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AN INVESTIGATION INTO SPECIFIC SEMINAL PLASMA PROTEINS AND THEIR EFFECT ON THE INNATE IMMUNE RESPONSE TO BREEDING IN THE MARE

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AN INVESTIGATION INTO SPECIFIC SEMINAL PLASMA PROTEINS AND THEIR EFFECT ON THE INNATE IMMUNE RESPONSE TO BREEDING IN THE MARE

DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the College of Agriculture, Food, and Environment at the University of Kentucky

BY

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2017

ABSTRACT OF DISSERTATION

The mare experiences a transient innate immune response to breeding, the resolution of which is crucial for optimal fertility. The majority of mares are able to modulate this inflammation in a timely fashion, but a subpopulation exists which fail to do so and are considered susceptible to persistent breeding-induced endometritis (PBIE). Seminal plasma has been shown to modulate aspects of this inflammation. Recently, two seminal plasma proteins have garnered interest for their immune modulating properties: cysteine-rich secretory protein-3 (CRISP-3) and lactoferrin. These proteins have been found to alter the binding between sperm and neutrophils based on sperm viability *in vitro*, but minimal work has evaluated their effect on endometrial mRNA expression of cytokines and inflammation in response to breeding. Experiments were performed to analyze the expression of equine CRISP-3. Found to be primarily synthesized in the ampulla of the vas deferens and to a lesser extent in the vesicular gland, CRISP-3 expression was only seen in the postpubertal stallion. Due to the effect of sperm viability on protein function *in vitro*, varying sperm populations were analyzed for their effect on gene expression in the uterus. It was determined that viable sperm suppressed the gene expression of the inflammatory modulating cytokine interleukin-6 (*IL-6*) in comparison to dead sperm. Next, the effect of CRISP-3 and lactoferrin on endometrial gene expression in the normal and susceptible mare was investigated. Neither protein had a significant effect on the mRNA expression of inflammatory cytokines in the normal mares at six hours post-breeding. In contrast, lactoferrin was found to significantly suppress the expression of the pro-inflammatory cytokine tumor necrosis factor (*TNF*)-α in susceptible mares. Due to this, lactoferrin was further analyzed as an immunomodulant for the treatment of PBIE. Susceptible mares were infused with varying doses of lactoferrin at six hours. post-breeding. Although not in a dose-dependent fashion, lactoferrin was found to decrease both fluid retention and neutrophil migration, in addition to suppressing the expression of the pro- inflammatory cytokine interferon gamma (*IFNγ*) and increasing the gene expression of the anti- inflammatory cytokine interleukin-1 receptor antagonist (*IL-1RN*). In conclusion, CRISP-3 expression occurs in secretory aspects of the male reproductive tract and appears to be up regulated after sexual maturation. Viability of spermatozoa affects the immune response to breeding and should be taken into consideration for experimental design and interpretation of data. The seminal plasma proteins CRISP-3 and lactoferrin have minimal effect on endometrial gene expression in normal mares, but lactoferrin suppresses the expression of *TNF* in susceptible mares. Finally, lactoferrin was found to function as a potent anti-inflammatory for the persistent inflammation seen in susceptible mares when administered post-breeding. This protein should be further investigated as a potential therapeutic for the treatment of persistent breeding-induced endometritis.

Keywords: Equine, Endometritis, Lactoferrin, CRISP-3, Uterus, Cytokine

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April 18th, 2017
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AN INVESTIGATION INTO SPECIFIC SEMINAL PLASMA PROTEINS AND THEIR EFFECT ON THE INNATE IMMUNE RESPONSE TO BREEDING IN THE MARE

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Date

This dissertation is dedicated to my father,

Dr. Nicholas J. Fedorka.

To the man who did not always appreciate the horses but always appreciated the science.

May you find comfort in knowing that I found somewhere to exist in the space between.

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CHAPTER 1

REVIEW OF THE LITERATURE

1.1 Abstract:

Breeding-induced endometritis has been extensively studied in the horse, with primary focus on its pathway to infertility and the potential therapeutics to avoid this outcome. The healthy equine uterus undergoes a transient innate immune response to breeding, allowing it to clear excess spermatozoa and bacteria in preparation for successful embryo survival and development. A sub-population of mares exists that are unable to do this in a timely fashion and are deemed susceptible to persistent breeding-induced endometritis (PBIE), which is one of the leading causes of equine infertility. Seminal plasma is a known mediator of the uterine inflammatory response of the uterus to spermatozoa. A population of proteins within the seminal plasma is potentially acting to both protect the viable spermatozoa, allowing them to reach the oviduct as well as to recruiting neutrophils to assist with the digestion and clearance of dead spermatozoa. These proteins may affect the immune response of the uterus by activating and mediating cytokines and growth factors, as well as stimulating the secretion of prostaglandins to aid in uterine contraction; therefore prompting the removal of excess contaminants from the uterus, preparing it for pregnancy. In this review, the inflammatory process of the uterus, as well as a subset of the constituents of seminal plasma that possibly affect it, will be discussed.

1.2 Introduction:

At the time of breeding, billions of sperm are deposited directly into the uterus of the mare along with a high volume of seminal fluid and potential contaminating bacteria. The components of the seminal plasma derive from the rete testis, epididymis, and the accessory sex glands, and are essential for sperm maturation and capacitation. Because seminal plasma is considered to be detrimental to sperm quality during long term storage under cooled or frozen conditions, the majority of seminal plasma is removed when semen is prepared for cryopreservation or cooling. However, current literature suggests that elements of seminal plasma are critical in regulating the immune response of the uterus, including but not limited to, the resolution of breeding-induced endometritis [1, 2]. The inflammatory response is characterized by an increase in the expression of pro-inflammatory cytokines that signal the recruitment of polymorphonuclear neutrophils (PMNs) into the uterine lumen [3-5]. Mares that are resistant to PBIE are able to fully clear excess spermatozoa, fluids, and bacteria from the uterus within 24-36 hours of breeding. A population of mares that are unable to clear this inflammation are considered susceptible to PBIE and they experience uterine inflammation for a considerable length of time post-insemination. Susceptible mares are thought to make up 10-15% of the broodmare population, causing the disease to be one of the greatest reproductive health concerns in veterinary medicine [6, 7]. Due to the residual state of the endometrium and uterine lumen at the time the embryo migrates to the uterus from the oviduct (approximately 5-6 days post-ovulation [8]), susceptibility may lead to lower conception rates [6]. Woodward *et al.* found a significant difference in the mRNA expression of key endometrial cytokines between susceptible and resistant mares, suggesting that a defective anti-inflammatory response may be involved in the pathophysiology of the

condition [9]. It has been suggested that seminal plasma plays a role in mediating this immune response to semen deposition into the uterus, with specific seminal plasma proteins potentially acting as key players [10, 11].

1.3. Persistent Breeding-Induced Endometritis:

The elimination of excess sperm and contaminants after insemination is a crucial aspect to preparing the uterus for embryo survival and development. The transient innate immune response begins within 0.5 hours following sperm deposition in the uterus, and the inflammation is resolved within 36 hours post-breeding [5]. The process begins with expression of pro-inflammatory cytokines, which are synthesized by the epithelial cells in addition to readily occurring within the native ejaculate. These cytokines recruit the inflammatory cells, including mononuclear cells such as macrophages and lymphocytes, in addition to the PMNs that represent the first line of defense to the invasion of spermatozoa, bacteria, and other foreign contaminants presented to the uterus at the time of breeding. The surge of PMNs to the site of inflammation within the uterus leads to phagocytosis of spermatozoa as well as an increased prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) production, which accounts for the myometrial contractions observed 4-12 hours post insemination [12, 13]. Susceptible mares are prone to this persistent inflammation due to a variety of reasons; with age, malformation of both internal and external reproductive organs, and the lack of a functional innate immune response all being potential risk factors [6, 9, 14, 15]. The prolonged uterine inflammation in susceptible mares has been found to be detrimental to the conceptus, creating an environment not conducive to embryo survival or implantation [16-18]. Previous literature had suggested that the inflammation was caused by bacteria inoculated into the uterus at the time of breeding,

but dual studies by Kotilainen *et al.* (1994) and Troedsson *et al.* (1995) found that aseptic inoculation of spermatozoa to the uterus caused a similar inflammatory response to that of bacteria [19, 20].

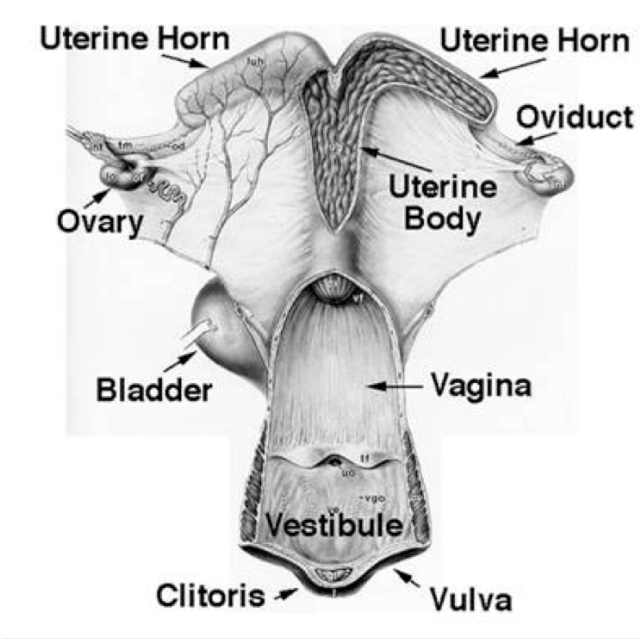
In regards to innate immunity, mares who are susceptible to PBIE were found to have a down-regulation of the endometrial gene expression for interleukin (*IL)-6*, interleukin-1 receptor antagonist *(IL-1RN),* and tumor necrosis factor (*TNF*)-α at 3 hours after *E.coli* inoculation, with a prolonged inflammatory response demonstrated by an up regulation of *IL-1β, IL-1RN*, and *IL-8* for over 72 hours post breeding. In contrast, resistant mares returned to baseline in their cytokine expression within 12 hours from breeding [21]. Concurrently with this study, Woodward *et al.* (2013) found that 6 hours after exposure to freeze-killed spermatozoa is a critical transitional time for the inflammatory response in both resistant and susceptible mares. Resistant mares had an increased expression of *IL-6, IL-1RN*, and *IL-10* at this time, when compared to susceptible mares [9]. Both studies agree that there is an inherent difference between resistant and susceptible mares in their ability to modulate the inflammatory response to breeding.

1.4 Uterine Defense and the Immune Response of the Uterus

1.4.1 Physical Barriers

The equine uterus is well protected from outer contamination by three specific barriers: the vulva, the cervix, and the vestibule or vaginal vault. In the normal mare, these barriers provide protection from fecal matter, bacterial infiltration, as well as air and urine pooling. During estrus, progesterone in circulation is low, allowing the vulva and the cervix to relax, thereby increasing the risk of uterine contamination. In the

normal mare, this risk is minimal and contaminants are easily expelled via myometrial contractions induced after exposure, as well as through an effective mucosal defense. If any of the three barriers are compromised, an increased risk of infection can occur. This occurs predominantly in older pluriparous mares, where poor perineal conformation is common. These factors induce a change in the overall position and angle of the vulva, which can then lead to an increased risk of fecal contamination. In addition, the routine deposition of spermatozoa into the uterus at the time of breeding is an additional cause of inflammation in the mare [4, 20, 22, 23]. This requires the uterus to have a unique immune response, one that will mount a rapid and potent response to bacterial pathogens, but be fairly tolerant to the spermatozoa that are introduced during breeding [24].



http://www.horsecoursesonline.com/college/repro/lesson_two_319_files

Figure 1.1 Equine female reproductive tract from a dorsal view [25].

1.4.2 The Innate Immune Response of the Uterus

The urogenital tract, which includes the uterus, is part of the mucosal immune system. The organs that make up this system all have a mucus-secreting epithelium that is the primary defense mechanism against foreign pathogens. Due to its physiological function of reproduction, this mucosal surface of epithelium is extremely permeable, and therefore of heightened risk to infection [26]. In addition to the pathogens that interact with the epithelium the mucosal immune system is also unique in that it is often exposed to foreign antigens that are not pathogenic. In the uterus, the spermatozoa as well as the proteins and enzymes within the seminal plasma are examples of this.

Although the uterus experiences both an innate and adaptive immune response, the innate immune response is both the first to defend, and the most potent defender. The innate immune system relies on different cell populations to enact specific activities. This includes the dendritic cells to act as antigen-presenting cells, while macrophages and neutrophils act to process antigens [27]. Antigens are detected by specific markers on their cell membranes, most commonly either pathogen-associated molecule patterns (PAMPs) or Toll-like receptors (TLR's), indicating that the antigen is in fact pathogenic. TLR's signal through ligand-receptor mediated pathways, which leads to the intracellular signaling and activation of PMNs and macrophages, as well as the recruitment and activation of the adaptive immune response [28, 29].

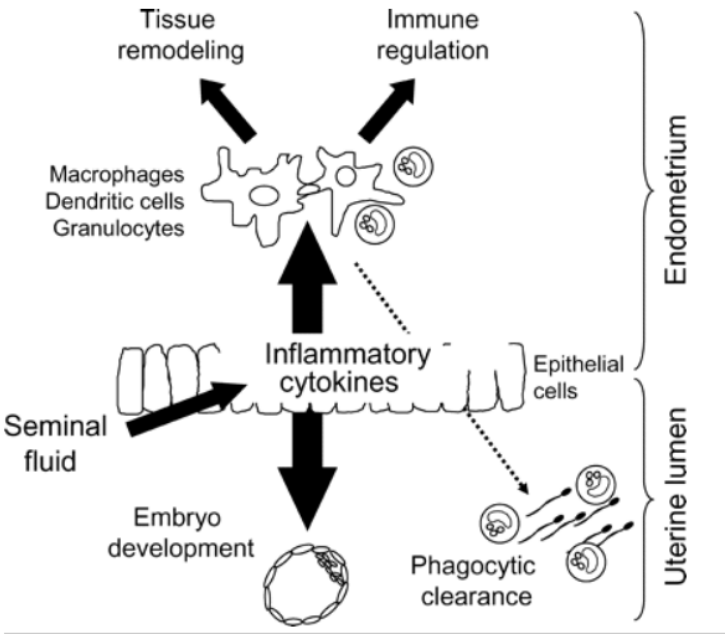


Figure 1.2 Schematic diagram of the innate immune response to breeding in the equine uterus. The deposition of semen activates the increased expression of inflammatory cytokines from the epithelial cells. These cytokines are multifunctional, and activate a number of immune cell types, including the macrophages, dendritic cells, granulocytes, and neutrophils. These cells than prepare the uterus for eventual embryo arrival and implantation. Figure used in permission by Dr. Robertson, and depicted in [30]

1.4.21 Epithelial Cells

Once activated, the TLR's activate the NFkβ pathway, which leads to the generation of pro-inflammatory molecules by the epithelial cells. These cells cover the mucosal surface and provide a physical barrier, preventing pathogens from getting further into additional aspects of the body. The epithelial cells are connected by tight junctions, which are regulated by steroids (estrogens and progestogens), calcium ions (Ca^{2+}), and numerous signaling molecules. These molecules majorly consist of lactoferrin, lysozyme, secretory leukocyte inhibitory protein (SLPI), and many cytokines [31-34].

It has been hypothesized that the primary role of the epithelial cells is to produce antimicrobial proteins in response to activated phagocytes [35]. In this study, activated

macrophages produced the cytokine *IL-1β*, and this increase induced the epithelial cells to heighten their production of the antimicrobial protein human beta defensin-2 (*HBD-2*). In conjunction to this study, Li *et al.* (2015) found that the intravenous infusion of the antimicrobial protein lactoferrin significantly suppressed synthesis of *IL-1β* after stimulation of inflammation in mice [36]. It appears that cytokines and antimicrobial proteins may function together either jointly or at least cohesively along similar pathways.

1.4.22 Cytokines

One example of the pro-inflammatory molecules that the epithelial cells produce is that of the cytokines and chemokines. This has been widely studied in the uterus of many species, with cytokines such as interleukin one (*IL-1*), interleukin six (*IL-6*), interleukin eight (*IL-8*), interleukin ten (*IL-10*), transforming growth factor beta (*TGF-β*), tumor necrosis factor (*TNF*)-α and -β, colony forming factor one (*CFF-1*), interferon (*IFN*)-α, -β, -τ, and –γ, and granulocyte macrophage colony stimulating factor (*GM-CSF*) reported [37-40]. In addition to the cytokines that have been noted in the uterus, prostaglandin E2 (PGE_2), prostaglandin F2 alpha ($PGF_2\alpha$), and inducible nitric oxide synthase (iNOS) were also increased in expression following the induction of inflammation in the uterine epithelium [33, 41, 42]. One of the first responders, *TNF*, is produced either by macrophages (*TNF-α*), or T cells (*TNF-β*). The interleukins (*IL-1* to *IL-17*) are involved in the interaction between lymphocytes and other leukocytes. Interferon's (*IFNα, β, γ, τ, ω*) are primarily synthesized in response to viral infection, but are also induced under immune and chemical stimulation. All cytokines and chemokines

are regulated by receptor expression, receptor antagonists, and other cytokines that may have stimulatory or antagonistic effects [43].

The cytokines have many downstream pathways, including the recruitment of immune cells, activation of hormones, and induction of myometrial contractility of the uterus. *TNF* is considered a first responder of the innate immune response, as it activates numerous downstream pathways. This pro-inflammatory cytokine is involved with the activation of the vascular endothelium, which increases membrane permeability, and therefore increases the entry of immunoglobulin's, complement, and other immune cells and mediators (Reviewed by [44]) . In a study done by Jacobs *et al.* (1993), it was shown that the pro-inflammatory cytokine *IL-1* was responsible for the activation of prostaglandin synthesis, which in turn stimulated myometrial contractility of the uterus [45]. *IL-1β* is also involved in the activation of lymphocytes, as well as localized tissue destruction. The pro-inflammatory cytokine *IL-8* is primarily responsible for the recruitment of PMN's due to its ability to up regulate cell-surface adhesion molecules, which enhances PMN adherence to the endothelial cells. In addition to this, *IL-8* is involved with the activation of lymphocytes and increased antibody production. *IL-6* is unique in that it has both pro- and anti-inflammatory abilities. At the beginning of an inflammatory response it acts through pro-inflammatory pathways, but then becomes critical in minimalizing PMN activation and instead recruits monocytes, leading to the resolution of inflammation. *IL-10* is an anti-inflammatory cytokine, as it prohibits monocytes from producing pro-inflammatory cytokines. *IL-1RN* is also anti-inflammatory, as it is a competitive inhibitor of the *IL-1* receptor, effectively blocking the pro-inflammatory activity of *IL-1β* [46].

In a study done by Woodward *et al.* (2013), it was found that in the healthy uterus, the synthesis of the pro-inflammatory cytokines (*IL-1β, IL-8, IFNγ*, and *TNF*) is increased within 2 hours after insemination, and peaks by six hours [9]. The expression of the anti-inflammatory cytokines (*IL-1RN, IL-10*) and inflammatory modulating cytokines (*IL-6*) was not as evident at two hours after insemination, but arose around six hours. In this study, the acute nature of inflammation was shown to occur at the 6-hour time point, as the key pro-inflammatory cytokines were relegated to basal levels at this time, while the anti-inflammatory cytokines were increased. This was hypothesized to be the cause of the acute and transient inflammation seen in the normal mare that is resistant to PBIE.

1.4.23 Immune Cells of the Innate Immune Response

One of the main functions of the pro-inflammatory cytokines is to stimulate, recruit, and activate the immune cells. PMNs, macrophages, and other leukocytes are the primary responders, and act swiftly to either digest or signal the elimination of foreign molecules. The first 24 hours of inflammation in the uterus appears to be regulated primarily by neutrophilic cells, and transitions to a monocytic cellular control after this time, with this transition controlled by the previously mentioned *IL-6* [46]. PMNs and macrophages are both phagocytic cells, but macrophages also function as antigen-presenting cells, whereas PMNs do not. Phagocytosis of microorganisms and foreign antigen such as spermatozoa occurs through a multistep process starting with chemotaxis via the chemokine *CXC8*. This begins by adherence of neutrophils through the activation of ICAM-1, followed by diapedesis of the neutrophils through the endothelial wall, which

involves many molecules, including LFA-1 and PECAM [3, 47-49]. This process enables the phagocytic immune cells to travel to the site of inflammation swiftly and efficiently.

In the uterus, the influx of PMNs occurs within 30 minutes after insemination, and this is simulated after the infusion of bacteria, seminal plasma, spermatozoa, or sterile solutions [5, 10, 20]. PMNs bind to microorganisms, including spermatozoa, via Neutrophil Extracellular Traps (NETs), as well as an unknown receptor/ligand mediated binding mechanism [10, 50]. Adhesion molecules such as the selectin family have been investigated as the ligand for the receptor/ligand binding mechanism, but their functionality was determined to be unaffected by the addition of antibodies specific to epitopes of their structure [51].

PMN phagocytosis appears to be regulated by proteins within the seminal plasma. *In vitro* studies have shown two seminal plasma proteins that modulate the interaction between PMNs and spermatozoa, cysteine-rich secretory protein-3 (CRISP-3) and lactoferrin. In a study done by Doty *et al.* (2011), CRISP-3 was found to selectively suppress the binding of PMNs to viable spermatozoa [11]. Conversely, a study done by Troedsson *et al.* (2014) demonstrated that lactoferrin increases the portion of PMNs which bind to spermatozoa, and this was further investigated and found to be selective towards dead spermatozoa [52]. Further research is required to investigate the functionality of these proteins *in vivo.*

The shift from a neutrophilic infiltration in the uterus to a monocytic resettlement is controlled by the expression of specific cytokines that recruit the two cell populations. As stated previously, PMNs are primarily activated by a heightened *IL-8/CXC8*

expression, which occurs early (<24 hours) in the inflammatory response. In contrast, monocytes are activated by monocyte chemotactic protein-1 (*MCP-1*), which increases in expression later in the response, and is maintained for numerous days (reviewed by [46]). *IL-6* may play a role in this transition, as it is able to activate endothelial cells to secrete both *IL-8* and *MCP-1* [53]. In the normal mare, PMN migration in the uterus is resolved within 24-48 hours, which is when macrophages begin to travel to the uterus. The macrophage invasion is then involved in angiogenesis, epithelial cell proliferation, and the restructuring on the endometrium as it prepares the endometrium to nurse an embryo [54]. Since all spermatozoa are removed before antigen-presenting cells enter the uterus, the development of an immunological memory against spermatozoa is avoided.

1.4.24 Prostaglandins

Another important aspect of the innate immune response to breeding is the contractility of the musculature of the uterus that is induced by an increase in prostaglandins synthesis. Prostaglandin F2 alpha ($PGF_2\alpha$) and prostaglandin E2 (PGE_2) are derived from arachidonic acid and are under the control of cyclooxygenase-2 (*COX-2*). These prostaglandins are produced in the male reproductive tract and secreted into seminal plasma, therefore describing their nomenclature. In addition to their synthesis in the accessory sex glands, they can also be produced by uterine cells as well as by activated PMNs that have migrated to the uterine lumen after breeding. These hormones are involved in a variety of phenomena in reproduction including ovulation, luteolysis, oviductal and uterine contractility, shedding of the uterine lining during menses, and involution of the uterus after parturition (Fuches, 1987).

The infusion of seminal plasma into the uterus activates further production of prostaglandins both PGE_2 and $PGF_2\alpha$, and this increase may affect the functionality of the ovary in the horse. In gilts, intra-uterine seminal plasma infusion increased the number of ovarian leukocytes at 34 hours in addition to having an effect on corpus luteum (CL) functionality [55]. In seminal plasma treated gilts, both plasma progesterone and the weight of the corpus luteum increased 5-9 days post-infusion. This was hypothesized to be due to the up regulation of *COX-2* transcription in response to seminal plasma infusion. *COX-2* is intrinsically involved in the production of prostaglandins, which can then access the ovary via the countercurrent articular circulation. This has not been determined in the equine ovary, which has different vasculature for which prostaglandins may travel to the ovary.

In addition to the role in luteolysis, prostaglandins play an integral role in the mechanical clearance of the uterus. This is crucial for uterine health after the deposition of semen, during parturition, as well as in the involution of the uterus post-partum. The dual functionality of $PGF_2\alpha$ and PGE_2 are critical in this, as $PGF_2\alpha$ has been shown to stimulate myometrial contractility, while PGE_2 acts to relax the cervix to allow for proper drainage from the uterus [49, 56].

In addition to the role in smooth muscle contractions, prostaglandins have been suggested to be involved in the disease process of endometrosis. Defined as degeneration of the endometrium and chronic fibrosis, an alteration in the mRNA expression of prostaglandin synthases has been described in addition to the production of prostaglandins in the endometrium of mares that suffer from this disease [57, 58].

Together, these hormones play pivotal roles in the resolution of inflammation after breeding.

1.4.3 The Adaptive Immune Response of the Uterus

In addition to the initial innate immune response, the uterus can also undergo an adaptive response to the presence of microorganisms. The uterus is unique in that it must mount this response to microorganisms, but maintain a low level of memory towards the repeated insult of spermatozoa. There exists a subpopulation of females that are unable to suppress this adaptive response, and in turn produce anti-sperm antibodies (ASAs), which have been suggested to be correlated with infertility [59]. A successful pregnancy is associated with a suppression of the adaptive immune response, including the expression of MHC molecules and T cells, allowing the embryo to remain viable in the uterus without being rejected (reviewed by [60]). Therefore, the production of ASAs, or any other aspect of the adaptive immune response, may result in lower fertility rates.

1.4.31 Immune Cells of the Adaptive Immune Response

In the adaptive response, macrophages and dendritic cells encounter foreign particles within the endometrium, bind, and then provide transportation to the lymph node for processing. This results in the activation of the Major Histocompatibility Complexes (MHC) Class I/II. Other cells of the adaptive immune response that have been found present in the uterus are the CD2+ (natural killer cell = NK cell), CD4+ (helper T cell), and the CD8+ (cytotoxic T cell) cells [61]. These cells function together to detect, process, and pursue foreign particles and organisms that infiltrate the body.

The adaptive immune response to breeding in agricultural animals has been most widely studied in the sow. Dual studies by Kaeoket *et al.* (2003) evaluated the effect of

inseminating pre-and post-ovulation on the immune cells that compose the adaptive response. The CD2+ cells were the most highly expressed cell in the surface and glandular epithelium and this was most notable within the first 24 hours after insemination [61, 62]. Upon investigation of the deeper tissues (subepithelial and glandular), the CD8+ were more present than the CD4+ cells, and this occurred later in the response, with the highest expression seen nineteen days after insemination. It was concluded in this study that the expression of these cell types was greater in the subepithelial layer later in the response, possibly due to the embryonic interaction with the surface of the epithelium at this time. The suppressed immune response within the surface epithelium may be intentional to enhance pregnancy outcome and lower the risk of rejection. In the equine uterus, T-cell populations have been investigated through immunohistochemistry. In a study by Tunón *et al.* (2000), it was shown that there is an increase in CD4+ but not CD8+ at 6 and 48 hours after insemination in the horse [63]. Further research is necessary to further describe the adaptive immune response in the uterus of the horse.

1.4.32 Anti-sperm antibodies

A hallmark of the adaptive immune response is the production of antibodies to the antigen present. Spermatozoa were found to be antigenic over a century ago, and in a small subpopulation anti-sperm antibodies (ASA) are present in circulation. In humans, ASA are detected in approximately 10% of infertile couples, but they are also found in 1.5% of fertile males and females (reviewed by [64]). Although largely correlated with infertility, minimal investigation has been conducted on their production, lifespan, and pathways.

Spermatozoa are produced in the seminiferous tubules of the male gonad, within which exists the blood-testis barrier. Created by interlinking sertoli cells, this provides a barrier between the premature spermatozoa and the immune cells that are constantly circulating through the system. This is essential to the production of viable spermatozoa as the spermatozoa are not present prior to puberty when the immune system is developing. Therefore, in males ASAs can be produced when the blood-testis barrier is damaged either by trauma, congenital abnormalities, or vasectomy [65].

ASAs are also produced in women; the production of which has been a topic of great debate for decades. In the 1920s, Baskin demonstrated that intramuscular immunization of previously fertile women with spermatozoa caused the production of ASAs [66]. However, repeated insemination of spermatozoa into the female reproductive tract did not cause the same response, indicating that the genitalia produce an inherently different response to the deposition of the antigen, or in this case, sperm. Although it is known that the female reproductive tract has a potent innate immune response consisting largely of a rapid influx of neutrophils and macrophages, it is widely believed that the factors that prevent antigenicity of spermatozoa are actually present within the seminal plasma, and specifically transforming growth factor beta (*TGF-β*) and prostaglandins. In a study by Stavnezer *et al.* (1995), it was shown that the production of ASA in the female mouse is dependent on *TGF-β* in a dose-dependent manner [67]. It was hypothesized that this was caused by the ability of *TGF-β* to inhibit B cell proliferation, which is an essential step in antibody production. The concentration of *TGF-β*, prostaglandins, and other mediators of the immune response within the ejaculate are variable, and this may be the reasoning behind a subpopulation of women producing ASAs.

In the horse, minimal work has been done on the effect of ASAs on fertility. In a case study done by Zhang *et al.* (1990), ASAs were found in a thoroughbred stallion suffering from infertility after testicular trauma [68]. A study by Kenney *et al.* (2000) found that inducible testicular degeneration causes an increase in ASA production, and that this lead to subfertility in stallions [69]. In this study, an ELISA was developed for the detection of ASAs, but minimal work has been published since.

1.5 The Role of Seminal Plasma in Breeding-Induced Endometritis

Unlike many other species where semen is deposited caudally to the cervix, spermatozoa and seminal plasma are deposited directly into the uterus of the horse at the time of breeding. Seminal plasma is the aqueous portion of the ejaculate, consisting of proteins, enzymes, electrolytes, and fluid derived from the accessory sex glands and epididymis of the stallion, which together make up a volume of 20-80 mL on average [10, 70]. Through the action of these proteins, enzymes, and metabolites, seminal plasma of the horse and pig has been shown to enhance sperm transport, hasten ovulation, and increase blood flow to the uterus and oviducts [71-73].

Seminal plasma is generally removed from spermatozoa to prepare for both the cryopreservation and cooling process involved in artificial insemination with cooled or frozen/thawed sperm. This can increase the duration of the inflammatory response to breeding, and possibly predispose mares to becoming susceptible to PBIE. In a study done by Alghamdi *et al.*, mares were inseminated with or without seminal plasma after having uterine inflammation induced, and found that only 1/22 (0.5%) of the mares conceived when seminal plasma was removed. Conversely, 17/22 (77%) of the mares that had seminal plasma in addition to the spermatozoa became pregnant [74]. It was

concluded that aspects of seminal plasma were critical to protecting spermatozoa from aspects of the immune system in order to achieve optimal fertility.

The effect of seminal plasma on the immune response has been studied in a variety of species, including mice, humans, and swine. It was found in mice that seminal plasma activates the expression of pro-inflammatory cytokines, including granulocyte-macrophage colony-stimulating factor (*GM-CSF*), interleukin-6 (*IL-6*), and several chemokines in the epithelium of the uterus [40, 75]. The expression of *GM-CSF* was also up regulated in the human epithelium, whereas in swine, *GM-CSF*, *IL-6*, and monocyte-chemotactic protein-1 (*MCP-1*) were the most notable pro-inflammatory cytokines to be activated after infusion with seminal plasma [76, 77]. Palm *et al*. (2008) found that seminal plasma caused an increase in the expression *of IL-1β, IL-6*, *TNF*, and *COX-2* at 12 hours post insemination in the endometrium of the horse, but noted that extender and phosphate buffered saline (PBS) caused similar results [78]. Unlike other studies into the immune response to breeding, this study only looked at the effect of treatment with semen extenders as well as seminal plasma without the addition of the spermatozoa, leaving for variation when comparing this response to that of what occurs at the time of breeding in the field.

1.6 Seminal Plasma Proteins

A primary constituent of seminal plasma are the proteins that are involved in many essential steps preceding fertilization including capacitation, regulation of the immune response to breeding, sperm transport and elimination, the establishment of the sperm reservoir at the oviduct, and gamete interaction and fusion [70]. Ranging from hormones to enzymes and proteinases, in addition to growth factors, the protein content

of the equine ejaculate is relatively low when compared to other species, at approximately 10 mg/mL. The majority of the seminal plasma proteins can be categorized into three groups; the fibronectin type II molecules (FN-2 type proteins), the cysteine-rich secretory proteins (CRISPs), and spermadhesins [70, 79]. Frazer and Bucci (1996) found a total of 14 proteins groups that were common to all stallions, and this was confirmed by the findings of a 2D-PAGE study by Brandon *et al.* (1999) [80, 81]. The first eight of these proteins to appear on the chromatography chart were categorized as HSP-1 to HSP-8 in chronological order of discovery.

1.6.1 FN-2 Type Proteins

The FN-2 type proteins, specifically HSP-1, HSP-2, and EQ-12 account for 70-80% of the total protein content of seminal plasma and are the equine ortholog to the major bovine heparin-binding proteins that play a role in sperm capacitation in cattle [82, 83]. HSP-1 and HSP-2 are produced by the ampulla of the vas deferens, whereas EQ-12 is synthesized in the caudal and corpus aspects of the epididymis [83]. The FN-2 type proteins are the most abundant seminal plasma protein in cattle, goats, and bison, although in the horse, a member of the CRISP family encompasses a large portion of the proteome [84-86]. These proteins are able to form oligomers due to their heparin-binding ability, forming larger aggregates of almost 90 kDa [87]. The most notable feature of these FN-2 type proteins is their ability to bind specifically with the phospholipids of the sperm membrane, which is mediated specifically by the choline lipids [88]. Widely studied in the bovine species, the bovine FN-2 type proteins, or BSPs, have been found to stimulate capacitation and induce the acrosome reaction [89]. Binding tightly to sperm at the post-acrosome and midpiece region via phospholipids, the BSPs travel through the

female reproductive tract with the ejaculate and appear to be involved with the development of the oviductal reservoir [83, 90]. It has been shown that bovine FN-2 type proteins are able to bind to phosphatidylcholine (PC) molecules that reside within the sperm plasma membrane, allowing the BSP-5 protein to actually penetrate the membrane [91]. When this occurs, it acts as a decapacitation factor, protecting the sperm from prematurely capacitating in the female reproductive tract (FRT). This protein also promotes capacitation once removed by increasing cholesterol efflux and phospholipid scrambling.

Recently, HSP-1 and HSP-2 have been shown to have chaperone activity, potentially protecting spermatozoa against damage [92]. Studies by Kumar *et al.,* these seminal plasma proteins were found to protect spermatozoa from oxidative, thermal, and chemical stress, and this was found to be potentially regulated by surfectants such as SDS and Tween-20 [92][93-95]. In addition, a pH switch was identified that control the chaperone-like activity of these seminal plasma proteins, indicating that their activity might be controlled by varying environments in the female reproductive tract [95]. Further research is required into the function of these proteins *in vivo*, and whether they may be critical for equine sperm capacitation.

1.6.2 Spermadhesins

The spermadhesin proteins have thus far only been found in ungulates, including the bull and horse, and have been extensively studied in swine. HSP-7 is the equine homologue of the spermadhesin AWN found in swine, which acts as a sperm-binding protein that is secreted in the caudal epididymis, and appears to play a role in fertilization [96]. One of the many proteins that are proposed to aid in sperm-zona pellucida

interactions, spermadhesin AWN-1 is synthesized in the tubuli recti, rete testis, and seminal vesicle epithelial cells of the porcine reproductive tract [97]. Most widely studied in swine, AWN-1 has phosphorylethanolamine-binding ability, which accounts for its ability to coat membranes and is a proposed mechanism for its ability to bind to zona pellucida [98].

In the bull, a similar seminal plasma protein is acidic seminal fluid protein, or aSFP. While structurally similar to porcine AWN-1, it lacks both the ability for sperm-coating as well as zona-pellucida binding [99]. The equine homologue, now known as HSP-7, or Equine AWN, shares 97.7% homology to porcine AWN-1, alluding to comparable activity [100]. Found in the spermatogonia, rete testis, ductus epididymis, as well as the seminal vesicles, it is a carbohydrate-binding protein that has been found to bind to intact equine zona pellucidae [100, 101]. Although it has been eluded that this protein may have a similar role in zona pellucida-sperm interactions as those seen in swine, this activity has not been investigated to our knowledge.

