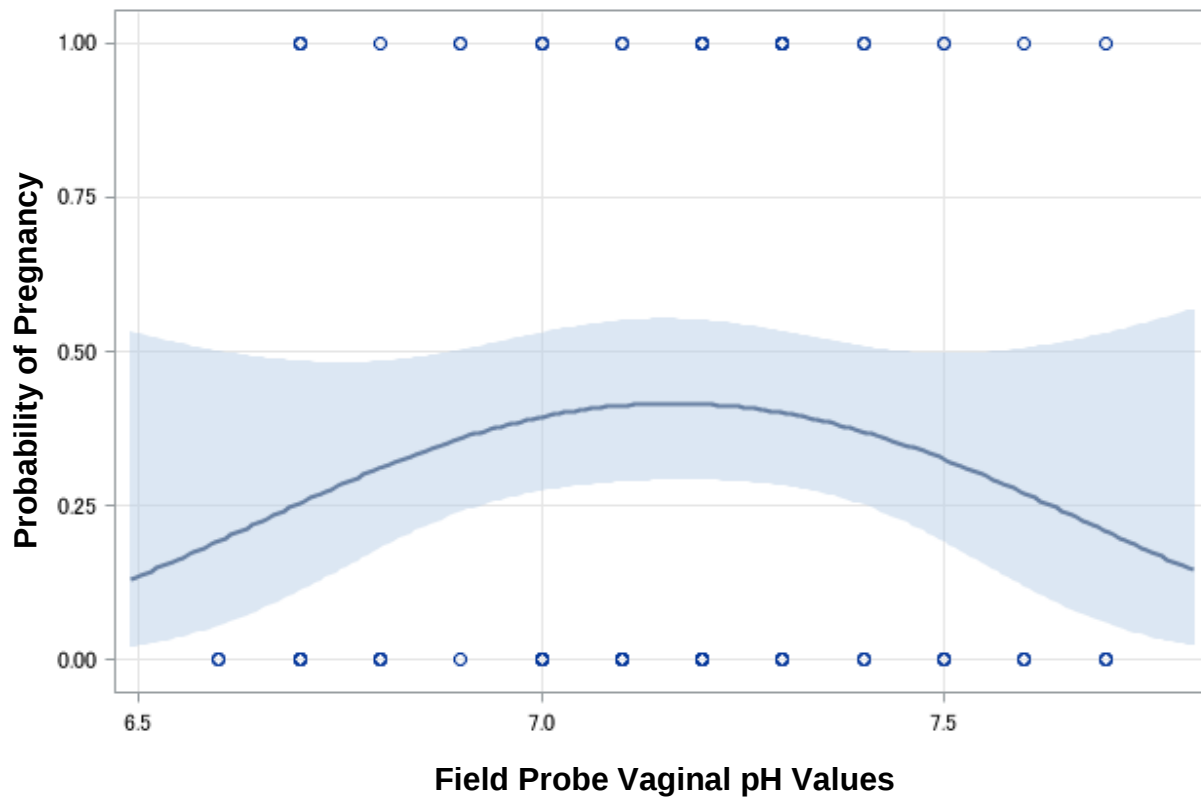


Figure 2.11 Likelihood plot predicting the probability of pregnancy for recipient animals over a vaginal pH range of 6.5 to 8. Slope = -3.44 ± 2.43 ; $P = 0.15$.

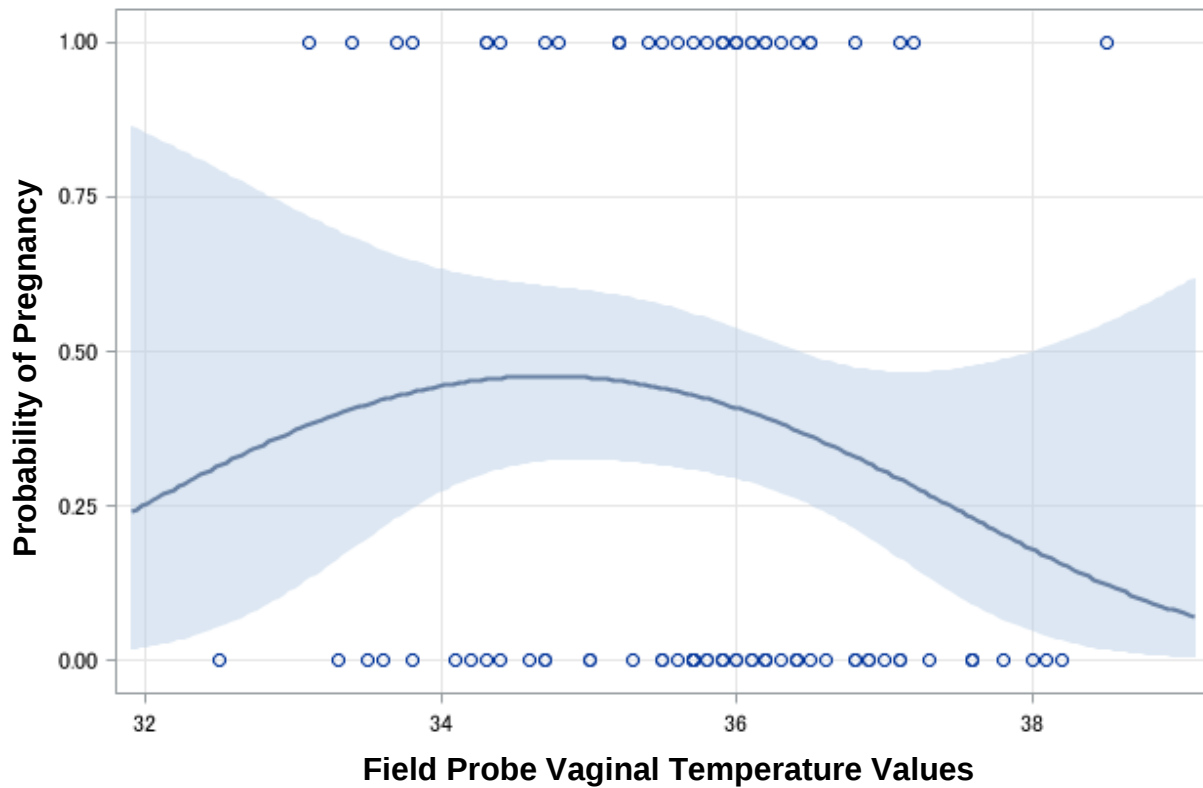


Figure 2.12 Likelihood plot predicting the probability of pregnancy for recipient animals over a vaginal temperature range of 32 to 39°C. Slope = -0.13 ± 0.12 ; $P = 0.30$.

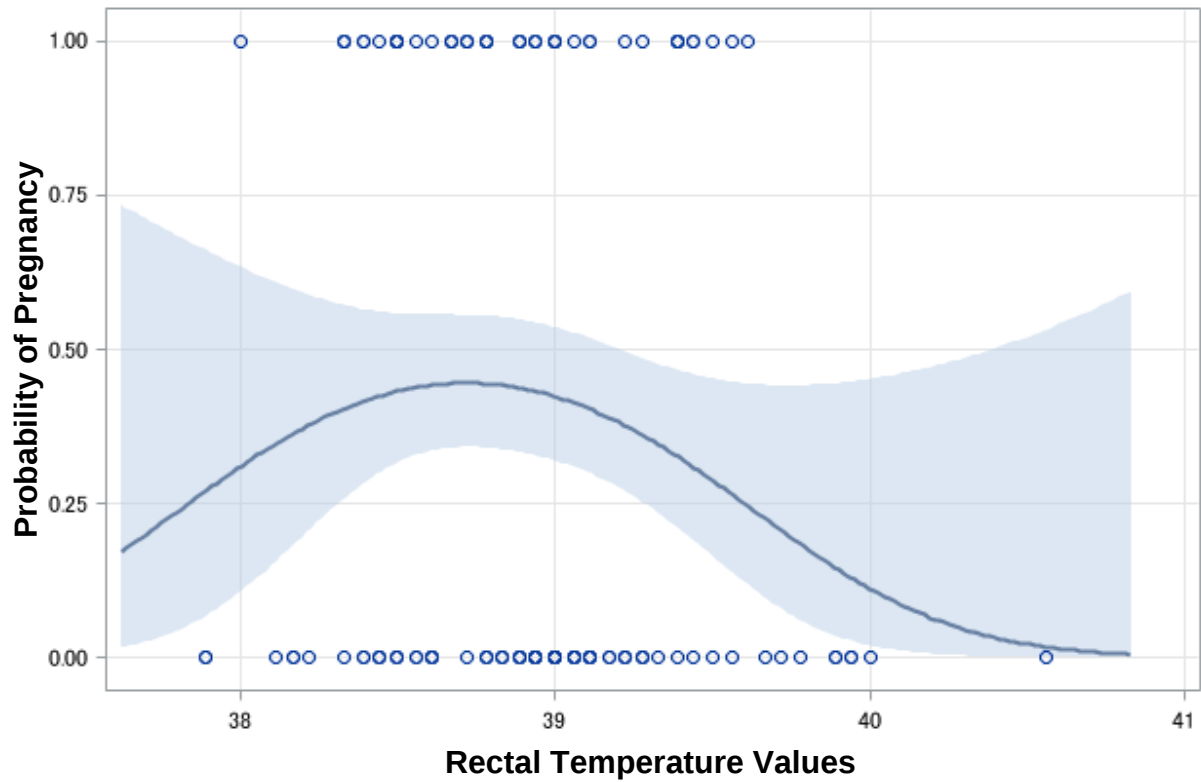


Figure 2.13 Likelihood plot predicting the probability of pregnancy for recipient animals over a rectal temperature range of 37 to 41°C. Slope = -1.13 ± 0.77 ; $P = 0.15$.

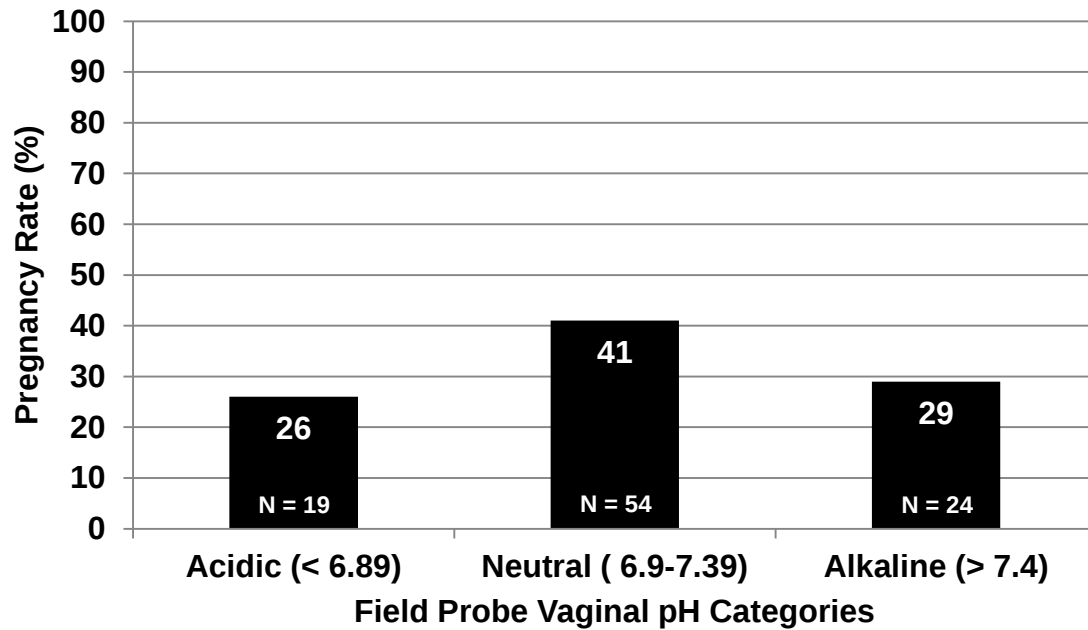


Figure 2.14 Pregnancy rate of recipient animals in which reproductive tract pH measurements were grouped into acidic, neutral, or alkaline pHs. Bars within treatments did not differ ($P = 0.42$). Pooled SEM = 0.09.