1.6.3 Cysteine-Rich Secretory Proteins

Recently, the CRISP family of proteins has gained interest in equine research due to the sizeable concentration found in the ejaculate of stallions. Multiple members of the CRISP family have been identified in the horse, including CRISP-1, CRISP-2, CRISP-3, and CRISP-4. Defined by the presence of 16 conserved cysteine residues that fold into two domains: a N-terminal CRISP with a CAP domain that contains six conserved cysteine residues, and the smaller C-terminal CRISP that contains 10 conserved cysteine residues [102]. While the CAP domain has been extensively studied, no known biological role has been found for it. Conversely, the C' terminal CRISP domain has

been found to play a role in the regulation of ion channels [103]. Transcripts for the CRISP proteins have been found throughout the reproductive tract of both mice and humans, with CRISP-1 found in the entire epididymis, CRISP-2 in the testis, epididymis, and seminal vesicles, and CRISP-3 in the epididymis, prostate, testis, and vas deferens [104]. CRISP proteins have been found bound to the sperm at a variety of locations, including the equatorial and post-acrosomal region, as well as the midpiece [105].

1.6.31 CRISP-1

CRISP-1, also referred to as Protein DE or AEG, has been studied extensively in rats with a focus on its ability to decapacitate spermatozoa. This allows the sperm in the epididymis to remain in a quiescent state, although the mechanism is unknown [106]. *In vitro* studies in the rat have shown that when recombinant CRISP-1 (rCRISP-1) is used in the media of zona-free rat eggs, the number of sperm that are able to penetrate is noticeably decreased, although the number of sperm that are able to simply bind to the egg remains the same [107]. A similar process has been described for both human and murine CRISP-1, implicating its involvement in gamete fusion via its binding to a complementary site on the egg [108, 109]. Another biological role of CRISP-1 is its involvement with protein tyrosine phosphorylation, an essential step in sperm capacitation. Studies done in CRISP-1 knockout mice have shown that *Crisp1-/-* mice had clearly lower levels of protein tyrosine phosphorylation than control mice, although they demonstrated similar levels of capacitation and maintained fertility [110]. Functionality of CRISP-1 has not been studied in the horse.

1.6.32 CRISP-2

Although CRISP-2 differs in only two amino acids from CRISP-1, its known biological function varies greatly. It has been shown that both mouse and human eggs

display complementary sites for CRISP-2, and this site may be shared with CRISP-1. The incubation of sperm with antibodies for CRISP-2 led to an inhibition of sperm penetration to the egg, although the number of sperm at the perivitelline layer was actually increased, leading the scientists to believe that CRISP-2 works at the sperm-egg membrane interaction level, and plays a direct part in gamete fusion [111].

1.6.33 CRISP-3

Although CRISP-1 and CRISP-2 have been found to play a role in male fertility, including assisting with the binding of the sperm to the oocyte and regulating motility, CRISP-3's only known biological activity lays in its ability moderate the immune defense of humans and rats [112, 113]. Transcripts for human CRISP-3 are found throughout the body, but predominantly in the salivary gland, pancreas, and prostate, which secretes CRISP-3 into the seminal plasma [114]. It is found in equine semen at a concentration of 0.3-1.3 mg/mL, and differs from human and murine CRISPs due to its lack of a N-glycosylation site [105].

It has been determined that equine CRISP-3 is a non-glycosylated protein that displays an isoelectric point of 8.0 [105, 115]. This differs from other members of the CRISP family, such as rat epididymal protein DE, mouse CRISP-1, and human neutrophil specific granule protein of 28 (SGP28) [116-118]. The most widely studied members of the family; these three proteins are all acidic or neutral glycoproteins. The proteins D and E, also known as CRISP-1, are N-glycosylated and highly homologous proteins [116, 119]. Equine CRISP-3 is similar to other CRISPs in that 14 of the 16 cysteine residues are located in the carboxyl terminus of the protein, but differs in that its N-terminal amino acid is blocked [105, 115]. It is proposed that amino acids 1-22 of the

gene represent a hydrophobic signal peptide, with the 23rd amino acid being glutamine, which is modified to glutamic acid. The mature equine CRISP-3 protein is 224 amino acids long, and this sequence shows 82% homology to human CRISP-2, 78% homology to guinea pig CRISP-2, and 77% homology to human CRISP-3 [105]. Within the CRISP-3 protein structure, all 16 cysteine residues are involved in disulfide bonds.

Research has investigated the use of CRISP-3 in the stallion ejaculate as an indicator for fertility, and its role in sperm/PMN interaction in the uterus. Hamann *et al.* (2007) found that the Hanoverian horse expresses 52 polymorphic sites for CRISP-3, and the E208K polymorphism in the transcript had a significant correlation with fertility in stallions [120]. Homozygosity for the specific genotype c.+622G, where a single nucleotide exchange resulted in an amino acid change from lysine to glutamine was found to be associated with increased fertility [120]. It was also recently shown that an abundance of CRISP-3 in the ejaculate is positively correlated with first cycle conception rates as well as high semen freezability [121, 122]. While CRISP-3 correlates with these aspects of fertility, the mechanism is unknown and requires further research.

Doty *et al.* (2013) demonstrated *in vitro* that the addition of equine CRISP-3 to PMNs and live spermatozoa caused a decreased amount of binding between PMNs and spermatozoa, and this was even greater than that of seminal plasma [11]. Conversely, when nonviable spermatozoa were used instead of live, this protection from phagocytosis was no longer present. CRISP-3's ability to provide selective protection of viable spermatozoa from the phagocytosis by PMNs would not only be of great importance for increased fertility but would also assist with effective clearance of the uterus post breeding [12].

1.6.34 CRISP-4

While CRISP-1, CRISP-2, and CRISP-3 have been identified in many mammalian species, CRISP-4 has only been found in the epididymis of murine species. Found to be the ortholog of human CRISP-1, it has been hypothesized to play a similar role in sperm-egg fusion. In a study done by Turunen *et al.* (2011), murine *iCre* knockins for the CRISP-4 gene were created to show that mice deficient of the CRISP-4 gene produced sperm that were unable to undergo the progesterone-induced acrosome reaction, as well as lower fertilization ability, although they did remain fertile in natural mating conditions [111]. In this study, it was also noted that less CRISP-4 deficient sperm were found bound to the oocyte when compared to wild type. These results lead the scientist to the conclusion that murine CRISP-4 plays a pivotal role in sperm-egg interactions.

1.6.4 Lactoferrin

A smaller portion of the protein fraction in the stallion ejaculate is made up of miscellaneous proteins that do not fall into the category of FN Type-II protein, spermadhesin, or CRISP. One of these proteins is lactoferrin, a seminal plasma protein that has been found to have a stimulatory effect on the binding of spermatozoa to PMNs [123]. Lactoferrin has been localized to the acrosome region and the sperm tail, but motile sperm showed no positive staining for the protein, suggesting that lactoferrin may bind selectively to non-viable spermatozoa [124]. Recent research suggests that this observation may involve a lactoferrin associated protein rather than lactoferrin, and this needs to be confirmed (unpublished observation). There is limited research on lactoferrin in the stallion, but it is known to be found in the seminal plasma of the stallion at an

average concentration of 157μg/mL and has been suggested to correlate with low semen freezability [122, 125].

With a molecular mass of 80kDa, lactoferrin is an iron chelator that is found throughout the body, including secretions such as semen, milk, and uterine luminal fluid, as well as in the secondary granules of neutrophils [126-128]. Lactoferrin has been found to act in a bactericidal fashion over a wide range of bacterial hosts, including *Streptococcus, Salmonella,* and *Escherichia [129]*. In addition, lactoferrin has been shown to suppress tumor growth and impede fungal infection development [130, 131].

Considered one of the principles in the first line of defense, lactoferrin has been found to affect many aspects of the immune system. Lactoferrin is able to stimulate macrophage function by binding to lactoferrin specific receptors on the cell membrane [132]. Via this binding, lactoferrin has been shown to suppress the activity of several pro-inflammatory cytokines, including *TNF, IL-6,* and granulocyte-macrophage colony-stimulating factor (GM-CSF) [133-136]. It has been demonstrated that lactoferrin functions through the NFκβ and MAP kinase pathways to affect the activation, differentiation, maturation, and function of many other immune cells, including natural killer (NK) cells and lymphocytes [137, 138].

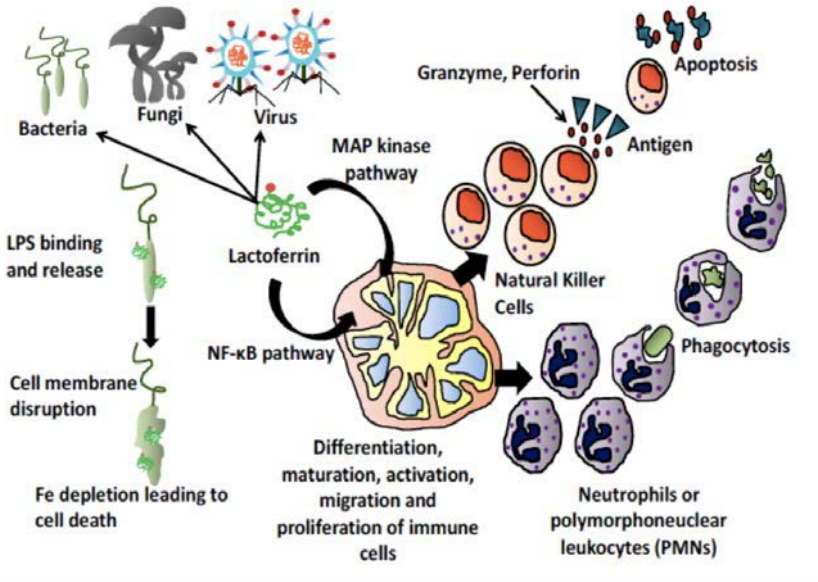


Figure 1.3 Schematic diagram of the role of lactoferrin in the innate immune response of humans. As described by Kanwar *et al.*, (2015) [139]. Lactoferrin plays an important role in the activation of immune cells, including the differentiation, maturation, activation, migration, proliferation and function of macrophages. Lactoferrin also promotes the cell-cell interaction and activation of PMNs and NK cells, therefore boosting the immune response.

Human lactoferrin has also been found to play an essential role in the Eppin Protein Complex (EPC), which is a part of the ejaculate coagulum that coats the spermatozoa immediately after ejaculation. The EPC is made up of lactoferrin, clusterin, and semenogelin, and is assembled on the surface of spermatozoa during the last step of spermiogenesis, acting to protect spermatozoa due to its bactericidal properties [140]. Research in humans also demonstrated that lactoferrin positively correlates with semen samples showing oligospermia as well as increased sperm motility and concentration [141-143].

Limited work has been done on lactoferrin in the horse. Lactoferrin has been shown to be up-regulated in the endometrium of the susceptible mare, indicating a role in

the immune response of the equine endometrium [144]. In addition, its synthesis was found to be hormonally regulated, as it was determined to be estrogen-dependent in the caudal aspect of the stallion epididymis [145].

1.6.5 Other

In addition to the three main families and lactoferrin, there exists many other seminal plasma proteins found in the ejaculate. Horse seminal protein-4 (HSP-4) has been found to relate to the calcitonin-like protein family. In humans and mice, calcitonin levels correlate with sperm motility, indicating that HSP-4 may be involved in equine sperm motility [146]. Horse seminal protein-6 (HSP-6) and horse seminal protein-8 (HSP-8) are thought to be isoforms of a single protein that is similar in structure to human prostate-specific antigen (PSA), which is involved in the cleavage of seminal coagulum [147]. While the gel fraction is removed during artificial insemination, in natural breeding and live cover, this would be an important role for a seminal plasma protein. Sperm protein 22 (SP22) was found to be primarily detected on the equatorial region of the sperm head. Environmental stress to spermatozoa associated with cryopreservation and cooling of semen altered the binding pattern of SP22 [148]. Although this protein is thought to be involved in fertility in rats, minimal work has been done on its effect on fertility in the horse [149]. Other proteins and enzymes, such as leptin, lipase, and angiotensin-converting enzyme have also been found to be secreted into the seminal plasma of stallions, but little is known on their function. With new technology developing on the purification and identification of proteins, future studies are expected on the proteomics of the equine ejaculate.

1.7 Steroid Regulation of Protein Secretion in the Male Reproductive Tract

1.7.1 Hormonal influences on male reproduction

In the male, testicular function and output is controlled by the hypothalamic-pituitary-gonadal axis, which involves the function of gonadotropin releasing hormone (GnRH), luteinizing hormone (LH), follicle stimulating hormone (FSH), androgens, estrogens, and inhibin. GnRH is released from the hypothalamus and travels in a pulsatile fashion to the anterior pituitary, where it binds to G-protein coupled receptors to stimulate the release of the gonadotropins, LH and FSH. The gonadotropins then travel via circulation to the testis, where LH stimulates the production of testosterone from the Leydig cells that surround the seminiferous tubules. Conversely, FSH binds to the Sertoli cells and stimulates the production of inhibin, activin, and androgen binding protein (ABP). While FSH regulates spermatogenesis during puberty, testosterone becomes the primary regulator of sperm production in the postpubertal male. Testosterone and inhibin then travel back to the hypothalamus via circulation in a primarily negative feedback loop.

Estrogens also play a role in testicular function, predominantly by regulating the secretion of LH into the system. While many estrogens have been detected in circulation of the stallion, estradiol-17β has been most studied. In a study done by Parlevliet *et al.* (2006) expression of estradiol-17β in the epididymis was found to be affected by age [150]. Prior work done by Roser *et al.* (1997) and Stewart (1998) demonstrated that estradiol-17β modulates the release of LH from the anterior pituitary, and that there were increasing levels of this hormone as age increased [151, 152]. It has also been shown that a population of infertile stallions have a significantly lower concentration of estrogens

than what is found in fertile stallions. It is evident that estrogens, specifically estradiol-17β, play an important role in stallion fertility and testicular function.

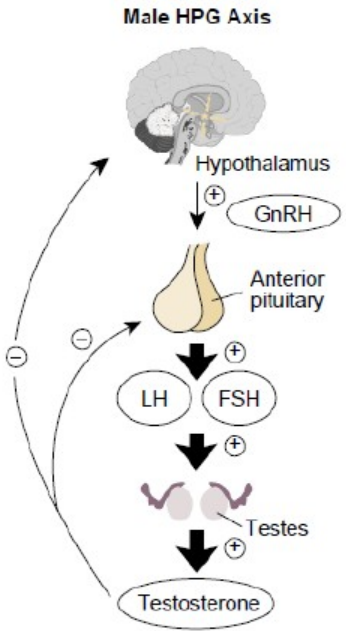


Figure 1.4 Schematic diagram of the male hypothalamic-pituitary-gonadal axis

1.7.2 Hormonal influences on seminal plasma proteins

It has long been known that steroid hormones play a role in the development and functionality of both the female and male reproductive systems. The removal of the testis via castration has been used to significantly suppress the production of testosterone, and this in turn may affect the synthesis and secretion of seminal plasma proteins [153]. In a study done in 1996 by McDowell *et al*., stallions were separated into three groups: intact stallions, geldings, and geldings supplemented with testosterone. It was seen that certain protein groups, specifically group 1 (60kDa, pI 7) and group 2 (23 kDa, pI 4-5) were more prominent in the stallions with testosterone in their systems, both the intact group as well as the supplemented. Conversely, the other three protein groups, group 3 (25-30 kDa, pI 5.5-6), group 4 (23 kDa, pI 7-8), and group 5 (15-20 kDa, pI 6-7.5) were more

prominent in the geldings who were not supplemented with testosterone, demonstrating that some proteins in the seminal plasma may be dependent on testosterone [154]. The authors suggested that more research was required to distinguish these proteins, as well as further investigate the androgen and estrogen dependency of each. Recently, a similar study was done in humans, where the seminal plasma protein content of men with hypogonadism was studied via mass spectrometry to determine if specific seminal plasma proteins were lacking in these patients, and testosterone supplementation was then performed to determine if any of the absent proteins were able to be reintroduced [155]. Thirty-three of the sixty-one proteins studied were not found in patients suffering from hypogonadism, and of these thirty-three, fourteen were detected after 6 months of testosterone supplementation. Some of the proteins identified were S-100 A9, lactoferrin, prolactin-inducible protein (PIP), prostatic acid phosphatase (PAP), carboxypeptidase E, and cystatin C, all of which have been shown to play a role in fertility. Their absence in the hypogonadic patients may explain the infertility related to the disease. Although it is known that seminal plasma proteins play a role in sperm maturation, capacitation, and fertilization, minimal work has been done on the effect of hormones on regulating protein synthesis in the horse.

1.7.21 Lactoferrin

Lactoferrin is found in the caudal and corpus regions of the epididymis in the horse. In a study done by Parlevliet *et al.* (2005), it was shown that the epididymis of the male horse is dependent on both androgens and estrogens. The concentration of testosterone in the testis and epididymis does not change as the horse matures, while the concentration of estrogens in all regions of the epididymis did increase with age,

suggesting that estradiol may play a role in the maturation of the epididymis, as well as its functionality [150]. It was recently shown that lactoferrin was not expressed in any aspect of the epididymis in pre-pubertal animals [145]. In a second study by Pearl and Roser, lactoferrin was further investigated through tissue culture of the epididymis [145]. It was determined that testosterone did not increase the amount of lactoferrin in the culture media, while estradiol-17β resulted in a significantly increased of this protein. It was concluded that estrogen appeared to regulate the secretion of lactoferrin from the epididymis of post-pubertal stallions. This correlates with previous research in humans and mice that demonstrated that lactoferrin upregulated in the epididymis of the immature mouse under estrogen supplementation [156, 157].

In the female, lactoferrin has been shown to be estrogen dependent in the human, monkey, mouse, rate, and equine endometrium [144, 158, 159]. In the equine model, Kolm *et al.* found that the stage of the estrous cycle in mares had a significant effect on the level of transcription for lactoferrin. Transcription was 5500-fold higher during estrus when compared to diestrus. It was also found in this study that mares susceptible to PBIE had increased levels of lactoferrin transcription throughout their cycle when compared to normal mares, and this reached statistical significance during proestrus. This increase in lactoferrin in the susceptible mare was found to correlate with the number of uterine glands and not the increased number of CD18+ leukocytes. These studies suggest that lactoferrin is estrogen dependent also in horses.

1.7.3 CRISP-3

Although found to be regulated by androgens in both the human and murine models, no studies have been performed on the androgen or estrogen regulation of

CRISPs in the horse. It was demonstrated in the murine model that castrated males showed a significantly decreased expression of CRISP-3 in the salivary gland than intact males [160]. This was further investigated via the administration of a gonadotropin-releasing hormone (GnRH) antagonist, where both CRISP-1 and CRISP-3 expression was markedly reduced in the salivary gland, while CRISP-2 expression in the testis was not effected [161]. In this same study, female mice were ovariectomised and supplemented with testosterone propionate before having their salivary glands analyzed by RT-PCR where CRISP-1 and CRISP-3 expression were notably increased, suggesting that both CRISP-1 and CRISP-3 are androgen dependent in the salivary gland, where they are primarily expressed in this species.

More notably, CRISP-3 is being used as an androgen dependent biomarker in diseases of the immune system in humans, such as Sjögren's Syndrome (SS). A disease of the exocrine glands that causes keratoconjunctivitis sicca (dry eyes) and xerostomia (dry mouth), it is primarily seen in older females [162]. In the human model, it has been shown that dehydroepiandrosterone (DHEA) and dihydrotestosterone (DHT) up regulate the expression of CRISP-3, and that this pathway seems to be defective in patients with SS [163]. Because patients with SS are unable to produce adequate amounts of DHEA and DHT, they are unable to produce normal amounts of CRISP-3 in the salivary gland, and physicians can use the expression of CRISP-3 as an androgen dependent biomarker to assist in their diagnosis of this disease.

1.8 Conclusions

Semen deposition occurs directly into the uterus of the mare, and this causes an innate transient immune response. Mares that are able to clear the resulting inflammation

within 24-36 hours are considered resistant to PBIE. However, a subset of mares are unable to clear the uterus from inflammation in a timely fashion, and this is an important cause of infertility in horses. This response is characterized by an influx of pro-inflammatory cytokines from the lumen, which recruit PMNs to begin the phagocytosis of excess spermatozoa as well as the trigger for synthesis of prostaglandins to initiate the contractility required to clear the uterus in preparation for embryo implantation. This neutrophil response is both rapid and transient, and the seminal plasma that carries the spermatozoa appears to play a key role in this action. Seminal plasma derived lactoferrin was shown to promote the binding of PMNs to dead sperm whereas the seminal plasma protein CRISP-3 was found to suppress this phagocytosis of viable sperm, and is theorized to help escort them to the oviduct in order to reach the ovulated oocyte. Therefore, we hypothesize that these select seminal plasma proteins will have an effect on aspects of the innate immune response to breeding in both the normal and diseased mare.

CHAPTER 2

Expression and localization of cysteine-rich secretory protein-3 (CRISP-3) in the prepubertal and postpubertal male horse

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2.1 Abstract

The seminal plasma protein cysteine-rich secretory protein-3 (CRISP-3) has been correlated with increased fertility and first cycle conception rates, and has been suggested to be involved in the immune response to breeding in the horse. Previous research demonstrated that equine CRISP-3 is located in both the ampulla of the vas deferens and the seminal vesicles. However, this was done with non-quantitative laboratory techniques. In humans and rodents, CRISP-3 has been described as an androgen-dependent protein, but the effect of androgens on the expression of CRISP-3 has not been investigated in the horse. The objectives of this study were to a) confirm and quantify the expression of CRISP-3 in the male equine reproductive tract, b) describe the localization of CRISP-3 within the specific tissues which express it, and c) determine if expression of CRISP-3 increases after puberty. We hypothesized that expression of CRISP-3 would be expressed in the ampulla of the vas deferens and the seminal vesicles, and expression would increase after puberty. Tissues were collected postmortem from three prepubertal colts (< six months) and six postpubertal stallions (> 3 years). Tissue samples were collected from the ampulla of vas deferens, seminal vesicles, bulbourethral gland, prostate gland, testis, as well as the cauda, corpus, and caput aspects of the epididymis. qPCR and immunohistochemistry (IHC) were performed using an equine specific CRISP-3 designed primer and monoclonal antibody. A mixed linear additive model was used to compare mRNA expression between age groups, and significance was set to $P<0.05$. There was a significant interaction between maturity and tissue type. mRNA expression of CRISP-3 was found primarily in the ampulla of the vas deferens with lesser expression in the seminal vesicles. Expression of CRISP-3 was higher in the postpubertal stallion when compared to the prepubertal colt for the ampulla ($P<0.0001$) and seminal vesicles ($P=0.0013$). IHC

showed that equine CRISP-3 is primarily located in the glandular aspects of both the ampulla of the vas deferens and the seminal vesicles, with staining concentrated in the cytoplasm of the epithelial cells that surrounded the glands of the mucosa. CRISP-3 was only observed in the postpubertal male horse, suggesting that puberty plays a role in activation of equine CRISP-3 expression.

2.2 Introduction

Seminal plasma proteins play an important role in many aspects of reproduction including sperm maturation and transport, fertilization, as well as the inflammatory response to breeding [11, 98, 164]. Previous studies have found that a portion of the protein content of seminal plasma is regulated by androgens and estrogens. In a study done by McDowell *et al.* (1996), it was found that certain seminal plasma protein groups were more prominent in the stallions with testosterone in their systems, as both the intact stallions and geldings that were supplemented with testosterone showed increased protein content [154]. Unfortunately, data on the specific proteomics of equine seminal plasma was unavailable at this time. It was therefore concluded that future studies should be performed on the effect of androgens on the specific proteins within seminal plasma, but minimal work has been done to date.

One seminal plasma protein of interest, cysteine-rich secretory protein-3 (CRISP-3), is found in the equine ejaculate at a higher concentration than in any other species studied (1mg/mL), and is associated with many markers of reproductive wellness [105]. CRISP-3 has been found to correlate with first cycle conception rates, and the modulation of polymorphonuclear neutrophil (PMN)-binding and phagocytosis of spermatozoa during the inflammatory response to breeding [11, 120, 121]. Although these studies

show CRISP-3 correlating with aspects of reproduction, there is minimal data demonstrating the pathway through which CRISP-3 acts.

In humans and rodents, CRISP-3 has been described as an androgen-dependent protein. In a study by Haendler *et al.* (1993), castrated male mice showed a significantly suppressed expression of CRISP-3 in the salivary gland in comparison to intact males [160]. This was further investigated via the administration of a gonadotropin-releasing hormone (GnRH) antagonist, causing both CRISP-1 and CRISP-3 expression to be markedly reduced in the salivary gland, while CRISP-2 expression in the testis was not affected [161]. When testosterone propionate was supplemented after the implementation of the GnRH antagonist, CRISP-3 expression was restored. In the same study, female mice were ovariectomised and supplemented with either estrogen or testosterone propionate. Testosterone propionate significantly increased the expression of CRISP-1 and CRISP-3, whereas estrogen had no effect, suggesting that both are solely androgen dependent.

While the salivary gland of the horse also has been found to express CRISP-3, our primary focus is on the expression of CRISP-3 within the reproductive tract. In other species, the protein is found predominantly in the epididymis and prostate, but previous research finds equine CRISP-3 primarily located in the ampulla of the vas deferens with a more moderate amount expressed in the seminal vesicles [105]. The equine differs from other species in the concentration of CRISP-3 synthesized, as well as the localization of its expression, making inferences with other species difficult to make.

Therefore, the objectives of this study were to a) confirm and quantify the amount of CRISP-3 mRNA transcript present in the ampulla and seminal vesicles of the mature stallion, b) describe the localization of CRISP-3 protein in the specific tissues via immunohistochemistry, and c) determine if CRISP-3 expression is up-regulated in the tissues of the postpubertal horses when compared to the prepubertal. We hypothesize that the pubertal transition in the male horse, which is controlled by an increased synthesis of androgens, will have an effect on the expression of equine CRISP-3 in the reproductive tract of the male horse.

2.3 Materials and Methods

2.31 Tissue Collection

For quantitative real-time polymerase chain reaction (qPCR) and immunohistochemistry (IHC), three prepubertal (<six months) and six postpubertal (>three years) animals were used. Reproductive tracts were collected from normal males of mixed breeds post mortem. Reproductive tracts were divided into eight parts: the ampulla of the vas deferens, bulbourethral gland, prostate gland, seminal vesicles, testis, and the caput, corpus and cauda of the epididymis. Tissues were fixed in 4% paraformaldehyde (w/v) for immunohistochemistry (IHC) or RNALater (Applied Biosystems, Carlsbad, CA, USA) for qPCR.

2.32 Quantitative Polymerase Chain Reaction Analysis

Total RNA was extracted from 50 mg of tissue using TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) as described by the manufacturer. Total RNA was precipitated using sodium acetate and isopropanol, resuspended in ddH_2O and DNAse treated (DNA-free™, Applied Biosystems), and then analyzed for quantity and quality via a NanoDrop® spectrophotometer (Thermo Scientific, Wilmington, DE, USA). RNA was

reverse transcribed and qPCR was performed as described by Fedorka *et al.* [165]. Briefly, 1.5 μg of RNA in 41.5 μL ddH_20 was reverse transcribed using Promega reagents; 0.5 μL AMV Reverse Transcriptase, 16 μL 5x RT Buffer, 1 μL RNAsin®, 16 μL MgCl, 4 μL dNTP, and 1μL Oligo(dT) Primer (Promega, Madison, WI, USA). Samples were incubated at 42°C for 60 minutes followed by 95°C for 5 min. Primer/probe sequences are shown in Table 2.1. cDNA was diluted 1:1 with ddH_20, and qPCR was performed using 4.5 μL of cDNA, 5μL of Sensimix™ II (Bioline, Tauton, MA, USA) and 0.5 μL of a custom primer/probe set from Applied Biosystems. Reactions were performed in duplicate with GAPDH as the reference gene using the ViiA 7 Real-Time PCR System (Applied Biosystems, Grand Island, NY, USA) [166]. Samples were incubated at 95°C for 10 min, followed by 45 cycles of 95°C for 15 sec and 60°C for 60 sec. PCR efficiencies were calculated using LinRegPCR (version 2013.0). Results were expressed as the mean relative quantification value (RQ) which were calculated using the $2^{-\Delta\Delta CT}$ method as described by Livak and Schmittgen, with the calibrator as the mean cycle threshold (ΔC_T) value of all prepubertal horses from the specific tissue utilized [167].

2.33 Immunohistochemistry

Immunohistochemical staining was performed using the monoclonal mouse anti-equine CRISP-3 antibody derived from the HL2175 line (University of Florida's Hybridoma Core Lab; Gainesville, Florida). Tissue sections of 5μm were cut from paraffin blocks. Slides were stained following the manufacturers protocols of the Bond Polymer detection systems (Leica Biosystems) as previously described by Klein *et al*. [168]. The liver from a mature intact male was used as a negative control. Briefly, this

involved an initial automated dewaxing and rehydration step, followed by heat-induced antigen retrieval (100°C for 20 min) with ethylenediaminetetraacetic acid (EDTA)-based ready-to-use solution (Leica Biosystems) at a pH of 8.8. The slides were then incubated with 3% hydrogen peroxide (5 min), diluted primary antibody (15-30 min), a post-primary blocking reagent to prevent nonspecific polymer binding (8 min), horseradish peroxidase-labeled polymer (8 min), and diaminobenzidine substrate (10 min).

The primary antibody was diluted 1:200 using Bond Primary Antibody Diluent (Leica Biosystems). Washing steps between each reagent were performed using Bond Wash Solution 10x Concentrate (Leica Biosystems) diluted to a 1x working solution with distilled water.

2.34 Statistical Analysis

For the qPCR data, analysis was performed using SAS 9.4 (SAS Institute INC., Cary, NC, USA). A mixed linear additive model with maturity and tissue type as fixed effects, and stallion as random was analyzed with proc glm. Data was tested for normality using both a Bartlett's and Modified Levine's test, and RQ values were square root transformed for normality. An analysis of variance was done on the main effects of maturity and tissue type with post hoc analysis performed using a Duncan's test. Significance was set to $P \leq 0.05$. Data are presented as the untransformed means ± the standard error of the mean.

2.4 Results

Using the $\Delta\Delta C_T$ method, expression levels of equine CRISP-3 analyzed by qPCR were calculated relative to the levels seen in prepubertal horses (Fig. 2.1). There was a significant interaction between maturity (prepubertal and postpubertal) and tissue when comparing the mRNA expression of CRISP-3 in the tissues of the reproductive tract

($P<0.0001$). The main effects were spliced out and analyzed individually, and a difference in the mRNA expression of CRISP-3 was observed in the postpubertal stallion ($P<0.0001$) but not the prepubertal colt ($P=0.0746$).

The tissue with the highest level of mRNA expression of CRISP-3 was the ampulla of the vas deferens. There was a significantly higher mRNA expression of CRISP-3 in the ampulla of the postpubertal stallion than the prepubertal colt ($P<0.0001$). A similar increase in the mRNA expression was seen in the seminal vesicles of the postpubertal stallion ($P=0.0013$). There were no significant differences in the level of mRNA expression of CRISP-3 when examining the bulbourethral gland, prostate gland, testis, or the cauda, corpus, or caput aspects of the epididymis, with minimal expression in both the prepubertal and postpubertal males seen (Fig 2.1).

Equine CRISP-3 immunolabelling was observed in the ampulla of the vas deferens of the postpubertal stallion (Fig 2.2A) as well as in the seminal vesicles (Fig 2.2B). A strong immunolabelling was detected in epithelial cells surrounding the glands of the ampulla. This staining was restricted to the cytoplasm of the epithelial cells and not detected in the nucleus (Figure 2.2E). The strongest immunolabelling was present in the mucosa directly surrounding the lumen of the ampulla, with minimal staining detected between the lumen and the muscularis. The majority of glands within the ampulla of the vas deferens of the postpubertal male horse stained positive for CRISP-3 (Fig 2.2A). The muscularis also showed strong immunolabelling for CRISP-3. The ampulla of the vas deferens in the prepubertal colt showed no labeling for CRISP-3 (Fig 2.2C).

The seminal vesicles showed expression for CRISP-3 primarily in the secretory epithelium of the mucosal folds (Fig 2.2B). Similar to the ampulla, this labeling occurred in the cytoplasm of the epithelial cells, but did not occur in the nucleus (Fig 2.3B). Immunolabelling of the seminal vesicles occurred throughout the tissue, and was present where glandular distribution was notable. This expression was not seen in the seminal vesicles of the prepubertal colt (Fig 2.2D). Immunolabeling was not observed in the other tissues examined, including bulbourethral gland, testis, as well as the cauda, corpus, and caput aspects of the epididymis in either the postpubertal stallion or prepubertal colt (data not shown).

The salivary gland of a postpubertal male also showed expression for CRISP-3 (Fig 2.3) while the prepubertal salivary gland did not. This was seen throughout the gland wherever glandular distribution was present. Similar to the other tissues, the cytoplasm of the cells stained positive, while the nuclei did not.

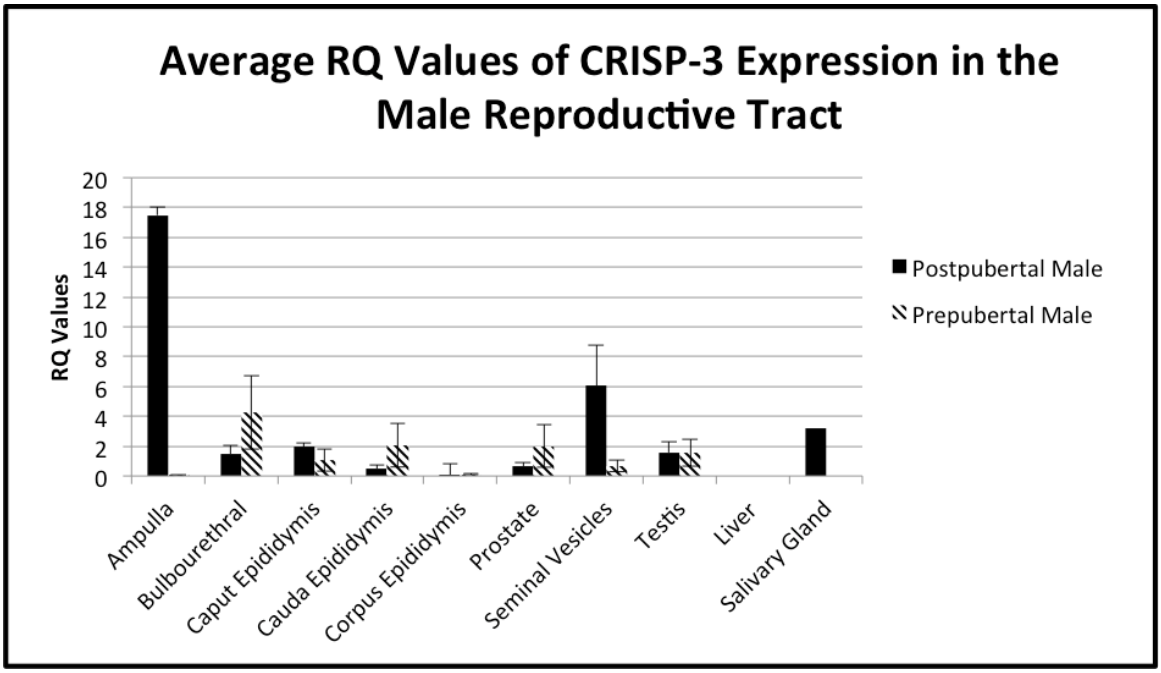

Figure 2.1: Relative Quantification (RQ) of mRNA expression of equine CRISP-3 in the male reproductive tract. Accessory sex glands, epididymis, and testis in prepubertal (<six months, n=3) and postpubertal (>three years, n=6) male horses. Along the x-axis is ampulla of the vas deferens, bulbourethral gland, caput epididymis, cauda epididymis, corpus epididymis, prostate gland, seminal vesicles, testis, liver (negative control), and salivary gland (positive control). The gene expressions are normalized to GAPDH and displayed as average RQ ± SEM. Significance was set to $P<0.05$, and differences in expression are indicated as an *.

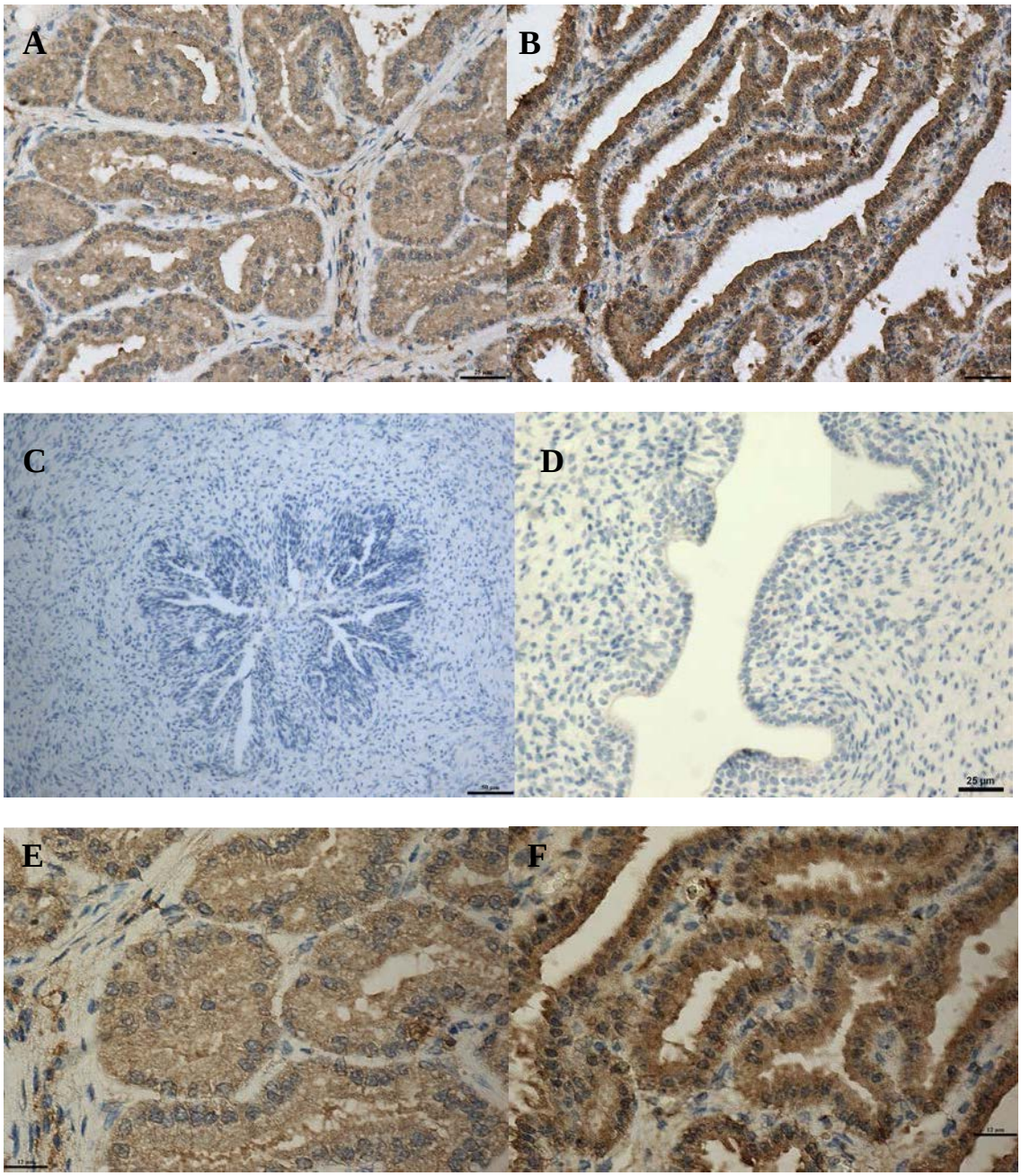

Figure 2.2: **Immunohistochemical staining for equine CRISP-3 in the ampulla of the vas deferens and the seminal vesicles.** Utilizing the monoclonal mouse anti-equine CRISP-3 antibody HL2175 at a dilution of 1:200. (A) ampulla of the postpubertal stallion at 20x, (B) seminal vesicles of the postpubertal stallion at 20x, (C) ampulla of the prepubertal stallion at 20x, (D) seminal vesicles of the prepubertal stallion at 20x, (E) ampulla of postpubertal stallion at 63x, and (F) seminal vesicles of the postpubertal stallion at 63x.

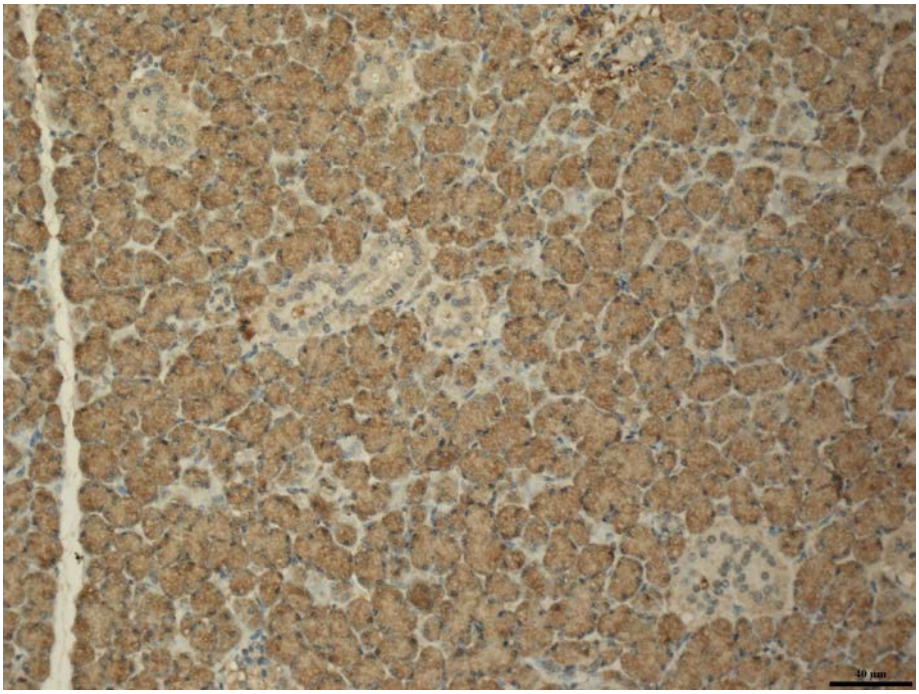

Figure 2.3: Immunohistochemical staining for equine CRISP-3 in the salivary gland of the postpubertal male horse. Utilizing the monoclonal mouse anti-equine CRISP-3 antibody HL2175 at a dilution of 1:200 at a magnification of 20x.

2.5 Discussion

The present study demonstrates that equine CRISP-3 is expressed primarily in the ampulla of the vas deferens of the mature male, and to a lesser degree the seminal vesicles. These findings corroborate with previous work done by Schambony *et al.* (1998) where both reverse transcriptase polymerase chain reaction (RT-PCR) and western blot demonstrated that mRNA as well as the protein expression were located in the ampulla of the vas deferens, the seminal vesicles, as well as the salivary gland of the adult male [105]. In our study, we utilized real time quantitative polymerase chain reaction (qPCR) and immunohistochemistry (IHC) to both confirm the previous study as well as quantify the expression. There was an increased mRNA expression of CRISP-3 in both the ampulla of the vas deferens as well as the seminal vesicles in the postpubertal

male when compared to the prepubertal colt. The expression of CRISP-3 in the ampulla was 300-fold higher in expression in the postpubertal male horse compared to the prepubertal male horse, 10-fold higher than the expression of the other tissues in the postpubertal male horse, 5-fold higher than the mature male salivary gland, and 3-fold higher than the expression of CRISP-3 in the seminal vesicles, the only other reproductive tissue studied that exhibited expression.

Equine CRISP-3 is of interest in the male reproductive tract due to its secretion in the seminal plasma being correlated with many markers of reproductive wellness. The genotype of stallions for the CRISP-3 gene has been shown to correlate with increased fertility. In a study by Hamann *et al.* (2007) it was shown that Hanoverian stallions express 52 polymorphic sites for CRISP-3, with the E208K polymorphism having a significant correlation with fertility. Homozygosity for the specific genotype c.+622G, where a single nucleotide exchange from lysine to glutamine occurs, was found to be responsible for the correlation with increased fertility [120]. A study done by Novak *et al.* (2010) found that increased amounts of equine CRISP-3 in the ejaculate correlated with increased first cycle conception rates [121]. Increased concentrations of CRISP-3 in the seminal plasma have also been shown to correlate with high semen freezability [122]. While many studies have indicated a role for CRISP-3 in stallion fertility and reproductive function, minimal information has elucidated the pathways that this protein is involved with, and therefore determining its expression, localization, and regulation is imperative.

IHC staining utilizing a monoclonal mouse anti-equine CRISP-3 antibody further confirmed the qPCR results. Expression of CRISP-3 was found in both the ampulla of

the vas deferens as well as the seminal vesicles of the postpubertal male. No staining for CRISP-3 was detected in any other postpubertal tissues, as well as any prepubertal male tissues. In the ampulla of the vas deferens, immunolabelling was detected in the mucosa directly surrounding the lumen of the ampulla, with the staining concentrated in the epithelial cells lining the glands. The staining intensity was similar in both the ampulla of the vas deferens and the seminal vesicles, which challenges our findings utilizing qPCR, where the ampulla was the major contributor to CRISP-3 expression. It should be noted that IHC represents a single moment in time, and is not a quantitative representation of protein expression. Both tissues showed immense staining within the glandular compartments, which may be suggestive of the secretory aspect of this protein.

The intense staining in the seminal vesicles as well as the ampulla of the vas deferens could correlate with the concentration of CRISP-3 that has previously been documented in the equine ejaculate [105]. In humans, CRISP-3 is primarily localized to the epididymis, with a more moderate amount in the prostate, testis, and vas deferens. As this study demonstrates, the localization of CRISP-3 in the equine male reproductive tract differs from other species. In addition, equine CRISP-3 is found in the ejaculate at an immense concentration of 1mg/ml, which is unique to the species, and differs greatly from the average concentration in the human ejaculate at 11ug/mL [105, 112]. It has been well documented that the accessory sex glands, in which the ampulla of the vas deferens is commonly included with, are the primary source of the fluid portion of the ejaculate. The immunolabelling of CRISP-3 within the glandular aspects of two of these secretory glands may be indicative of its uniquely heightened concentration in the equine ejaculate.

There was negligible detection of equine CRISP-3 in any of the tissues investigated of the prepubertal male. This indicates that equine CRISP-3 may be similar to murine and human CRISP-3 in its regulation by androgens [160, 161, 169]. In a study by Haendler *et al.* (1997), male mice were treated with the gonadotropin-releasing hormone (GnRH) antagonist Ac-DNapAla-DClPhAla-DPyrAla-Ser-Tyr-DCtly-Leu-Lys(Mor)-Pro-DAla-NH_2, and then supplemented with testosterone propionate (TP) to determine androgen dependency [161]. It was determined in the study that CRISP-3 was androgen dependent, as its expression increased with testosterone supplementation. In the same study, female mice were ovariectomized and supplemented with either estrogen (E2) or TP to evaluate both androgen and estrogen dependency. The expression of CRISP-3 in female mice was found to be androgen dependent, but not estrogen dependent.

This has been further investigated in the human, where two regions within the human CRISP-3 gene share strong homology with androgen response elements (ARE's). In a study by Laine *et al.* (2007), human CRISP-3 mRNA expression in the salivary gland was found to be controlled by dihydroepiandrosterone (DHEA) [169]. Because of its regulation by androgens, human CRISP-3 is now being utilized as a biomarker for androgen-dependent diseases, such as Sjögrens Syndrome and prostate cancer [169, 170]. The present study demonstrated that puberty, and its concurrent increase in both androgens and estrogens, plays a role in the expression of equine CRISP-3 in the male reproductive tract. Further studies are required to elucidate if equine CRISP-3 expression is actually regulated specifically by either androgens or estrogens.

In conclusion, equine CRISP-3 is found primarily in the ampulla of the vas deferens, with a more moderate expression in the seminal vesicles of the mature male reproductive tract. In both tissues, it is found in the cytoplasm of the epithelial cells, which line the glands, as well as secreted within the glands of the mucosa. Its localization to the accessory sex glands may be contributory in the immense concentration that it is found in the average equine ejaculate. Expression of both protein as well as mRNA transcript was exclusive for the postpubertal male horse, with negligible expression in the prepubertal colt. This indicates that equine CRISP-3 may be similar to murine and human CRISP-3, that are both androgen dependent, but further investigation is required to confirm this.

2.6 Acknowledgements

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CHAPTER 3

Immune response of the equine uterus after insemination with live versus dead spermatozoa

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3.1 Abstract

The innate immune response to breeding in the mare is essential for effective uterine clearance of excess spermatozoa and bacteria. The resulting response is modulated via a cascade of signaling molecules, specifically cytokines. The effects of freeze-killed spermatozoa, extender, and bacteria on pro- and anti- inflammatory cytokine mRNA gene expression have been compared within and between susceptible and resistant mares. However, data is lacking on the possibility of a difference in the immune response following insemination with live versus dead spermatozoa. Therefore, the objective of this study was to examine the expression of *IL-1β, IL-1RN, IL-6, IL-8, and IL-10* in endometrial tissue after insemination with either $1x10^9$ progressively motile or $1x10^9$ freeze-killed sperm. We hypothesized that there would be no difference in the immune response of the uterus after insemination with either live or dead spermatozoa. Semen was collected and pooled from 2 stallions, centrifuged to remove seminal plasma, and resuspended in 30 mL Equipro extender (Minitube of America, Verona, WI). Four mares were inseminated in estrus over two consecutive cycles in randomized order. Culture and cytology examinations were performed prior to insemination to rule out the presence of existing inflammation. An endometrial biopsy was obtained at the base of the uterine horn at 6 hours post-breeding for qPCR analysis of select pro- and anti- inflammatory cytokines. A one-way ANOVA was used to compare mRNA expression between the treatment groups, and significance was set to $P<0.05$. The mRNA expression of *IL-6* was increased when mares were infused with dead in comparison to live sperm ($P=0.049$). No significant difference was found between the treatment groups for the mRNA expression of *IL-1β, IL-1RN, IL-8,* and *IL-10*. The cause of the increased expression of

IL-6 following insemination with dead sperm was not determined under the condition of this study. A possible explanation is the loss or relocation of sperm proteins that are involved in regulation of uterine inflammatory responses. In conclusion, there was a limited but significant effect on the innate immune response of the uterus following breeding with varying inseminants.

3.2 Introduction

In the horse, the deposition of semen directly into the uterus at the time of breeding causes a transient innate immune response. Described as breeding-induced endometritis, this inflammation begins by an increase in the expression of pro-inflammatory cytokines. These cytokines then signal the recruitment of immune cells such as the polymorphonuclear neutrophils (PMNs), which bind to and digest excess spermatozoa and contaminating pathogens. Exposed to spermatozoa by either the neutrophil extracellular traps (NETs) or an unidentified receptor/ligand mechanism, the PMNs create large cellular aggregates that are proposed to interfere with sperm transport [50]. This is followed by an increased synthesis of anti-inflammatory cytokines, which are thought to resolve this inflammation. The majority of mares are able to accomplish this resolution within 24-36 hours after breeding, and these mares are considered resistant to persistent breeding-induced endometritis (PBIE) [5, 49]. In contrast, it was found that 10-15% of the thoroughbred mare population in Central Kentucky are unable to resolve this inflammation in a timely fashion, and are considered susceptible to the disease of PBIE [6].

Numerous studies have evaluated the differences between and within the inflammatory process of the resistant and susceptible mare. In a study by Woodward *et al.*, it was shown that susceptible mares experienced an inherently different innate immune response to breeding than the resistant [9]. Six hours after the insemination of freeze-killed sperm susceptible mares had significantly suppressed mRNA expression of the anti-inflammatory cytokines interleukin (*IL)-10* and IL-1 receptor antagonist (*IL-1RN*), as well as the inflammatory modulating cytokine *IL-6*. Christoffersen *et al.* (2012) found that resistant mares have an up-regulation of the pro-inflammatory cytokines *IL-1β, IL-8, IL-6,* and tumor necrosis factor (*TNF*)-α at three hours after the inoculation with *Escherichia coli* [21]. In addition, many studies have shown the effect of immunomodulators, ecbolics, and lavage, on the severity of PBIE, with bacterial inoculation, as well as fresh, cooled, and frozen semen being used as the inducer of inflammation [171-173]. In addition, lipolysaccharide has been utilized to induce uterine inflammation in other species [36].

A variety of foreign molecules including bacteria, seminal plasma, spermatozoa, as well as sterile solutions have been shown to cause an inflammatory response in the uterus of the mare [174, 175]. Numerous studies have investigated this response by examining the expression of signaling molecules such as cytokines, enzymes, and immune modulating cells, but with considerable variation between insemination protocols, time points, and outcomes.

Minimal research has investigated the effect of varying insemination or inflammation inducing protocols on the immune response. It has been shown that artificial insemination with frozen semen causes an increase in leukocyte count, and this

was hypothesized to be due to the increase in dead sperm within the insemination dose of frozen semen [19]. In addition, we have recently shown that the viability of spermatozoa has an effect on PMN binding. However, Katila *et al.* (1997) demonstrated that the viability of sperm had no effect on neutrophil numbers, endometrial edema, or vaginal discharge at five hours after breeding [176]. With minimal, and at times contrasting, work on this subject, further investigation is required. Therefore, the objective of this study was to evaluate the effect of inseminating live versus dead spermatozoa at six hours after breeding on the expression of selected cytokines associated with inflammation.

3.3 Materials and Methods

3.3.1 Insemination of Live Versus Dead Spermatozoa

3.3.11 Mares

Four reproductively normal mares of mixed breeds and age (5-20 yr) qualified for the study and were kept on grass pasture with grain supplementation and access to water and minerals *ad libitum* at the University of Kentucky's Maine Chance Farm in Lexington, KY, USA. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Kentucky (protocol number 2013-1070).

3.3.12 Semen Collection and Isolation of Fresh Spermatozoa

Semen was collected from two stallions by the use of a Missouri model artificial vagina (Nasco, Fort Atkinson, WI, USA) equipped with a gel filter (Animal Reproductive Systems, Chino, CA, USA). Only samples with >60% progressively motile sperm were utilized. Semen was centrifuged at 1600xg for 5 min, and the supernatant was removed. After centrifugation, sperm samples were adjusted to a total concentration of $1x10^9$ spermatozoa in 30mL Equipro® extender (MOFA Global, Verona, WI, USA). The live spermatozoa treatment was kept at room temperature (23-25°C) until insemination, but

for no longer than 15 min. The freeze-killed treatment was processed through freeze/thaw cycles to render the sperm non-viable. At least twice, the aliquots were exposed to -20°C and then brought back to room temperature repeatedly. Freeze-killed samples were stored at -20°C, and thawed slowly to room temperature (23-25°C) before insemination.

3.3.13 Insemination of Spermatozoa

Mares were examined daily via rectal palpation and ultrasonography of their reproductive tracts for follicular development, endometrial edema, as well as uterine and cervical tone. When the presence of a preovulatory follicle was noted (>35mm) combined with reduced uterine tone, increased endometrial edema, and a relaxed cervix, mares were evaluated for the presence or absence of inflammation by endometrial cytology and bacterial cultures [177]. A positive cytology was defined as greater than 2 PMNs observed for every 100 epithelial cells, and a positive culture defined as any bacterial growth after 24 hours of incubation. Only mares clear of inflammation were inseminated. Over the course of two estrous cycles, mares were inseminated with one of the following treatments in randomized order: (1) $1x10^9$ progressively motile spermatozoa in 30mL milk-based extender; or (2) $1x10^9$ freeze-killed spermatozoa in 30mL milk-based extender. Mares received 3000 IU of human chorionic gonadotropin (hCG; Intervet International B.V., Boxmeer Holland) intravenously at the time of insemination to standardize the interval between insemination and ovulation. Endometrial biopsies were collected six hours after insemination with sterile alligator jaw biopsy forceps and the endometrium was stored in RNALater® (Applied Biosystems, Carlsbad, CA, USA) overnight at 4°C, and then transferred to -20°C until further processing. Mares were monitored for ovulation daily, treated with 7.5 mg of intramuscular prostaglandin F_2a

(PGF_2a; Lutalyse, Pfizer, New York, NY, USA) at 7 days post ovulation, and submitted to subsequent treatment in the following estrus.

3.3.2 Quantitative Polymerase Chain Reaction Analysis

Total RNA was extracted from 50mg of endometrial tissue using TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) as described by the manufacturer. Total RNA was precipitated using sodium acetate and isopropanol, resuspended in ddH_2O and DNAse treated (DNA-free™, Applied Biosystems) and then analyzed for quantity and quality via a NanoDrop® spectrophotometer (Thermo Scientific, Wilmington, DE, USA). RNA was reverse transcribed and qPCR was performed as described by Woodward *et al.* [9]. Briefly, 1.5µg of RNA in 41.5 µL ddH_20 was reverse transcribed using Promega reagents; 0.5 µL AMV Reverse Transcriptase, 16 µL 5x RT Buffer, 1 µL RNAsin®, 16 µL MgCl, 4 µL dNTP, and 1µL Oligo(dT) Primer (Promega, Madison, WI, USA). Samples were incubated at 42°C for 60 minutes followed by 95°C for 5 min. cDNA was diluted 1:1 with ddH_20, and qPCR was performed using 4.5 µL of cDNA, 5µL of Sensimix™ II (Bioline, Tauton, MA, USA) and 0.5µL of a custom primer/probe set from Applied Biosystems. Reactions were performed in duplicate with Beta-actin (ACTB) as the reference gene using the ViiA 7 Real-Time PCR System (Table 3.1: Applied Biosystems, Grand Island, NY, USA). Samples were incubated at 95°C for 10 min, followed by 45 cycles of 95°C for 15 sec and 60°C for 60 sec. PCR efficiencies were calculated using LinRegPCR (version 2013.0). Results were expressed as the mean relative quantification value (RQ) which were calculated using the $2^{-\Delta\Delta CT}$ method as described by Livak and Schmittgen, with the calibrator as the mean cycle threshold (ΔC_T)

value of the negative control (LRS and $1x10^9$ spermatozoa) collection from all mares [167].

3.3.3 Statistical Analysis

Data were analyzed using SigmaPlot 12.3 (SyStat, San Jose, CA, USA) and log transformed for normality. Comparisons were made between treatments using the mean ± the standard error via a one-way ANOVA with post hoc analysis performed using a Fisher's protected least significant difference (LSD) test and significance was set to $P \leq 0.05$. Data are presented as the mean ± the standard error of the mean.

3.4 Results

The endometrial mRNA expression for the pro-inflammatory cytokines *IL-1RN*, *IL-1β, IL-8,* and *IL-10* were not significantly altered between treatment groups (Fig 3.1). There was however, a reduction of expression of the inflammatory modulating cytokine *IL-6* in the live spermatozoa treated group when compared to the dead spermatozoa treated group (P=0.049) (Fig 3.2).

Time of ovulation was noted for all cycles after insemination. All of the mares ovulated within 36 hours after hCG was administered. None of the mares had a positive culture or cytology at the time of insemination, and therefore no rest cycles were required. None of the mares presented with fluid retention 24 hours after insemination.

Table 3.1 Dual hydrolysis primer/probe set sequences for the detection of equine mRNA. Primer/probe sets were designed using Assays-By-Design (Applied Biosystems)

Cytokine	Forward primer sequence 5'-3'	Reverse primer sequence 3'-5'	Probe sequence
EqACTB	CTGGACTTCGAGCAGGAGATG	CGTCGGGCAGCTCGTA	CCGCGGCCTCCAGCT
EqIL1β	CCGACACCAGTGACATGATGA	ATCCTCCTCAAAGAACAGGTCATTC	ATTGCCGCTGCAGTAAG
EqIL1RN	AGTTGCTGGATACTTGCAAGAATCA	GAGTCCCAGGAATAGAGCATCAG	CATCTATCTTCTCTTGTAATTTA
EqIL6	GGATGCTTCCAATCTGGGTTCAAT	TCCGAAAGACCAGTGGTGATTTT	ATCAGGCAGGTCTCCTG
EqIL8	GCCGTCTTCCTGCTTTCTG	CCGAAGCTCTGCAGTAATTCTTGAT	CAACCGCAGCTTCAC
EqIL10	ATGCCCCAGGCTGAGAAC	CGGAGGGTCTTCAGCTTTTCC	CCAGACATCAAGGAGCACG

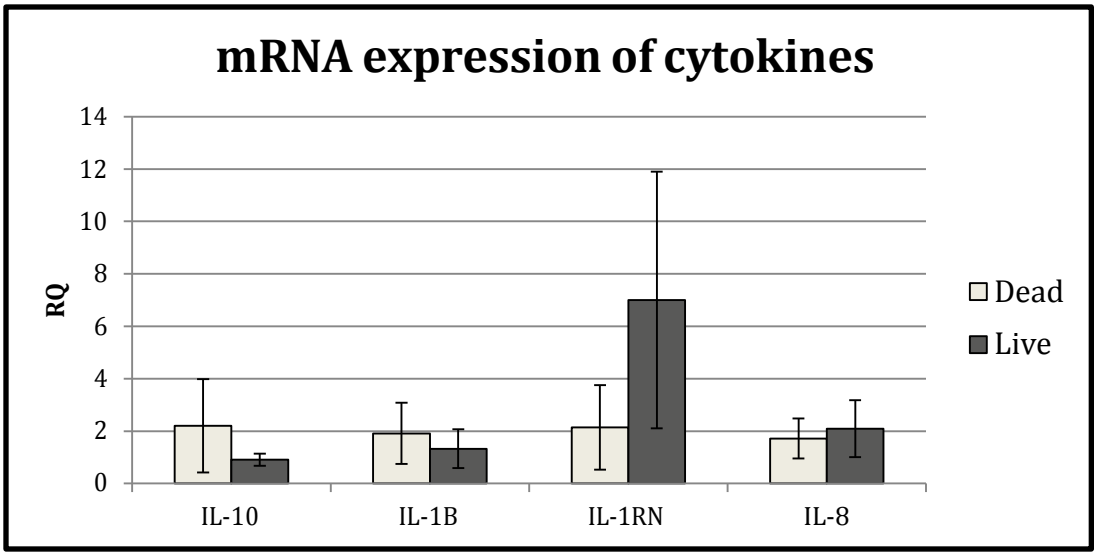

Figure 3.1: Relative quantification (RQ) of mRNA transcripts of endometrial gene transcripts. Specifically *IL-10, IL-1β, IL-1RN*, and *IL-8* after insemination with live or dead spermatozoa. The gene expressions are normalized to *β-actin* and displayed as RQ

± SEM. RQ values describe fold changes in each mare compared to the Δ*C*T value. There were no significant differences in any of the cytokines studied.

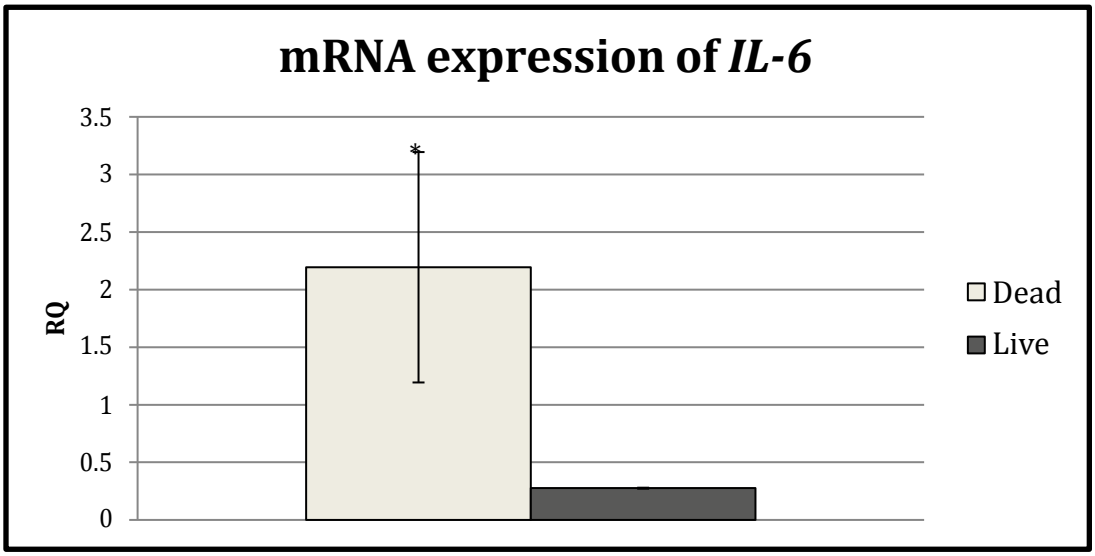

Figure 3.2: **Relative quantification (RQ) of mRNA transcript of endometrial *IL-6* after insemination with live or dead spermatozoa.** The gene expressions are normalized to β-actin and displayed as RQ ± SEM. RQ values describe fold changes in each mare compared to the ΔΔ*C*T value. Significance set to $p < 0.05$ and * indicates significant differences between treatments. Insemination of dead spermatozoa resulted in a significant increase in the endometrial mRNA expression of *IL-6*.

3.5 Discussion

The equine uterus undergoes a transient innate immune response to the deposition of semen at the time of breeding. Considerable work has gone into investigating this response to breeding by utilizing varying models and obtaining contrasting results. Many models have been applied to this topic in the research setting, including the infusion of common bacteria, with others studying the inflammatory response through the insemination of either fresh or freeze-killed sperm. In addition, there exists the variable of the addition or exclusion of seminal plasma, varying extenders, and suspended in sterile solutions. With such variable methodology, extrapolating results to compare reports can be difficult.

While minimal work has gone into the comparison of these contrasting methods on the immune response, some of the individual infusions and insemination protocols have been studied. The utilization of frozen semen in artificial insemination practices has been shown to significantly increase the leukocyte numbers, and this was found to be dependent on the added volume and concentration of spermatozoa [19]. Due to the high population of dead sperm (as high as 70%) seen post-thaw in the frozen semen insemination dose, it was also hypothesized that this increase in leukocyte numbers was due to the presence of the nonviable sperm. Therefore, Katila (1997) evaluated the effect of live versus dead sperm on a variety of outcomes, and found no significant effect of sperm viability on endometrial edema, neutrophil numbers, or vaginal discharge at 5 hours post-breeding. It was concluded that the insemination of dead sperm had no effect on the inflammation seen post-breeding.

Recently, the signaling pathways involved in the innate immune response to breeding have been investigated. Uterine inflammation is activated by an increase in the expression of pro-inflammatory cytokines, specifically interleukin-1β (*IL-1β*), and tumor necrosis factor (*TNF*)-α, which peak around two hours post-breeding [9]. *IL-1β*, in addition to other pro-inflammatory cytokines such as interleukin-8 (*IL-8*) then signal the migration of the PMNs to the uterine lumen to begin phagocytosis of excess sperm and bacteria. At this time, the uterus also begins undergoing myometrial contractions, signaled by an increase in prostaglandin-F2-alpha ($PGF_2\alpha$). Finally, inflammation is resolved by an increase in the expression of the anti-inflammatory cytokines, specifically interleukin-1 receptor antagonist (*IL-1RN*), and interleukin-10 (*IL-10*), in addition to the inflammatory modulating cytokine interleukin-6 (*IL-6*) [9, 46, 178]. While this increase

in anti-inflammatory cytokines is most prominent at six hours post breeding, full resolution of residual inflammation occurs within 24-36 hours after the deposition of semen [9, 179].

In this study, the insemination of freeze-killed sperm significantly increased the endometrial mRNA expression of the inflammatory modulating cytokine *IL-6*. Initially seen as pro-inflammatory early in the inflammatory response, at approximately 24 hours after the deposition of semen, *IL-6* is essential for the transition from a neutrophilic response to a monocytic response, therefore modulating the preliminary inflammatory. While we are uncertain what instigates the significant increase in the endometrial mRNA expression of this cytokine, it may be correlated with the decreased functionality of the remaining seminal plasma proteins within the insemination dose post-thaw. While the majority of the seminal plasma was removed via centrifugation, a small portion remained in the insemination dose. Recent research has found two specific seminal plasma proteins, lactoferrin and cysteine-rich secretory-protein 3 (CRISP-3) to be involved in the binding of sperm to PMNs based on their viability [11, 123]. Studied *in vitro*, lactoferrin was shown to significantly increase the number of PMNs bound to dead sperm, while CRISP-3 was found to increase the binding of PMNs to live. Under the confines of this study, the presence and functionality of these specific proteins *in vivo* was not evaluated, but their role in the innate immune response *in vitro* should be considered.

In conclusion, the insemination of freeze-killed sperm significantly increased the endometrial mRNA expression of the inflammatory modulating cytokine *IL-6* in comparison to live. To our knowledge, this is the first report suggesting that the viability of sperm may have a significant effect on the innate immune response seen in the uterus

of mare post-breeding. While we are unsure what causes this increase at six hours post-breeding, it may explain the variability amongst results in the study of uterine inflammation when utilizing varying protocols. Future research is needed to fully assess the causation and implications of this effect.

3.6 Acknowledgements

This work was funded by the Janet H. Koller Endowment for Equine Research. The authors would like to thank Mr. Lynn Ennis, Mr. Kevin Gallagher, and the dedicated farm staff of the University of Kentucky's Maine Chance Farm for their animal care and assistance. We would also like to thank Dr. Anthony Claes and Dr. Gabriel Davolli for assistance in collecting stallions.

CHAPTER 4

The effect of cysteine-rich secretory protein-3 (CRISP-3) and lactoferrin on endometrial cytokine expression after breeding in the normal mare

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4.1 Abstract

The equine uterus undergoes a transient innate immune response after breeding, also known as breeding-induced endometritis. The deposition of spermatozoa triggers the expression of pro-inflammatory cytokines, which results in the migration of polymorphonuclear neutrophils (PMNs) into the endometrium and the uterine lumen. Select seminal plasma proteins, specifically cysteine-rich secretory protein 3 (CRISP-3) and lactoferrin, have been shown to affect the activity of the PMNs, either by suppressing (CRISP-3) or promoting (lactoferrin) the phagocytosis of spermatozoa based on their viability *in vitro*. Conjointly, many components of inseminate, including seminal plasma, bacteria, and spermatozoa itself, have shown to have an effect on the expression of endometrial cytokines after breeding. The objective of this study was to determine if select proteins affect the mRNA expression of endometrial cytokines after insemination. Six mares were bred during four consecutive estrous cycles with treatments in randomized order of: 1mg/mL CRISP-3, 150 ug/mL lactoferrin, seminal plasma, or Lactated Ringer's Solution (LRS) to a total volume of 10mL combined with $1x10^9$ progressively motile spermatozoa pooled from two stallions. Six hours after treatment, an endometrial biopsy was obtained for qPCR analysis. No treatment effects were found for the mRNA expression of *IL-1β, IL-6, IL-8, IL-10, TNF,* and *IFNγ*, while lactoferrin significantly suppressed the mRNA expression of *IL-1RN* when compared to LRS. In conclusion, the seminal plasma proteins CRISP-3 and lactoferrin have minimal effect on the expression of select endometrial cytokines at 6 hours post breeding.

4.2 Introduction

Breeding-induced endometritis is a transient inflammatory response to spermatozoa that are deposited into the equine uterus at the time of breeding.

Characterized by an increase in the expression of pro-inflammatory cytokines, a subset of which signal the recruitment of polymorphonuclear neutrophils (PMNs) into the uterine lumen, this inflammatory response begins within 30 minutes after insemination [3-5]. Neutrophils are exposed to spermatozoa through neutrophil extracellular traps (NETs), and an unidentified receptor/ligand mechanism, which may interfere with sperm transport to the oviduct [50]. The reproductively healthy mare is able to fully clear the inflammation and excess spermatozoa, fluids, and bacteria from the uterus within 24 to 36 hours after breeding, and is deemed resistant to persistent breeding-induced endometritis (PBIE) [4, 5]. Mares who fail to clear this transient inflammatory response are considered susceptible to PBIE, which persists for more than 72 hours after insemination, and may therefore be present when the embryo descends from the oviduct into the uterine lumen at 5 days after fertilization [4, 180]. Due to this lengthy inflammatory response, these mares have lower fertility rates, and are considered a great economic burden to the equine industry [181].

Mares resistant to PBIE have been found to have an up regulation in the expression of the anti-inflammatory cytokines interleukin-1 receptor antagonist (*IL-1RN),* interleukin-6 *(IL-6),* and *IL-10* when compared to susceptible mares [9]. The authors concluded that six hours after insemination represents a critical time point in the further development of the inflammation, as it showed the most marked difference in the up regulation of anti-inflammatory cytokines. They also concluded that the inflammatory response to insemination in susceptible and resistant mares were notably different, suggesting that susceptible mares have a defective ability to mount a transient innate immune response in the uterus.

Numerous studies have demonstrated that seminal plasma plays a role in the modulation of the innate immune response to breeding [74, 174, 182]. In a study done by Palm *et al*, seminal plasma without spermatozoa caused an up regulation in the mRNA expression of *IL-1β, IL-6*, tumor necrosis factor-α *(TNF-α)*, as well as cyclooxygenase-2 (*COX-2)* in the equine endometrium, and similar findings have been reported for mice, swine, and humans [75-78, 183]. All species showed an up regulation of granulocyte-macrophage colony-stimulating factor (*GM-CSF*), and both the mouse and swine also showed an increase in the expression of *IL-6*. In addition, seminal plasma appears to influence PMN-chemotaxis *in vitro* [184]. This data suggest that seminal plasma is involved in the inflammatory response to semen in the uterus, but no known mechanism has been described. While the inflammatory response to breeding has been widely studied, including the response to seminal plasma itself, less information is available on the components of the seminal plasma. Recently, two seminal plasma proteins have been identified for their activity on the interaction of PMNs and sperm *in vitro*; cysteine-rich secretory protein-3 (CRISP-3) and lactoferrin.