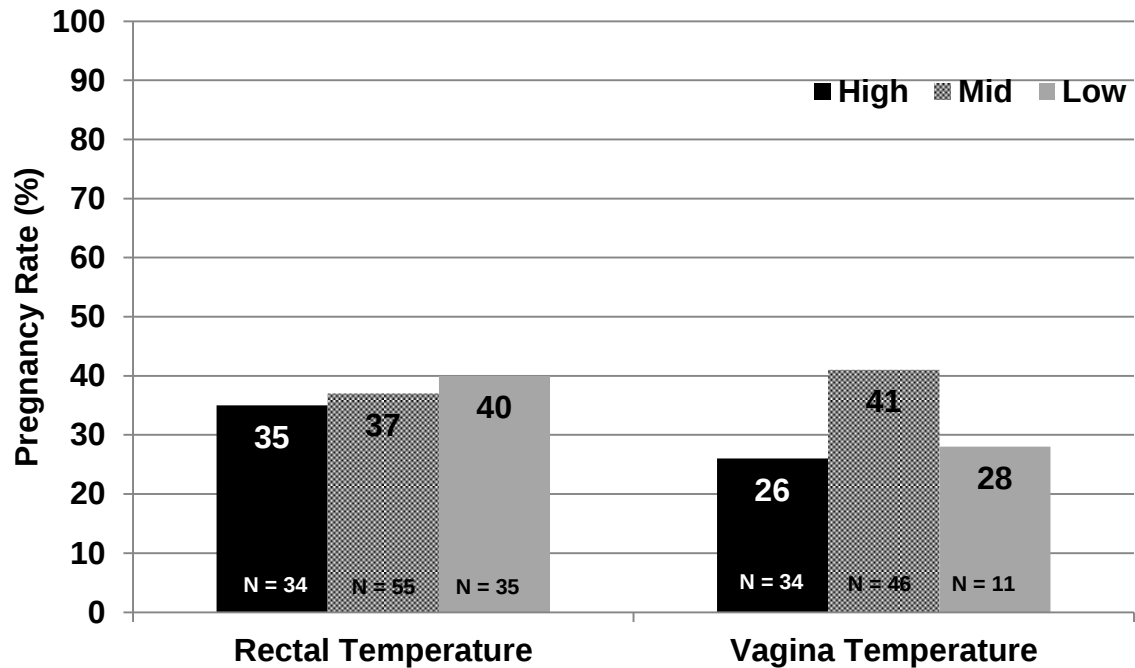


Figure 2.15 Pregnancy rates of recipients based on temperature of the rectum, vagina, temperature values separated into groups. For rectal temperature high = 39.1 and higher, middle = 38.5 to 39, and low = 38.4 and lower; vagina temperature high = 36 and higher, middle = 34 to 35, and low = 33 and lower. Bars within groups did not differ ($P = 0.92$ and 0.16 , rectum and vagina). Pooled SEM = 0.07 and 0.06 , rectum and vagina, respectively.

Pregnancy rate based on location of embryo transfer differed when the embryo was deposited in the upper third (nearest the ovary), middle third (curvature of the horn), or lower third (near the bifurcation) of the uterine horn ($P = 0.08$; figure 2.16). Specifically, the means differed when comparing the upper third to the middle and lower third regions. Similarly, transfer score impacted pregnancy rates ($P = 0.07$, figure 2.17); specifically between transfer score 1 versus 3 ($P = 0.07$; figure 2.17). Finally, the time required (in minutes) to complete embryo transfer and collect data had a significant influence on pregnancy rates. Pregnancy rates of recipients were lower when more than 14 minutes was required compared to cows that required 10 to 13 minutes and less than 9 minutes ($P = 0.03$; figure 2.18). No other models or variables analyzed influenced pregnancy rates.

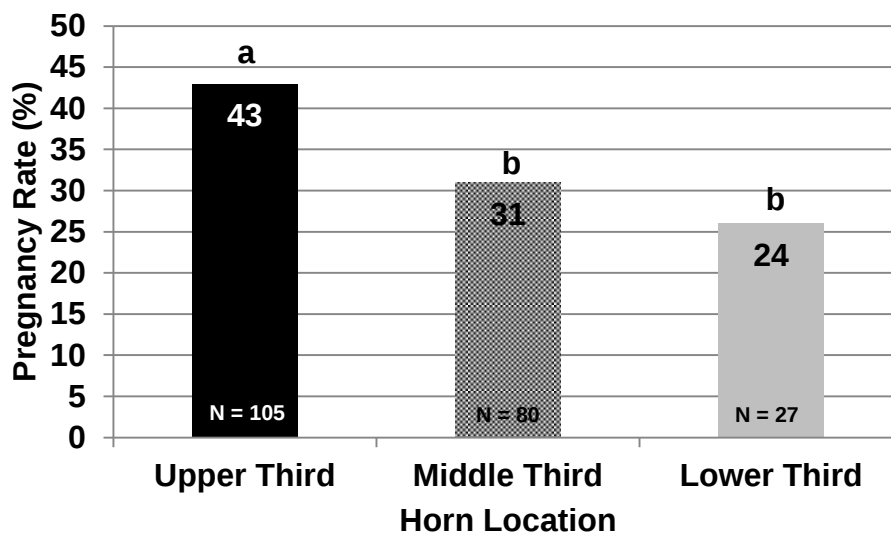


Figure 2.16 Pregnancy rate of recipient animals based on horn location in which the embryo was transferred. Bars within treatments with different superscripts differed (^{a,b} $P = 0.08$). Pooled SEM = 0.06.

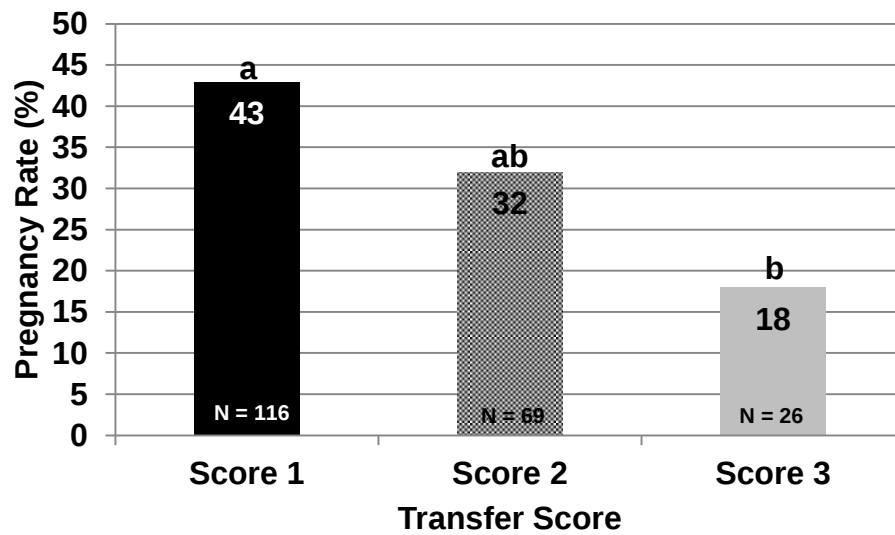


Figure 2.17 Pregnancy rate of recipient animals based on transfer scores. Bars within treatments with different superscripts differed (^{a,b} $P = 0.07$). Pooled SEM = 0.06.

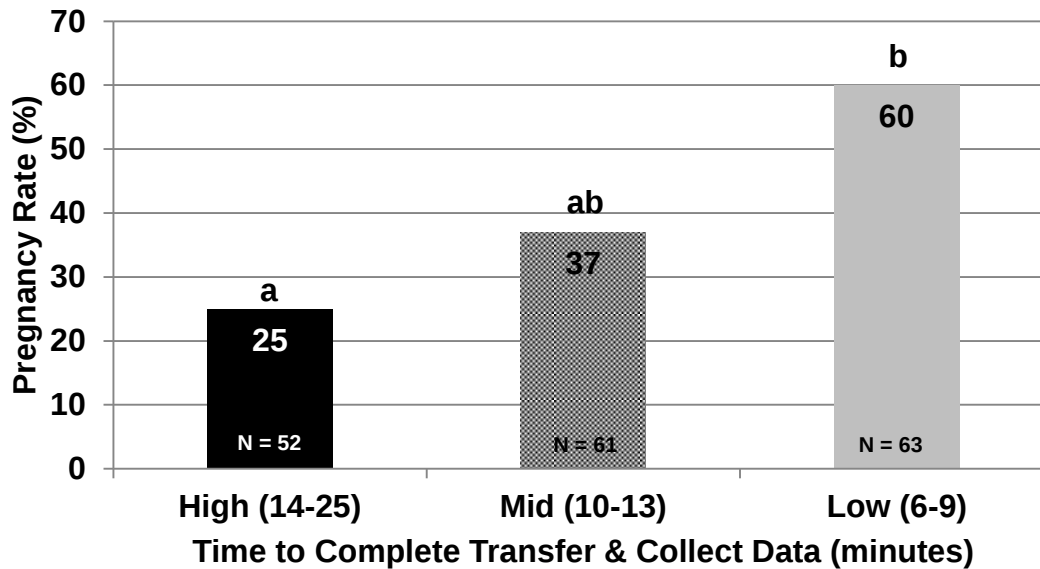


Figure 2.18 Pregnancy rate of recipient animals based on time (minutes) required to complete transfer and collect data. Bars within treatments with differ (^{a,b}, $P = 0.02$). Pooled SEM = 0.07.

Discussion

The primary effort of the current research was to develop uterine selection criteria for embryo transfer recipients based on uterine and vaginal pH and temperature measurements. Criteria for current recipient selection is based on display of estrus in conjunction with the presence of a corpus luteum (Spell *et al.* 2001b). However, this selection criterion alone is not sufficient to identify prospective recipient animals. A great deal of variability exists in recipients ability to establish a pregnancy (McMillan 1998; Stroud and Hasler 2006). The key component of the recipient absent for selection is characterization of the uterine environment. One aspect of this dynamic organ that can influence the ability to establish and maintain a pregnancy is the pH and temperature of the reproductive tract. Both pH and temperature are known to have detrimental effects on fertility in *in vivo* and *in vitro* settings (Putney *et al.* 1988; Putney *et al.* 1989; Elrod and Butler 1993; Edwards *et al.* 1998; Squirrell *et al.* 2001; Ocon and Hansen 2003; Karen *et al.* 2011). Despite this, no characterization of the bovine recipient's uterus for these parameters exist for the specific time and site of transfer. Thus, the current effort developed a uterine probe system that would allow for the measurement of the

ipsilateral uterine horn to the ovary containing a corpus luteum. These measurements would be performed both at the site and time of transfer in the ipsilateral uterine horn to gain a better understanding of the site in which the embryo is being deposited. In addition, this probe would also allow for the characterization of the vaginal environment and test for associations between the two locations.

Experiment 1: Determination of uterine to vaginal relationships with uterine probe

Utilizing the uterine probe, the average pH and temperature values of the ipsilateral uterine horn and vagina for recipients establishing pregnancy versus those that did not were determined. These results are similar to others that have characterized the pH of the bovine uterine body on day 7 of the estrous cycle (Elrod and Butler 1993; Elrod *et al.* 1993). Elrod and Butler (1993) documented that the uterine body pH values will decline at estrus to more acidic values and will return to pH values more similar to a physiological normal level (~7.3). The values of the current experiment are slightly higher but could be explained by different locations and differences in pH probes used. Furthermore, previous efforts have determined that a failure to return to a physiological normal pH at day 7 has resulted in decreased pregnancy rates in artificially inseminated dairy heifers (Elrod and Butler 1993). Similar negative effects have also been documented in superovulated embryo donor cattle as indicated by reduced embryo quality at time of collection (McEvoy *et al.* 1997). These, experiments utilized altered crude protein levels of the diet to induce alterations in uterine pH. The current experiment utilized cattle maintained on the same diet and could explain no differences in pH values for pregnant versus non-pregnant recipients.