CRISP-3 is a 26kDa protein that is found at a concentration of greater than 1mg/mL in seminal plasma, which makes it the most abundant seminal plasma protein in the equine ejaculate, differing from other species [105]. Stallion CRISP-3 positively correlates with many markers of reproductive wellness, including high semen freezability, first cycle conception rate, as well as increased fertility rates [120-122]. Furthermore, Doty *et al* suggested that equine CRISP-3 is involved in the selective transport of live sperm in the female reproductive tract, possibly by its suppression of the binding of PMNs to live spermatozoa [11]. This protective effect against PMN digestion

and elimination of live spermatozoa may explain CRISP-3's association with increased fertility, but no *in vivo* studies to confirm this have been reported.

Lactoferrin, an 80 kDA iron chelating protein with bactericidal properties, is found in most secretions in the body, including semen, saliva, and milk, as well as the epithelial lining of a variety of organs including the genital tract [141]. Lactoferrin is present in the stallion ejaculate at a concentration of approximately 150 ug/mL, and has been found to negatively correlate with stallion semen freezability as well as positively correlating with oligospermia in men [122, 125, 141]. In addition, we have previously observed that lactoferrin stimulates the binding of PMNs to dead spermatozoa *in vitro* in concentrations approximating those in semen [123]. It was suggested that the increased phagocytosis of nonviable spermatozoa in the presence of lactoferrin could assist with the clearance of the uterus post-insemination, readying it for survival, and eventual implantation, of the embryo.

Although the effect of seminal plasma on the inflammatory response to breeding has been reported previously, investigations on the role of specific proteins within the seminal plasma are limited. Therefore, the objective of this study was to determine the effect of CRISP-3 and lactoferrin on the mRNA expression of select cytokines that have previously been investigated in the endometrium of the uterus at six hours post-insemination after sperm induced inflammation.

4.3 Materials and Methods

4.3.1 Insemination of Seminal Plasma Proteins

4.3.11 Classification of Mares

Reproductively normal mares were screened for resistance to persistent mating induced endometritis (PBIE) based on the following criteria: a) an endometrial biopsy that was examined for periglandular fibrosis, inflammatory cells, glandular distribution and lymphatic lacunae, and scored a I to IIa according to the Kenney and Doig scale [185], b) a negative culture and cytology at 0 and 48 hours pre- and post- insemination, and c) no intrauterine fluid accumulation at 48 hours post-insemination . Six mares of mixed breeds and age (5-20 yrs) qualified for the study and were kept on grass pasture with grain supplementation and access to water and minerals *ad libitum* at the University of Kentucky's Maine Chance Farm in Lexington, KY, USA. For classification of resistance to PBIE, mares were inseminated in estrus with $1x10^9$ freeze-killed spermatozoa suspended in 30 mL EquiPro® extender (>35 mm follicle, presence of uterine edema, and relaxed cervix). All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Kentucky under the protocol number 2013-1070.

4.3.12 Semen Collection and Isolation of Fresh Spermatozoa

Semen was collected from two stallions by the use of a Missouri model artificial vagina (Nasco, Fort Atkinson, WI, USA) equipped with a gel filter (Animal Reproductive Systems, Chino, CA, USA). Semen was collected fresh on the day of insemination, and only samples with >50% progressively motile sperm were utilized. Semen from two stallions was pooled and centrifuged at 1,400x g for 15 min, and seminal plasma was discarded. The sperm pellet was washed in lactated ringer's solution (LRS), and

centrifuged again at 1,400x g for another 15 min. After centrifugation, sperm samples were adjusted to a concentration of $1x10^9$ spermatozoa in LRS and kept at room temperature (23-25°C) until insemination, but for no longer than 15 min.

4.3.13 Preparation of Seminal Plasma and Proteins

Seminal plasma was prepared by centrifugation of fresh semen pooled from two stallions immediately after collection at 1,400x g for 15 min at room temperature to remove the spermatozoa. Ten mL of seminal plasma was added to the $1x10^9$ spermatozoa dose immediately prior to insemination so that all seminal plasma samples were fresh. Human recombinant lactoferrin (ProSpec, East Brunswick, NJ, USA) was reconstituted in LRS at a concentration of 150 μg/mL (the average concentration found in the natural stallion ejaculate) and stored at -20°C until thawed at 38°C immediately before insemination [125]. Biological activity of human recombinant lactoferrin was confirmed by comparing its effect to equine lactoferrin on equine sperm-PMN binding via flow cytometry (Figure 4.3), following the protocol previously described by Doty *et al*. [11] . Equine CRISP-3 was obtained from ejaculates pooled from five stallions and purified as described by Doty *et al.* [11], then stored with proteinase inhibitors at a concentration of 1mg/mL (the concentration found on average in the natural stallion ejaculate) in LRS at -20°C until thawed at 38°C via immediately before insemination [105].

4.3.14 Insemination of Seminal Plasma Proteins

Mares were examined daily via rectal palpation and ultrasonography of their reproductive tracts for follicular development, endometrial edema, as well as uterine and cervical tone. When the presence of a pre-ovulatory follicle was noted (>35mm) alongside the reduction in uterine and cervical tone and the presence of endometrial edema, mares were evaluated for the presence or absence of inflammation by endometrial cytology and bacterial cultures

[177]. Endometrial cytology was performed using a cytology brush, and uterine culture performed with a cytologic swab (MOFA Global; Verona, WI, USA). Negative culture was defined as less than two neutrophils per five fields at × 400 magnification. Negative culture defined as complete absence of bacterial growth on a blood agar plate at 24 hours of incubation at 32ºC. Only mares clear of inflammation were inseminated, and in consecutive cycles with treatment in randomized order, each serving as their own control. The treatments were: (1) $1x10^9$ spermatozoa in 10mL seminal plasma as the positive control; (2) $1x10^9$ spermatozoa in 10mL LRS as the negative control; (3) $1x10^9$ spermatozoa and 1mg/mL equine CRISP-3 resuspended in 10mL LRS; (4) $1x10^9$ spermatozoa and 150μg/mL human recombinant lactoferrin resuspended in 10mL LRS. Mares received 3000 IU of human chorionic gonadotropin (hCG; Intervet International B.V., Boxmeer Holland) intravenously at the time of insemination to standardize the interval between insemination and ovulation. Endometrial biopsies were collected at six hours after insemination using a sterile alligator jaw biopsy punch and the endometrium was stored in RNALater® (Applied Biosystems, Carlsbad, CA, USA) overnight at 4°C, and then moved to -20°C until further processing. Mares were monitored for ovulation and fluid retention daily and treated with 7.5 mg of intramuscular prostaglandin $F_2\alpha$ ($PGF_2\alpha$; Lutalyse, Pfizer, New York, NY, USA) at 7 days post-ovulation and treated in the subsequent estrous with no rest.

4.3.2 Quantitative Polymerase Chain Reaction Analysis

Total RNA was extracted from 50mg of endometrial tissue using TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) as described by the manufacturer. Total RNA was precipitated using sodium acetate and isopropanol, resuspended in ddH_2O and DNAse

treated (DNA-free™, Applied Biosystems) and then analyzed for quantity and quality via a NanoDrop® spectrophotometer (Thermo Scientific, Wilmington, DE, USA). RNA was reverse transcribed and qPCR was performed as described by Woodward et al [9]. Briefly, 1.5µg of RNA in 41.5 µL ddH_20 was reverse transcribed using Promega reagents; 0.5 µL AMV Reverse Transcriptase, 16 µL 5x RT Buffer, 1 µL RNAsin®, 16 µL MgCl, 4 µL dNTP, and 1µL Oligo(dT) Primer (Promega, Madison, WI, USA). Primer/probe sequences are shown in Table 1. Samples were incubated at 42°C for 60 mins followed by 95°C for 5 min. cDNA was diluted 1:1 with ddH_20, and qPCR was performed using 4.5 µL of cDNA, 5µL of Sensimix™ II (Bioline, Tauton, MA, USA) and 0.5µL of a custom primer/probe set from Applied Biosystems. Reactions were performed in duplicate with β-actin (ACTB) as the reference gene using the ViiA 7 Real-Time PCR System (Applied Biosystems, Grand Island, NY, USA). Samples were incubated at 95°C for 10 min, followed by 45 cycles of 95°C for 15 sec and 60°C for 60 sec. PCR efficiencies were calculated using LinRegPCR (version 2013.0). Results were expressed as the mean relative quantification value (RQ) which were calculated using the $2^{-\Delta\Delta CT}$ method as described by Livak and Schmittgen, with the calibrator as the mean cycle threshold (ΔC_T) value of the negative control (LRS and $1x10^9$ PMS) collection from all mares [167].

4.3.3 Statistical Analysis

Data were analyzed using SigmaPlot 12.3 (SyStat, San Jose, CA, USA) and log transformed for normality. Comparisons were made between treatments using the mean ± the standard error via a one-way ANOVA with post hoc analysis performed using a

Fisher's protected LSD test and significance was set to $P \leq 0.05$. Data are presented as the mean ± the standard error of the mean.

4.4 Results

The mRNA expression for the pro-inflammatory cytokines *IL-1β, IL-8, IFNγ*, and *TNF* were not significantly altered between treatment groups (Figure 4.1a-d). Furthermore, we did not observe any significant alteration between treatment groups with regards to mRNA expression for the modulating cytokine *IL-6,* or the anti-inflammatory cytokine *IL-10* (Figure 4.1e and 1f). There was however, a reduction of expression of the anti-inflammatory cytokine *IL-1RN* in the lactoferrin treated group when compared to the LRS treated control group (P=0.033). Furthermore, there was a tendency towards a decrease in mRNA expression of *IL-1RN* after treatment with seminal plasma when compared to LRS (P=0.059) (Figure 4.2).

Time of ovulation was noted for all cycles after insemination. All of the mares ovulated within 36 hours after hCG was administered. None of the mares had a positive culture or cytology at the time of insemination, and therefore no rest cycles were used. None of the mares presented with fluid retention 24 hours after insemination.

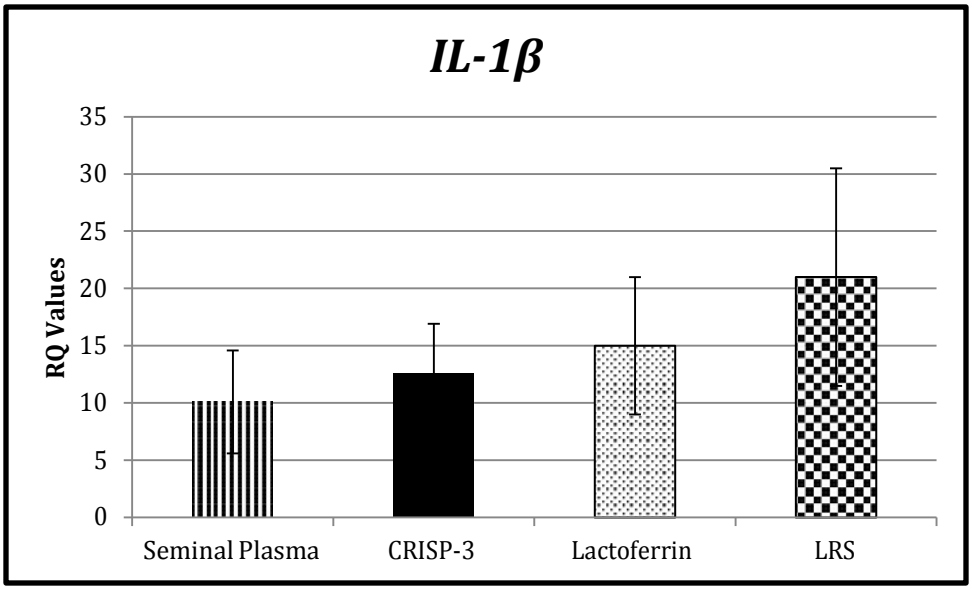

Figure 4.1a-f: Relative quantification (RQ) of mRNA transcripts of endometrial a) *IL-1β*, b) *IL-8*, c) *IFNγ*, d) *TNF*, e) *IL-6*, and f) *IL-10*. As analyzed after insemination with CRISP-3, lactoferrin, LRS, or seminal plasma. The gene expressions are normalized to β-actin and displayed as RQ ± SEM. There were no significant differences noted in any treatment.

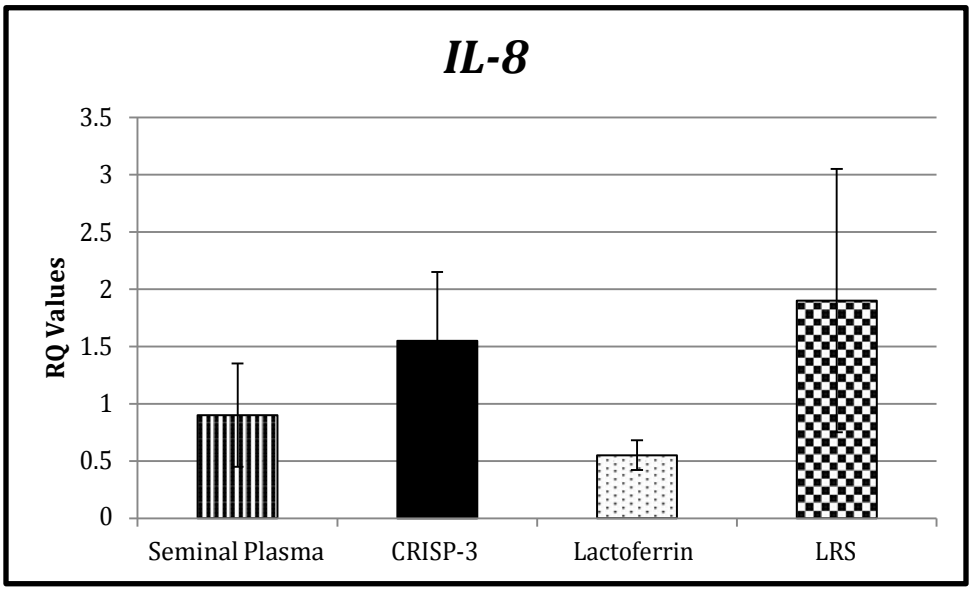

Figure 4.1b

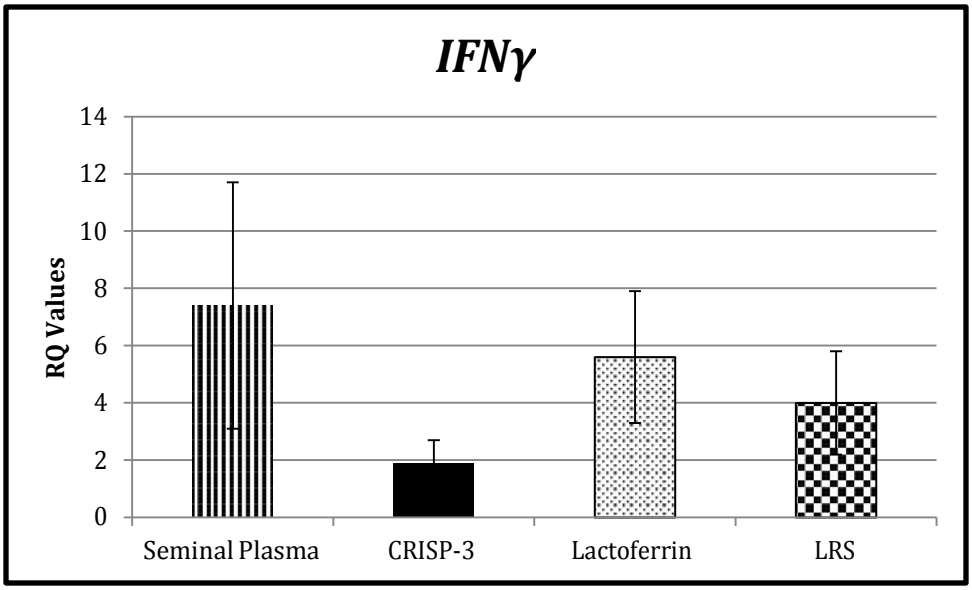

Figure 4.1c

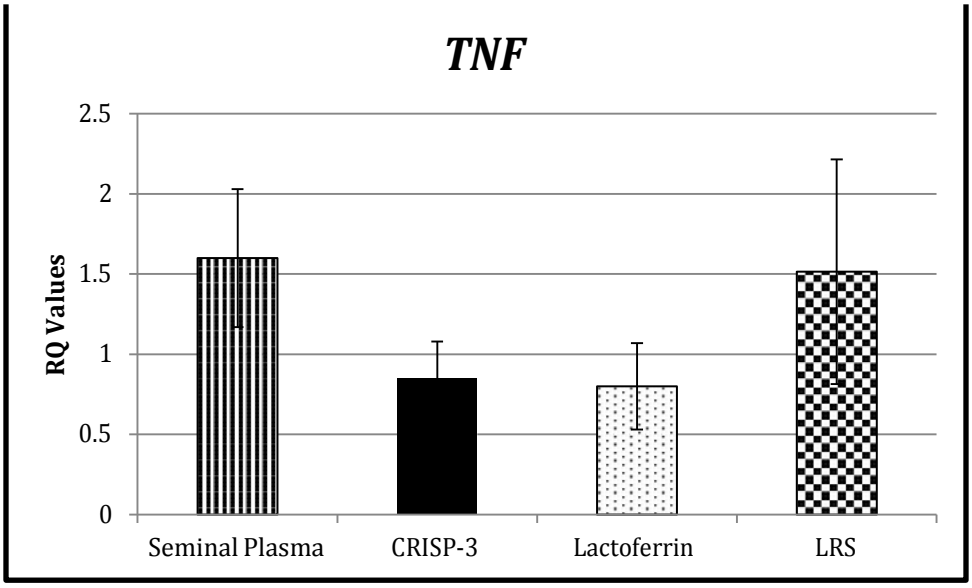

Figure 4.1d

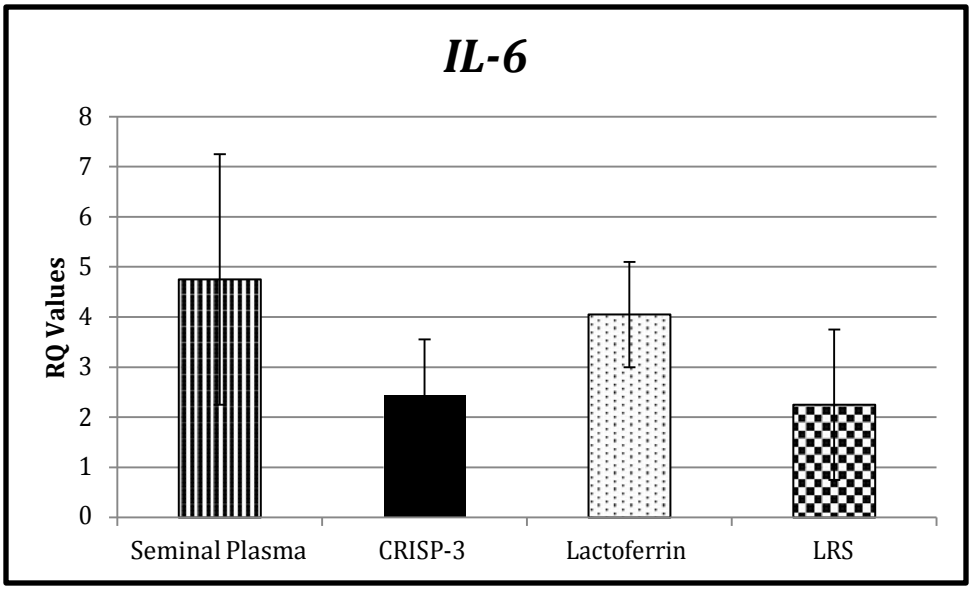

Figure 4.1e

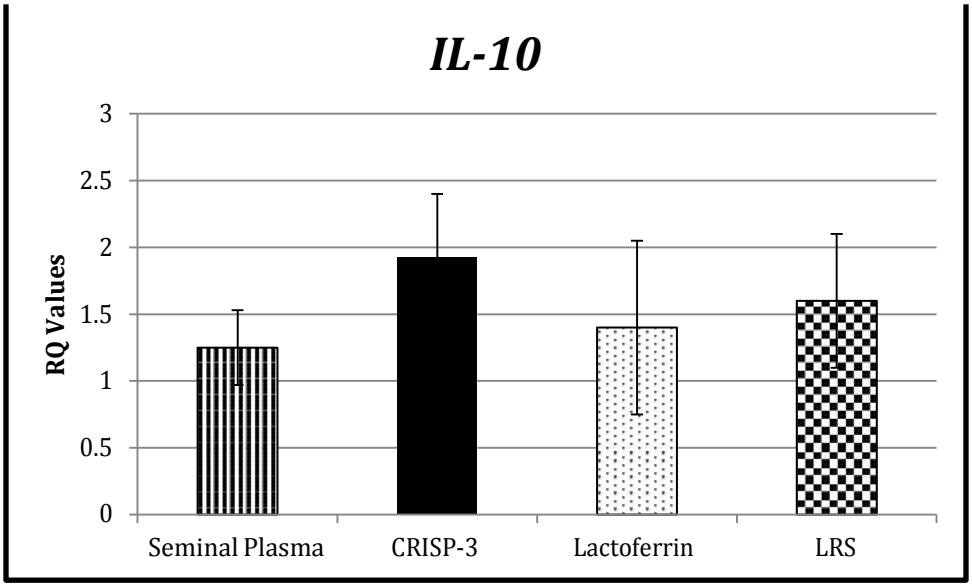

Figure 4.1f

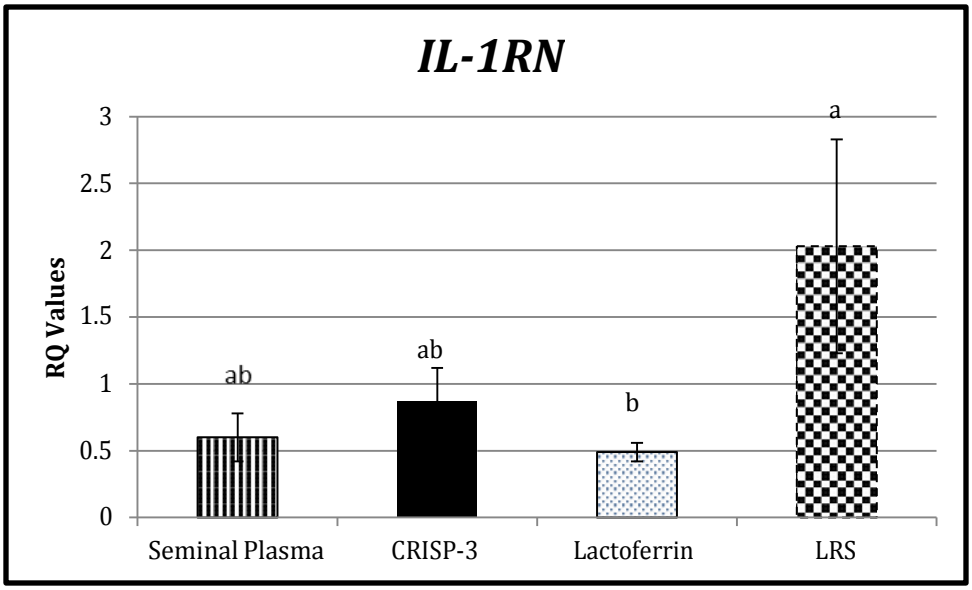

Figure 4.2: **Relative quantification (RQ) of mRNA transcripts of endometrial IL-1RN in mares after insemination with CRISP-3, lactoferrin, LRS, and seminal plasma.** The gene expressions are normalized to β-actin and displayed as RQ values ± SEM. RQ values describe fold changes in each mare compared to the Δ*C*T value. Significance set to $p < 0.05$ and different letters (a,b) indicate significance among treatments. Exogenous lactoferrin significantly suppressed the expression of IL-1RN in comparison to LRS.

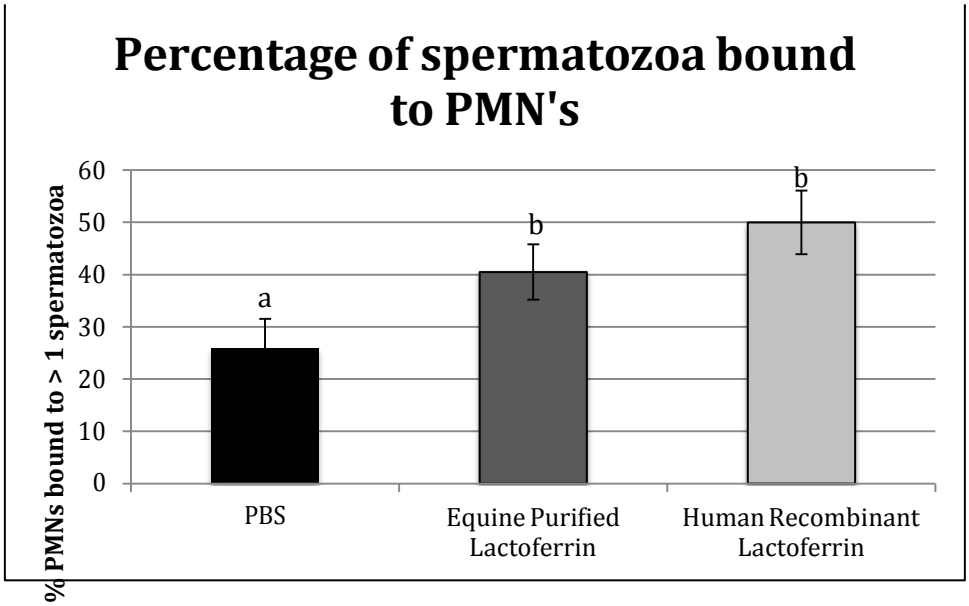

Figure 4.3: Percentage of spermatozoa bound to PMN's. Utilizing flow cytometry with the addition of treatments PBS, equine purified lactoferrin, and human recombinant

lactoferrin. Significance set to $P < 0.05$ and different letters (a,b) indicate significance among treatments.

Table 4.1: Dual hydrolysis primer/probe set sequences for the detection of equine mRNA. Primer/probe sets were designed using Assays-By-Design (Applied Biosystems) [9].

Cytokine	Forward primer sequence 5'-3'	Reverse primer sequence 3'-5'	Probe sequence
EqACTB	CTGGACTTCGAGCAGGAGATG	CGTCGGGCAGCTCGTA	CCGCGGCCTCCAGCT
EqIL1β	CCGACACCAGTGACATGATGA	ATCCTCCTCAAAGAACAGGTCATTC	ATTGCCGCTGCAGTAAG
EqIL1RN	AGTTGCTGGATACTTGCAAGAATCA	GAGTCCCAGGAATAGAGCATCAG	CATCTATCTTCTCTTGTAATTTA
EqIL6	GGATGCTTCCAATCTGGGTTCAAT	TCCGAAAGACCAGTGGTGATTTT	ATCAGGCAGGTCTCCTG
EqIL8	GCCGTCTTCCTGCTTTCTG	CCGAAGCTCTGCAGTAATTCTTGAT	CAACCGCAGCTTCAC
EqIL10	ATGCCCCAGGCTGAGAAC	CGGAGGGTCTTCAGCTTTTCC	CCAGACATCAAGGAGCACG
EqINFG	AGCAGCACCAGCAAGCT	TTTGCGCTGGACCTTCAGA	ATTGAGATTCCGGTAAATG
EqTNF	TTACCGAATGCCTTCCAGTCAAT	GGGCTACAGGCTTGTCACTT	CCAGACACTCAGATCAT

4.5 Discussion

Although CRISP-3 and lactoferrin may be involved in the interaction between uterine PMNs and their binding and phagocytic actions on spermatozoa, the effect on the mRNA expression of the studied pro- and anti- inflammatory cytokines in the uterus appears to be minimal. These cytokines have previously been investigated in the endometrium at six hours post-insemination and determined to be important to the resolution of inflammation seen after breeding [9]. In particular, a critical increase of *IL-6*, *IL-10*, and *IL-1RN* at this time point suggests they are important for a timely resolution of the transient breeding induced inflammation. The expression of pro-inflammatory cytokines, and their signaling activities, act as the first line of defense in response to exposure to an antigen, as they initiate inflammatory cell recruitment; thus triggering the migration of PMNs to the endometrial tissue and into the uterine lumen in response to breeding [5, 30]. In previous studies on endometrial cytokine expression, seminal plasma and the associated proteins were removed by centrifugation in order to reduce variation caused by seminal plasma [9]. The current study was conducted to determine if seminal plasma, or selected seminal plasma proteins with known biological activity, had a regulatory role on cytokine expression.

To minimize variability, all mares included in this study were classified as resistant to PBIE via a breeding challenge in advance of the main experiment. Although it has been shown that spermatozoa trigger the inflammatory response, it is not clear if there is a stallion effect on the uterine inflammatory response to semen [6]. To further minimize the potential effect of a single stallion, semen from two stallions was therefore pooled, and the seminal plasma was removed via centrifugation. All treatments were

applied in random order in consecutive estrous cycles. It is not known if repeated inseminations in consecutive estrous cycles have residual effect on endometrial inflammatory cytokines in resistant mares. However, previous observations under similar experimental conditions, demonstrate a short duration of both pro-inflammatory and inflammatory modulating cytokines after insemination [9].

Inclusion of seminal plasma and LRS to the insemination dose had no effect on the expression of the cytokines studied. These results are contradictory to a previous study, as Palm *et al.* found that infusion of seminal plasma into the uterus at estrus caused an increase in the expression of interleukins (*IL)-1β* and *-6*, tumor necrosis factor-α (*TNF-α*) and cyclooxygenase-2 (*COX-2*) [78]. Differences between studies may be explained by variations in the experimental protocols that were employed by the different studies. The study by Palm *et al* was conducted without sperm-induced inflammation at the time of treatment, as mares were not inseminated. Furthermore, the study was conducted as a comparison of endometrial cytokine expression between biopsies taken after treatments when the mares were in estrous versus biopsies prior to treatment when the mares were in diestrous. Due to the differing experimental designs, it may be difficult to compare results between the two studies.

Lactoferrin is a bactericidal and a known iron chelator found in a variety of secretions, including milk, saliva, semen, as well as neutrophils. Kolm *et al* found that the transcription for lactoferrin in the endometrium was increased in mares susceptible to PBIE compared to resistant mares during proestrous. They concluded that this could be due to the increased influx of uterine PMN's in these mares, as they often have an ongoing inflammation, and lactoferrin exists within the granules of the PMN's [144]. It

is believed that lactoferrin acts as a first line of defense in the immune system due to its bactericidal activities. Under the conditions of the present study, *IL-1RN* was significantly suppressed by the addition of lactoferrin to inseminate, in comparison to the treatment of LRS. There was also a trend toward a significant suppression in the suppression of *IL-1RN* in the treatment of seminal plasma (0.059) in comparison to LRS, which is of importance, as seminal plasma would hypothetically contain lactoferrin. Previous studies have demonstrated that lactoferrin can inhibit the production of cytokines *IL-6, IL-1β,* and *TNF-α*, but none of these studies were conducted in endometrial tissue or with the addition of a sperm challenge [186, 187].

IL-1RN is the receptor antagonist of *IL-1α* and *IL-1β*, both of which are pro-inflammatory cytokines that are primarily responsible for the induction of fever, but also for the activation of vascular endothelium, activation of lymphocytes, as well as the signaling of the acute phase response [188]. Previous studies have shown that *IL-1RN* plays a critical role in modulating the signaling activity of *IL-1*, which if not regulated, can cause tissue damage [189]. It has also been shown that *IL-1RN* plays a critical role in normal reproductive function [189, 190]. In the horse, *IL-1RN* expression was found to be increased at six hours post-insemination in the mare resistant to PBIE when compared to the susceptible mares [9]. In this study, the expression of *IL-1RN* was significantly decreased when the mares were treated with lactoferrin when compared to the negative control, and there was also a trend towards a decline in the expression of *IL-1RN* for the treatment of seminal plasma (P=0.059). The suppression of *IL-1RN* expression in the endometrium when the uterus is treated with lactoferrin and seminal plasma is surprising, since the inflammation is a natural response to breeding and seminal plasma is a

physiological component within the ejaculate. Furthermore, a modulation of mating induced endometritis appears to be necessary to resolve the inflammation in a timely fashion and prevent a persistent inflammation that may interfere with fertility. Future investigation in this area is needed to fully explain this observation.

The treatment of CRISP-3 had no effect on the expression of the targeted cytokines in the endometrium when compared to LRS or seminal plasma. This protein is found in the stallion ejaculate at concentration of greater than 1mg/mL, and this large amount is significantly greater than the concentration found in other species [105]. Therefore, it is hypothesized to play a critical role in many aspects of reproduction, including sperm transport, the immune response, and fertility, although limited data are available. The only known documented role of CRISP-3 is its suppression of the binding of PMNs to live spermatozoa in a dose dependent manner with the most noticeable suppression at a concentration of >125µg/mL *in vitro* [11]. Previous studies have correlated CRISP-3 with both increased fertility and first cycle conception rate [120, 121]. In the study done by Hamann *et al.,* Hanoverian stallions who demonstrated homozygosity for the polymorphism c.+622G>A SNP in the CRISP-3 gene were found to have increased fertility rates, while Novak *et al.* found that stallions of mixed breeds who had an abundance of CRISP-3 in their ejaculate had increased first cycle conception rates ($r=0.495$, $P=0.027$). Doty hypothesized that the proteins correlation with fertility could be due to CRISP-3's role in protecting spermatozoa from phagocytosis [11]. Further research is necessary to elucidate a pathway for which CRISP-3 can correlate with these functions.

In this study, seminal plasma and its proteins were removed from spermatozoa by centrifugation, and washing of the pellet in LRS. It was not determined if any lactoferrin or CRISP-3 were still bound to spermatozoa after this treatment. Schambony *et al* (1998) found that CRISP-3 binds tightly to the sperm head and midpiece, based on a salt wash of 0.5 mol NaCl, which leaves the protein intact [191]. Preliminary data in Schambony's study showed that the amount of CRISP-3 still present on the sperm after this salt wash directly correlated with the fertility of the stallion. In the present study, spermatozoa was centrifuged twice at 1,400x g for a total of 30 min, but due to its strong binding, CRISP-3 may not have been completely dislodged by this protocol, potentially causing variability within the results. There has also been work done on the binding of lactoferrin to spermatozoa after centrifugation, which alludes to the fact that it is also bound tightly (unpublished). This may lead to variability within the amount of protein-sperm interactions within the inseminate. However, the addition of CRISP-3, lactoferrin, or seminal plasma should have overcome most of this variability. Furthermore, it is not known if and how sperm-bound lactoferrin and CRISP-3 regulate endometrial mRNA expression of cytokines compared to our hypothesis regarding unbound proteins in seminal plasma. The results of the study are therefore, limited to evaluating the effect of exogenous unbound CRISP-3 and lactoferrin in seminal plasma/media on endometrial mRNA cytokine expression.

In conclusion, while lactoferrin and CRISP-3 may moderate the activity of PMN's and their elimination of sperm from the lumen of the uterus, minimal effect on the mRNA expression of cytokines in the endometrium of the uterus after the induction of inflammation was demonstrated. Lactoferrin was the only treatment with any significant

suppression of cytokine expression, as it suppressed the mRNA expression of the anti-inflammatory cytokine *IL-1RN* when compared to the negative control of LRS. Further investigations are needed to fully determine the biological mechanism for these specific seminal plasma proteins and their physiological roles.

4.6 Acknowledgements

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CHAPTER 5

The effect of cysteine-rich secretory protein-3 (CRISP-3) and lactoferrin on endometrial cytokine expression after breeding in the mare susceptible to persistent breeding-induced endometritis (PBIE)

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5.1 Abstract

In the horse, breeding induces a transient endometrial inflammation. A subset of mares are unable to resolve this inflammation, and they are considered susceptible to persistent breeding-induced endometritis (PBIE). Select seminal plasma proteins cysteine-rich secretory protein-3 (CRISP-3) and lactoferrin have been shown to affect the innate immune response to sperm *in vitro*. The objective of this study was to determine if the addition of CRISP-3 and lactoferrin at the time of insemination had an effect on the mRNA expression of endometrial cytokines in susceptible mares after breeding. Six mares classified as susceptible to PBIE were inseminated during four consecutive estrous cycles with treatments in randomized order of: 1mg/mL CRISP-3, 150 ug/mL lactoferrin, seminal plasma (positive control), or lactated Ringer's solution (LRS; negative control) to a total volume of 10mL combined with $1x10^9$ spermatozoa pooled from two stallions. Six hours after treatment, an endometrial biopsy was obtained for qPCR analysis of selected genes associated with inflammation (pro-inflammatory cytokines interleukin (*IL)-1β, IL-8*, tumor necrosis factor *(TNF)-α,* interferon *(INF)-γ*, anti-inflammatory cytokines *IL-1RN* and *IL-10*, and inflammatory modulating cytokine *IL-6).* Seminal plasma treatment significantly increased the mRNA expression of *IL-1β* (P=0.019) and *IL-8* (P=0.0068), while suppressing the mRNA expression of *TNF* (P=0.0013). Lactoferrin also suppressed the mRNA expression of *TNF* (P=0.0013). In conclusion, exogenous lactoferrin may be considered as one modulator of the complex series of events resulting in the poorly regulated pro-inflammatory response seen in susceptible mares.

5.2 Introduction

There is a transient innate immune response in the equine uterus after the deposition of semen at the time of breeding. The majority of mares can clear this inflammation and are considered resistant to persistent breeding-induced endometritis (PBIE). A subset of mares cannot modulate this immune response in a timely fashion, and are therefore considered susceptible to PBIE. It is thought that this occurs in 10-15% of mares, and is a large economic burden on the equine breeding industry [6, 15].

The innate immune response to semen begins with the production of pro-inflammatory cytokines, which recruit circulating polymorphonuclear neutrophils (PMNs) to the endometrium and into the uterine lumen. In the equine uterus, PMNs are present in the endometrium and uterine lumen within 30 minutes after breeding [3, 49, 192]. Neutrophils are exposed to spermatozoa by both neutrophil extracellular traps (NETs) and an unidentified receptor/ligand mechanism, which may create large cellular aggregates and interfere with sperm transport to the oviduct [50]. Within 6 hours of insemination, there is an increase in the synthesis of the anti-inflammatory cytokines interleukin (*IL*)-*1RN* and *IL-10*, in addition to the modulating cytokine *IL-6*, which are thought to be involved in resolving the inflammation within 24 to 36 hours [9].

Susceptible mares were shown to have an impaired anti-inflammatory and modulatory cytokine response to breeding, as well as an increased expression of nitric oxide synthase (iNOS) and an associated increase in the intra-uterine synthesis of nitric oxide (NO) when compared to resistant mares [9, 193]. As a result, inflammation is still present in the uterus at 24 and 72 hours after insemination in susceptible mares [9, 21,

179]. All of these factors may explain the delayed uterine clearance present in mares that are susceptible to PBIE.

Seminal plasma, or the fluid portion of the ejaculate, contains a mixture of enzymes, amino acids, hormones, and proteins. While it serves as a vehicle for the sperm, it also plays multiple roles in the uterine immune response to breeding. For example, seminal plasma was shown to increase the number of eosinophil's that migrate to the uterus after breeding [78]. Seminal plasma also modulates the binding of PMN's to spermatozoa and recently the specific proteins involved in this interaction were elucidated. Cysteine-rich secretory protein-3 (CRISP-3) was found to suppress the binding of PMNs to live sperm, while lactoferrin appears to increase the number of dead sperm bound to PMNs *in vitro* [11, 123]. However, the mechanism by which this occurs is unknown.

CRISP-3, a 26kDa glycoprotein, is found in the equine ejaculate as the most abundant seminal plasma protein (concentration of approximately 1mg/mL), which is unique to the equine [105]. Primarily synthesized in the ampulla, this protein has been shown to correlate with many markers of fertility, including first cycle conception rates and high semen freezability [121, 122, 191]. CRISP-3 also has high homology with pathogenesis-related (PR) proteins from plants, which are induced in response to foreign particles [112]. The only documented biological role of CRISP-3 in the equine ejaculate is in the modulation of PMN binding to spermatozoa, suggesting that this protein may play a role in the uterine immune response to breeding [11].

Lactoferrin is found in the equine ejaculate at an average concentration of 150 ug/mL [125]. This 80kDa protein is present in a variety of tissues and secretions throughout the body, including colostrum, seminal plasma, the epididymis, endometrium, and neutrophilic granules [125, 144, 145, 194, 195]. It is thought to be bactericidal due to its ability to chelate free iron, required for bacterial metabolism [196]. In the stallion, little is known about lactoferrin, but it has been shown to positively correlate with poor semen freezability, as well as increased sperm concentration and total sperm output [122, 194]. In other species, research on lactoferrin has focused on its anti-inflammatory properties. In the mouse, lactoferrin suppresses the expression of the pro-inflammatory cytokines *IL-1β,* as well as tumor necrosis factor (*TNF*)-*α* after lipopolysaccharide-induced endometritis [36]. In humans, lactoferrin is garnering interest as a therapeutic for immune diseases such as Crohn's Disease, certain types of cancer, and asthma [197-200]. We have previously observed that lactoferrin suppresses the expression of the anti-inflammatory cytokine *IL-1RN* in mares resistant to PBIE [201].

While the effect of seminal plasma on the uterine inflammatory response has been reported previously, investigations into the roles of specific proteins within seminal plasma are limited. CRISP-3 and lactoferrin appeared to have minimal effect on the mRNA expression of both pro- and anti-inflammatory cytokines in the normal mare [201]. However, it is known that mares susceptible to PBIE have an inherently different immune response to breeding in comparison to the resistant mare. Therefore, the objective of this study was to determine the effect of exogenous CRISP-3 and lactoferrin on the mRNA expression of select cytokines in the endometrium of the susceptible mare at six hours after insemination.

5.3 Material and Methods

5.3.1 General Experimental Procedure

5.3.12 Classification of Mares

Ten mares with documented history of infertility were screened for susceptibility to persistent breeding-induced endometritis (PBIE) based on the following criteria: a) endometrial histopathology characterized by periglandular fibrosis, inflammation, glandular distribution and lymphatic lacunae (Category IIb to III) [202], b) negative culture and cytology at 0 hours, c) positive culture and cytology at 96 hours post-breeding, and d) intrauterine fluid accumulation at 96 hours after artificial insemination during estrous with $1x10^9$ freeze-killed sperm extended in 30 mL EquiPro® extender (>35 mm follicle, presence of uterine edema, and relaxed cervix). A positive cytology was defined as greater than 2 PMNs observed for every 100 epithelial cells, and a positive culture defined as any bacterial growth after 24 hours of incubation on blood agar at 37°C. Out of the ten mares that were screened for the condition, six mares of mixed breeds and age (10-25 yr) qualified as susceptible to PBIE for the study. All mares were kept on grass pasture with grain supplementation and access to water and minerals *ad libitum* at the University of Kentucky's Maine Chance Farm in Lexington, KY, USA. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Kentucky (protocol number 2013-1070).

5.3.13 Semen Collection and Isolation of Fresh Spermatozoa

Semen was collected from two stallions by the use of a Missouri model artificial vagina (Nasco, Fort Atkinson, WI, USA) equipped with a gel filter (Animal Reproduction Systems, Chino, CA, USA). In order to minimize variations due to the proportion of live versus dead sperm in the insemination dose, only samples with >50% progressively

motile sperm were utilized. In addition, variability was minimized by collecting semen from two stallions, which was then pooled and layered on 15 mL of AndroColl-E (MOFA Global, Verona, WI, USA). Semen was centrifuged at 300xg for 20 min, and the supernatant was removed. The sperm pellet was washed in lactated Ringer's solution (LRS) and centrifuged again at 300xg for another 15 min. After centrifugation, sperm samples were adjusted to a concentration of $1x10^9$ spermatozoa in LRS and kept at room temperature (23-25°C) for approximately 15 min prior to insemination.

5.3.14 Preparation of Seminal Plasma and Proteins

Seminal plasma was harvested fresh from each pair of ejaculates to be used on the day of insemination. Seminal plasma was prepared by centrifugation of fresh semen pooled from two stallions immediately after collection at 1400xg for 15 min at room temperature. Ten milliliters of seminal plasma was added to $1x10^9$ sperm immediately prior to insemination so that all seminal plasma samples were fresh. Human recombinant lactoferrin (hrLF) (ProSpec, East Brunswick, NJ, USA) was reconstituted in LRS at a concentration of 150 μg/mL (the average concentration found in the stallion ejaculate) and stored at -20°C until thawed at 38°C immediately before insemination [125]. Biological activity of human recombinant lactoferrin was previously confirmed by comparing its effect to equine lactoferrin on equine sperm-PMN binding via flow cytometry [201]. Equine CRISP-3 was obtained from ejaculates pooled from five stallions and purified as described by Doty *et al.* [11], then stored at a concentration of 1mg/mL (the concentration found on average in the natural stallion ejaculate) in LRS at -20°C until thawed at 38°C immediately before insemination .

5.3.15 Insemination of Seminal Plasma Proteins

Mares were examined daily via rectal palpation and ultrasonography of their reproductive tracts for follicular development, endometrial edema, as well as uterine and cervical tone. When the presence of a preovulatory follicle was noted (>35mm) combined with reduced uterine tone, increased endometrial edema, and a relaxed cervix, mares were evaluated for the presence or absence of inflammation by endometrial cytology and bacterial cultures [177]. Endometrial cytology was performed using a cytology brush, and uterine culture performed with a cytologic swab (MOFA Global; Verona, WI, USA). Negative cytology was defined as less than two neutrophils per 100 epithelial cells, with PMNs identified through the use of the Diff-Quik stain as described by [203]. Negative culture defined as complete absence of bacterial growth on a blood agar plate at 24 hours of incubation at 38ºC. Only mares clear of inflammation were inseminated. If inflammation was observed, a rest cycle was implemented and mares were treated with 20 IU of intravenous oxytocin (Oxytocin Injection, Biomeda-MTC Animal Health Inc., Lavaltrie, Quebec, CAN), lavage, and intra-uterine infusion of antibiotics based on culture results. Over the course of four estrous cycles, mares were inseminated with one of the following treatments in randomized order: (1) $1x10^9$ spermatozoa in 10mL seminal plasma (positive control); (2) $1x10^9$ spermatozoa in 10mL LRS (negative control); (3) $1x10^9$ spermatozoa and 1mg/mL equine CRISP-3 resuspended in 10mL LRS; (4) $1x10^9$ spermatozoa and 150 μg/mL hrLF resuspended in 10mL LRS. Mares received 3000 IU of human chorionic gonadotropin (hCG; Intervet International B.V., Boxmeer Holland) intravenously at the time of insemination to standardize the interval between insemination and ovulation. Endometrial biopsies were collected six hours after insemination with sterile alligator jaw biopsy forceps and the endometrium was stored in RNALater® (Applied Biosystems,

Carlsbad, CA, USA) overnight at 4°C, and then transferred to -20°C until further processing. Mares received 20 IU of intramuscular oxytocin (Oxytocin Injection, Biomeda-MTC Animal Health Inc., Lavaltrie, Quebec, CAN) immediately after the biopsy procedure. Mares were monitored for ovulation daily and treated with 7.5 mg of intramuscular prostaglandin F_2a (PGF_2a; Lutalyse, Pfizer, New York, NY, USA) at 7 days postovulation and submitted to subsequent treatments in the following estrus.

5.3.2 Quantitative Polymerase Chain Reaction Analysis

Total RNA was extracted from 50mg of endometrial tissue using TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) as described by the manufacturer. Total RNA was precipitated using sodium acetate and isopropanol, resuspended in ddH_2O and DNAse treated (DNA-free™, Applied Biosystems) and then analyzed for quantity and quality via a NanoDrop® spectrophotometer (Thermo Scientific, Wilmington, DE, USA). RNA was reverse transcribed and qPCR was performed as described by Woodward *et al.* [9]. Briefly, 1.5µg of RNA in 41.5 µL ddH_20 was reverse transcribed using Promega reagents; 0.5 µL AMV Reverse Transcriptase, 16 µL 5x RT Buffer, 1 µL RNAsin®, 16 µL MgCl, 4 µL dNTP, and 1µL Oligo(dT) Primer (Promega, Madison, WI, USA). Samples were incubated at 42°C for 60 minutes followed by 95°C for 5 min. cDNA was diluted 1:1 with ddH_20, and qPCR was performed using 4.5 µL of cDNA, 5µL of Sensimix™ II (Bioline, Tauton, MA, USA) and 0.5µL of a custom primer/probe set from Applied Biosystems. Reactions were performed in duplicate with Beta-actin (ACTB) as the reference gene using the ViiA 7 Real-Time PCR System (Applied Biosystems, Grand Island, NY, USA). Equine specific primer/probes for interleukin (*IL)-1β, IL-1RN, IL-6, IL-8, IL-10,* tumor necrosis factor *(TNF)-α*, and interferon (*INF)-γ* genes were developed

using Assay By-Design (Applied Biosystems) (Table 3.1). Samples were incubated at 95°C for 10 min, followed by 45 cycles of 95°C for 15 sec and 60°C for 60 sec. PCR efficiencies were calculated using LinRegPCR (version 2013.0). Results were expressed as the mean relative quantification value (RQ) which were calculated using the $2^{-\Delta\Delta CT}$ method as described by Livak and Schmittgen (2001), with the calibrator as the mean cycle threshold (ΔC_T) value of the negative control (LRS and $1x10^9$ spermatozoa) collection from all mares.

5.3.3 Statistical Analysis:

Data were analyzed using SAS 9.4 (SAS Institute Inc., Cary, NC, USA). Data was assessed for normality using a Shapiro-Wilkes test and equal variances with a Bartlett's test. The model was assessed using a general linear additive model, with treatment and cytokine as fixed effects, and mare as a random effect. All significant interactions were spliced for analysis. Comparisons were made between treatments using the mean ± the standard error and post hoc analysis performed using a Fisher's protected LSD test and significance was set to $P<0.05$. Data are presented as the mean ± the standard error of the mean.

5.4 Results

All mares ovulated within 48 hours of administration of hCG. Only one mare required intrauterine infusions of 100mg/mL gentamicin (GentaFuse, Henry Schein Animal Health, Melville, NY, USA) and a rest cycle and resumed treatment on the following cycle. There was a significant interaction between the effect of treatment and that of cytokine mRNA expression ($P<0.001$).

The treatment of seminal plasma significantly increased mRNA expression of the pro-inflammatory cytokines *IL-8* (P=0.0068) and *IL-1β* (P=0.0192) in comparison to the treatment with lactoferrin, CRISP-3, and the negative control of LRS (Figure 5.1a and 5.1b). Both seminal plasma and lactoferrin significantly suppressed the mRNA expression of the pro-inflammatory cytokine *TNF* (0.0013) when compared to LRS treated control group or CRISP-3 (Figure 5.2). There was no effect of treatment on the mRNA expression of the anti-inflammatory endometrial cytokines *IL-10*, *IL-1RN*, as well as the inflammatory modulating cytokine *IL-6* for any treatment (Figure 5.3a). There was also no significant effect of treatment on the pro-inflammatory cytokine interferon gamma (*IFN*)-γ (Figure 5.3b).

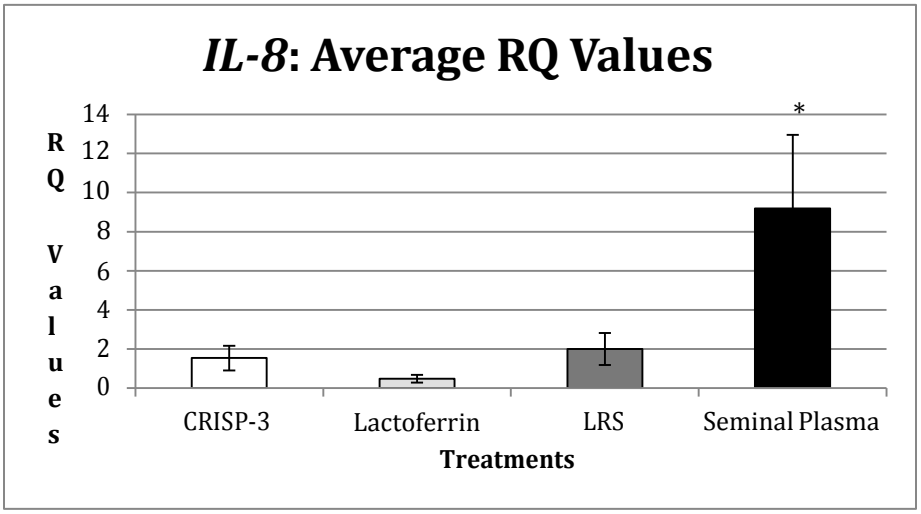

Figure 5.1a: **Relative Quantification (RQ) of endometrial mRNA transcripts of *IL-8* in mares after insemination with CRISP-3, lactoferrin, LRS, and seminal plasma.** The gene expressions are normalized to *ACTB* and displayed as RQ values ± SEM. RQ values describe fold changes in each mare compared to the ΔCt value. Significant difference from other treatments is indicated as * and set to $p < 0.05$.

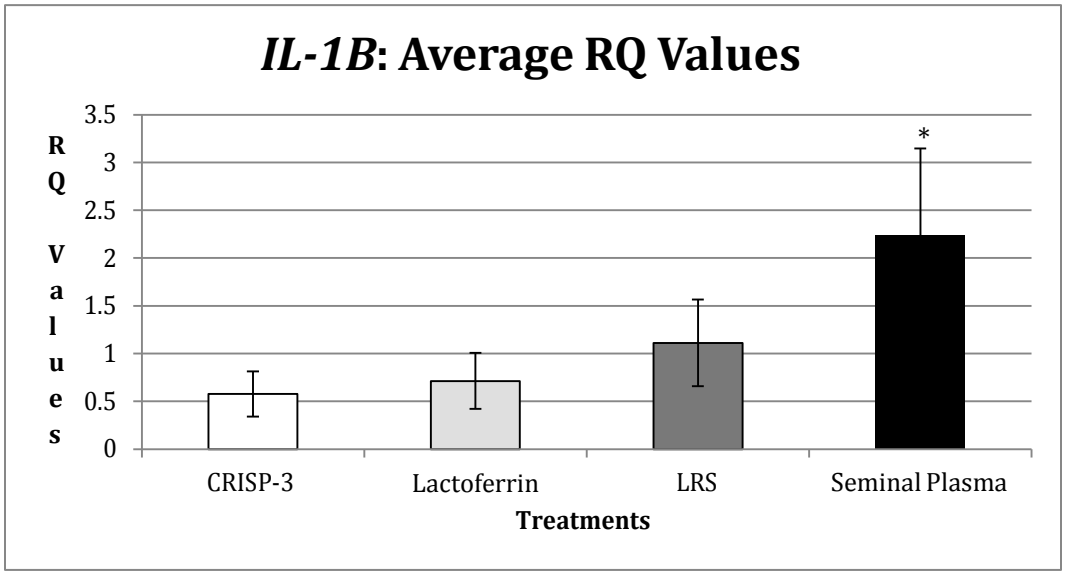

Figure 5.1b: Relative Quantification (RQ) of endometrial mRNA transcripts of endometrial *IL-1β* in mares after insemination with CRISP-3, lactoferrin, LRS, and seminal plasma. The gene expressions are normalized to *ACTB* and displayed as RQ values ± SEM. RQ values describe fold changes in each mare compared to the ΔCt value. Significant difference from the other treatments is indicated as * and set to $p < 0.05$.

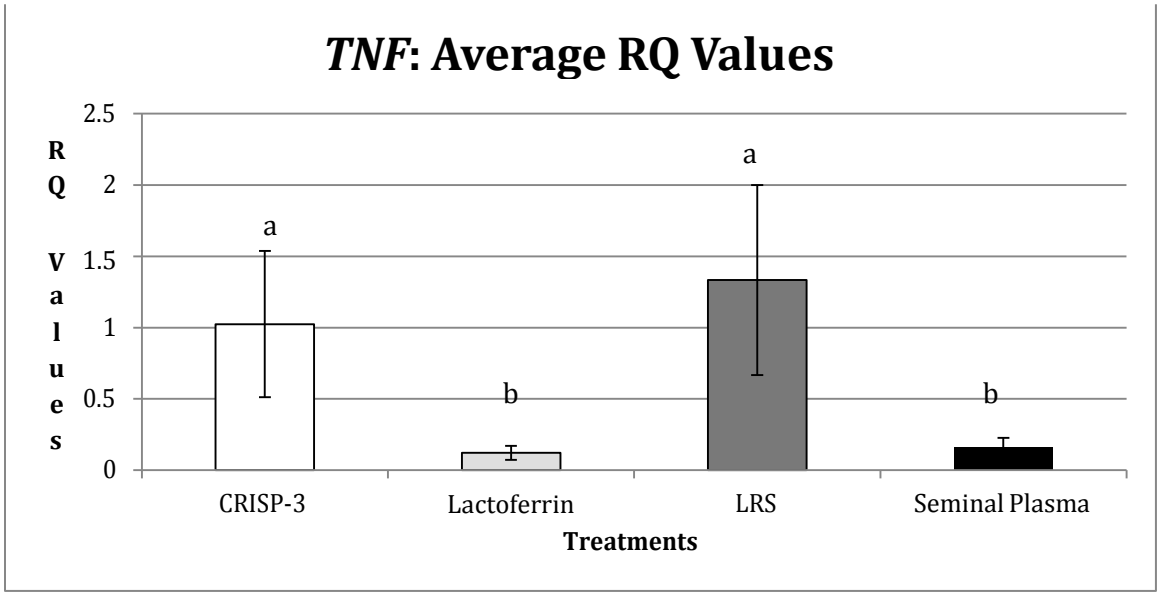

Figure 5.2: Relative Quantification (RQ) of endometrial mRNA transcripts of endometrial *TNF* in mares after insemination with CRISP-3, lactoferrin, LRS, and seminal plasma. The gene expressions are normalized to *ACTB* and displayed as RQ values ± SEM. RQ values describe fold changes in each mare compared to the ΔCt

value. Different letters (a,b) indicate significant differences among treatment groups, set to $p < 0.05$.

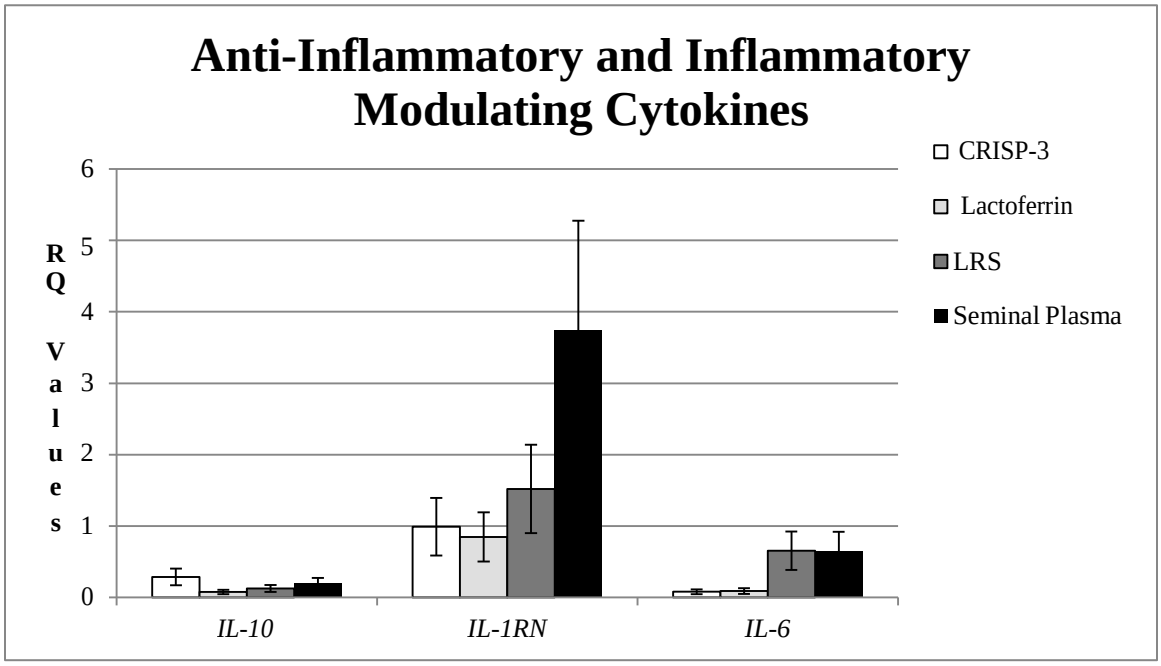

Figure 5.3a: **Relative quantification (RQ) of endometrial mRNA transcripts of the anti-inflammatory cytokines *IL-10, IL-1RN,* and *IL-6* in mares after insemination with CRISP-3, lactoferrin, LRS, or seminal plasma.** The gene expressions are normalized to ACTB, and displayed as RQ values ± SEM.

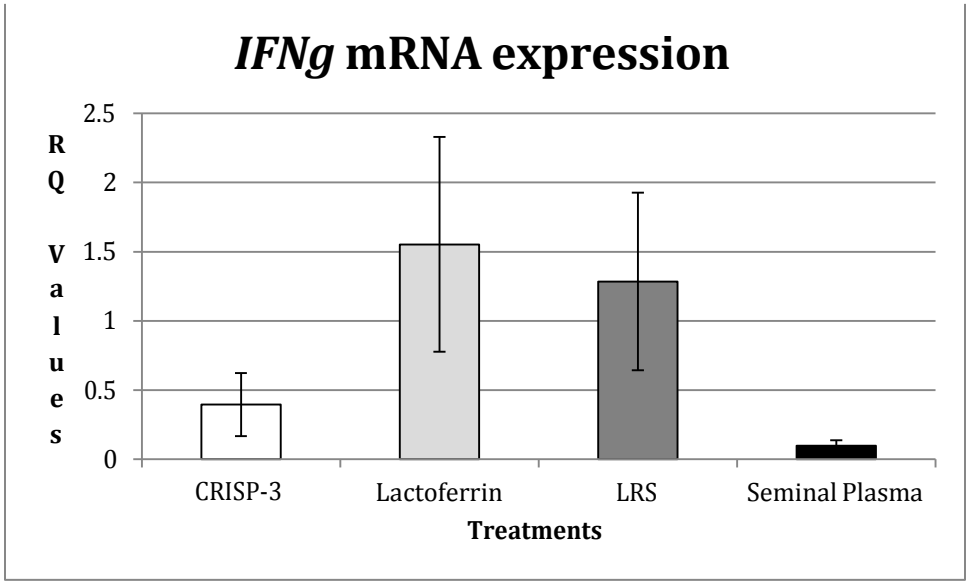

Figure 5.3b: Relative quantification (RQ) of endometrial mRNA transcripts of the pro-inflammatory cytokine interferon gamma (*IFNγ*) in mares after insemination of CRISP-3, lactoferrin, LRS, or seminal plasma. The gene expressions are normalized to ACTB, and displayed as RQ values ± SEM.

5.5 Discussion

In this study, we evaluated the effect of seminal plasma proteins CRISP-3 and lactoferrin on the mRNA expression of select cytokines after breeding in the mare. Seminal plasma increased the mRNA expression of the pro-inflammatory cytokines interleukin (*IL)-8* and *IL-1β*, while suppressing the mRNA expression of tumor necrosis factor (*TNF*)-*α*. In addition, lactoferrin significantly suppressed the mRNA expression of *TNF* at six hours post-breeding. To our knowledge, this is the first report on the ability of a specific seminal plasma protein to modulate the innate immune response to breeding in the susceptible mare.

Seminal plasma and its proteins were removed from the spermatozoa by single layer centrifugation (SLC) and the pellet was washed in lactated Ringer's solution (LRS). Androcoll-E has been found effective in separating sperm-protein interactions in the boar and has been used in other protocols as a means to remove seminal plasma from equine spermatozoa [204, 205]. It was not determined whether lactoferrin and CRISP-3 were still bound to spermatozoa after this treatment. While we are uncertain if Androcoll-E was sufficient in completely separating the seminal plasma proteins CRISP-3 and lactoferrin, previous data indicates that the amount of seminal plasma proteins still adhered to spermatozoa are significantly reduced in comparison to the addition of native seminal plasma.

Numerous studies have found seminal plasma to be beneficial to reproduction, and yet the majority of it is removed during artificial insemination with cooled and frozen

spermatozoa, due to the negative effect of seminal plasma on the viability of semen after thawing [206]. While the addition of seminal plasma significantly increased the mRNA expression of the pro-inflammatory cytokines *IL-8* and *IL-1β* at six hours post-breeding, it also suppressed the mRNA expression of *TNF*, another pro-inflammatory cytokine. This may be explained by the pathway of the innate immune response; as *TNF* is a first responder [44], with *IL-8* being signaled to activate downstream. Although *IL-1β* is also considered an initial responder to the detection of pathogens [207], the two cytokines function through different receptors and utilize different downstream pathways, possibly explaining the contrasting effect of seminal plasma. In addition, this study did not determine the concentration of lactoferrin and CRISP-3 in the native seminal plasma used. Both proteins are found in the natural ejaculate with large variability in concentration, and this may have led to some variability in the results.

Studies have shown that seminal plasma plays a role in breeding-induced endometritis. Fewer PMNs were recovered from the uterus after breeding when seminal plasma was present in the inseminate, in comparison to extended semen [175]. One study induced inflammation in mares before breeding either with or without seminal plasma and found that a significantly larger number of mares achieved a pregnancy when bred with sperm in seminal plasma [74]. Similar to this study, seminal plasma has been found to increase the expression of pro-inflammatory cytokines in response to breeding in the mouse, human, and swine models [77, 208, 209]. It is important that the beneficial aspects of seminal plasma in the horse are elucidated so that these factors may be reintroduced at the optimal concentration to the mare at the time of breeding when using cooled and frozen semen.

During the innate immune response, there is a balance between the pro- and anti-inflammatory cytokines. Anti-inflammatory cytokines modulate the pro-inflammatory response, which if left uninhibited, can lead to tissue damage and prolonged inflammation [178, 189]. Susceptible mares have an impaired modulatory cytokine response at six hours after breeding [9], and this subpopulation of mares also had up-regulated transcription of *IL-1β*, *IL-6*, and *TNF* at 24 hours after breeding [21]. These mares also have increased neutrophilic migration into the lumen of the uterus at 48 hours post breeding in comparison to resistant mares [210]. Lactoferrin is thought to act as a first line of defense in the innate immune response, mediating both of these factors; the expression of cytokines, as well as neutrophil recruitment and signaling [36, 123]. In the current study, the addition of exogenous lactoferrin to the insemination dose significantly suppressed the mRNA expression of the pro-inflammatory cytokine *TNF* in comparison to the negative control of LRS, as well as the treatment of CRISP-3. This observation correlates with recent work on endometritis in the mouse, where lactoferrin was shown to inhibit the uterine protein expression of *TNF* and *IL-1β* in a dose dependent manner [36].

Lactoferrin is an estrogen-dependent uterine protein [211] which is bactericidal due to its binding to free-iron [212]. In addition to iron chelation, the loop region within the protein is thought to be responsible for binding to lipopolysaccharide (LPS) residues on gram-negative bacteria [213]. Lactoferrin also modulates the innate immune response in other tissues by suppression of the pro-inflammatory cytokines *TNF* and granulocyte-macrophage colony-stimulating factor, as well as the inflammatory modulating cytokine *IL-6* [36, 133-135]. Lactoferrin promotes the binding of PMN's to nonviable spermatozoa *in vitro*, possibly contributing to their digestion and elimination [123]. Our research

appears to be the first to examine the effects of exogenous lactoferrin on the innate immune response in the uterus of susceptible mares.

TNF is released by activated monocytes and macrophages and signals and regulates the synthesis of other cytokines, immune cells, and inflammatory mediators [214]. In the equine uterus, *TNF* is produced by the stromal and epithelial cells of the endometrium, and expression of *TNF* is regulated by ovarian steroids [215]. Numerous studies have demonstrated that *TNF* is significantly increased during endometritis in multiple species. In the mouse, both *TNF* and *IL-1β* are significantly increased after LPS-induced endometritis, and this was further confirmed in the buffalo [36, 216]. In a study done by Woodward *et al.* (2013), TNF was found to be increased within 2 hours after insemination of freeze-killed sperm, with the resistant mare population having a significantly higher expression of this cytokine in comparison to the susceptible [9].

While studies have shown that equine CRISP-3 significantly correlates with many markers of reproductive efficiency, this protein had no effect on the cytokines that were evaluated under the conditions of this study. CRISP-3 is primarily synthesized in the ampulla of the vas deferens and in the salivary gland, and to a lesser degree in the seminal vesicles [105]. Due to its high homology with pathogenesis-related proteins from plants, which are induced in response to foreign materials, this protein has been thought to act within the immune response in the species that express it [112]. The only reported effect that CRISP-3 has on the immune response to breeding of the horse is in its suppression of PMN binding to viable spermatozoa *in vitro* [11]. In a recent study, it was shown that CRISP-3 had no effect on the expression of select endometrial cytokines in response to breeding in the normal mare [201]. This protein is hypothesized to play a

role in the protection of viable spermatozoa from neutrophilic phagocytosis, and therefore may enact its abilities earlier than six hours after breeding. It is known that neutrophils are present within thirty minutes after insemination, and sperm transport in the uterus is complete within 4 hours after breeding [217, 218]. Therefore, the effect of CRISP-3 may occur much earlier in the innate immune response.

In conclusion, seminal plasma had an overall effect on the endometrial expression of select cytokines that make up the innate immune response to breeding. The treatment of a specific seminal plasma protein, lactoferrin, caused a significant suppression in the endometrial mRNA expression of the pro-inflammatory cytokine *TNF*. A suppression of *TNF* by exogenous lactoferrin has also been documented in multiple other species during the disease process of endometritis. This is the first report in which lactoferrin was shown to alter the pro-inflammatory response in mares that are susceptible to PBIE. Results from this study warrant further investigation into the role of lactoferrin as an inflammatory modulator of breeding-induced endometritis, and a potential role as a therapeutic immune modulator for mares that are susceptible to PBIE.

5.6 Acknowledgements

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CHAPTER 6

The anti-inflammatory effect of exogenous lactoferrin on breeding-induced inflammation in susceptible mares when administered post-breeding

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6.1 Abstract

The deposition of semen into the uterus of the horse induces a transient innate immune response that lasts 24-36 hours in the normal mare. There exists a subset of mares that are unable to resolve this inflammation in a timely manner, and are classified as susceptible to the disease of persistent breeding-induced endometritis (PBIE). Lactoferrin is a protein of interest as a potential therapeutic for this persistent inflammation due to its anti-inflammatory and bactericidal properties. The addition of human recombinant lactoferrin (hrLF) to the insemination dose was previously shown to suppress mRNA expression of the pro-inflammatory cytokine tumor necrosis factor (*TNF*)-α at 6 hours after insemination, but no studies have shown the effect of lactoferrin when infused post-breeding. Therefore, the objectives of this study were to (1) assess the safety of intra-uterine infusion of hrLF, (2) evaluate the effect of intrauterine infusion of hrLF post-breeding as a modulator of the immune response to breeding in the susceptible mare, and (3) determine the most effective concentration of hrLF. For the first experiment four normal mares received an intrauterine infusion of 500μg/mL hrLF resuspended in 10mL lactated Ringer's solution (LRS) and heart rate, rectal temperature, and respiration were evaluated. For the second experiment, six mares classified as susceptible to PBIE were bred during estrous with $500x10^6$ progressively motile sperm comprised of the ejaculates from two stallions, which were centrifuged over Androcoll-E to remove seminal plasma. Each insemination dose was resuspended in 30mL lactated Ringer's solution (LRS). Six hours after breeding, a 1L LRS uterine lavage was performed prior to treatments. Four treatments were administered over four consecutive estrous cycles in randomized order of: 10mL LRS (vehicle control), 50μg/mL hrLF resuspended in 10mL LRS, 250μg/mL hrLF resuspended in 10mL LRS, and 500μg/mL hrLF resuspended in 10mL LRS.

Twenty-four hours after breeding the mares were evaluated via transrectal ultrasonography for fluid retention. A low volume uterine lavage (250mL LRS) was performed and the effluent was evaluated for polymorphonuclear neutrophils (PMNs). Finally, an endometrial biopsy was obtained for qPCR analysis of selected inflammatory cytokines. Lactoferrin had no significant overall effect on vital signs. The addition of hrLF (50μg/mL, 250μg/mL, 500μg/mL) did not affect the amount of fluid detected post-breeding, but significantly suppressed the ratio of PMNs to epithelial cells at all three concentrations compared to controls. In addition, all three concentrations of hrLF increased the mRNA expression of the anti-inflammatory cytokine interleukin-1 receptor antagonist (*IL-1RN*), while the 50μg/mL dose significantly suppressed mRNA expression of the pro-inflammatory cytokine interferon gamma (*IFNγ*). In conclusion, the infusion of hrLF post-breeding was found to modulate the inflammatory response to breeding in the mare, and appears to be most effective at the 50μg/mL concentration.

6.2 Introduction

The equine uterus experiences a transient innate immune response to the deposition of semen. The inflammation is initiated rapidly by an increase in the expression of pro-inflammatory cytokines, which recruit immune cells such as polymorphonuclear neutrophils (PMNs). This is followed by an up-regulated expression of anti-inflammatory cytokines, which signal for a modulation of pro-inflammatory cytokine activation. Following migration of PMNs into the uterus, the release of prostaglandin F2 alpha ($PGF_2\alpha$) induces myometrial contractions, and excess semen and contaminants are eliminated from the uterus through the dilated cervix [219]. In the normal mare, this inflammation begins as rapidly as 0.5 hrs after breeding, but is resolved

within 24-36 hrs. However, a subset of mares are unable to resolve the inflammation on their own, and consequently develop a persistent breeding-induced endometritis (PBIE) [220]. Susceptibility to PBIE is thought to occur in 10-15% of Thoroughbred mares after breeding, and is considered a large financial burden to the equine breeding industry [221].