Similarly, the average ipsilateral uterine horn, vagina, and rectal temperature values between pregnant and non-pregnant animals were documented in the current study. Previous efforts have determined that elevated body temperature is detrimental to embryo quality and fertility in cattle (Putney *et al.* 1988; Putney *et al.* 1989). Additionally, *in vivo* and *in vitro* research efforts have further characterized that heat stress applied during differing stages of embryo development reduce the embryo's ability to undergo crucial developmental steps or reach the blastocyst stage of development (Putney *et al.* 1988; Putney *et al.* 1989; Edwards *et al.* 2009). A

temperature in excess of 41°C has been deemed sufficient heat stress level to cause this reduction in fertility (Edwards *et al.* 2009; Sakatani *et al.* 2012). Within the current research, average temperature levels neared 39°C but did not exceed this point. Therefore, it is not surprising that temperature results of any location within the reproductive tract did not indicate a difference between pregnant or non-pregnant recipients. Similar for pH, these animals were all maintained in similar environments during early spring months (March and April). These cooler time points may not have induced sufficient heat stress to reduce fertility. In addition, past research indicated that heat stress earlier rather than later in embryonic development increased the detrimental heat effects (Hansen 2002; Edwards *et al.* 2009). This concept fits with embryo transfer in that morula or blastocyst stage embryos being transferred may not be as susceptible to heat stress when compared to 2 cell or 4 cell embryos.

One intriguing aspect of the current results is the rather alkaline environment of the vagina versus the ipsilateral uterine horn. This difference can be noted in results of average pH values (figure 2.4). This effect is further explored by results of average pregnancy rates based on pH groups (figure 2.6). Though pregnancy rates were not different across groups some general tendencies can be noted. Within the vaginal pH groups, it appears that more recipients became pregnant when the vaginal pH was more alkaline (high group). However, the opposite effect is noted in the ipsilateral uterine horn pH groups. The lower pH value group appears to have a higher pregnancy rates. It is important to realize that in these results, the low ipsilateral uterine horn group is actually of similar values to those reported by Butler and Elrod (1993), and thus may not be surprising.

Additionally, when temperatures are separated into groups and pregnancy rates are evaluated, general tendencies can again be noted. Though not statistically significant, the general data trends are interesting. First, it appears that the highest pregnancy rates were achieved in the recipients with the highest rectal temperatures. This result is surprising based on all the previous efforts detailing negative effects of heat stress on fertility. The difference in the current study and previous efforts is the fact that the negative impacts of heat stress were seen on developing embryos. Thus, the argument can be made that the recipient's temperature does not impact the embryo's

developmental ability (Hansen 2002). In fact it is a common practice in heat stressed dairy animals to utilize embryo transfer as a means to bypass heat stress reduction in pregnancy rates (Hansen and Aréchiga 1997). Alternatively, perhaps these cows had only a short duration of heat stress in response to processing that did not sufficiently alter the uterine environment. In conjunction with this, the possibility arises that rectal temperature is not a good indicator of vaginal or uterine horn temperatures. The opposite trends in results are seen for pregnancy rates in temperature groups in the vagina and ipsilateral uterine horn. The more thermoneutral animals appear to have a higher pregnancy rate as expected.

The statement that rectal temperatures may not be indicative of vaginal or uterine horn temperatures is interesting. Furthermore, it is interesting to think that the vaginal environment could predict the uterine environment of the ipsilateral uterine horn. Therefore, correlation analysis was performed for these variables between locations. The current results indicate that the ipsilateral uterine horn pH and temperature was highly correlated to vaginal pH and temperature (table 2.1). However, the same cannot be said for rectal temperature and ipsilateral uterine horn temperature correlation ($r = 0.04$, $P = 0.67$, data not shown). This finding is intriguing since it is not uncommon to measure rectal temperature as a reflection of whole body temperature. However, this may be misleading and overestimate the temperature of organs and locations closer to the body core. To our knowledge, this experiment is the first to measure pH and temperature of both locations and establish that correlations do exist.

Ultimately, discovery of the ipsilateral uterine horn to vaginal pH correlation and temperature is extremely important for future efforts and the following two experiments. In experiment one, a subset of recipient animals were maintained as positive controls to assess the effects of uterine measurement techniques on pregnancy rates. The current results indicate that measuring the uterine pH significantly reduced the pregnancy rates in those recipients compared to the positive control recipients (figure 2.8). In fact, the average pregnancy rate of the pH measured recipients is reduced to an unacceptable level by production standards. Therefore, in future experiments, measuring uterine pH and temperature is not feasible. However, the determination of vaginal and ipsilateral uterine horn relationships can be used to bypass this affect. The decreased pregnancy

rates in this group could be attributed to multiple factors. However, most significant of these could be increased secretions of prostaglandin F_{2α} (PGF_{2α}). PGF_{2α} is known to be increased following manipulation of the reproductive tract (Schallenberger *et al.* 1989) and to have negative impacts on embryos (Scenna *et al.* 2005).

Experiment 2: Validation of uterine to vaginal relationships with field probe

The main objective of experiment two was to further validate the association of uterine horn pH and temperatures to vaginal pH and temperature and develop a measurement system that would be more applicable in a field setting (a field probe). It was important to be able to develop the field probe and then test it by measuring both the uterine horn and vagina with the same field probe. This would eliminate any difference between probes and further validate the previous relationship results seen in experiment one. To allow for the field probe to be used in both uterine horns and the vagina, experiment two was performed using abattoir collected bovine reproductive tracts. Significant selection pressure and criteria was used to identify only tracts that would most resemble that of an embryo transfer recipient animal. From these tracts the general characterizations of the pH and temperature values of each location was determined (table 2.2). Values for pH significantly differed when vaginal pH was compared to ipsilateral and contralateral uterine horns. The mean pH values are more acidic in experiment two compared to one but the same pattern of difference between the vagina and uterine horns is present. In general the vaginal pH is higher compared to the uterine horn pH values, while pH differences were not seen between the two horns.

The more acidic pH values of experiment two can be explained by a switch to anaerobic cellular energy production. It is well characterized in the meat science literature that tissue pH values decrease following harvest (Cross 1979; Jeleníková *et al.* 2008). This drop is due in part to removal of oxygen delivery to muscle and a switch to anaerobic respiration as a cellular energy source (Zeikus 1980; Clark 1989). Several of these anaerobic respiration by products can impact the pH of the surrounding environment and ultimately trigger a decrease in tissue pH (Zeikus 1980; Clark 1989). In fact, efforts performed to evaluate the effects of decreasing pH on tenderness values in beef carcasses reported pH values similar to those in the current study (Jeleníková *et*

al. 2008). Specifically, the pH values 45 minutes post mortem appear very similar to current reported values in experiment two. However, to our knowledge, this concept has not been discussed elsewhere as a potential factor to lower pH values of the reproductive tract. Research presented by Wehrend et al. (2003) employing similar procedures reported similar pH values to the current values within varying compartments of the reproductive tract. Conversely, temperature differences were not seen between locations in experiment two, but do appear lower compared to the means reported in experiment one. This simply could be an artifact of the time between animal harvest, removal of the reproductive tract and data collection. However, specific differences between locations were not found in experiment one either. Thus from that aspect both experiments are in agreement.

Furthermore, pH correlations between the ipsilateral uterine horn (side with ovary possessing a corpus luteum), contralateral uterine horn (side opposite ovary with a corpus luteum) and the vagina (table 2.2) were validated. Similarly, the temperatures of the ipsilateral uterine horn, contralateral uterine horn, and vagina were all correlated (table 2.2). However, temperatures were not correlated to pH values of any location. Again, beyond the results of experiments one and two, this is the only known experiment describing the pH and temperature relationships between the uterine horns and vagina with the same probe and in the same tract or animal. Other efforts have assessed the pH values of varying locations of the reproductive tract across the estrous cycle (Elrod and Butler 1993; Elrod *et al.* 1993; Hugentobler *et al.* 2004; Perry and Perry 2008b; Perry and Perry 2008a). However, the direct relationships between uterine horn and vagina have not been made.

Experiment 3: The relationship of vaginal pH to pregnancy status of recipients

The objective of experiment three was to assess the relationship of vaginal pH on pregnancy rates in recipient animals using the field probe developed and validated in experiment two. This was essential to prevent the need to directly measure the uterine environment and reduce pregnancy rates seen in experiment one. Experiment three revealed no difference in vaginal pH between recipients that established pregnancy versus non-pregnant recipients (figure 2.9). To our knowledge, no data exist in the

literature assessing the effects of vaginal pH on pregnancy or the normal pH ranges for the vagina across the bovine estrous cycle. However, when compared to day 7 uterine pH results of others (Elrod and Butler 1993; Elrod *et al.* 1993), they are similar. However, in comparison to vaginal values in experiment one, these averages are drastically more acidic. This could be a factor of two different probes being used between the experiments. Probes will respond differently between types and across days (Swain 2010a). However, confidence is placed in the results due to validation efforts of experiment two and daily calibrations revealing low inter- and intra- cv readings.

Similarly, average rectal and vaginal temperatures did not differ between pregnant and non-pregnant recipients. As mentioned previously, heat stress effects on bovine fertility have been characterized (Putney *et al.* 1988; Putney *et al.* 1989; Hansen 2002; Edwards *et al.* 2009). The average temperature values for rectal temperature could be classified as borderline sufficient for causing heat stress while the vaginal temperatures are well below the established threshold for inducing heat stressed negative effects on embryos. In experiment three, recipients were managed in similar fashions and thus, pH and temperature differences were not artificially induced through experimental influences. This could also explain why no differences were seen between groups.

However, to further evaluate the effects of rectal temperature, vaginal pH, and vaginal temperature; regression models were used to predict the probability of establishing pregnancy based on these variables. The vaginal pH model indicated an optimal range between 6.9 and 7.4 for improved pregnancy rates in recipient animals (figure 2.11). Thus, vaginal pH groups were established and evaluated for effects on pregnancy rates (figure 2.14). These results were not significantly different, but indicated a higher pregnancy rate when recipients had a neutral vaginal pH. Again, no data existed in the literature for vaginal pH effects on pregnancy rates. However, applying the uterine pH fertility results of others (Elrod and Butler 1993; Elrod *et al.* 1993), this may not be surprising. However, in comparison to experiment one, these pH values are closer to neutral pH rather than alkaline.

As for rectal temperature and vaginal temperature, regression analysis also predicted optimal ranges for improved pregnancy probability (figures 2.12 and 2.13 respectively). When rectal temperature is separated into high, middle, and low temperature groups no differences are seen in pregnancy rates (figure 2.15). However, in general the lower temperature recipients had a higher pregnancy rate. These data are more in line with the previous results of heat stress inducing negative effects on fertility. In addition, no significant differences were seen in the vaginal temperature groups, but in general the middle range group had a higher pregnancy rate (figure 2.15). Again, this would indicate that heat stress and maybe even cold stress had a negative impact on fertility.

Utilizing the combined data sets for experiments one and three, differences existed in pregnancy based on transfer location within the uterine horn (figure 2.16), transfer score (figure 2.17), and time to complete transfer and collect data (time in cow, figure 2.18). The accepted technique is to place the embryo into the deepest location possible within the ipsilateral uterine horn to the ovary possessing a corpus luteum (Robertson *et al.* 2008). However, inherent to the embryo transfer technique is the inability to reach the upper third of the ipsilateral uterine horn each time an embryo is transferred. This is due to obstructions in the tract, behavior of the cow, or technician experience or fatigue to name a few. However, it has been characterized that pregnancy is increased when uterine synchrony matches the stage of embryo development (Pope 1988) and reviewed by Barnes (2000). Thus, it would allow that the proper location for a day 7 embryo would be the caudal regions (closest to the ovary) of the uterine horn and would provide a greater chance for pregnancy establishment and current data would support this. Based on previous efforts it has been determined that reproductive tract secretions vary across the tract and the estrous cycle (Hugentobler *et al.* 2004; Hugentobler *et al.* 2007). Thus, the environment of the caudal uterine region may provide a more ideal location for continued embryonic development. In contrast, other efforts have revealed no influence of tract location on pregnancy rates in embryo transfer recipients (Bó *et al.* 2011).