The presence of PMNs and retention of inflammatory fluid in the uterus can persist in susceptible mares for greater than 96 hrs; therefore potentially interfering with embryo survival at the time of migration from the oviduct into the uterine lumen. It has been shown that the susceptible mares have a different innate immune response after insemination compared to resistant mares [9]. At six hours post breeding, resistant mares were observed to have an increased expression of mRNA for the anti-inflammatory cytokines interleukin-10 (*IL-10*) and interleukin- 1 receptor antagonist (*IL-1RN*). In addition, mRNA expression of the inflammatory modulating cytokine interleukin-6 (*IL-6*) was also up regulated at this time. In contrast, susceptible mares did not demonstrate the same level of up regulation of mRNA for anti-inflammatory cytokines at this time point. Therefore, a deficient anti-inflammatory response in susceptible mares may contribute to prolonged inflammation, and six hours after breeding appears to represent a critical time point for this transition.

The pathology of PBIE has long been studied, with a multitude of therapeutic options proposed to help modulate the induced inflammation [171, 173, 222]. Options have ranged from post-breeding infusion of antimicrobials and ecbolics, to uterine lavage, acupuncture, and immune-modulators. Recently, the seminal plasma protein lactoferrin was observed to have an immune-modulating effect in mares suffering from

PBIE [223]. Lactoferrin is found in the body in a variety of tissues and secretions including breast milk, epididymis, endometrium, in addition to its presence in seminal plasma at an average concentration of 150μg/mL in the stallion [125]. It is thought to be both bactericidal and anti-inflammatory in numerous species including humans, mice, and buffalo [36, 195, 224]. Due to its ability to chelate the free iron that is required for the metabolism of gram-negative bacteria, lactoferrin has been found to disrupt the formation of biofilm [196]. In the horse lactoferrin has been shown to be involved in sperm elimination from the mare's reproductive tract by increasing sperm-PMN binding in the horse *in vitro* [225]. *In vivo*, Kolm *et al.* (2006) found that susceptible mares have a significantly up regulated expression of lactoferrin in the endometrium which is not correlated with the number of CD 18 positive leukocytes, but rather the number of uterine glands [144]. In addition, exogenous lactoferrin added at the time of breeding was found to suppress mRNA expression of the pro-inflammatory cytokine tumor necrosis factor (*TNF*)-α in the endometrium at six hours after insemination when compared to the addition of lactated Ringer's solution (LRS) in the horse [226], and similar results have also been reported in the murine model [36]. It was concluded that future studies in the horse were necessary to evaluate the therapeutic effect of exogenous lactoferrin on inflammation in a more clinical setting.

Although lactoferrin is found throughout the body, the safety of intra-uterine infusion has not been reported. In addition, the efficacy of exogenous lactoferrin when administered as a therapeutic post-breeding has not been investigated. Therefore, the objectives of this study were to 1) determine if intra-uterine administration of exogenous lactoferrin is detrimental to the overall health and reproductive wellbeing of the horse, 2)

evaluate the effect of lactoferrin on the innate immune response to breeding in mares susceptible to PBIE when infused six hours after insemination, as well as 3) determine the most effective concentration of exogenous lactoferrin to be used as a modulator of the persistent inflammation seen in mares susceptible to PBIE. We hypothesized that the addition of human recombinant lactoferrin would be both a safe and effective modulator to the persistent inflammation seen in mares susceptible to PBIE.

6.3 Materials and Methods

6.3.1 Safety of Lactoferrin

Reproductive cycles of four reproductively normal mares were followed via transrectal ultrasonography. Once determined to be in diestrus (presence of a corpus luteum, toned cervix and lack of endometrial edema) an endometrial biopsy was obtained with sterile alligator jaw biopsy forceps and placed in 10% formalin to serve as a pre-treatment reference according to the Kenney and Doig scale based on inflammation, fibrosis, lymphatics, and glandular distribution [227]. In the following estrous cycle (>35mm follicle, presence of uterine edema, relaxed cervix), mares resting vitals were monitored, including 1) heart rate, 2) rectal temperature, and 3) respiratory rate. Mares were then infused transcervically with 500μg/mL exogenous human recombinant lactoferrin (hrLF) in 10mL lactated Ringers solution (LRS). This concentration of hrLF (500μg/mL) was utilized as it was 3-fold higher than the average concentration found in the normal stallion ejaculate and would be considered the highest concentration evaluated in the second experiment [125]. Vitals were evaluated immediately post-infusion, 30 min post-infusion, and 90 min post-infusion. In the following diestrus (5-9 days post-ovulation), a

second endometrial biopsy was obtained with sterile alligator jaw biopsy forceps and placed in 10% formalin to serve as a post-treatment reference for inflammation, fibrosis, lymphatics, and glandular distribution.

6.3.2 General Experimental Procedure

6.3.21 Classification of Mares

Eight mares with documented clinical history of infertility were screened for susceptibility to PBIE. This was based on the following criteria: a) endometrial histopathology characterized by the presence of one or more of the following 1) periglandular fibrosis, 2) inflammation, 3) glandular distribution, and 4) lymphatic lacunae consistent with Kenney category IIb to III) [228], b) negative culture and cytology pre-breeding, c) positive culture and cytology at 96 hours post-breeding, and d) intrauterine fluid accumulation at 96 hours after artificial insemination during estrous (>35 mm follicle, presence of uterine edema, and relaxed cervix) with $1x10^9$ freeze-killed sperm extended in 30 mL EquiPro® extender (MOFA Global, Verona, WI). A positive cytology was defined as greater than 2 PMNs observed for every 100 epithelial cells, and a positive culture defined as any bacterial growth after 24 hours of microbial culture. Six mares of mixed breeds and age (5-20 yr) qualified for the study and were kept on grass pasture with grain supplementation with access to water and minerals *ad libitum* at the University of Kentucky's Maine Chance Farm in Lexington, KY, USA. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Kentucky (protocol number 2013-1070).

6.3.22 Semen Collection and Insemination

Semen was collected from two stallions with a Missouri model artificial vagina (Nasco, Fort Atkinson, WI, USA) equipped with a gel filter (Animal Reproduction Systems, Chino, CA, USA). Only samples with >50% progressively motile sperm at the time of collection were utilized. To minimize variability, semen from two stallions was pooled and layered on 15 mL of AndroColl-E (MOFA Global). Semen was centrifuged at 300xg for 20 min, and the supernatant was removed. After centrifugation, sperm samples were adjusted to a concentration of 500 x 10^6 spermatozoa in 30 mL LRS and kept at room temperature (23-25°C) for approximately 15 min prior to insemination.

Mares were examined daily via transrectal palpation and ultrasonography of their reproductive tracts for follicular development, endometrial edema, as well as uterine and cervical tone. When the presence of a preovulatory follicle was noted (>35mm) combined with reduced uterine tone, increased endometrial edema, and a relaxed cervix mares were evaluated for the presence or absence of inflammation by endometrial cytology and bacterial cultures [229]. Endometrial cytology was performed using a cytology brush, and uterine culture performed with an endometrial swab (MOFA Global; Verona, WI, USA). Negative cytology was defined as less than two neutrophils per five fields at × 400 magnification. Negative culture defined as complete absence of bacterial growth on a blood agar plate at 24 hours of incubation at 37ºC. Only mares clear of inflammation were inseminated. If inflammation was observed, a rest cycle was implemented and mares were treated accordingly. Over the course of four estrous cycles, mares were inseminated as described above and received 3000 IU of human chorionic

gonadotropin (hCG; Intervet International B.V., Boxmeer Holland) intravenously to standardize the interval between insemination and ovulation.

6.3.23 Lactoferrin Infusion

Six hours after insemination, a uterine lavage was performed with 1L lactated Ringers solution (LRS). In brief, the vulva and perineum were scrubbed with 0.5% chlorhexidine three times and rinsed with water. A uterine catheter was then passed through the cervix and into the uterus by an examiner wearing a sterile sleeve. The end of the catheter was attached to a bag containing 1L LRS, the entirety of which was infused into the body of the uterus. Fluid was retrieved from the uterus via gravity. Immediately after lavage, the mares were infused with one of the following treatments in randomized order: (1) 10mL LRS (negative control); (2) 50μg/mL human recombinant lactoferrin (hrLF) resuspended in 10mL LRS (3) 250μg/mL hrLF resuspended in 10mL LRS; and (4) 500μg/mL hrLF resuspended in 10mL LRS. Human recombinant lactoferrin (ProSpec, East Brunswick, NJ, USA) was stored at -20°C until thawed at 38°C immediately before insemination [125]. Biological activity of human recombinant lactoferrin was previously confirmed by comparing its effect to purified equine lactoferrin on equine sperm-PMN binding via flow cytometry [230].

6.3.25 Evaluation of the innate immune response and sample collection

Twenty-four hours after insemination (18 hours after treatment), mares were examined via transrectal ultrasonography to evaluate the uterus for fluid retention. Fluid was measured by the largest circumference of retention. A low volume lavage was performed according to LeBlanc *et al*. (2007). In brief, a uterine catheter was then passed through the cervix and into the uterus by an examiner wearing a sterile sleeve. The end of the catheter was attached to a bag containing 250mL LRS, the entirety of which was infused

into the body of the uterus. The uterus was then manipulated by rectal palpation for 30 seconds to distribute fluid throughout. The fluid was drained into the sterile bag by gravity flow. A resuspended aliquot of 40mL was retrieved from the sample and centrifuged at 300xg for 10 min. All but 5 mL of the supernatant was decanted and the pellet was resuspended in the remaining supernatant. Finally, 20µL of the resuspended pellet was placed on a microscope slide to create a cellular smear. An endometrial biopsy was then collected with sterile alligator jaw biopsy forceps and the endometrium was stored in RNALater® (Applied Biosystems, Carlsbad, CA, USA) overnight at 4°C, and then transferred to -20°C until further processing. Mares received 20 IU of intramuscular oxytocin (Oxytocin Injection, Biomeda-MTC Animal Health Inc., Lavaltrie, Quebec, CAN) immediately after the biopsy procedure. Mares were monitored for ovulation daily and treated with 7.5mg of intramuscular prostaglandin $F_2\alpha$ ($PGF_2\alpha$; Lutalyse, Pfizer, New York, NY, USA) at 7 days post-ovulation and submitted to subsequent treatments in the subsequent estrus.

6.3.3 Quantitative Polymerase Chain Reaction Analysis

Total RNA was extracted from 50mg of endometrial tissue using TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) as described by the manufacturer. Total RNA was precipitated using sodium acetate and isopropanol, resuspended in ddH_2O and DNAse treated (DNA-free™, Applied Biosystems) and then analyzed for quantity and quality via a NanoDrop® spectrophotometer (Thermo Scientific, Wilmington, DE, USA). RNA was reverse transcribed and qPCR was performed as previously described by Fedorka *et al.* (2016) [226]. Briefly, 1.5 µg of RNA in 41.5 µL ddH_20 was reverse transcribed using Promega reagents; 0.5 µL AMV Reverse Transcriptase, 16 µL 5x RT Buffer, 1 µL

RNAsin®, 16 μL MgCl, 4 μL dNTP, and 1μL Oligo(dT) Primer (Promega, Madison, WI, USA). Samples were incubated at 42°C for 60 min followed by 95°C for 5 min. cDNA was diluted 1:1 with ddH_20, and qPCR was performed using 4.5 μL of cDNA, 5μL of Sensimix™ II (Bioline, Tauton, MA, USA) and 0.5μL of a custom primer/probe set from Applied Biosystems. Primer sequences were designed using the TaqMan® Gene Expression System (Thermo Fischer, Wilmington, DE, USA) (Table 3.1). Reactions were performed in duplicate with beta-actin (ACTB) as the reference gene using the ViiA 7 Real-Time PCR System (Applied Biosystems, Grand Island, NY, USA). Samples were incubated at 95°C for 10 min, followed by 45 cycles of 95°C for 15 sec and 60°C for 60 sec. PCR efficiencies were calculated using LinRegPCR (version 2013.0). Results were expressed as the mean ΔΔCT, following the method described by Livak and Schmittgen [167].

6.3.4 Statistical Analysis

Data were analyzed using SAS 9.4 (SAS Institute Inc., Cary, NC, USA). Data was assessed for normality using a Shapiro-Wilkes test and equal variances with a Bartlett's test. For the assessment of vitals, data was analyzed with a general linear model (*proc glm*), with individual vitals as fixed effects and mare as a random effect. For assessing the effect of lactoferrin on biopsy grade, data was analyzed using the nonparametric McNemar's test. When evaluating the effect of treatment on PMN:epithelial cell ratio and fluid retention, a general linear model (*proc glm*) was used with treatment as a fixed effect and mare as random. Finally, for the qPCR data a general linear model (*proc glm*) was assessed with treatment and cytokine as fixed effects, and mare as a random effect. All significant interactions were spliced for analysis. Comparisons were made between

treatments using the mean ± the standard error with post hoc analysis performed using a Fisher's protected least significant difference's (LSD) test with significance set to $P \leq 0.05$. Data are presented as the mean ± the standard error of the mean.

6.4 Results

When assessing the effect of lactoferrin on endometrial biopsy score, no mare lowered in score according to the Kenney and Doig scale. Lactoferrin had an initial effect on temperature, heart rate, and respiration, with a significant increase noted for all three outcomes immediately post infusion (Fig. 6.1). However, this rise decreased back to at or below pre-treatment levels within thirty minutes post-infusion for all three outcomes evaluated.

There was no significant effect of lactoferrin treatment on the area of fluid encompassed in the uterus at 24 hours post breeding (Fig. 6.2a). There was however, a trend towards significance when evaluating the effect of hrLF on fluid retention if the individual concentrations were analyzed as one variable, with hrLF decreasing the circumference of intrauterine fluid at 18 hours post-treatment (P=0.0941) (Fig. 6.2b). The infusion of hrLF significantly decreased the PMN:epithelial cell ratio seen in a post-breeding uterine cytology (P=0.0397). When the concentrations were analyzed individually, no significant difference in PMN:epithelial cell ratio was seen amongst concentrations of hrLF (Fig. 6.3). All concentration of hrLF had a marked decrease on PMN:epithelial cell ratio in comparison to the control infusion of LRS, with 50μg/mL hrLF (P=0.021), 250μg/mL hrLF (P=0.008), and 500μg/mL hrLF (P=0.038) respectively.

The effect of lactoferrin on mRNA expression of cytokines in the endometrium was evaluated at 24 hours after insemination. There was a significant effect of treatment

on interleukin-1 receptor antagonist (*IL-1RN*) (P=0.049), with all three concentrations of hrLF increasing mRNA expression of the anti-inflammatory cytokine in the endometrium (Fig. 6.4). Lactoferrin also had a significant effect on mRNA expression of the pro-inflammatory cytokine interferon gamma (*IFNγ*), with the treatment of 50µg/mL hrLF significantly suppressing the mRNA expression of *IFNγ* in comparison to LRS (Fig. 6.5). No significant difference was noted for either of the increased concentrations (250µg/mL or 500µg/mL). There did not appear to be an effect of lactoferrin infusion on of the other cytokines studied (*IL-1β, IL-6, IL-8, IL-10, TNF*) in comparison to the control of LRS.

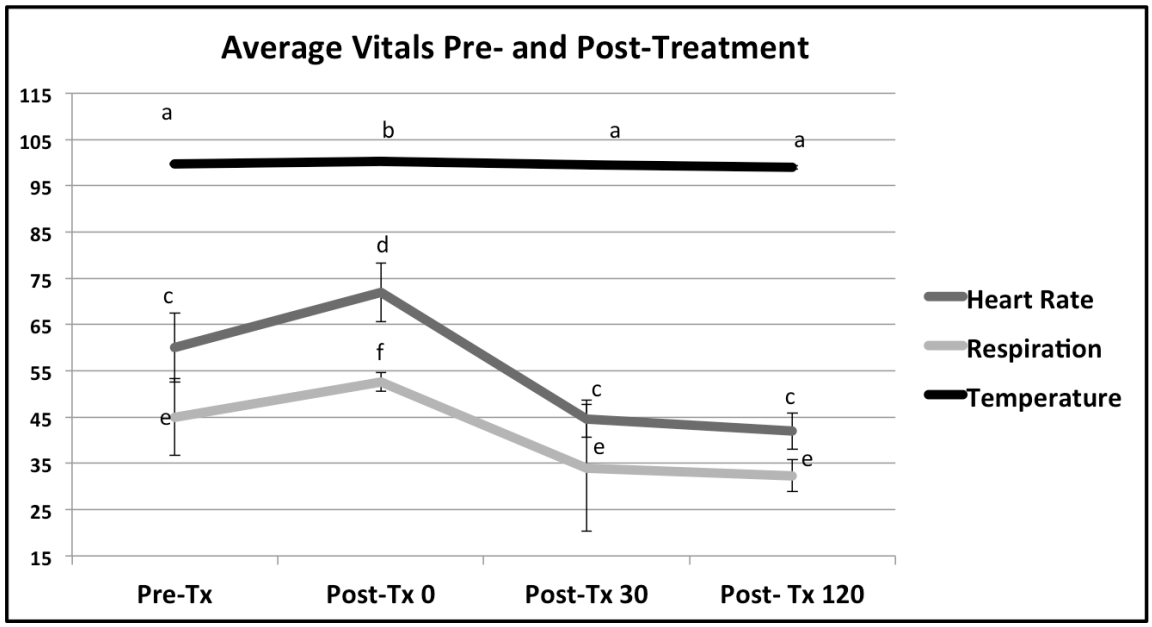

Figure 6.1: The effect of intrauterine infusion of lactoferrin on vitals. Heart rate, respiration, and temperature were evaluated on normal mares before the intra-uterine infusion of 500ug/mL hrLF resuspended in 10mL lactated Ringers solution (LRS). This was repeated immediately post-infusion, in addition to 30 and 120 min post-infusion. There was a significant increase in all three vitals immediately post-infusion with all returning to below resting within 30 min post-infusion.

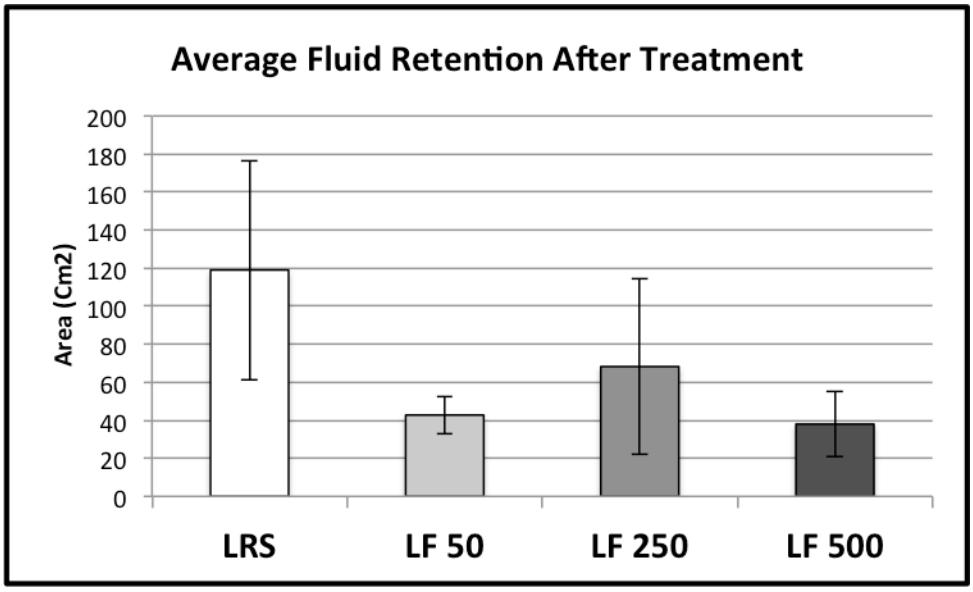

Figure 6.2a: The effect of varying concentrations of hrLF infusion on uterine fluid. Area (cm^2) uterine fluid evaluated via transrectal ultrasonography at 24 hours post-insemination. There were no significant differences found when comparing the infusion of hrLF to that of LRS.

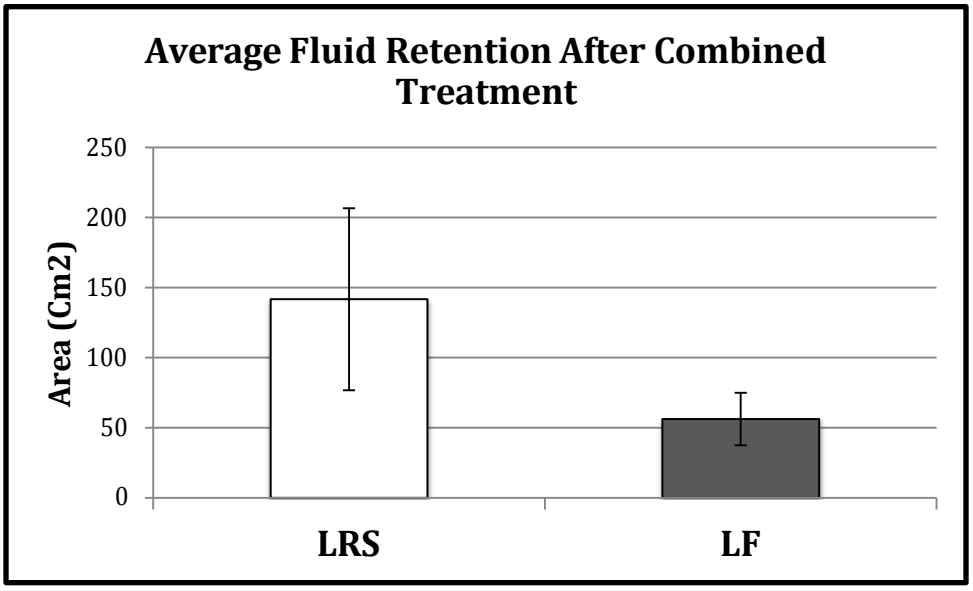

Figure 6.2b. The effect of total hrLF infusion on uterine fluid. Area (cm2) inflammatory uterine fluid evaluated via transrectal ultrasonography at 24 hours post-insemination. hrLF tended to decrease the circumference of fluid retention when individual concentrations of hrLF were combined into one variable (P=0.0941).

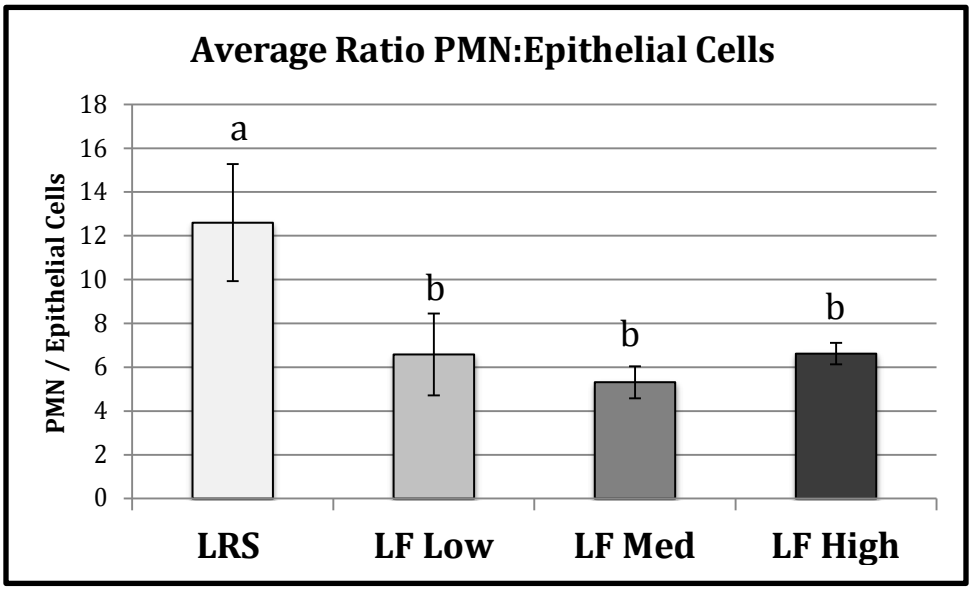

Figure 6.3: The effect of hrLF infusion on the ratio of PMN's to epithelial cells. PMN migration evaluated via low volume lavage at 24 hours post-breeding. Cells were counted using a cellular smear stained in Diff Quik. 10 views at 40x were evaluated, different letters (a,b) signify significant differences . All three concentrations of hrLF (50µg/mL, 250µg/mL, and 500µg/mL) significantly decreased the ratio of PMNs to epithelial cell numbers.

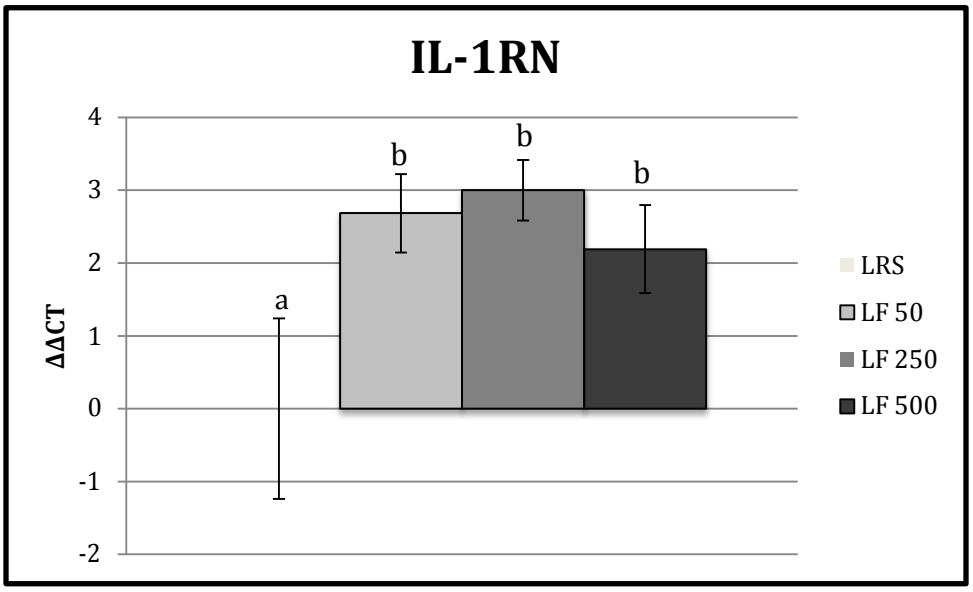

Figure 6.4: **ΔΔCT of endometrial mRNA transcripts of *IL-1RN* in mares 18 hours after infusion with exogenous hrLF.** The gene expressions are normalized to ACTB and displayed as ΔΔCT values ± SEM with LRS as the calibrator. Different letters (a,b) indicate significant differences among treatment groups, set to $p < 0.05$.

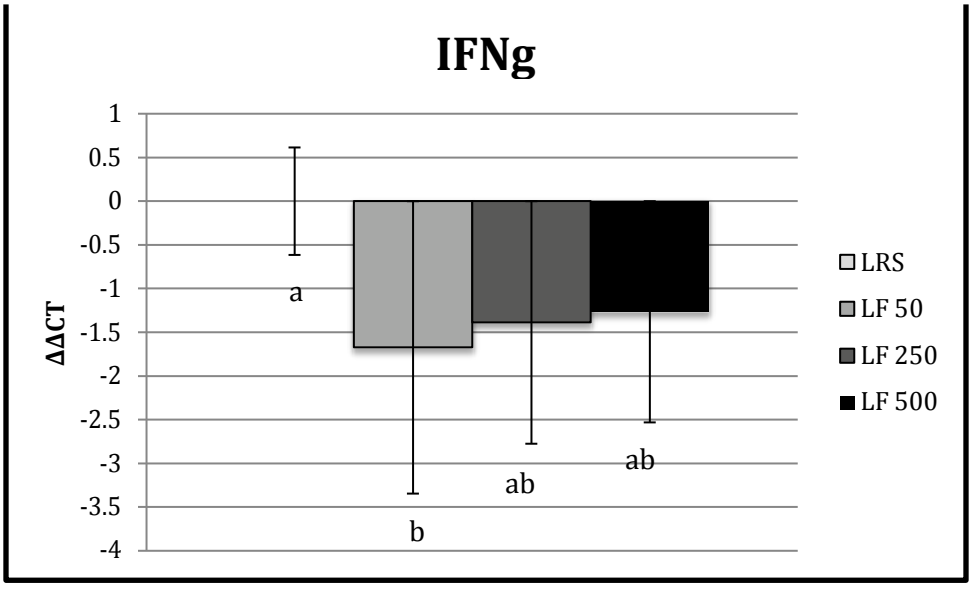

Figure 6.5: **ΔΔCT of endometrial mRNA transcripts of *IFNγ* in mares 18 hours after infusion with exogenous hrLF.** The gene expressions are normalized to *ACTB* and displayed as ΔΔCT values ± SEM with LRS as the calibrator. Different letters (a,b) indicate significant differences among treatment groups, set to $p < 0.05$.

6.5 Discussion

In this study, exogenous human recombinant lactoferrin (hrLF) was found to modulate the innate immune response seen after breeding in the susceptible mare. When administered in estrous, hrLF was found to have no overall detrimental effect on the vital signs evaluated, with rectal temperature, heart rate, and respiration all returning back to basal levels or below within 30 min post-infusion. Intrauterine infusion of hrLF was also shown to significantly effect many aspects of the inflammation to breeding. To our

knowledge, this is the first report of a seminal plasma protein altering the innate immune response to breeding in susceptible mares when infused at 6 hours after insemination.

Lactoferrin is an estrogen-dependent protein found to be bactericidal due to its ability to chelate free iron, enabling its usage to suppress a wide rage of gram-negative bacteria such as *Staphylococcus aureus*, which has been reported as a common uterine pathogens [213]. In addition, lactoferrin has been shown to function as an anti-inflammatory on many markers of innate immunity. A study by Machnicki *et al.* (1993), found lactoferrin to significantly decrease the serum concentration of tumor necrosis factor (*TNF*) and interleukin-6 (*IL-6*) after induction of inflammation by intravenous lipopolysaccharide (LPS) injection in the mouse [135]. Another murine study found the intravenous infusion of lactoferrin to significantly suppress the synthesis of both interleukin-1β (*IL-1β*) and tumor necrosis factor (*TNF*) after lipopolysaccharide (LPS)-induced endometritis [36].

In the horse, lactoferrin has been shown to affect the innate immune response both *in vitro* and *in vivo*. Lactoferrin was found to significantly increase the number of polymorphonuclear neutrophils (PMNs) bound to sperm, and specifically nonviable sperm *in vitro* [225]. *In vivo*, lactoferrin has also been shown to modulate the innate immune response to breeding in mares suffering from persistent breeding-induced endometritis. When added at the time of breeding, 150μg/mL hrLF was found to significantly suppress the endometrial mRNA expression of the pro-inflammatory cytokine *TNF* at six hrs after breeding [226]. While the literature supports the basis of lactoferrin affecting many markers of the innate immune response, to our knowledge the

present study is the first to assess the ability of lactoferrin to modulate persistent breeding-induced inflammation in the equine uterus.

In this study, the addition of hrLF significantly decreased the ratio of polymorphonuclear neutrophils (PMNs) to epithelial cells post-breeding. Elevated numbers of PMNs are found within the uterine lumen as rapidly as 30 mins post-insemination, and PMN numbers peak approximately 4-8 hours after breeding [231]. In the susceptible mare, PMN migration into the uterus can persist upwards of 96 hours post-breeding; and the resolution of this is paramount for optimal fertility [232]. It has been shown that the addition of seminal plasma to the insemination dose suppresses the PMN infiltration at 24 hours after breeding, but research into the specific components of seminal plasma which play a role in this suppression is lacking [175]. In the current study, the migration of PMNs was evaluated at 24 hours after insemination, and analyzed between varying concentrations of hrLF added at 6 hours post-breeding in contrast to the negative control of lactated Ringers solution (LRS). When mares were infused with LRS, the percentage of PMNs to every 100 epithelial cells was 12.6%, a value comparable to previous literature. Cocchia *et al.* (2012) evaluated chronically infertile mares for neutrophil migration through a variety of techniques and found the average ratio of PMNs to epithelial cells after low-volume lavage to be 10.67 [233]. In this study, the addition of exogenous hrLF was found to significantly suppress the PMN:epithelial cell ratio at all three concentrations (50μg/mL, 250μg/mL, and 500μg/mL) and to below the average seen in other reports (6.5, 5.6, and 6.3 respectively). While there was no effect on the specific concentration of hrLF infused, the suppression on this hallmark of the innate immune response at 24 hours after breeding is intriguing.

In addition to its effect on PMN migration, the infusion of hrLF significantly affected the endometrial mRNA expression of inflammatory cytokines. In particular, all three concentrations of hrLF infusion at six hrs post-breeding significantly increased the mRNA expression of interleukin-1 receptor antagonist (*IL-1RN*) in comparison to LRS. *IL-1RN* is a competitive inhibitor of the receptor for the pro-inflammatory cytokine interleukin-1 (*IL-1/IL-1β*), therefore acting as an anti-inflammatory in response [234]. *IL-1β*, in addition to tumor necrosis factor (*TNF*), are initial responders to the detection of foreign particles and pathogens to the system. Both *IL-1β* and *TNF* stimulate the production of interleukin-6 (*IL-6*), which is critical for the transition from a neutrophilic to monocytic invasion. Woodward *et al.* (2013), found that susceptible mares have a significantly lower endometrial mRNA expression of *IL-1RN* in comparison to resistant mares at 6 hours post-breeding, and it was suggested that this suppression of the anti-inflammatory response was explanatory to the prolonged inflammation seen in the susceptible mare [9]. The infusion of exogenous hrLF at six hours post-breeding appears to modulate this suppression of the anti-inflammatory response to breeding, and may assist with the resolution of the prolonged inflammatory state of the uterus that occurs in these mares.

In addition to its effect on *IL-1RN*, the infusion of 50μg/mL of exogenous hrLF also significantly suppressed the endometrial mRNA expression of interferon gamma (*IFNγ*). A pro-inflammatory cytokine, *IFNγ* is a critical component in the activation of mononuclear phagocytes, and specifically the macrophage population [235]. *IFNγ* is also critical in inducing antigen-presenting cells to produce increasing amounts of other pro-inflammatory cytokines, such as *TNF* and interleukin-12 (*IL-12*). In addition to its effect

on the immune response, researchers have found *IFNγ* to play a role in pregnancy development and maintenance in other species. A study by Szekeres-Bartho *et al.* (1990) found that when *IFNγ, TNF*, and *IL-2* were injected into non-abortion prone mice, their risk of miscarriage significantly increased [236]. In addition, increased levels of *IFNγ* expression were found in endometrial biopsies of pigs that contained retarded, though viable, conceptuses [237]. The role of *IFNγ* in equine pregnancies has not been investigated.

Although lactoferrin showed a significant affect on the signaling molecules of the innate immune system, no significant difference was seen in the level of inflammatory fluid when exogenous lactoferrin was infused. All three doses of hrLF appeared to lower the circumference of fluid retention in the susceptible mare in comparison to LRS, but this was not found to be significant. When the individual concentrations of hrLF were combined as a single variable of treatment, there was a trend towards a significant decrease in the circumference of inflammatory fluid retention when hrLF was added in contrast to that of LRS (P=0.0941). We believe that the low number of mares evaluated in this study, in addition to the high variability seen in fluid grades across mares as well as between cycles contribute to the lack of significance, and should be noted.

In conclusion, the infusion of exogenous human recombinant lactoferrin at six hours post-breeding was found to be both a safe and potent modulator of the prolonged inflammation seen in the mare suffering from persistent breeding-induced endometritis. The intrauterine infusion of hrLF in normal mares in estrous did not have a clinically relevant effect on the heart rate, temperature, or respiration under the conditions of this study. When administered post-breeding, the intrauterine infusion of all three

concentrations of hrLF post-lavage in mares susceptible to PBIE significantly suppressed the ratio of PMNs to epithelial cells. In addition, all three concentrations of hrLF significantly increased the endometrial mRNA expression of the anti-inflammatory cytokine *IL-1RN*, with the concentration of 50μg/mL hrLF also decreasing the endometrial mRNA expression of the pro-inflammatory cytokine *IFNγ*. To our knowledge, this is the first report in which the administration of exogenous lactoferrin significantly altered the immune response of the susceptible mare at 24 hours post breeding. Results from this study show that hrLF is an effective modulator of the inflammation seen after breeding in the mare that is susceptible to PBIE, and future research is required to see its effect on fertility.

6.6 Acknowledgements

This work was funded by the Janet H. Koller Endowment for Equine Research. The authors would like to thank Mr. Lynn Ennis, Mr. Kevin Gallagher, and the dedicated farm staff of the University of Kentucky's Maine Chance Farm for their animal care and assistance and the University of Kentucky's Veterinary Diagnostic Laboratory for their processing of samples. In addition, the authors would like to thank Mr. Seth Kruger for his assistance in stallion collections and animal work.