As mentioned above, the ability to reach the upper third of the ipsilateral uterine horn is based on many factors during the embryo transfer procedure that may increase

the amount of manipulation in the uterus. Transfer scores can be used as a means to access this difficulty of reaching the upper third of the ipsilateral uterine horn. Transfer scores are subjectively assigned by the technician and may be an indicator of technician experience as well. Varying results exist in the literature on technician success across programs and species (Schoolcraft *et al.* 2001; Hasler 2006; Bó *et al.* 2011). In the current study, technician was not a factor and would agree with results from Bo and co-workers (2011), while a retrospective study in humans indicate a difference in pregnancy success based on technician skill (Schoolcraft *et al.* 2001). In experiment three, a trend existed between transfer scores (easiest to most difficult, figure 2.16). Embryo transfer techniques require manipulation of the reproductive tract and as a negative consequence greater prostaglandin $F_{2\alpha}$ can be produced (Schallenberger *et al.* 1989; Wann and Randel 1990). Difficult transfers resulting in a higher numeric score may lead to increased prostaglandin $F_{2\alpha}$.

Inherently, embryo transfer techniques require additional time compared to artificial insemination. The technician must accomplish several steps prior to the embryo being deposited into the ipsilateral uterine horn. Again, this requires manipulation of tract and general increased stress on the recipient as a whole. Current data indicated a significant effect of time required to complete embryo transfer and data collection on pregnancy rates of recipient animals. Specifically, higher pregnancy rates was achieved in recipients that required less time complete the transfer and data collection process. Taken together, the effects of location, transfer score, and time in cow suggest that an embryo transfer practitioner must not only be precise in embryo placement but care to reduce excessive manipulation of the uterus is imperative along with speed of the transfer procedure.

Conclusion

To our knowledge, this is the first effort to develop a uterine probe to establish relationships and associations of uterine and vaginal environments. In turn, this allowed effects of pH and temperature to be evaluated for influences on bovine embryo transfer recipient pregnancy rates. Currently, experiment one indicated that uterine pH, uterine temperature, vaginal pH, vaginal temperature, and rectal temperature average values

were not different in pregnant and non-pregnant recipients. Furthermore, the uterine pH and temperature were correlated to the vaginal pH and temperature. However, measuring uterine pH is severally detrimental to recipient pregnancy rates. Therefore, experiment two developed and validated a field probe revealing that ipsilateral pH and temperature, contralateral pH and temperature, and vaginal pH and temperature were correlated. This allowed the field probe to be used in experiment three to indicate that vaginal pH, vaginal temperature, and rectal temperatures were not different between pregnant and non-pregnant recipients. However, optimal ranges for all three appear to exist for improving the likelihood of pregnancy establishment by recipient animals. Finally, location of embryo deposit, embryo transfer score, and time to complete transfer and collect data (time in cow) all significantly impacted recipient pregnancy rates.

Chapter 3. Association of Bacteria Presence on Uterine pH in Cattle

Abstract

The bovine reproductive tract serves a critical role in supporting the development of an early embryo. Perturbations in the uterine environment in the form of bacterial infections that may alter the pH of the uterine environment may prevent successful pregnancy establishment in embryo transfer recipient animals. Therefore, the hypothesis was tested that increased uterine bacteria loads in the ipsilateral horn to the ovary with a corpus luteum and the vagina would influence the pH of the ipsilateral horn. Bovine reproductive tracts ($n = 43$) from a local abattoir were identified based on the presence of an approximately day 7 corpus luteum in conjunction with no visible signs of uterine or vaginal infections. Swabs for bacteria culture along with pH and temperature values were collected from both horns and vagina. Swab samples were cultured for aerobic, gram negative, and anaerobic bacteria growth on respective agar plates. Aerobic and gram negative colonies were counted initially at 24 hours and again at 48 hours (plates with no or slight growth), while anaerobic colonies were counted following 72 hours of incubation. Together, this allowed variable analysis for correlation, regression and ANOVA models to be performed. Ipsilateral horn pH was correlated to contralateral and vaginal pH ($r = 0.63$ and 0.59 , respectively, $P < 0.0001$). Furthermore, ipsilateral temperature was correlated to contralateral and vaginal temperature ($r = 0.81$ and 0.81 , respectively, $P < 0.0001$). Specific effects of types of bacteria growth revealed that only anaerobic growth impacted pH in ANOVA and Regression models ($P = 0.05$ and $P = 0.02$, respectively). However, a regression model evaluating the same effect within the ipsilateral horn failed to indicate an anaerobic bacteria impact on pH ($P = 0.29$). Groups based on amount of anaerobic growth were established and pH was different when more growth was present ($P < 0.001$) across all locations. However, when locations were separated no difference in growth levels impacted pH in the ipsilateral horn or the vagina. Similar models for aerobic and gram negative bacteria growth indicated no influence on pH values. Furthermore, vaginal bacteria did not affect ipsilateral horn pH values. However, the pH values of the horns and vagina differed in all models analyzed ($P < 0.0001$), specifically the vaginal pH differed from both the ipsilateral and contralateral pH. Sample date did impact the pH values ($P = 0.0002$) and was included in all models of analysis. Progesterone concentrations were not

associated with pH values. A single negative correlation existed between progesterone concentration and vaginal aerobic bacteria growth ($r = -0.34$, $P = 0.03$), anaerobic bacteria means did not differ based on level or progesterone concentration. Finally, luteal volume (based on measurements of the exposed corpus luteum crown) and progesterone were correlated (0.34 , $P = 0.0002$). Thus, pH values were not influenced by bacteria within the ipsilateral uterine horn, while anaerobic bacteria growth only influences vaginal pH. Furthermore, vaginal bacteria growth is not indicative of the uterine environment.

Introduction

Clinical and subclinical uterine infections have been demonstrated to reduce fertility in cattle, leading to economically important losses in production (Azawi 2008; Sheldon *et al.* 2008a; Sheldon *et al.* 2009). As many as 40% of dairy cows experience clinical uterine infections within 1 week postpartum (Sheldon *et al.* 2009). This figure is potentially low since it does not include subclinical cows or beef cattle that are not as frequently examined postpartum as dairy cattle. Another estimate by Griffin *et al.* (1974a) estimates that 90% of uteri have bacterial invasion within the first 10 days postpartum. Additional estimates state that as many as 50% of postpartum cows still have a subclinical infection 50 to 60 days postpartum (Sheldon *et al.* 2008b). These subclinical cows pose potential problems for production as many may go unnoticed and untreated, leading to decreased fertility.

Bacterial infections have been implicated in reducing fertility in cattle (Peter *et al.* 1989; Battaglia *et al.* 1999; Battaglia *et al.* 2000; Williams *et al.* 2001; Sheldon *et al.* 2002). The disruptions can occur at both the hypothalamic-pituitary (Peter *et al.* 1989; Battaglia *et al.* 1999; Battaglia *et al.* 2000; Williams *et al.* 2001) and the local ovarian-reproductive tract levels (Sheldon *et al.* 2002; Herath *et al.* 2007). Bacterial byproducts can interfere with the signaling cascade of gonadotropin releasing hormone (GNRH) from the hypothalamus to induce a luteinizing hormone (LH) surge from the pituitary required for ovulation (Battaglia *et al.* 1999; Battaglia *et al.* 2000). In addition, endotoxins have been recovered from ovarian follicular fluid (Sheldon *et al.* 2002). When endotoxin is found in follicular fluid, decreased levels of estrogen have been

reported and may impede the feedback system of estrogen to promote GnRH (hypothalamus) and subsequent LH (pituitary) release (Sheldon *et al.* 2002). Furthermore, bacterial infections have been demonstrated to reduce the size and number of growing and dominant follicles on the ovary ipsilateral to the horn possessing the infection (Sheldon *et al.* 2002). In combination it is evident that bacterial infections can decrease fertility. However, most of this information is from clinically infected cows and does not provide a characterization of the natural microflora of the reproductive tract. Additionally, this information does not indicate effects of bacteria on natural functions of the uterus in either a clinical or subclinical infectious state.

This characterization of subclinical infection effects may hold important relevance for embryo transfer recipient animals. Typically, recipient animals are multiparous animals and are targeted beginning approximately 45 to 50 days postpartum to begin attempting synchronization and embryo transfer. Therefore the above estimation indicating that 50% of cows still possess subclinical infections at day 50 postpartum poses potential problems for recipients. Unfortunately, current selection methods for recipients do not possess criteria to identify animals that have a subclinical infection. The current selection tools for recipients are limited to estrus display and presence of a corpus luteum (CL) (Spell *et al.* 2001b) but these tools do not solve the issues currently seen in reduced pregnancy rates (McMillan 1998; Stroud and Hasler 2006).

In combination with bacterial specific fertility impacts, altered uterine pH effects on fertility have been explored (Elrod and Butler 1993; Elrod *et al.* 1993; Butler *et al.* 1996). These efforts indicate that fertility is reduced in bovine animals when pH is altered from, specifically when pH levels are more acidic at day 7 of the estrous cycle compared animals with a more neutral pH. Thus the question arises if bacterial presence can alter the pH environment of the uterus. To our knowledge only limited data exists characterizing pH and bacteria presence in the bovine uterus (Ozenc *et al.* 2010). This effort only characterized the presence of bacteria and pH of the uterus across cycle stages and did not characterize the vaginal environment. Thus, with specific regard to recipient selection, this combined characterization is important as potential selection criteria.

Therefore, the focus of the current research effort was aimed at more precisely characterizing the environment of bovine reproductive tracts during the early luteal phase. Specifically, more effort was targeted to evaluate the environment of the vagina in conjunction with the ipsilateral and contralateral uterine horn. The first effort was to determine if bacteria were present in clinically normal appearing reproductive tracts. Second, determine if bacterial presence have effects on pH values in these locations. Finally, determine the ability of vaginal environments to predict the environment of the ipsilateral uterine horn.

Material and Methods

Bovine reproductive tracts (n= 43) were obtained from a local abattoir in conjunction with a companion research effort (chapter 2, experiment 2). Tracts were recovered approximately 15 to 45 minutes after exsanguination and identified using visual evaluation for pregnancy status and presence of an approximately day 7 corpus luteum (CL) using a method similar to Ireland and co-workers (1980). Furthermore, tracts were selected on a gross normal appearance to avoid potential cases of clinical metritis. The side, size, color, and external presence of a cavity were documented for each CL. Following characterization of CLs, bacteria swab samples and pH readings were collected in three compartments of the reproductive tract (upper third of the contralateral and ipsilateral horns in relation to the CL and vagina). Each location was first swabbed with an iodine scrub solution and allowed to briefly dry. Then a scalpel blade was used in a stabbing motion to pierce the tract in each location. The scalpel was then rotated and incision was made using an “in to out” motion. The incision was held open with a large set of tweezers and a Copan liquid amies elution swab (ESwab, Copan, Murrieta, CA, USA) was inserted in the opposite direction as to the cut to prevent contamination of the sample. The swab was returned to the holding tube and medium, then stored on ice until returning to the lab. Next, pH readings were taken in the opposite direction and within a few centimeters of the bacteria swab site. A 2 cell pH probe (Thermo Scientific, Beverly, MA, USA) and Orion 3 Star portable pH meter (Thermo Scientific) were used to capture pH readings. A pH reading was recorded every 15 seconds for one minute and a temperature reading recorded at the end of one minute. Between measurement

collections the probe was rinsed with de-ionized water. Prior to use each day, the probe and meter were calibrated in known standards of 4, 7, and 10 pH (Fisher Scientific, Suwanee, GA, USA) and the calibration slope was recorded (inter- coefficients of variations were 1.2%). Between tracts, the pH probe was again rinsed with de-ionized water and maintained in a standard solution of 7 pH (Fisher Scientific).

Blood samples were collected from each animal (jugular vein during exsanguination), maintained on ice, centrifuged, and serum stored for progesterone analysis at -20° C.

Following return to the laboratory, dilutions and bacteria culture were performed using modified techniques from those of Otero et al. (2000) and Wang et al. (2013). Serial dilutions were made for the vagina on all cows (n=43), uterine horn serial dilutions were made only on the first sample date (n=2 cows), thereafter, dilutions were made only if colony counts were “to numerous to count” and if sample was still viable. Ten-fold serial dilutions (-1 dilutions) were made using 100 µL of sample diluted into 900 µL of phosphate buffered saline (PBS). To obtain a twenty-fold dilution (-2 dilution) 100 µL of the -1 sample was diluted into 900 µL of PBS. The same technique was performed when a thirty-fold dilution was required. Following dilutions, 100 µL of each sample was plated on Trypticas soy agar plates supplemented with 5% sheep blood (aerobic bacteria growth; Becton Dickinson, Sparks, MD, USA), McConkey agar plates (gram negative bacteria growth; REMEL, Lenexa, KS, USA), and anaerobic pea blood agar plates supplemented with vitamin K (anaerobic bacteria growth; REMEL). Bacteria were allowed to culture for approximately 24h at 37° C for aerobic and gram negative bacteria growth. Any plate showing no or slight growth at 24h was allowed to culture for an additional 24h and then reevaluated. Anaerobic plates were cultured for approximately 72h in BD GasPak EZ Anaerobe gas generating pouch systems (Becton Dickinson) at 37° C prior to colonies being counted.