CHAPTER 7

GENERAL DISCUSSION

Persistent breeding-induced endometritis (PBIE) in the horse is considered an economic burden to the breeding industry. While multifactorial in causation, its incidence has been shown to correlate with increasing age, worsening endometrial biopsy score, poor perineal conformation, and parity [14, 15, 238-240]. Immense research has been conducted on the disease process of PBIE in addition to therapeutics with which to resolve it in a timely fashion, but considerable variability exists within the results. This may be due to the inconsistencies found within protocols for the induction of inflammation, screening of mares for disease risk, in addition to the endpoints evaluated and the time points they are performed.

The constituents of the insemination dose vary tremendously between management protocols, stallions and methods of breeding. In the live cover or natural breeding situations, the sperm is supported by the addition of both seminal plasma and the gel fraction. The gel fraction is eliminated during most semen collection practices through filtration, and the seminal plasma removed via centrifugation or dilution to a minimal amount prior to cryopreservation or cooling [241]. While the removal of seminal plasma may be beneficial to the longevity and viability of sperm, its presence has been documented as beneficial to the immune response seen post-breeding in the mare [1, 74]. The role of specific aspects of seminal plasma on inflammation post-breeding is

lacking, with limited data to support their functionality *in vivo*. *In vitro*, seminal plasma proteins have been found to modulate specific markers of inflammation, including the bioactivity of PMNs. While lactoferrin was found to significantly increase PMN binding to dead sperm [123] and CRISP-3 was found to suppress PMN binding to live sperm [11], the data within this dissertation is the first to look into their effect *in vivo*.

To properly understand the mechanisms and pathways with which these select seminal plasma proteins act additional information is required. In Chapter 2, the effect of puberty on the expression of CRISP-3 in the male reproductive tract was evaluated. There exists generous literature to support the role of androgens and estrogens on the synthesis and expression of seminal plasma proteins, but minimal work has been done on this specific constituent of the proteome in the horse. In Chapter 2, it was determined that puberty plays a role in the activation of CRISP-3 expression, as both mRNA and protein expressions were increased in the postpubertal male. Unfortunately, under the confines of this experiment, it remains uncertain if this is due to the activation of androgens and/or estrogens that occurs at the onset of puberty, or if additional variables were involved. It also became apparent in this chapter that CRISP-3 is primarily produced in the glandular aspects of the secretory glands of the reproductive tract. We hypothesize that this is what causes the immense concentration of this protein found in the stallion ejaculate, approximately 1mg/mL, which is considerably more than what is seen in other species [105].

A crucial aspect of the pathology of persistent breeding-induced endometritis is within the innate immune response. Pivotal research identified differing inflammatory gene expression of key cytokines when comparing the resistant and susceptible mare

populations [9, 21]. Unfortunately, there exists vast differences in methodology in comparable studies, and therefore the effect of inseminating live versus dead sperm on inflammatory gene expression was evaluated. The results from the experiment in Chapter 3 confirmed the hypothesis that the insemination protocol affected the results of the study, as the infusion of live sperm caused a significantly different response in comparison to that of freeze-killed sperm. With a suppression of the inflammatory modulating cytokine interleukin-6 (*IL-6*), this result suggests that the protocol used to induce inflammation, and its effect on the outcomes studied should be considered when interpreting data. Although some researchers choose to induce inflammation through the intrauterine infusion of non-physiological components, such as freeze-killed sperm or bacterial pathogens, this study demonstrates that the variable of inseminant may have an effect on the outcomes reported, and potentially stray from the physiological state in a normal breeding operation.

Previous studies found that six hours post-breeding is a key time point for the diverging immune response between that of susceptible and resistant mares to occur [9]. At this time, resistant mares were found to have a significant increase in the expression of both the anti-inflammatory and inflammatory-modulating cytokines. In contrast, the susceptible mares failed to mount this response, and it was hypothesized that this failure to launch an anti-inflammatory response was the cause of the prolonged inflammation noted. This pivotal study was performed with the infusion of freeze-killed sperm to induce inflammation, and with the seminal plasma reduced significantly from its original volume. As noted, it has been shown that seminal plasma may play a role in the inflammatory response to breeding, although minimal attention has been paid to the

specific proteins within the ejaculate. Two proteins of interest are that of lactoferrin and CRISP-3, both of which have been shown to modulate the binding activity of PMNs to spermatozoa *in vitro*. Therefore, the dual objectives of Chapters 4 and 5 were to determine if the addition of these two proteins at the time of breeding had an effect on the gene expression of key cytokines in either the resistant or susceptible mare. While neither protein was found to have a substantial effect on the genes studied in resistant mares, the addition of lactoferrin was found to significantly suppress the expression of the cytokine tumor necrosis factor (*TNF*)-α in susceptible mares. The suppression of this pro-inflammatory signaling molecule at 6 hours post-breeding indicates that the seminal plasma protein lactoferrin may function as an anti-inflammatory in response to insemination. Minimizing the concentration of this protein via centrifugation for artificial insemination practices therefore may explain the increased risk of endometritis when using cryopreserved semen in the field.

The pathway which inherent lactoferrin acts in the equine uterus has not been reported. Our proposed pathway is described in Figure 7.1. We hypothesize that the deposition of semen in the uterus at the time of breeding induces an increase in the expression of pro-inflammatory cytokines *IL-1β* and *TNF* from the epithelial cells of the endometrium. These cytokines are considered the first responders of the innate immune response and are activated rapidly after the detection of foreign materials. This increase then signals an increased production of *IL-8*, which activates the migration of PMNS from the endometrium to the uterine lumen. This is thought to occur within 0.5hr after breeding, and peaks at 2 hrs. [242]. Lactoferrin, which is present in the seminal plasma of the ejaculate, has been found to increase the binding of PMNs to nonviable sperm [52].

However, it is unknown how lactoferrin mediates this action, as no specific receptor for the protein has been detected on neutrophils. It has also been shown that the granules of neutrophils contain lactoferrin, and therefore the enhancement of phagocytic activity may be due to the functionality of the neutrophil-derived protein, as the phagosome is created through the extrusion and entrapment of foreign materials via the granules of the neutrophils. The presence of granulitic lactoferrin within the phagocytic cell itself may contribute to this bactericidal activity.

While the initial cellular immune response to breeding is predominantly a neutrophilic one, this has been shown to shift to a monocytic invasion around 24 hours after insemination, and this is mediated by the dual function of *IL-6*. It is hypothesized that lactoferrin may bind to macrophages present, as lactoferrin specific receptors have been found on the phagocytic cells in the bovine and human model [139]. The monocytes predecessor, macrophages are responsible for the production of cytokines such as *TNF* through the activation of the NFkβ pathway [243], and the inhibition of this action by lactoferrin may explain the suppression of *TNF* at 6 hours post-breeding. The latent suppression of *TNF* at 6 hours post-breeding could then potentially have a negative feedback on production of *IL-8*. As *IL-8* is the primary stimulant for PMN migration, this suppression will be followed by a decrease in PMN infiltration, and a resolution of inflammation. In the normal mare, this occurs within 24-36 hours, and lactoferrin appears to play an important role in this pathway. In the susceptible mare, a supplemental addition of this protein at the time of breeding may be beneficial to stimulate this feedback.

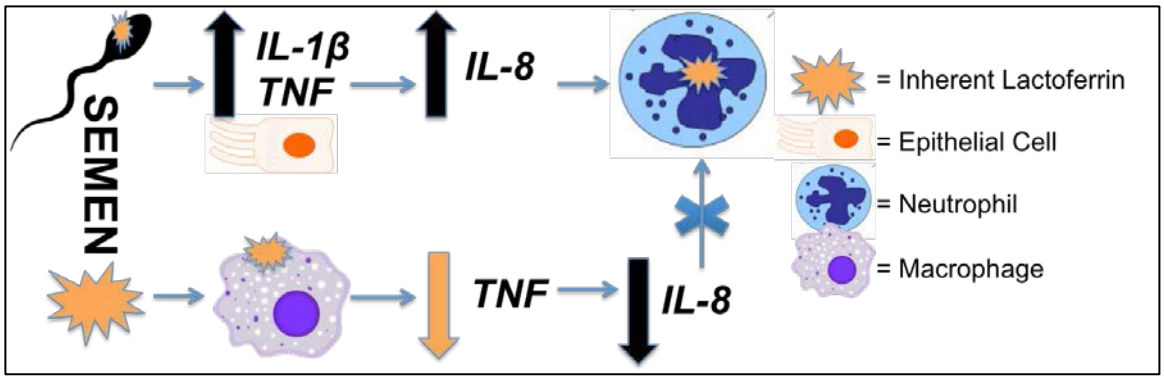

Figure 7.1: **Schematic diagram of the inherent lactoferrin pathway**. Orange arrows indicate inherent lactoferrin effect, as caused by the effect of lactoferrin within seminal plasma. It is hypothesized that the deposition of semen directly into the uterus activates an increased expression in the pro-inflammatory cytokines, resulting in an increased neutrophilic migration. Lactoferrin is thought to increase PMN binding, although the receptor/ligand has not been determined. Lactoferrin is hypothesized to bind to activated macrophages and suppress the synthesis of TNF. This decrease in TNF may then suppress the production of IL-8, resulting in the cessation of the neutrophilic infiltration. This indicates that lactoferrin may play an essential role in the activation of the pro-inflammatory response in addition to the negative feedback required for the checks and balances of the innate immune system.

Although the majority of seminal plasma is removed before cryopreservation of semen, it is left whole for practices of live cover and most artificial insemination protocols using fresh semen. In addition, a subset of thoroughbred mares bred under live cover conditions also experience persistent breeding-induced endometritis, as noted in a study by Zent *et al.* (2005). This disease was shown to occur in 10-15% of the thoroughbred population in Kentucky, indicating that the addition of seminal plasma proteins at the time of breeding does not offer total inhibition of the prolonged inflammation from occurring. The susceptible mares experience a persistent inflammatory response, which left uninhibited, can lead to infertility and possibly chronic endometrial fibrosis if unattended to. Therefore, in Chapter 6 we investigated the use of

exogeous lactoferrin as a potential modulator of persistent inflammation in the uterus. Although not in a dose-dependent fashion, lactoferrin was found to act as an anti-inflammatory when infused post-breeding, the mechanism for which is described in Figure 7.2. The suppression of PMN infiltration, fluid retention, and dual suppression of pro-inflammatory cytokines (*IFNγ*) while stimulating anti-inflammatory cytokine production (*IL-1RN*) in the diseased mare indicates a potential pathway for exogenous lactoferrin to prepare the uterus for embryo arrival and future implantation. However, future studies are required to examine its affect on fertility.

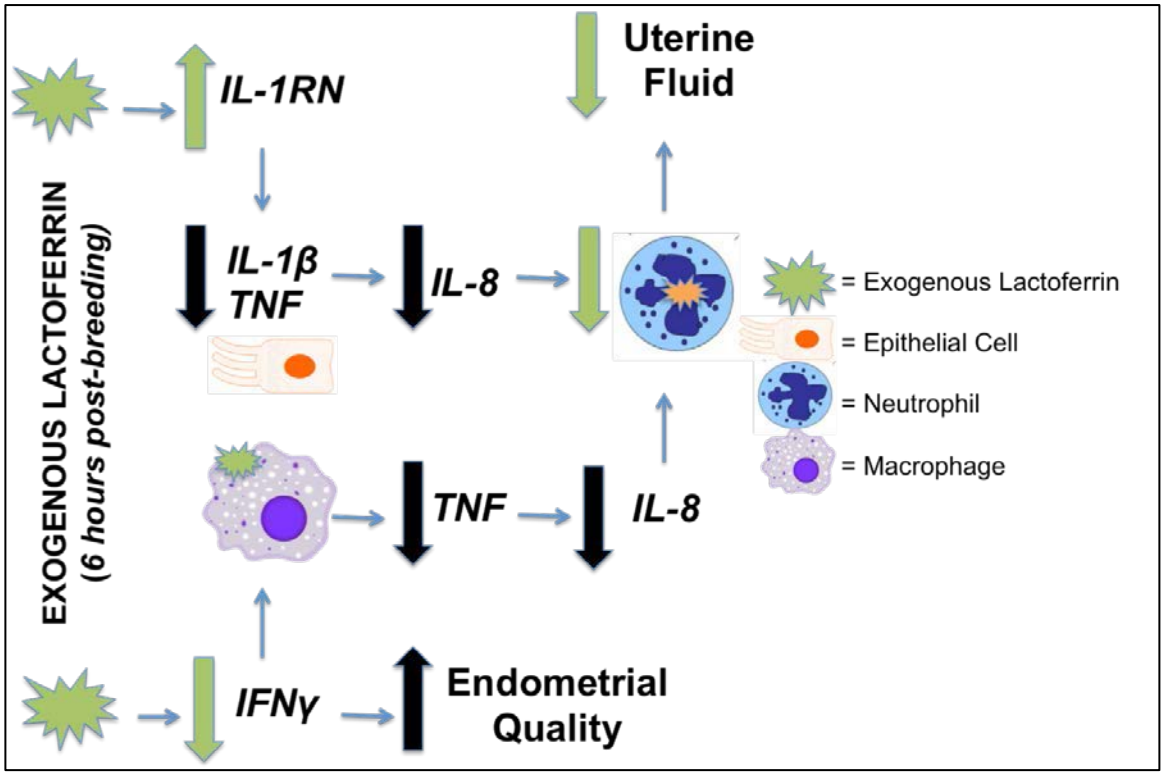

Figure 7.2 Schematic diagram of the exogenous lactoferrin pathway. In the susceptible mare, persistent inflammation can remain in the uterus for upwards of 96 hours. Green arrows indicate effect of exogenous lactoferrin as described in the dissertation, and black indicating hypothesized outcomes as indicated in previous

literature. Exogenous lactoferrin administered at 6 hours post-breeding acts as an anti-inflammatory, dually suppressing the expression of the pro-inflammatory cytokine *IFNγ* while increasing the expression of the anti-inflammatory cytokine *IL-1RN*. This activity functions cohesively to both decrease the activity of *IL-1β* while also further suppressing the macrophage-stimulated *TNF* synthesis. This suppression may inactivate the *IL-8* pathway to neutrophilic infiltration, effectively decreasing the neutrophilic presence in the uterine lumen. In addition, exogenous lactoferrin was found to suppress uterine fluid. In other species, a down-regulated expression of *IFNγ* is imperative for the endometrium to achieve pre-implantation quality.

The proteomics of the stallion ejaculate deserve further investigation into their functionality, in addition to the pathways that regulate them. While the majority of seminal plasma is removed from the insemination dose in many artificial insemination practices, we have shown that certain proteins may be beneficial to the resolution of inflammation seen post breeding, and their reinstatement at the time of breeding may be beneficial. In addition, the proteins found capable of affecting the immune response should be further pursued as biologically derived immunomodulators. If found effective, biologics that are intrinsic to the ejaculate may serve as better options for the treatment of inflammation when compared to nonsteroidal anti-inflammatories (NSAIDs), antimicrobials, and steroids as they are less likely to induce contraindicated effects.

In conclusion, lactoferrin suppresses numerous aspects of the pro-inflammatory pathway of the innate immune response, serving as an innately produced anti-inflammatory in the ejaculate. Increased understanding into the functionality of both lactoferrin, in addition to other proteins within the seminal proteome, may further improve our management of broodmares.

Appendices

Appendix A: The effect of the gonadotropin-releasing hormone (GnRH) antagonist acyline on the synthesis of CRISP-3 in the equine ejaculate

A.1: Background

The effect of androgens and estrogens on seminal plasma proteins is of interest to further determine the pathways with which these proteins function. Recently, the third generation GnRH antagonist acyline (Ac-D2Nal-D4Cpa-D3Pal-Ser-4Aph(Ac)-D4Aph(Ac)-Leu-Lys(Ipr)-Pro-DAla-NH_2) was investigated in the horse. Numerous studies have shown that acyline suppresses the HPG axis in canines and humans, and it has been used as a therapeutic in androgen dependent diseases [244-246]. Acyline was recently found to suppress the hypothalamic-pituitary-gonadal (HPG) axis for upwards of five days in the stallion [247], significantly decreasing the production of steroid hormones; including testosterone, estrogen, luteinizing hormone (LH) and follicle stimulating hormone (FSH). This suppression was reversible, and within 10-15 days after the end of treatment, the levels had replenished to their normal values. It was also shown in this study that total protein content of the seminal plasma was significantly lowered in the stallions treated with acyline, but the individual proteins within the seminal plasma were not evaluated.

While the androgen dependency of the seminal plasma protein CRISP-3 has been reported in the murine and human model, no data can be found to support this in the equine. Therefore, the objective of this study was to investigate the effect of the third generation GnRH antagonist acyline on the synthesis of CRISP-3 in the seminal plasma of the mature stallion. We hypothesize that androgens, and their decreased synthesis due

to the serial injections of acyline, will have a negative effect on the synthesis of equine CRISP-3 in equine seminal plasma.

A.2: Materials and Methods

A2.1 Animals and Sample Collections

All animal research and sample collection was conducted according to Davolli *et al.* (2016) [247].

A2.2: Western Blotting

Protein concentration in seminal plasma was determined with coomassie (Bradford) protein assay kit (#23200, Thermo Scientific, Kalamazoo, MI, USA) as previously described by Bradford [248]. Seminal plasma was then resuspended to a concentration of 100μg/mL in 50% 10 mM Tris (Bio-Rad, Hercules, CA, USA) (pH 7.2), 47.5% Laemmli Sample Buffer (Invitrogen, Carlsbad, CA, USA), and 2.5% b-mercaptoethanol (MP Biomedicals, Solon, OH, USA), mixed vigorously for 30 s, and boiled at 90–100°C for 5 min. From each sample, 50ng of protein was loaded onto a 12% polyacrylamide gel and electrophoresed at 200 V for 40min, then the proteins were transferred to an Immun-Blot PVDF Membrane (Fisher Scientific, Pittsburgh, PA, USA) at 100 V for 1 h in a transfer buffer consisting of 70% deionized water, 20% methanol (Fisher Scientific) and 10% Tris/Glycine buffer (Fisher Scientific). Following transfer, the membranes were rinsed well in deionized water and blocked using 5% non-fat dry milk (Carnation, Wal-Mart, Lexington, KY, USA) in 0.1% Tween-20 (Sigma) (PBS-T) for 1h. The membranes were washed three times in 1x PBS for 5 min each, then incubated overnight with 0.1% Tween-20 (Sigma) (PBS-T) with 3% bovine serum

albumin (Eastman Kodak Co., Rochester, NY, USA) (BSA) and primary antibody against CRISP-3 at a dilution of 1:5000. The next day, following incubation in primary antibody, the membranes were washed three times in 1x PBS-T for 5 min each, then incubated for 1 h in 1x PBS- T and anti-rabbit whole molecule horse- radish peroxidase-conjugated (HRP) secondary antibody (A-3415) (Zeiss) at a dilution of 1:5000, followed by washing three times in 1% PBS-T for 5 min each. The membranes were treated with SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA) for 5 min prior to developing on high performance chemiluminescence film (Applied Biosystems, Foster City, CA, USA) using a Kodak X-OMAT 1000A Processor (Eastman Kodak Co.).

A.3: Results

The decrease of testosterone and estrone sulfate in the serum of stallions had no significant effect on the production of CRISP-3 in the seminal plasma. Although a level of decrease was noted on certain days (refer to +50 day), this was not due to treatment, as controls also experienced a suppression of CRISP-3 synthesis at that specific time point (Fig A.1). These results may be due to seasonal variations as the experiment was conducted during the months of August-December.

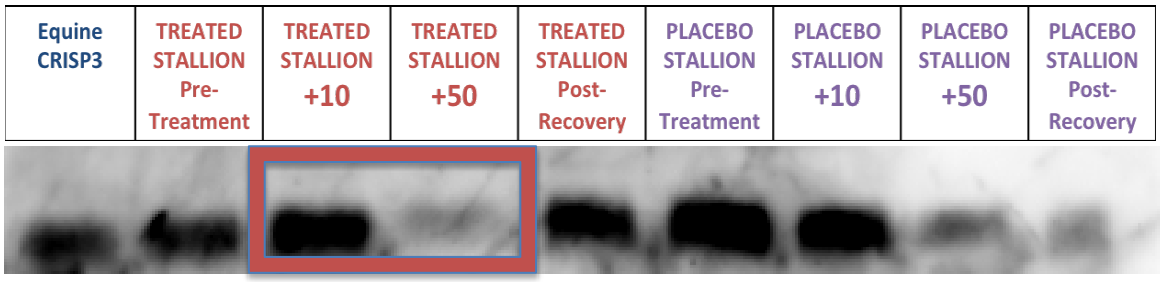

Figure A1.1: Western blot of eCRISP-3 as evaluated pre- during and post-serial acyline administration. No significant decrease in CRISP-3 synthesis in the seminal plasma was noted across stallions, with large variability seen between stallions and days.

A.4: Discussion

In this experiment, the effect of a gonadotropin-releasing hormone (GnRH) antagonist on the synthesis of equine CRISP-3 in the ejaculate was evaluated. The expression and synthesis of CRISP-3 has been found to be hormonally regulated in a variety of species, including the murine and human models. Although data suggests that it is puberty dependent in the horse [223], no research has gone into the specific endocrine markers which affect its expression and synthesis. Although the third generation GnRH antagonist acyline was determined to be an effective and reversible suppressant of both androgens and estrogens in the stallion, it had minimal effect on the synthesis of CRISP-3 [247]. In the study by Davolli *et al.* (2016), acyline was found to significantly decrease the total protein content in the ejaculate. While it is intriguing that the total protein content was suppressed, but not CRISP-3 synthesis, it should be noted that the antibody used to detect equine CRISP-3 in this project was a polyclonal antibody raised in rabbits against purified protein. When attempted for usage in an enzyme-linked immunosorbent assay (ELISA), this antibody demonstrated tremendous inter- and intra-assay variability, and therefore its sensitivity and specificity is questionable. In addition, it is difficult to extrapolate the exact causative function of any of these hormones on the synthesis of seminal plasma proteins in the confines of this study, as both testosterone and estrone sulphate were suppressed. This may have contributed to the results of this study, as although CRISP-3 was found to be testosterone dependent in other species, estrogen is a known mediator of the synthesis of other seminal plasma proteins, including lactoferrin [145]. Future work should consider this quandary, and assess the effects of the

specific steroid hormones individually on the synthesis of equine CRISP-3 in the stallion ejaculate. In addition, antibodies should be re-developed against a protein of higher purity or of recombinant form to further increased the specificity and sensitivity.

Appendix B: The development of an equine specific CRISP-3 enzyme-linked immunosorbent assay (ELISA)

B.1: Background

The use of an enzyme-linked immunosorbent assay (ELISA) has become common practice in research and diagnostics due to its high precision and accuracy at measuring protein, hormone, and antibody concentrations in a variety of fluids. One protein, equine cysteine-rich secretory protein-3 (CRISP-3) has gained interest due to both its high homology with plant pathogenesis-related (PR) proteins in addition to its ability to suppress the binding between polymorphonuclear neutrophils (PMNs) to viable spermatozoa *in vitro*, leading to the hypothesis of its involvement in the immune response to breeding [11]. In a study by Novak *et al.* (2010) it was found that the abundance of CRISP-3 in the ejaculate was positively correlated to high first cycle conception rates, but no causative effect was found [121]. Because of this, our ability to assess total CRISP-3 content in the ejaculate may be beneficial in predicting future fertility potential of a stallion. Previously, a competitive indirect ELISA was postulated to detect equine CRISP-3 [249], but the protocol described was not replicable in the confines of our laboratory. Therefore, the objective of this study was to produce and validate an additional ELISA for the detection of CRISP-3 in equine seminal plasma with minimal variability and great repeatability.

There are three types of ELISAs: direct, indirect, and sandwich. The specific

steps of each style of ELISA differ, but all utilize the specific binding between antigens and their antibodies to detect the presence of a molecule of interest [250]. Finally, a colorimetic reaction takes place between an enzyme conjugated to an antibody and substrate, and the measurement of a target substance can be performed through the use of optical density values.

ELISA's involve the stepwise addition of reagents and the reactions between them upon a solid phase-bound substance, usually a plastic microwell plate. The three basic systems of ELISA are all based off of this same principle application, with varying degrees of difference. In the direct ELISA, which is considered the most basic platform, an antigen is diluted into either a high pH bicarbonate or carbonate buffer or a basic pH solution such as PBS and allowed to attach passively to the plastic of the microwell plates. After incubation, the plate is washed thoroughly by flooding and emptying the wells of the plate using a neutral solution (PBS or TBS). Once washed, antibodies are added that are raised against the specific antigen and are conjugated to substrate. These conjugated antibodies are diluted in a substance that will interfere with passive binding but not interfere with the direct binding between antibody and antigen. These diluents are referred to as blocking buffers, and commonly are bovine serum albumin (BSA) or nonfat freeze dried milk (NFDM). The antibody is allowed to incubate for a specific amount of time, and this is again followed by a washing step. Finally, a suitable substrate that reacts with the conjugate of the antibody is added. This will evolve into a colorimetric that can be quantified be the use of spectrophotometer reading at the appropriate wavelength.

The initial steps of an indirect ELISA are similar to that of a direct system, with

the exception that an additional antibody is utilized. Following the coating of antigen and washing steps, an unlabeled antibody specific for the coating antigen is added in a blocking buffer and incubated for a specific time. This is washed to remove excess and then a second anti-species antibody is added. These secondary antibodies are raised against the immunoglobulins of the primary antibody species and are then conjugated to the detecting substrate. The final steps are similar to the direct ELISA, with the development of color that can be detected through the use of a spectrophotometer. The addition of the second antibody allows for the addition of a number of antisera to be utilized, making them easy to use. Unfortunately, this also leads to an increase in nonspecific binding in the individual sera (Crowther 2001).

The sandwich ELISA can be further divided into two subsets of platforms: the direct sandwich ELISA, and the indirect sandwich ELISA. In the direct sandwich ELISA, specific non-conjugated antibodies coat the plate and then antigen is added following the washing and blocking steps. Following this, the antigen is captured by a secondary antigen-specific antibody that is conjugated to substrate. This second antibody can be the same as the first in terms of species it was raised in, or it can differ. The steps following the capture of antigen are identical to the previous systems described. This system is limited in a variety of ways, including that the antigen must have at least two antigenic sites, or epitopes. It is also limited in specificities since a single enzyme-conjugated antibody is used. It is recommended that only one of the antibodies be monoclonal so as to not limit the detection of the antigen.

In the indirect sandwich ELISA, the first steps are identical to that of a direct sandwich ELISA. A specific antibody coats the plate, and following wash and blocking

steps, the antigen of interest is added and incubated. Following the addition of antigen, a second non-conjugated antibody is added. Finally, an anti-species conjugated tertiary antibody is added to detect the secondary non-conjugated antibody. The bound tertiary antibody is then detected as in the previously described systems. This system increases the specificity of detection, but is also rather expensive due to the number of antibodies needed to achieve results.

In addition to the three differing systems, it is also possible to increase or decrease capture and specificity of the platform by choosing either a monoclonal or polyclonal antibody. The antibodies generated in the natural immune response or after immunization are a mixture of molecules that contain different specificities and affinities [251]. Some of this is due to the immunization of a variety of epitopes of antigen, but antibodies raised against a single antigenic determinant can still be heterogeneous. This can be overcome by fusing spleen cells from the specific species immunized against antigen to that of a myeloma to produce a hybrid cell, or hybridoma, that can proliferate indefinitely and produce antibodies specific for the antigen used for immunization. Due to the fact that these hybridomas are clones derived from the fusion with a single B cell, they produce antibodies that are identical in structure, including in their antigen-binding site. These antibodies are therefore called monoclonal antibodies due to their specificity for a single epitope. In contrast, polyclonal antibodies are developed through the immunization against a specific antigen, but without the hybridization of the clones. Therefore, polyclonal antibodies maintain their heterogenicity and are less specific for a single epitope of antigen.

Throughout the process of the development of an ELISA specific for the detection

of equine cysteine-rich secretory protein-3 (CRISP-3), numerous systems, antibodies, reagents, blocking solutions, wash buffers, and substances were evaluated. Each was assessed at varying dilutions and concentrations, and yet no true detection system was found to possess both minimal variability and high repeatability. While initially following the protocol previous described by Connor (2009), it was noted that this led to a complete lack of colorimetric detection as written, and troubleshooting began. The following is a description of the variables assessed and their respective outcomes.

B.2: Attempts to develop and validate an equine-specific CRISP-3 ELISA

B.2.1 Protocol as per Connor (2010)

The initial protocol for the quantification of equine CRISP-3 in the seminal plasma of stallions was performed as described by [249]. In brief, an indirect competitive ELISA was used, utilizing the Nunc Maxisorpt 96-well plates. Each plate was coated with 1μg/ml CRISP-3 diluted in PBS. Coating incubation was overnight at 4ºC. After completion of coating, plates were washed using WellWash and were blocked with a volume of 200 μl 1% BSA. After incubation via shaking for one hour at room temperature, the plate was washed again. Every plate included a standard curve ranging from 10μg/ml to 25ng/ml purified equine CRISP-3, a total binding measurement as well as a non-specific binding measurement. Seminal plasma samples were diluted for analysis, ranging from 1:2400 to 1:307200 in a dilution curve. Each dilution was duplicated within the plate and added to the plate at a volume of 50μL. The mouse anti-equine monoclonal antibody HL2175 was diluted from glycerol stock solution to a dilution of 1:80,000 and 50μl of antibody was added to appropriate wells of the plate. The

plate was incubated for one hour, shaking at room temperature and then washed. Following washing, 50µl of rabbit anti-mouse IgG secondary antibody conjugated to alkaline phosphatase (AP) was added at a dilution of 1:1000 to each well. The secondary antibody incubated for one hour, shaking at room temperature. Following washing, P-Nitrophenyl-phosphate (pNPP; 200µL of 1mg/mL) was used as a substrate and added to each well. The pNPP was hydrolyzed to p-nitrophenol by the AP and detected spectrophotometrically at 405nm. The concentration of the resulting enzymatic product was inversely proportional to the amount of the antigen present in the sample.

B.2.2 Troubleshooting the equine CRISP-3 ELISA

No color developed after repeated attempts following the protocol as described by Connor, and potential adjustments were investigated. It was noted upon purchase of the secondary antibody conjugated to alkaline phosphatase that researchers were advised against diluting any alkaline phosphatase linked enzyme in PBS due to its potential inhibition on enzymatic activity.

Determination of Coating Buffer and Binding Absorption of Plates

Immulon® Microtiter™ (medium binding) and Nunc® Maxisorp (high binding) 96-well plates were analyzed for binding efficacy of antigen and background noise. Immulon® Microtiter™ (medium binding) was chosen due to low background noise and coefficient of variability amongst negative controls (Fig 1).

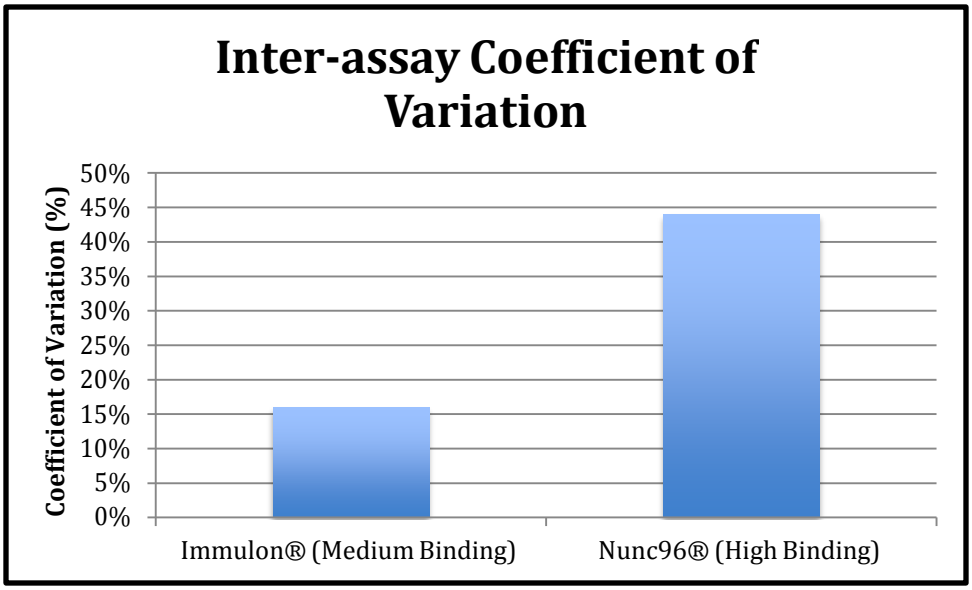

Figure B.1: Inter-assay Coefficient of Variation's (CV) of blank wells, as averaged over 5 plates. Immulon® Microtiter™ (medium binding) had an acceptable albeit high inter-assay CV of 15.6%, while Nunc Maxisorp (high binding) plates had an inter-assay CV of 44%.

Utilizing the Immulon® Microtiter™ 96-well plates, varying concentrations of purified equine CRISP-3 ranging from 50ng/mL to 10µg/mL were diluted in one of the following diluents: 1) 1x PBS (Fischer Scientific), 2) 1x TBS (0.2 M Tris-HCL + 5 mM MgCl2), pH 7.6 buffer (Trizma; Sigma Aldrich), or 3) carbonate buffer (50mM, pH 9.6: 15mM Na_2CO_3, 35 mM $NaHCO_3$) to assess binding efficacy and well overload. No color was noted when using either PBS or TBS, and 1ug/mL of purified equine CRISP-3 in carbonate buffer was chosen as the coating antigen (Fig. 2).

In addition, BSA (1%, w/v) in 0.2 M Tris buffer solution was used for diluting all antigen (both purified CRISP-3 and seminal plasma), the primary antibody, and the secondary antibody. Tris buffer solution was the buffer chosen for the ELISA

spectrophotometric measurements due to the lack of color development when PBS was utilized due to its denaturing by alkaline phosphatase. The washing solution (TBS-T) was used after each assay step and was prepared by adding 0.05 Tween 20 (v/v) to the 1x TBS.

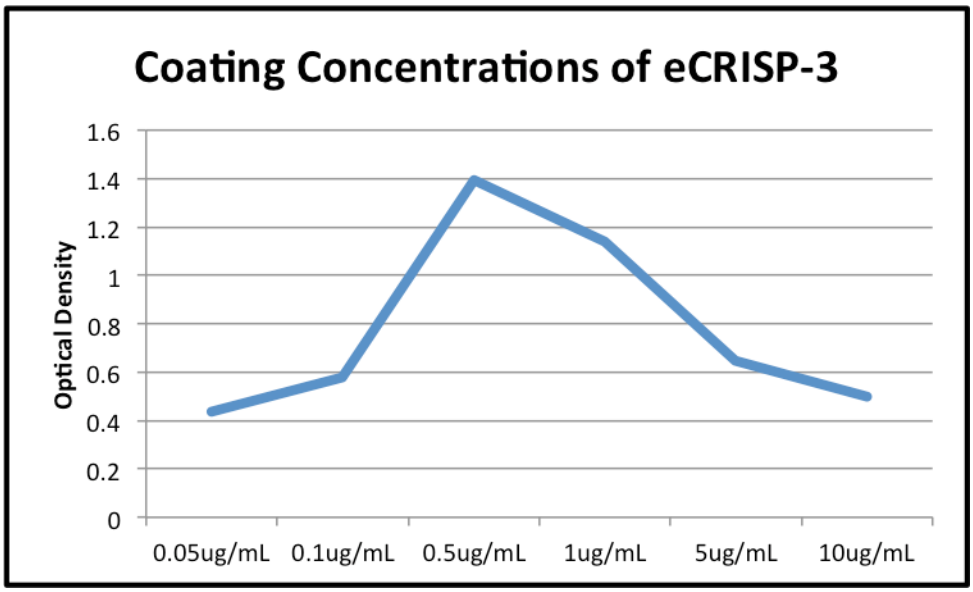

Figure B.2: Analyzing varying coating concentrations of purified equine CRISP-3. An increase in OD was noted from 50ng/mL upwards to 1,000ng/mL. Any higher concentration of eCRISP-3 was found excessive and resulted in wasted protein.

Determination of Blocking Buffer

Both BSA (1% w/v) and NFDM (5%, w/v) in 0.2 M Tris buffer solution were evaluated for use as blocking reagent. NFDM (5% w/v) was selected as the blocking buffer due to its low optical density evaluated on duplicated negative control wells across plates (n=8), in addition to its low intra-assay correlation of variation (CV) (Fig 3). Negative controls were defined as duplicated wells where no antigen was applied to the coating stage, and used to evaluate percent binding, as they indicated any background noise seen across wells.