To calculate the number of colonies in the original sample, zero dilution samples were multiplied by 10 to return the estimate to milliliter (mL) basis, while the serial dilutions were multiplied by their respective dilution factor and then multiplied by 10 to return the estimate to mL basis. In cases when counts were performed at both 24 and 48h, the average colony count for both time points was calculated for analysis. Plates

with an estimated colony count greater than 250 were categorized as “too numerous to count”.

Radioimmunoassays for progesterone (intra- coefficients of variations were 5.99%) were performed with a Coat-A-Count progesterone kits (Siemens, USA). Cows with progesterone levels below 1ng/ml at time of harvest were removed from analysis (n=4). The following variables were produced and analyzed from the current study: sample collection date, tract size score (Young *et al.* 2010), corpus luteum volume, progesterone concentration, ipsilateral horn pH and temperature, contralateral horn pH and temperature, vagina pH and temperature, aerobic bacteria growth (ipsilateral horn, contralateral horn, and vagina), anaerobic bacteria growth (ipsilateral horn, contralateral horn, and vagina), and gram negative bacteria growth (ipsilateral horn, contralateral horn, and vagina). Luteal volume was estimated using techniques previously described (Lüttgenau *et al.* 2011), pH values from each of the 4 time points (15, 30, 45, and 60 seconds) were pooled and averaged for each location measured. Reproductive tract compartment temperatures were all recorded in Celsius.

Model and variable selection utilized a “put and take” method to test for bacterial presence differences in tract locations and bacterial influence on pH values of tract locations. All analysis was performed using SAS 9.4 (SAS Institute, Cary, NC). Means were analyzed with generalized mixed model analysis of variance (GLIMMIX, SAS 9.4; least significant difference technique) while generalized linear models (SAS 9.4, SAS Institute) were used for regression models. Bacteria models were normalized using a log 10 transformation with a transformation value of 1. Results are presented as least squares means (back transformed means) plus standard error of the means (ANOVA analysis) and slope estimates plus standard error of significant variables (linear regression analysis). Correlation analysis was performed using Pearson correlation in SAS 9.4 (SAS Institute, Cary, NC) and presented as the correlation with resulting *p* values. Sample date did influence mean pH values and thus was including as a random effect in all models.

Results

Presence of bacteria in varying compartments of the reproductive tract

Aerobic, anaerobic, and gram negative bacteria was present in all locations sampled (figure 3.1). In addition, vaginal growth of aerobic, anaerobic, and gram negative was significantly greater compared to contralateral and ipsilateral uterine horn growth (figure 3.1). However, aerobic, anaerobic, and gram negative growth did not differ between the contralateral and ipsilateral uterine horns (figure 3.1).

Determination of bacterial presence on pH and temperature

First, the general characterization of the pH in the uterine horns and vagina was assessed to establish pH differences due to location regardless of bacteria presence. The location main effect indicated that vagina pH differed from the horns while the horns were not different from each other ($P < 0.0001$, table 3.1). Linear model analysis determined that anaerobic bacteria presence effected pH values in the reproductive tract ($P = 0.02$, figure 3.2). However, the removal of vagina and contralateral horn revealed that bacteria did not affect the pH of the ipsilateral horn ($P = 0.29$, figure 3.3). Anaerobic bacteria did affect the pH of the reproductive tract ($P < 0.0001$, figure 3.4), but not when separated by locations ($P = 0.18$ and 0.43 , ipsilateral and vagina, respectively)

Linear models revealed no effect of aerobic or gram negative bacteria on pH values of the reproductive tract ($P = 0.7$ and 0.6 , respectively). Similarly, generalized mixed models indicate no difference in tract pH values when aerobic or gram negative bacteria are present ($P = 0.29$ and 0.2 , respectively). In contrast, the location main effects in both models indicated mean pH differences between tract compartments ($P = 0.00023$ and $P < 0.0001$, aerobic and gram negative, respectively, table 3.1). Specifically, the vaginal pH differed from both horns, while the horns were not different from each other (table 3.1). A correlation analysis of vaginal pH with ipsilateral horn bacteria growth (all three types of bacteria) was performed to depict ability of vaginal pH to predict uterine infections (table 3.2). These tests revealed no correlation between vaginal pH and ipsilateral horn growth on any of the three types (table 3.2). A similar correlation analysis was performed using vaginal temperature correlated to ipsilateral

horn bacteria growth on all three plate types. Again, this analysis revealed no correlations (table 3.2). Furthermore, correlation analysis of ipsilateral horn pH and temperature to ipsilateral horn bacteria growth resulted in no correlations between variables (table 3.2). Finally, effect of vagina bacterial growth (all three types) on ipsilateral horn pH was analyzed using correlations and linear regression. Bacteria growth in the vagina is not correlated to ipsilateral horn pH (table 3.3) and regression analysis yielded no difference in ipsilateral pH values across varying levels of aerobic ($P = 0.72$, data not shown), anaerobic ($P = 0.33$, data not shown), or gram negative ($P = 0.46$, data not shown) bacteria growth.

Sample date did have an effect on ipsilateral ($P = 0.0002$; figure 3.5) and vaginal ($P = 0.04$; figure 3.6) pH values. However sample date did not affect ipsilateral aerobic ($P = 0.47$), anaerobic ($P = 0.92$), or gram negative ($P = 0.86$) bacteria growth (data not shown). Furthermore, sample date did not affect vaginal aerobic ($P = 0.47$), anaerobic ($P = 0.93$), or gram negative ($P = 0.50$) bacteria growth (data not shown). Tract size score did not affect ipsilateral ($P = 0.18$, figure 3.7) or vagina ($P = 0.44$, figure 3.7) pH values. Tract score also did not affect vagina ($P = 0.55, 0.76, 0.53$, aerobic, anaerobic, gram negative, respectively, figure 3.8) or ipsilateral ($P = 0.54, 0.92, 0.62$, aerobic, anaerobic, gram negative, respectively, figure 3.9) bacteria growth.

Analysis of progesterone influences on reproductive tract pH and bacteria growth

Progesterone concentrations differed across cows ($P < 0.0001$) and ranged from 1 to 11 ng/mL. Based on previous anaerobic effects on pH, progesterone effects on anaerobic bacteria was assessed and showed no difference in growth between high, mid, or low progesterone concentration groups in the ipsilateral horn and the vagina ($P = 0.45$ and 0.16 , respectively, figure 3.10). Correlation analyses of progesterone concentration indicated no correlation to ipsilateral horn or vagina pH, ipsilateral horn bacteria growth, or vagina anaerobic and gram negative bacterial growth (table 3.5). Progesterone concentration was positively correlated to luteal volume (0.34 , $P = 0.0002$, table 3.5) and negatively correlated to vagina aerobic bacteria growth (-0.35 , $P = 0.03$, table 3.5). ANOVA models did not reveal an effect of progesterone on reproductive tract pH values ($P = 0.42$; data not shown). Additionally, progesterone concentration did not

influence aerobic ($P = 0.62$, data not shown), anaerobic ($P = 0.57$, data not shown), or gram negative ($P = 0.99$, data not shown) bacteria growth.

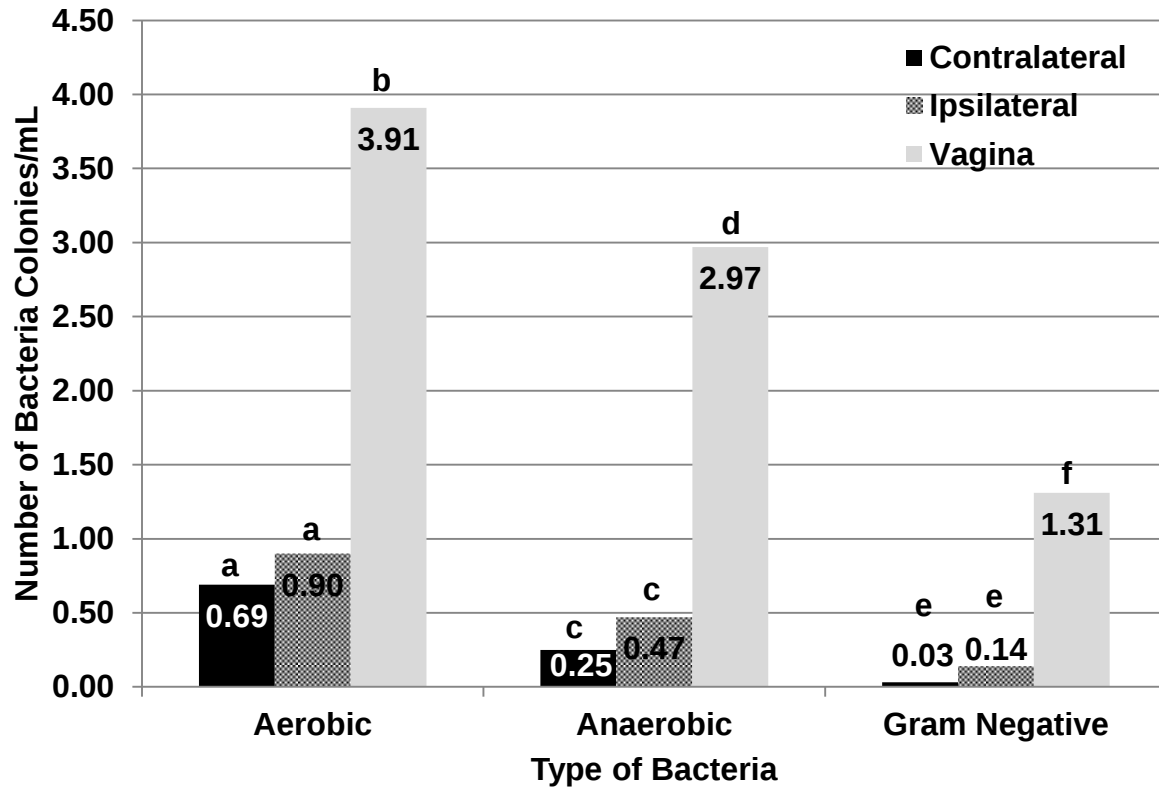


Figure 3.1 Bacteria growth by type of bacteria within compartments of the reproductive tract. Bars within treatments with different superscripts differed (^{a,b;c,d;e,f} $P < 0.0001$). Pooled SEM = 0.21, 0.17, 0.09 (aerobic, anaerobic, gram negative, respectively).

Table 3.1. Reproductive tract pH values across various locations.

	Location		
	Vagina	Ipsilateral Horn	Contralateral Horn
Reproductive tract location	6.72 ^{a*}	6.35 ^{b*}	6.4 ^{b*}
Model			
Aerobic	6.79 ^c	6.34 ^d	6.37 ^d
Anaerobic	6.9 ^e	6.34 ^f	6.36 ^f
Gram Negative	6.72 ^g	6.34 ^h	6.32 ^h

Values within rows with different letters differed (^{a,b} $P < 0.001$, ^{c,d} $P = 0.0023$, ^{e,f,g,h} $P < 0.0001$). Pooled SEM = 0.005, 0.006, 0.006, 0.006 (reproductive tract location, aerobic, anaerobic, and gram negative, respectively).

*Data also utilized in Chapter 2, Experiment 2.

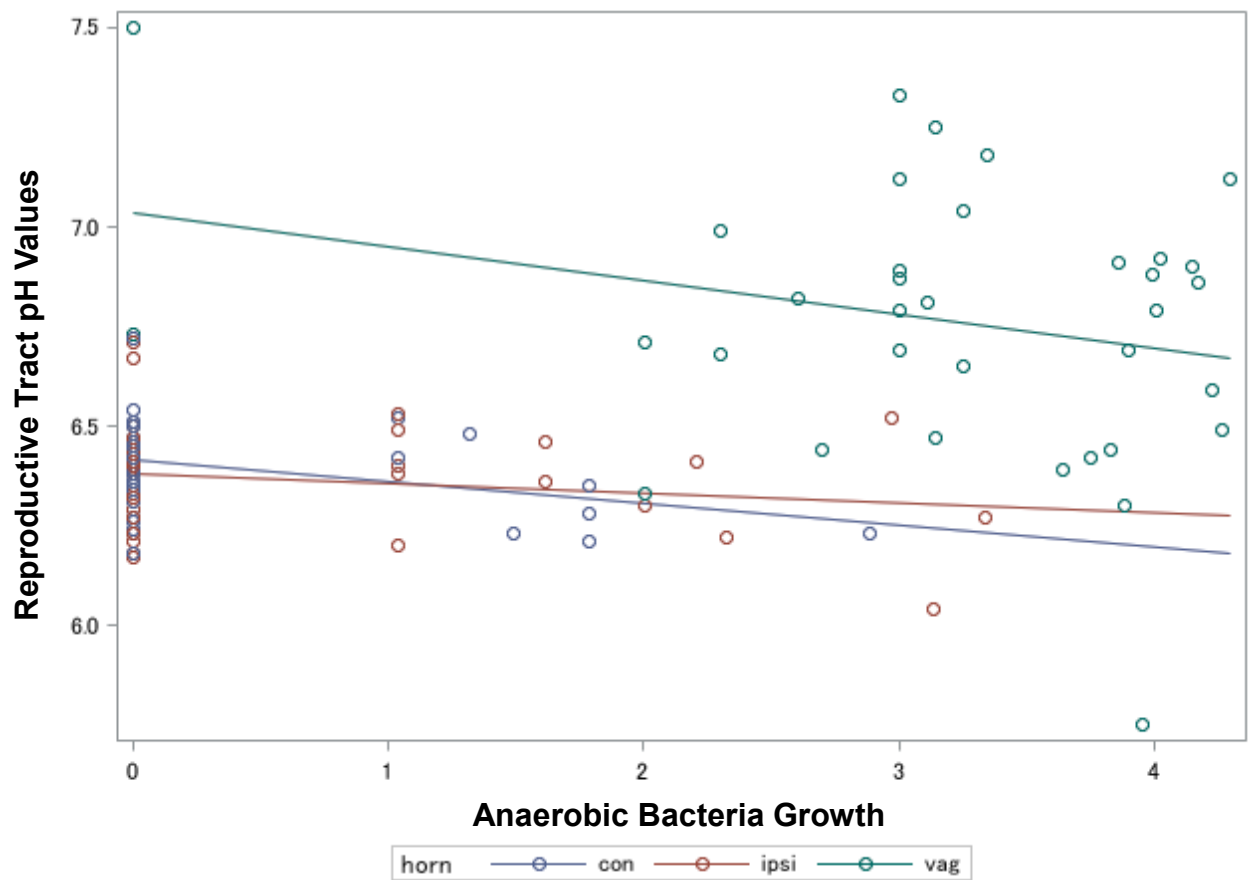


Figure 3.2 Regression analysis results of anaerobic bacteria growth influence on pH values of specified reproductive tract compartments. The slope of the lines differed ($P = 0.02$).

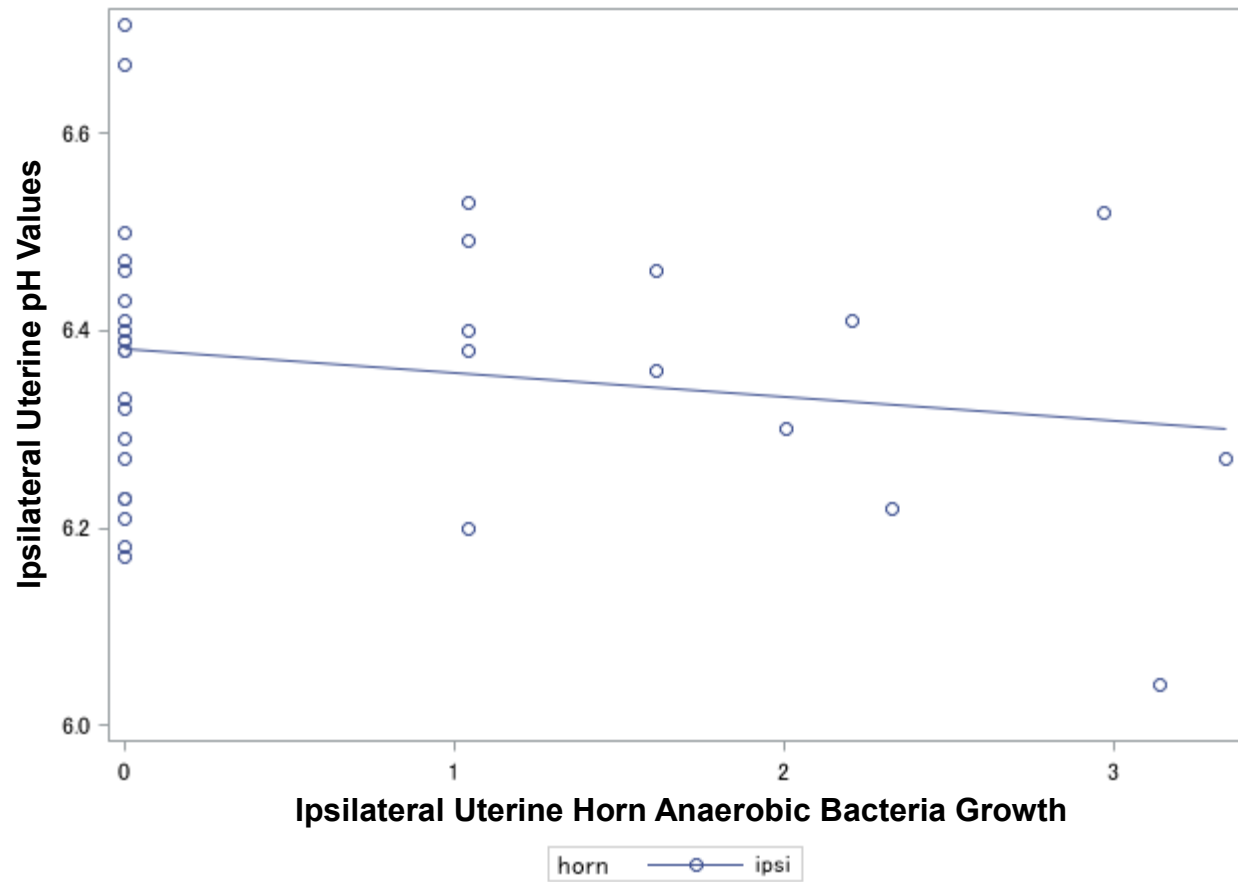


Figure 3.3 Regression analysis results of ipsilateral horn anaerobic bacteria growth influence on ipsilateral horn pH values. The slope of the lines did not differ ($P = 0.29$).

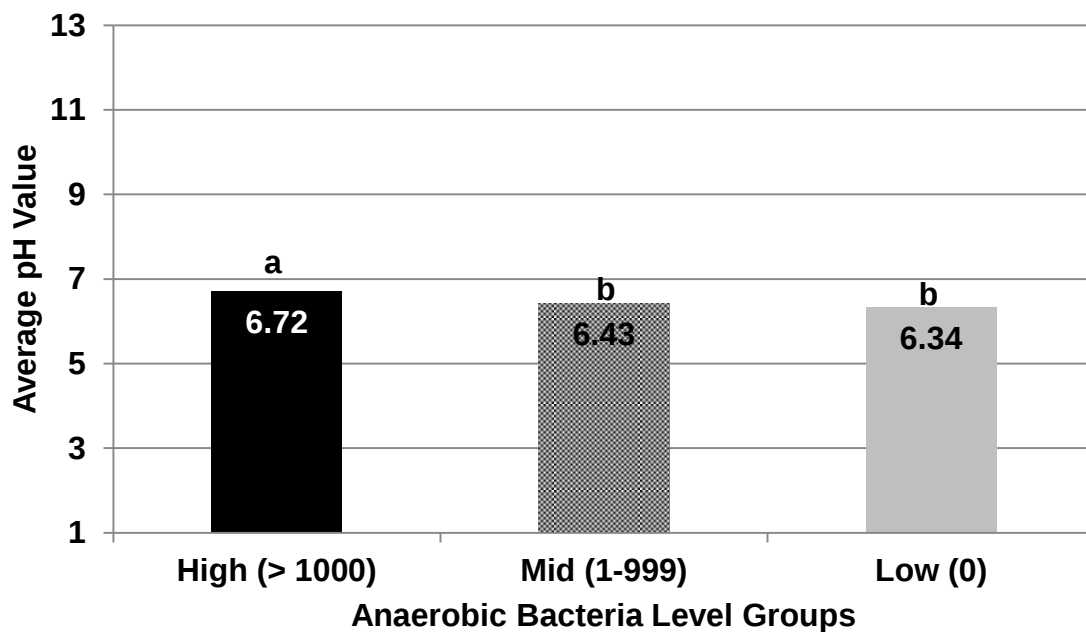


Figure 3.4 Average pH values within groups determined by anaerobic bacteria load across all three tract locations. Bars with different letters differed (^{a,b} $P < 0.0001$). Pooled SEM = 0.05.

Table 3.2. Correlation and associated *p* values of vaginal pH, vaginal temperature and ipsilateral horn temperature with bacteria load within selected compartments of the bovine reproductive tract.

Ipsilateral Horn Bacteria	Vagina pH		Vagina Temperature		Ipsilateral pH		Ipsilateral Temperature	
	Correlation	<i>P</i> Value	Correlation	<i>P</i> Value	Correlation	<i>P</i> Value	Correlation	<i>P</i> Value
Aerobic	0.11	0.48	-0.07	0.66	-0.02	0.9	0.08	0.61
Gram negative	-0.02	0.90	-0.14	0.39	-0.23	0.16	0.03	0.82
Anaerobic	-0.06	0.75	-0.28	0.1	-0.18	0.3	-0.12	0.47

Table 3.3. Correlations of vagina bacteria growth with ipsilateral horn pH values.

Vaginal Bacteria	Ipsilateral pH	
	Correlation	<i>P</i> Value
Aerobic	-0.02	0.92
Gram negative	0.10	0.53
Anaerobic	-0.09	0.60

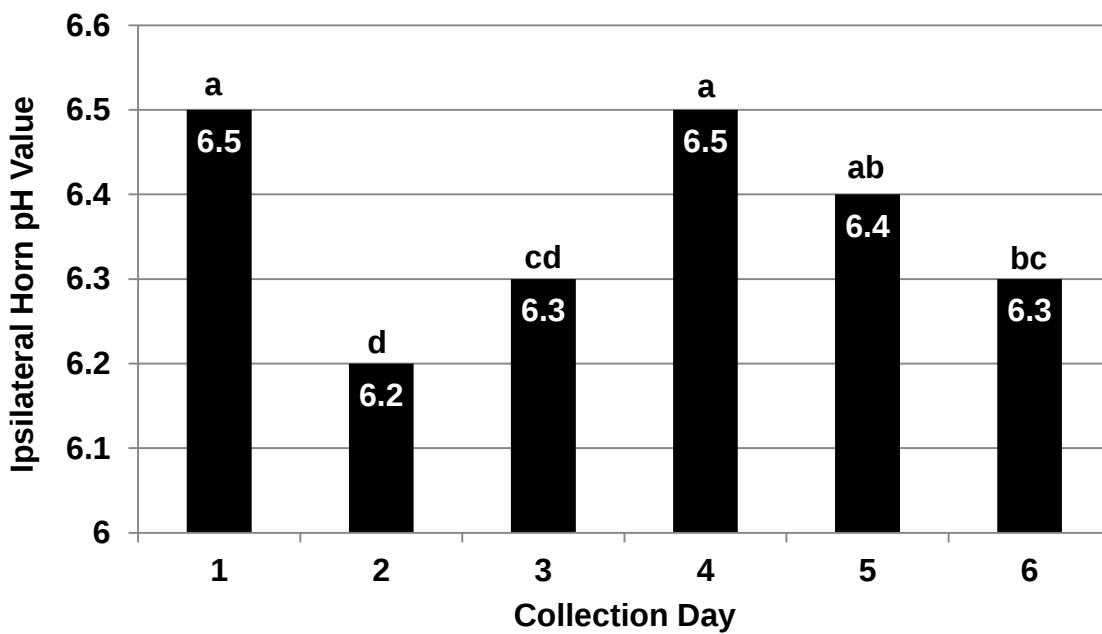


Figure 3.5 Ipsilateral horn pH values based collection day. Bars with different superscripts differed (^{a,b,c,d} $P = 0.0002$). Pooled SEM = 0.05.

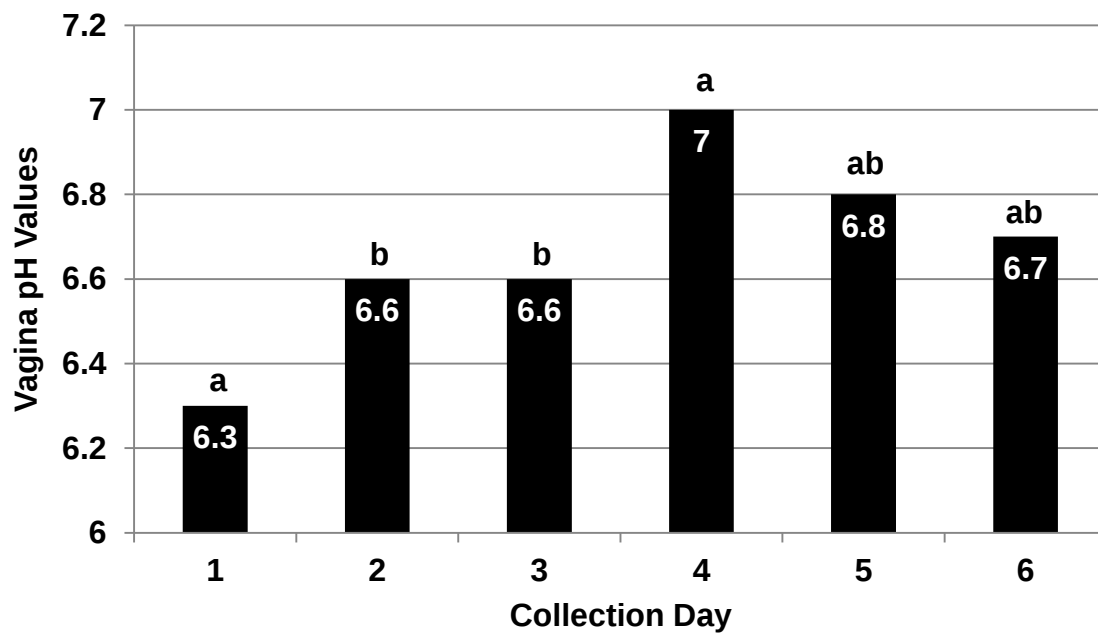


Figure 3.6 Vaginal pH values based on collection day. Bars with different superscripts differed (^{a,b} $P = 0.04$). Pooled SEM = 0.14.

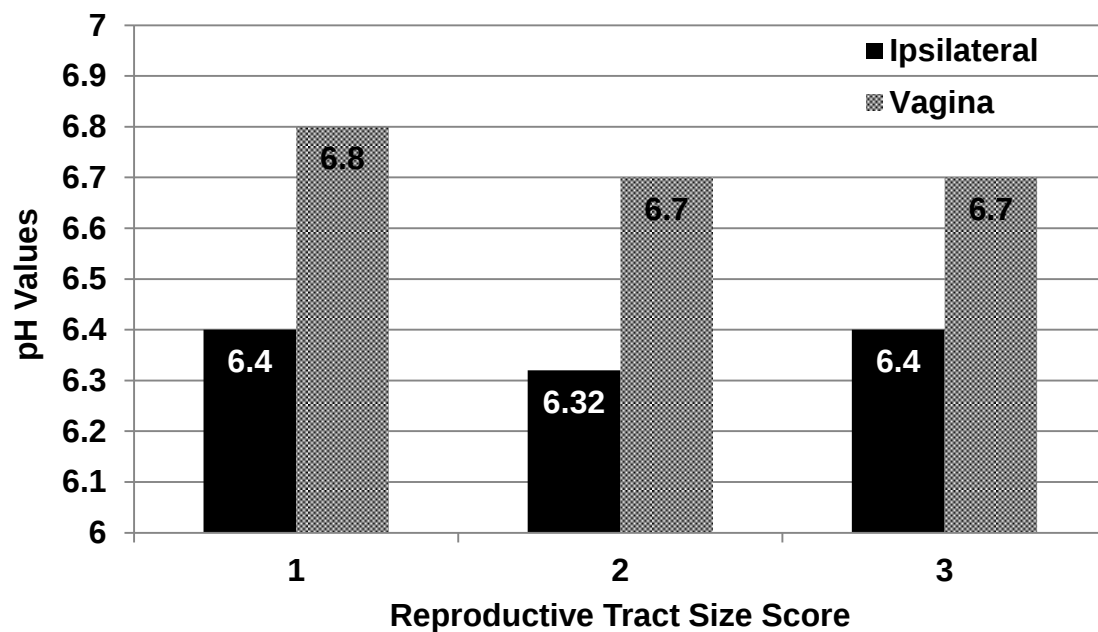


Figure 3.7 Impact of reproductive tract size score on ipsilateral and vaginal pH values. Bars within treatments did not differ ($P = 0.39, 0.53$; ipsilateral and vagina, respectively). Pooled SEM = 0.05, 0.12 (ipsilateral and vagina, respectively).

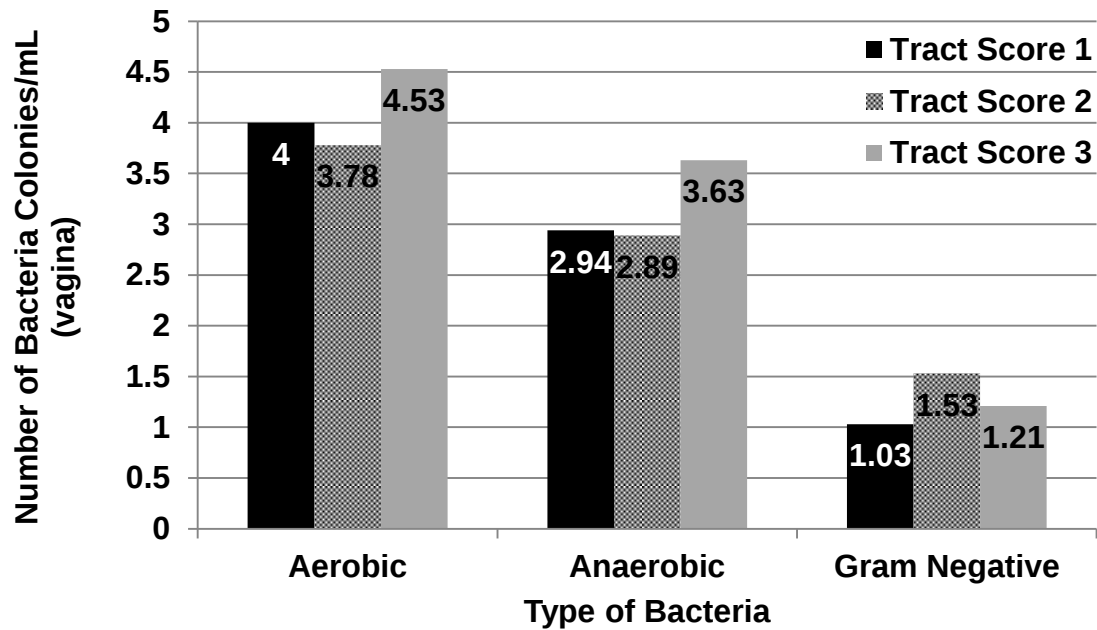


Figure 3.8 Impact of reproductive tract size score on vagina bacteria colony growth. Bars within treatments do not differ ($P = 0.55, 0.76, 0.53$; aerobic, anaerobic, gram negative, respectively). Pooled SEM = 0.42, 0.60, 0.45 (aerobic, anaerobic, gram negative, respectively).

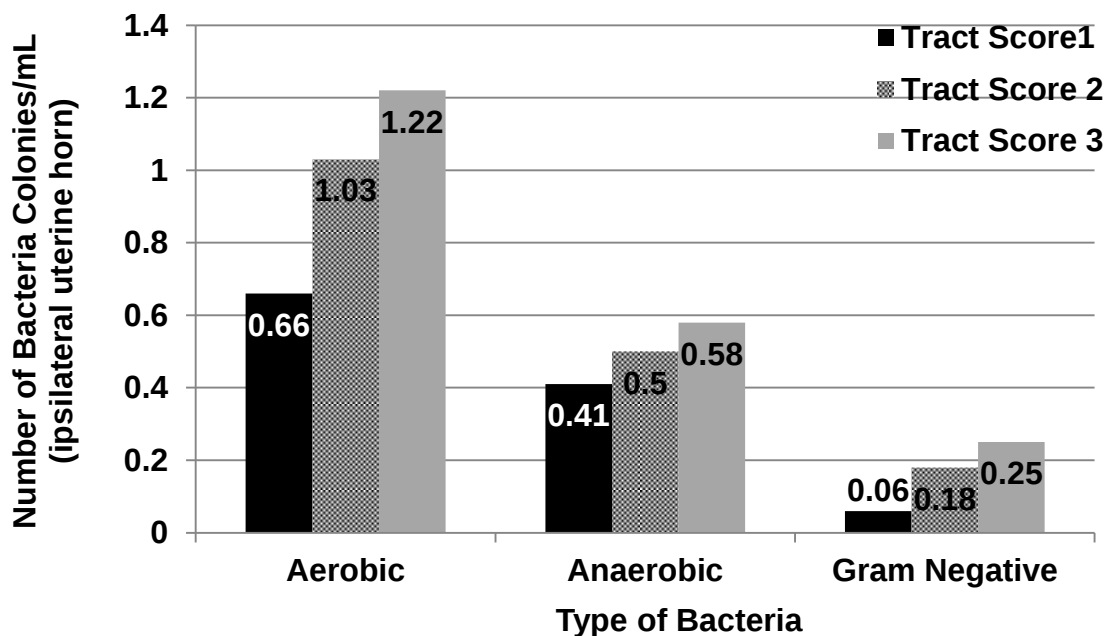


Figure 3.9 Impact of reproductive tract size score on ipsilateral horn bacteria colony growth. Bars within treatments do not differ ($P = 0.55, 0.92, 0.62$). Pooled SEM = 0.41, 0.30, 0.15 (aerobic, anaerobic, gram negative, respectively).

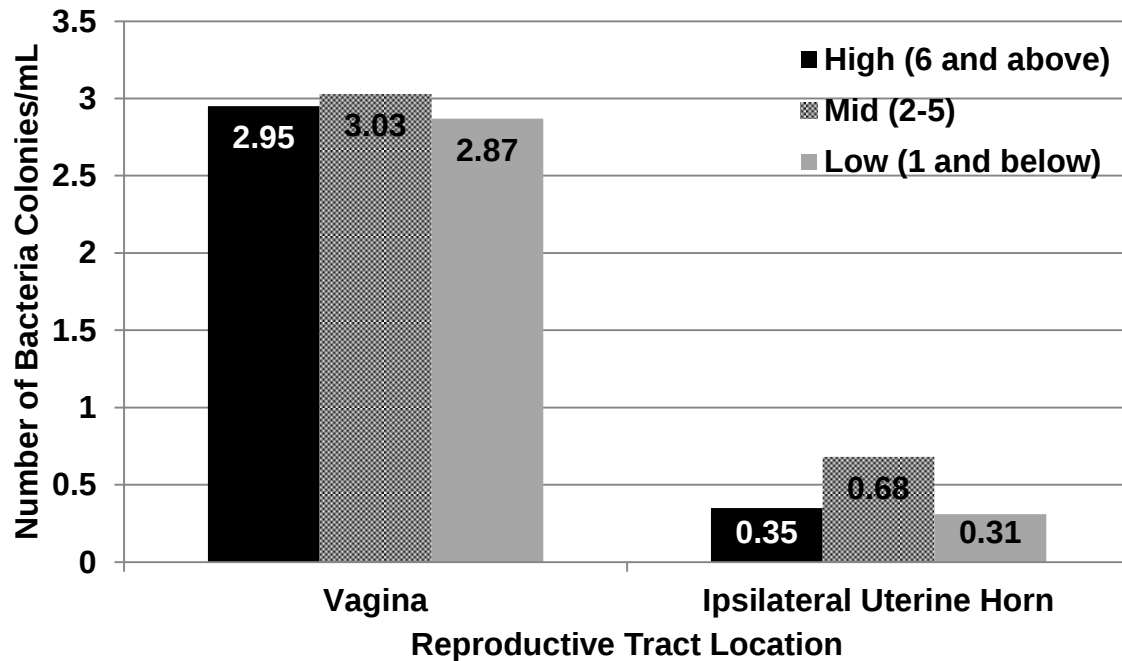


Figure 3.10 Impact of progesterone level (ng/mL) on vagina and ipsilateral uterine horn bacteria colony growth. Bars within treatments do not differ ($P = 0.16$ and 0.45 , respectively). Pooled SEM = 0.45 and 0.23 (vagina and ipsilateral uterine horn, respectively).

Table 3.4. Correlation and associated *p* values of serum progesterone concentration with selected pH values, corpus luteum volume, and bacteria growth variables.

	Progesterone Concentration	
	Correlation	<i>P</i> Value
Ipsilateral horn pH	-0.006	0.97
Vagina pH	0.09	0.58
Ipsilateral horn aerobic	0.05	0.76
Ipsilateral horn anaerobic	0.05	0.81
Ipsilateral horn gram negative	0.05	0.75
Vagina aerobic	-0.35	0.03
Vagina anaerobic	0.12	0.54
Vagina gram negative	-0.06	0.70
Corpus luteum volume	0.34	0.0002

Discussion

Presence of bacteria in varying compartments of the reproductive tract

Initially it was important to establish and characterize the microflora of the uterine and vaginal environment. To date only one other report exists in the literature assessing the microflora of the uterine horns (Ozenc *et al.* 2010). However, no reports have been published assessing the normal microflora of the vagina in comparison with the uterus. Both pieces of information are important for bovine recipient animal selection. The typical bovine recipient animal is a postpartum animal and these animals have been proven to harbor bacteria out to days 40 to 50 postpartum (Sheldon *et al.* 2008b). In addition many of these animals at this time point have subclinical infections that are difficult to detect (Sheldon *et al.* 2008b). Therefore, the potential exists for embryos to be transferred into recipients that have subclinical infections.

The current results indicate that bacteria were present to some degree in all locations. Bacterial presence in the uterus is consistent with other reports (Sheldon and Dobson 2004b; McDougall 2005; Ozenc *et al.* 2010). Furthermore, variability in the number of uteri that contain bacteria existed in these experiments as well. This was also in agreement with the current results of low uterine bacterial numbers in general. However, low uterine bacterial numbers was surprising considering that in the current

experiment the vagina had significantly more bacteria when compared to the uterine horns. A possible explanation for this can be due to the role and function of the cervix to filter bacteria from entering the uterus (Roberts 1978). The cervical structure may provide the uterus protection from the bacteria microflora of the vagina. Alternatively, clinically normal reproductive tracts were specifically selected in the current study. Thus, reproductive tracts utilized may be indicative of the normal uterine microflora. If a clinical infection had been present, increased bacteria growth the uterus may have been discovered.

Determination of bacterial presence on pH and temperature

Knowing that bacteria are present in the uterus in visibly normal appearing tracts; the next component was to identify effects of bacterial presence on pH or temperature in the uterine horns and vagina. Previous efforts have characterized the pH of the uterine body and horns (Chapter 2; Elrod and Butler 1993; Elrod *et al.* 1993; Rhoads *et al.* 2004; Perry and Perry 2008b; Perry and Perry 2008a; Karen *et al.* 2011). However, only limited research exists characterizing the effect of bacteria on uterine pH (Ozenc *et al.* 2010). However, past research evaluated the uterine environment and not the relationship of bacteria and pH between the uterus and the vagina. Thus an additional part of the current experiment was to evaluate the influence of bacteria on pH in varying locations of the reproductive tract. To assess this, pH values across locations regardless of bacteria was established to determine if pH differences existed. Similar to a companion research (Chapter 2, experiment 1), pH values were different between the vagina and ipsilateral uterine horn. However, differences between the uterine horns did not differ and agree with other efforts in this regard (Hugentobler *et al.* 2004; Ozenc *et al.* 2010). However, uterine horn pH values were significantly different from that of the vagina. The values reported in the current study were similar to pH values reported by others in the same location of reproductive tracts (Wehrend *et al.* 2003). That vagina values were higher than the uterine horns was somewhat surprising given the acidic nature of urine. Perhaps, cells of the uterus experience a faster postmortem shift to anaerobic energy production resulting in an increase in lactic acid. If so, this would

mimic the postmortem decline in pH seen in the meat science literature (Cross 1979; Jeleníková *et al.* 2008).

In regard to bacterial influence on pH value, only anaerobic bacteria had an effect in a linear model. Specifically, it appears that vaginal pH is the most influenced by bacterial presence. When only ipsilateral horn bacterial presence was evaluated, no effects on ipsilateral horn pH were revealed. However, Ozec and co-workers (2010) reported differences due to specific bacteria species within the uterus. Perhaps specific species have more of an influence on the local pH values. The current study only cultured bacteria based on general categories of aerobic, anaerobic, and gram negative and specific species were not differentiated.

However, pH differences were seen when groups were established based on anaerobic bacteria loads (figure 3.4). The tracts with more than 1000 colonies had a higher pH value compared to tracts in the other two groups. However, no differences in pH values were seen in either the ipsilateral uterine horn or vagina when separated out individually. Research by Ozec and co-workers (2010) indicate that pH differences do exist across bacterial species. Potentially, bacterial species may alter their local pH environment due to cellular activities (reviewed by Zeikus 1980; Clark 1989). Conversely pH may simply provide a local environment more conducive for growth of that species. In the current research the latter may be true. The pH of the vagina was higher regardless of bacteria presence and when bacteria were analyzed with pH more bacterial growth is present in the vagina.

Therefore, it may not be surprising that correlation results indicated that vaginal and ipsilateral environments were not correlated to each other. Specifically, vaginal bacteria growth did not depict the bacterial or pH values of the ipsilateral uterine horn. This aspect of the current research held importance for development of additional embryo transfer recipient selection criteria. It appears that the two environments are not connected, at least in the case of clinically normal appearing tracts. Again, based on the previous discussion of the cervix this may not be surprising (Roberts 1978). However, in cases of clinically infected animals, the environments may be more closely associated (Lewis 1997b; Sheldon and Dobson 2004b; McDougall 2005). The fact that clinically normal appearing tracts were specifically selected in the current study may have

influenced this, but also may provide further evidence of the normal microflora characteristics.

The pH values in the current study were affected by collection date although bacteria growth was not. It is surprising for date to impact one but not the other. Multiple factors could impact daily pH readings in the present study. Though samples were collected during four consecutive weeks in June, temperature could have impacted pH values (Swain 2010b). The temperature effect could have been directly on the cow prior to harvest or due to slight variations in plant temperature where samples were recorded. Additionally, past nutrition of the cattle could have played a role as well. Cattle in a negative energy balance being fed excessive amounts of crude protein (Elrod and Butler 1993) may have been harvested in greater numbers on various dates. Unfortunately, temperature readings and past nutritional status were not available for the current study.

The size of the reproductive tract did not have a statistical effect on either bacterial presence or pH values in the current experiment. It has been speculated in the past that reproductive tract size may have an influence on pregnancy success in artificial insemination programs (Young *et al.* 2010). However, previously unknown was the pH and bacterial presence of these larger tracts. The current experiment indicates that pH and size of these tracts may not be the factor leading to decreased pregnancy rates.

Analysis of progesterone influences on reproductive tract pH and bacteria growth

Progesterone is known to possess anti-immune properties that prevent natural antimicrobial mechanisms from fighting infections (Black *et al.* 1953; Rowson *et al.* 1953; Lewis 2003). Thus, as progesterone increases, the ability to combat infections decrease and in turn an increase in bacterial growth should be present. However, this is not the case in the current research. Possibly, duration of increased progesterone levels were not sufficient to promote increased bacterial growth. Reproductive tracts were selected based on the presence of a day 7 CL. Thus, progesterone concentrations were possibly still increasing and not reached maximal levels normally seen later in the estrous cycle (Henricks *et al.* 1970). However, more likely is the fact that specific

selection criteria for reproductive tracts in the present research targeted clinically normal appearing tracts. The animals from which these tracts were selected may have previously removed the bacterial infections through normal immune or postpartum mechanisms.

Conclusion

Bacteria were present in clinically normal appearing bovine reproductive tracts with a day 7 CL. However, presence of bacteria did not impact pH values of the uterine horn ipsilateral to the CL; the uterine horn associated with transfer of embryos in recipient animals. Vaginal bacteria growth was not directly correlated to ipsilateral uterine horn pH and bacteria presence. However, vaginal pH values were correlated to ipsilateral horn pH and can be used to predict this measure without attempting to directly measure the uterine horn. This serves a practical commercial application for bovine researchers and practitioners.

Research Conclusions

The ultimate goal of these research efforts was to identify physiological traits that could be used to distinguish and identify bovine recipient animals, prior to embryo transfer, which would achieve the greatest pregnancy rates. The recipient animal is one of the biggest challenges and sources of failure (both in fertility and economic terms) within the bovine embryo transfer industry. The current research measured various parameters of the bovine recipient animal's reproductive tract at the time and location of transfer to help better characterize the local environment the embryo is relying on for sustained development. The fact that uterine pH impacts bovine fertility is not a new concept but its application to the embryo transfer recipient animal is novel.

From this research, the realization that this pH influence has potentially negative impacts on pregnancy establishment holds exciting implications for the commercial embryo transfer industry. However, measuring the uterus of every cow is not practical and thus the establishment of pH correlations between the vagina and uterus provides the most potential for future product development. In addition, the established vaginal to uterine horn correlation was accomplished with two different probes. The latter option provides exciting field applications that remove the need for intrauterine measurements. Thus, as we move forward in this research area, measurements only need to be taken in the vagina to determine a recipient's suitability for embryo transfer. The largest limitation to the current efforts is the limited cow numbers. However, this was an accepted limitation and further validation should be the goal moving forward.

Furthermore, the current microbial data are still important and applicable to the commercial embryo transfer and artificial insemination industries. The reproductive tracts selected in the current research were similar to those that would receive an embryo in the commercial embryo transfer industry. It is evident that the vagina is a viable reservoir for bacterial growth and that in health appearing reproductive tracts the uterus is protected by the cervix. Thus, each time an insemination or transfer occurs we potentially transfer bacteria into the uterus with our current techniques. This realization underscores the required use of protective sheaths over both AI and ET guns during these procedures. This extra care would prevent transfer of bacteria from the vagina into the cervix and from cow to cow.

The current data offer exciting new understanding of the bovine reproductive tract and offers additional focus areas for future efforts. In addition, it offers an additional selection tool that can be used in the embryo transfer industry. Thus, practitioners and producers who utilize this information as an additional tool may hopefully improve pregnancy rates, production efficiency, and profitability.

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Vita

David Allen Roper was born on October 13, 1982 in Heidenheimer, TX and was raised on a small cattle operation. David was active in 4-H and FFA prior to graduating from Little-River Academy High School in 2001 and enrolling in Texas A&M University. He obtained his Bachelors of Science degree in Animal Science in May 2005. He then worked for Ultimate Genetics as a Marketing Representative prior to enrolling at the University of Tennessee to pursue a Masters and PhD in Bovine Reproduction. David worked under the guidance of F. Neal Schrick during his graduate career and served as a Lecturer in the Animal Science Department. In this role, David trained and coached the UT Livestock Judging Team. He completed his Masters of Science degree in May 2009 and his PhD in December 2014.