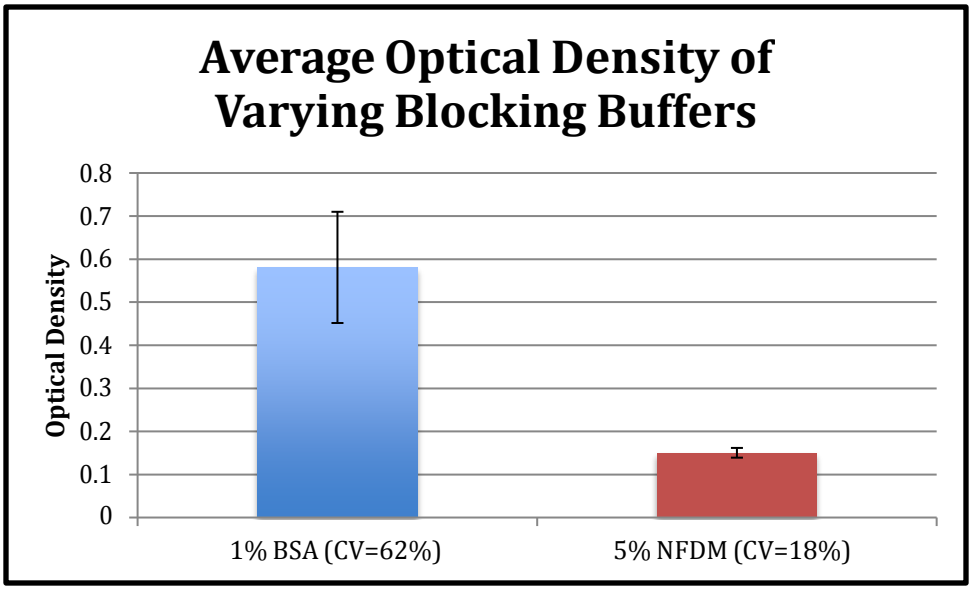

Figure B.3: Average optical density of varying blocking buffers. Note the increased OD and intra-assay CV when 1% BSA was utilized in comparison to 5% NFDM.

Selection of primary anti-equine CRISP-3 antibody

The initial antibody selected for use as a primary anti-equine antibody was a monoclonal mouse anti-equine antibody raised at the University of Florida's Hybridoma Core Lab utilizing standard protocols to develop monoclonal antibody production as described by Doty *et. al*. The specific antibody chosen was selected due to the highest functionality as evaluated via western blot and raised from the HL2175 cell line, herein referred to as simply HL2175 [249]. Both HL2212 and HL2199 were investigated as potential primary antibodies and resulted in a lack of detection of purified CRISP-3 via western blot. HL2175 was titrated and found to be of highest functionality at a dilution of 1:80,000. At this dilution, optimal color was developed and resulted in the production of standardization, with low intra-assay CV's. Unfortunately, repeatability of this protocol was unacceptably low, which resulted in high inter-assay CV's (33%) (Fig 4).

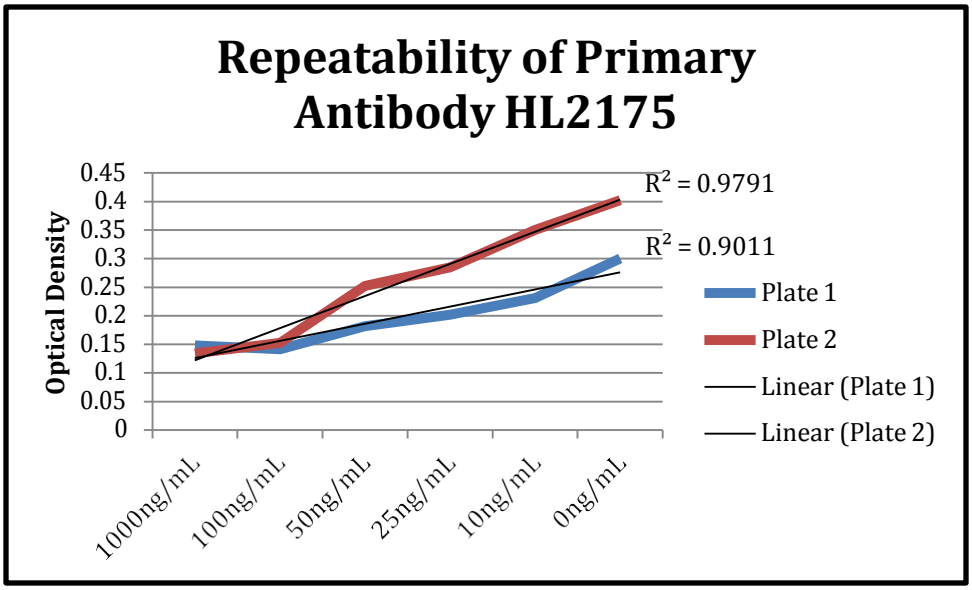

Figure B.4: Repeatability of ELISA protocol utilizing monoclonal mouse anti-equine antibody HL2175.

Because of this variability, a second antibody was developed. Purified equine CRISP-3 was obtained as described by Doty *et al.* (2011) and sent for antibody development at Thermo Fischer Laboratories. A polyclonal rabbit-anti equine CRISP-3 antibody (875) was produced from a single animal and raised for 57 days. Titration of this antibody found the optimal dilution to range from 1:400 to 1:1,600, and 1:1,000 (Fig 5a) was selected as the dilution to minimize materials used for each plate. Unfortunately, the 875 antibody also resulted in considerable variability and lacked repeatability, resulting in high intra- and inter-assay CV's (32% and 29% respectively (Fig 5b/5c).

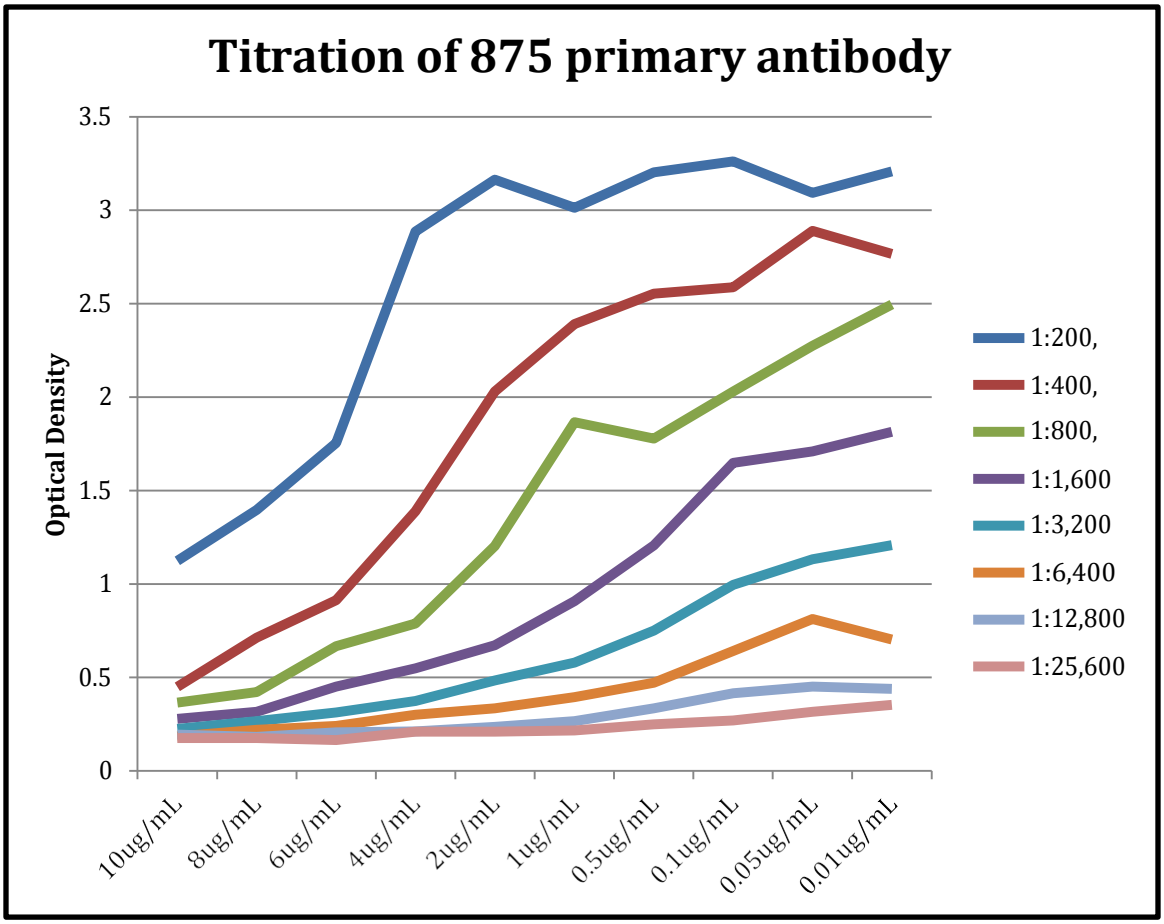

Figure B.5a: Titration of polyclonal rabbit anti-equine CRISP-3 antibody. The best range of titration was found from between 1:400 to 1:1,600, with the higher dilution being chosen to minimize amount of antibody necessary per plate.

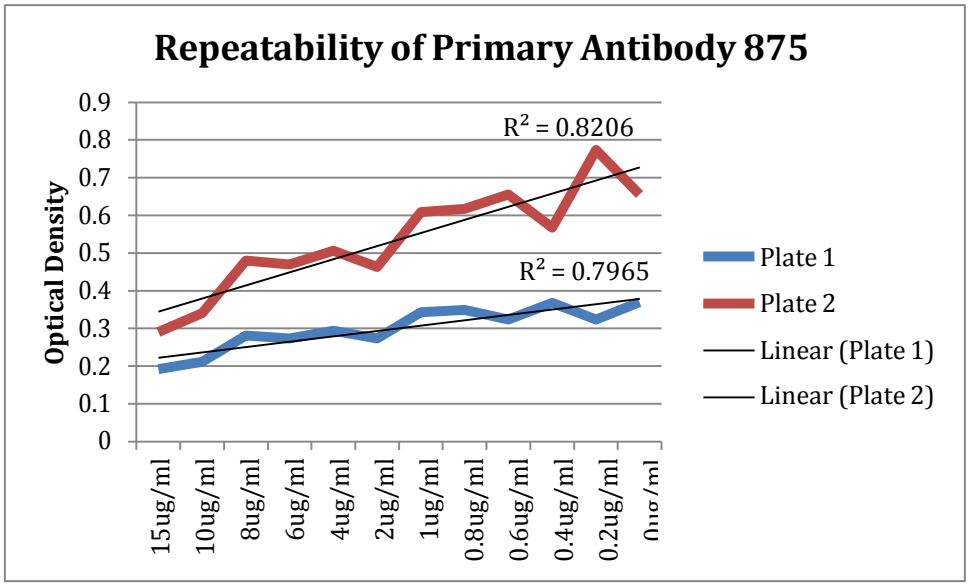

Figure B.5b: Repeatability of ELISA protocol utilizing polyclonal rabbit anti-equine antibody 875

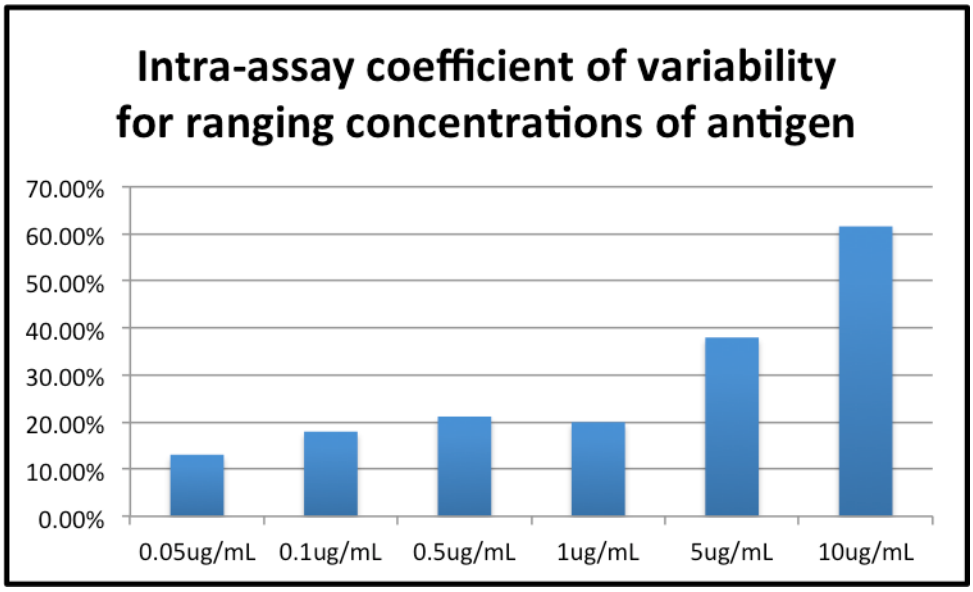

Figure B.5c. Intra-assay CV's for varying concentrations of antigen added. This was analyzed in replicates of 16.

Secondary Antibody, Conjugated Substrate, and Detection System

Both goat anti-rabbit IgG and rat anti-rabbit IgG conjugated to alkaline phosphatase were assessed as capture antibodies with minimal variability between the two. The secondary antibody was applied at the manufacturers recommend dilution of 1:1,000 in BSA (1%, w/v) in 0.2 M Tris buffer solution. P-Nitrophenyl-phosphate (pNPP) tablets (3 mM *p*-nitrophenyl phosphate (NPP) + 0.05 M Na2CO3 + 0.5 m M M gC l2; Sigma Aldrich) were diluted in P-NPP buffer at a concentration of 1mg/mL and were used as the substrate diluted. In this detection system, pNPP is hydrolyzed to p-nitrophenol by the AP, and transforms to a yellowish hue. ELISA plates were read using a 405nm filter to detect pNPP.

B.3: Conclusions

In conclusion, we were unsuccessful in constructing an indirect competitive ELISA for the detection of equine CRISP-3 in seminal plasma. Great effort was applied to this platform, with unacceptably low repeatability and high levels of both intra- and inter-assay variability detected. The initial application of the protocol as described within the thesis of Connor produced no colorimetric response, possibly caused by the use of phosphate buffered saline (PBS) as the diluent. Alkaline phosphatase acts by cleaving the phosphate groups through dephosphorylation, and would therefore disrupt the molecular structure of PBS. Because of this, further steps to troubleshoot the development of this platform were performed with Tris buffered saline (TBS) as the diluent.

The use of TBS as the diluent was still applied to the initial antibody produced to detect equine CRISP-3. This monoclonal mouse anti-equine antibody was raised as described by Doty *et al.,* and was determined effective at detecting equine CRISP-3 in a western blot [11]. Unfortunately, when we applied this antibody to an ELISA platform, a high level of inter-assay variability was detected. A second antibody was produced, a polyclonal rabbit-anti equine antibody which also was validated via western blot. It should be noted that when the monoclonal antibody was analyzed on western blot, two bands were detected at roughly 26 and 28 kDa in size. When the polyclonal antibody was evaluated, a single band was noted, at approximately 28kDa. Previous work by Doty *et al*. on the monoclonal antibody stated that the dual bands were present due to glycosylation of the native protein, which was also noted in the human CRISP-3 protein [11, 252]. In contrast, the single band detected when the polyclonal antibody was utilized correlates with previous literature. A study by Schambony *et al.* (1998) sequenced purified equine CRISP-3 from

seminal plasma and determined it to not have an N-linked glycosylation site [105]. The contrast in band structure was not determined and is therefore, unknown. Monoclonal antibodies detect a specific region on the protein of interest and may cross react with any other protein that shares a similar epitope, therefore offering lower sensitivity but with heightened specificity. The polyclonal antibody will allow for the entire protein to be detected, as numerous immunoglobulins are raised from varying regions of the protein. Although this allows for greater sensitivity, it also increases the chance of cross reactivity, as numerous epitopes can cross react with similar proteins.

Some of the variability may lie within the purification of the equine CRISP-3. Equine CRISP-3 shares considerable homology to both human and murine CRISP-3 (78% and 82%), and recent literature points to the difficulties with purifying this protein in other species. Equine CRISP-3 shares similar structure with other CRISP-3's in that the mature protein contains 16 cysteine residues, with 14 cysteines clustered in the carboxy-terminal, exhibiting similar spacing characteristics to that found in the rat, human, and mouse [105]. As Anklesaria et al. (2016) reported, these 16 cysteines lead to a considerable amount of disulfide linkages, the purification of which can lead to protein misfolding and the formation of protein aggregates [253]. In addition, Magdaleno et al (2005) reported that the protein was folded into a compact structure, making access to various epitopes difficult [115]. This protein misfolding and structure may contribute to the considerable variability noted in both the production of a standard curve in addition to the monoclonal and polyclonal antibodies, all of which utilize the purified equine CRISP-3. It may also contribute to the ability of either antibody to detect equine CRISP-3 through the utilization of a western blot, which detects denatured protein and not whole. To minimize the

variability caused by this purification, it would perchance be beneficial to develop a recombinant equine CRISP-3, or at least validate the purity of the purified protein.

In addition, the platform used may have affected the variability within the results. The development of a conjugated secondary antibody for equine CRISP-3 for use in a direct sandwich ELISA could possibly help minimize the variability seen in the indirect competitive platform. In addition, an unconjugated secondary antibody may have assisted in developing an indirect sandwich platform. The addition of a second antibody specific for the antigen of interest, in this case, equine CRISP-3, could potentially minimize variability, as numerous epitopes are being detected instead of the singular epitope that was detected with the monoclonal antibody. The heightened concentration found on average of this protein in the ejaculate may play a role in the lack of sensitivity of the platform investigated, and the additional antibody may help increase the sensitivity of detection.

In conclusion, we were unable to develop or validate an ELISA for the detection of CRISP-3 in the equine ejaculate. While a competitive indirect ELISA was assessed with both monoclonal and polyclonal antibodies added, unacceptable variability was seen throughout the process, indicating the lack of repeatability or use for research or commercial settings. It is believed that consideration should be applied towards obtaining a protein of higher purity or even a recombinant protein for the development of a more specific antibody, as well as the development of a secondary antibody for the development of a sandwich ELISA. Neither of those objectives was performed within this dissertation.

References:

1. Troedsson, M.H., et al., *The role of seminal plasma in post-breeding uterine inflammation.* J Reprod Fertil Suppl, 2000(56): p. 341-9.
2. Amann, R.P., M.J. Cristanelli, and E.L. Squires, *Proteins in stallion seminal plasma.* J Reprod Fertil Suppl, 1987. **35**: p. 113-20.
3. Katila, T., M. Celebi, and E. Koskinen, *Effect of timing of frozen semen insemination on pregnancy rate in mares.* Acta Vet Scand, 1996. **37**(3): p. 361-5.
4. Troedsson, M.H.T., *Uterine clearance and resistance to persistent endometritis in the mare.* Theriogenology, 1999. **52**(3): p. 461-71.
5. Katila, T., *Onset and duration of uterine inflammation of mares with fresh semen.* Biol Reprod Mono, 1995. **1**: p. 515-517.
6. Zent, W., M. Troedsson, and J. Xue. *Postbreeding uterine fluid accumulation in a normal population of Thoroughbred mares: a field study.* in *Proc of the Annul Convention of the AAEP*. 1998.
7. Traub-Dargatz, J.L., M.D. Salman, and J.L. Voss, *Medical problems of adult horses, as ranked by equine practitioners.* J Am Vet Med Assoc, 1991. **198**(10): p. 1745-7.
8. Battut, I., et al. *When do equine embryos enter the uterine cavity? An attempt to answer*. in *Havemeyer Foundation Monograph Series No. 3: Proceedings of the 5th International Symposium on Equine Embryo Transfer*. 2001. Newmarket: R&W Publications.
9. Woodward, E.M., et al., *Endometrial inflammatory markers of the early immune response in mares susceptible or resistant to persistent breeding-induced endometritis.* Reproduction, 2013. **145**(3): p. 289-96.
10. Troedsson, M.H., et al., *Components in seminal plasma regulating sperm transport and elimination.* Anim Reprod Sci, 2005. **89**(1-4): p. 171-86.
11. Doty, A., et al., *Equine CRISP3 modulates interaction between spermatozoa and polymorphonuclear neutrophils.* Biol Reprod, 2011. **85**(1): p. 157-64.
12. Troedsson, M.H., I.K. Liu, and B.G. Crabo, *Sperm transport and survival in the mare: a review.* Theriogenology, 1998. **50**(5): p. 807-18.
13. Troedsson, M.H., I.K. Liu, and M. Thurmond, *Immunoglobulin (IgG and IgA) and complement (C3) concentrations in uterine secretion following an intrauterine challenge of Streptococcus zooepidemicus in mares susceptible to versus resistant to chronic uterine infection.* Biol Reprod, 1993. **49**(3): p. 502-6.
14. Hemberg, E., N. Lundeheim, and S. Einarsson, *Retrospective study on vulvar conformation in relation to endometrial cytology and fertility in thoroughbred mares.* J Vet Med A Physiol Pathol Clin Med, 2005. **52**(9): p. 474-7.
15. Ricketts, S.W. and S. Alonso, *The effect of age and parity on the development of equine chronic endometrial disease.* Equine Vet J, 1991. **23**(3): p. 189-92.
16. Reilas, T., et al., *Relationship between embryo recovery rate and uterine lavage fluid composition in postpartum mares.* Reprod Nutr Dev, 2000. **40**(4): p. 383-91.
17. Adams, G.P., et al., *Effect of uterine inflammation and ultrasonically-detected uterine pathology on fertility in the mare.* J Reprod Fertil Suppl, 1987. **35**: p. 445-54.
18. Hill, J. and R. Gilbert, *Reduced quality of bovine embryos cultured in media conditioned by exposure to an inflamed endometrium.* Aust Vet J, 2008. **86**(8): p. 312-6.
19. Kotilainen, T., M. Huhtinen, and T. Katila, *Sperm-induced leukocytosis in the equine uterus.* Theriogenology, 1994. **41**(3): p. 629-36.
20. Troedsson, M.H.T., et al., *Mechanism of Sperm-Induced Endometritis in the Mare.* Biol Reprod, 1995. **52**: p. 133-133.

21. Christoffersen, M., et al., *Inflammatory responses to induced infectious endometritis in mares resistant or susceptible to persistent endometritis.* BMC Vet Res, 2012. **8**: p. 41.
22. Caslick, E.A., *The vulva and vulvo-vaginal orifice and its relation to genital tracts of the thoroughbred mare.* Cornell Vet, 1937. **27**: p. 178-187.
23. Delahanty, D.D., *Surgical correction of contributory causes of uterine disease in the mare.* J Am Vet Med Assoc, 1968. **153**(12): p. 1563-6.
24. Head, J.R. and R.E. Billingham, *Concerning the immunology of the uterus.* Am J Reprod Immunol Microbiol, 1986. **10**(3): p. 76-81.
25. Ginther, O.J., *Reproductive Biology of the Mare: Basic and Applied Aspects*. Vol. 1. 1979, Dallas, TX: O.J. Ginther.
26. Singh, J., et al., *The immune status of the bovine uterus during the peripartum period.* Vet J, 2008. **175**(3): p. 301-9.
27. Kutteh, W.H., et al., *Secretory immune system of the female reproductive tract. II. Local immune system in normal and infected fallopian tube.* Fertil Steril, 1990. **54**(1): p. 51-5.
28. Akira, S. and K. Takeda, *Functions of toll-like receptors: lessons from KO mice.* C R Biol, 2004. **327**(6): p. 581-9.
29. Yang, R.B., et al., *Signaling events induced by lipopolysaccharide-activated toll-like receptor 2.* J Immunol, 1999. **163**(2): p. 639-43.
30. Robertson, S.A., *Seminal fluid signaling in the female reproductive tract: lessons from rodents and pigs.* J Anim Sci, 2007. **85**(13 Suppl): p. E36-44.
31. Ganz, T. and R.I. Lehrer, *Antibiotic peptides from higher eukaryotes: biology and applications.* Mol Med Today, 1999. **5**(7): p. 292-7.
32. Kayisli, U.A., N.G. Mahutte, and A. Arici, *Uterine chemokines in reproductive physiology and pathology.* Am J Reprod Immunol, 2002. **47**(4): p. 213-21.
33. Quayle, A.J., *The innate and early immune response to pathogen challenge in the female genital tract and the pivotal role of epithelial cells.* J Reprod Immunol, 2002. **57**(1-2): p. 61-79.
34. Sullivan, D.A. and C.R. Wira, *Hormonal regulation of immunoglobulins in the rat uterus: uterine response to multiple estradiol treatments.* Endocrinology, 1984. **114**(2): p. 650-8.
35. Pioli, P.A., et al., *Lipopolysaccharide-induced IL-1 beta production by human uterine macrophages up-regulates uterine epithelial cell expression of human beta-defensin 2.* J Immunol, 2006. **176**(11): p. 6647-55.
36. Li, W., et al., *Lactoferrin suppresses lipopolysaccharide-induced endometritis in mice via down-regulation of the NF-kappaB pathway.* Int Immunopharmacol, 2015. **28**(1): p. 695-9.
37. Robertson, S.A., G. Mayrhofer, and R.F. Seamark, *Ovarian steroid hormones regulate granulocyte-macrophage colony-stimulating factor synthesis by uterine epithelial cells in the mouse.* Biol Reprod, 1996. **54**(1): p. 183-96.
38. Kaushic, C., et al., *Infection of polarized primary epithelial cells from rat uterus with Chlamydia trachomatis: cell-cell interaction and cytokine secretion.* Am J Reprod Immunol, 2000. **44**(2): p. 73-9.
39. Robertson, S.A., *Control of the immunological environment of the uterus.* Rev Reprod, 2000. **5**(3): p. 164-74.
40. Robertson, S.A., G. Mayrhofer, and R.F. Seamark, *Uterine epithelial cells synthesize granulocyte-macrophage colony-stimulating factor and interleukin-6 in pregnant and nonpregnant mice.* Biol Reprod, 1992. **46**(6): p. 1069-79.

41. Jacobs, A.L., et al., *Secretion and hormonal regulation of interleukin-6 production by mouse uterine stromal and polarized epithelial cells cultured in vitro.* Endocrinology, 1992. **131**(3): p. 1037-46.
42. Wira, C.R., J.E. Stern, and E. Colby, *Estradiol regulation of secretory component in the uterus of the rat: evidence for involvement of RNA synthesis.* J Immunol, 1984. **133**(5): p. 2624-8.
43. Tizard, I. and Y. Ni, *Use of serologic testing to assess immune status of companion animals.* J Am Vet Med Assoc, 1998. **213**(1): p. 54-60.
44. Kalliolias, G.D. and L.B. Ivashkiv, *TNF biology, pathogenic mechanisms and emerging therapeutic strategies.* Nat Rev Rheumatol, 2016. **12**(1): p. 49-62.
45. Jacobs, A.L. and D.D. Carson, *Uterine epithelial cell secretion of interleukin-1 alpha induces prostaglandin E2 (PGE2) and PGF2 alpha secretion by uterine stromal cells in vitro.* Endocrinology, 1993. **132**(1): p. 300-8.
46. Kaplanski, G., et al., *IL-6: a regulator of the transition from neutrophil to monocyte recruitment during inflammation.* Trends Immunol, 2003. **24**(1): p. 25-9.
47. Frank, T., et al., *Phagocytosis in the uterus: A review.* Theriogenology, 1983. **20**(1): p. 103-10.
48. Killingbeck, J. and G.E. Lamming, *Influence of uterine secretions on phagocytosis.* Nature, 1963. **198**: p. 111-2.
49. Troedsson, M.H., *Uterine clearance and resistance to persistent endometritis in the mare.* Theriogenology, 1999. **52**(3): p. 461-71.
50. Alghamdi, A.S. and D.N. Foster, *Seminal DNase frees spermatozoa entangled in neutrophil extracellular traps.* Biol Reprod, 2005. **73**(6): p. 1174-81.
51. Alghamdi, A.S., et al., *Equine sperm-neutrophil binding.* Biol Reprod, 2015. **92**(4): p. 94.
52. Troedsson, M.H.T., et al., *Equine seminal plasma derived lactoferrin regulates binding of polymorphonuclear neutrophils (PMNs) to spermatozoa.* JEVS, 2014. **34**(1).
53. Romano, M., et al., *Role of IL-6 and its soluble receptor in induction of chemokines and leukocyte recruitment.* Immunity, 1997. **6**(3): p. 315-25.
54. Tizard, I.R., *Macrophage-cytophilic antibodies and the functions of macrophage-bound immunoglobulins.* Bacteriol Rev, 1971. **35**(4): p. 365-78.
55. Gangnuss, S., et al., *Seminal plasma regulates corpora lutea macrophage populations during early pregnancy in mice.* Biol Reprod, 2004. **71**(4): p. 1135-41.
56. Shemesh, M., et al., *Functional importance of bovine myometrial and vascular LH receptors and cervical FSH receptors.* Semin Reprod Med, 2001. **19**(1): p. 87-96.
57. Szostek, A.Z., et al., *mRNA transcription of prostaglandin synthases and their products in the equine endometrium in the course of fibrosis.* Theriogenology, 2012. **78**(4): p. 768-76.
58. Szostek, A.Z., et al., *Impairment of the interleukin system in equine endometrium during the course of endometrosis.* Biol Reprod, 2013. **89**(4): p. 79.
59. Gupta, K.G. and A.K. Garg, *Presence of antisperm antibodies in fertile and infertile persons.* Acta Obstet Gynecol Scand, 1975. **54**(5): p. 407-10.
60. Segerson, E.C. and P.K. Beetham, *Immunosuppressive macromolecules of endometrial and conceptus origins in livestock species.* J Reprod Immunol, 2000. **48**(1): p. 27-46.
61. Kaeoket, K., E. Persson, and A.M. Dalin, *Influence of pre-ovulatory insemination and early pregnancy on the distribution of CD2, CD4, CD8 and MHC class II expressing cells in the sow endometrium.* Anim Reprod Sci, 2003. **76**(3-4): p. 231-44.
62. Kaeoket, K., E. Persson, and A.M. Dalin, *Influence of post-ovulatory insemination on sperm distribution, pregnancy and the infiltration by cells of the immune system, and*

the distribution of CD2, CD4, CD8 and MHC class II expressing cells in the sow endometrium. J Vet Med A Physiol Pathol Clin Med, 2003. **50**(4): p. 169-78.
63. Tunon, A.M., et al., *T-cell distribution in two different segments of the equine endometrium 6 and 48 hours after insemination.* Theriogenology, 2000. **54**(6): p. 835-41.
64. Chamley, L.W. and G.N. Clarke, *Antisperm antibodies and conception.* Semin Immunopathol, 2007. **29**(2): p. 169-84.
65. Alexander, N.J., *Vasectomy and vasovasostomy in rhesus monkeys: the effect of circulating antisperm antibodies on fertility.* Fertil Steril, 1977. **28**(5): p. 562-9.
66. Baskin, M.J., *Temporary sterilization by injection of human spermatozoa: a preliminary report.* Am J Obstet Gynecol, 1932. **24**: p. 892–897.
67. Stavnezer, J., *Regulation of antibody production and class switching by TGF-beta.* J Immunol, 1995. **155**(4): p. 1647-51.
68. Zhang, J., S.W. Ricketts, and S.J. Tanner, *Antisperm antibodies in the semen of a stallion following testicular trauma.* Equine Vet J, 1990. **22**(2): p. 138-41.
69. Kenney, R.M., et al., *Possible role of autoimmunity to spermatozoa in idiopathic infertility of stallions.* J Reprod Fertil Suppl, 2000(56): p. 23-30.
70. Topfer-Petersen, E., et al., *The role of stallion seminal proteins in fertilisation.* Anim Reprod Sci, 2005. **89**(1-4): p. 159-70.
71. Claus, R., et al., *Replenishment of AI-doses with oestrogens in physiological amounts: effect on sow prolificacy in a field trial.* Zentralbl Veterinarmed A, 1989. **36**(10): p. 797-800.
72. Bollwein, H., C. Sowade, and R. Stolla, *The effect of semen extender, seminal plasma and raw semen on uterine and ovarian blood flow in mares.* Theriogenology, 2003. **60**(4): p. 607-16.
73. Weitze, K.F., et al., *Interaction between Inseminate, Uterine and Ovarial Function in the Sow: I. Influence of seminal plasma and oestrogens in the inseminate on intragenital sperm transport, time of ovulation and fertility results in gilts.* Reproduction in Domestic Animals, 1990. **25**(4): p. 191-196.
74. Alghamdi, A.S., D.N. Foster, and M.H. Troedsson, *Equine seminal plasma reduces sperm binding to polymorphonuclear neutrophils (PMNs) and improves the fertility of fresh semen inseminated into inflamed uteri.* Reproduction, 2004. **127**(5): p. 593-600.
75. Robertson, S.A., et al., *Cytokine-leukocyte networks and the establishment of pregnancy.* Am J Reprod Immunol, 1997. **37**(6): p. 438-42.
76. Robertson, S.A., et al., *Granulocyte-macrophage colony-stimulating factor promotes glucose transport and blastomere viability in murine preimplantation embryos.* Biol Reprod, 2001. **64**(4): p. 1206-15.
77. O'Leary, S., et al., *Seminal plasma regulates endometrial cytokine expression, leukocyte recruitment and embryo development in the pig.* Reproduction, 2004. **128**(2): p. 237-47.
78. Palm, F., et al., *Influence of different semen extenders and seminal plasma on PMN migration and on expression of IL-1beta, IL-6, TNF-alpha and COX-2 mRNA in the equine endometrium.* Theriogenology, 2008. **70**(5): p. 843-51.
79. Kareskoski, M. and T. Katila, *Components of stallion seminal plasma and the effects of seminal plasma on sperm longevity.* Anim Reprod Sci, 2008. **107**(3-4): p. 249-56.
80. Frazer, G.S. and D.M. Bucci, *SDS-PAGE characterization of the proteins in equine seminal plasma.* Theriogenology, 1996. **46**(4): p. 579-91.

81. Brandon, C.I., et al., *Two-dimensional polyacrylamide gel electrophoresis of equine seminal plasma proteins and their correlation with fertility.* Theriogenology, 1999. **52**(5): p. 863-73.
82. Calvete, J.J., et al., *Amino acid sequence of HSP-1, a major protein of stallion seminal plasma: effect of glycosylation on its heparin- and gelatin-binding capabilities.* Biochem J, 1995. **310 (Pt 2)**: p. 615-22.
83. Ekhlasi-Hundrieser, M., et al., *Structural and molecular characterization of equine sperm-binding fibronectin-II module proteins.* Mol Reprod Dev, 2005. **70**(1): p. 45-57.
84. Manjunath, P. and M.R. Sairam, *Purification and biochemical characterization of three major acidic proteins (BSP-A1, BSP-A2 and BSP-A3) from bovine seminal plasma.* Biochem J, 1987. **241**(3): p. 685-92.
85. Villemure, M., C. Lazure, and P. Manjunath, *Isolation and characterization of gelatin-binding proteins from goat seminal plasma.* Reprod Biol Endocrinol, 2003. **1**: p. 39.
86. Boisvert, M., et al., *Isolation and characterization of gelatin-binding bison seminal vesicle secretory proteins.* Biol Reprod, 2004. **70**(3): p. 656-61.
87. Calvete, J.J., et al., *Characterisation of the conformational and quaternary structure-dependent heparin-binding region of bovine seminal plasma protein PDC-109.* FEBS Lett, 1999. **444**(2-3): p. 260-4.
88. Calvete, J.J., et al., *Isolation and characterization of heparin- and phosphorylcholine-binding proteins of boar and stallion seminal plasma. Primary structure of porcine pB1.* FEBS Lett, 1997. **407**(2): p. 201-6.
89. Therien, I., G. Bleau, and P. Manjunath, *Phosphatidylcholine-binding proteins of bovine seminal plasma modulate capacitation of spermatozoa by heparin.* Biol Reprod, 1995. **52**(6): p. 1372-9.
90. Gwathmey, T.M., G.G. Ignotz, and S.S. Suarez, *PDC-109 (BSP-A1/A2) promotes bull sperm binding to oviductal epithelium in vitro and may be involved in forming the oviductal sperm reservoir.* Biol Reprod, 2003. **69**(3): p. 809-15.
91. Jois, P.S., et al., *Functional characterization of the domains of the bovine binder of SPerm 5 (BSP5) protein.* Reprod Biol Endocrinol, 2015. **13**: p. 64.
92. Kumar, C.S. and M.J. Swamy, *HSP-1/2, a major horse seminal plasma protein, acts as a chaperone against oxidative stress.* Biochem Biophys Res Commun, 2016. **473**(4): p. 1058-63.
93. Kumar, C.S., et al., *Fluorescence investigations on choline phospholipid binding and chemical unfolding of HSP-1/2, a major protein of horse seminal plasma.* J Photochem Photobiol B, 2016. **158**: p. 89-98.
94. Kumar, C.S. and M.J. Swamy, *Differential modulation of the chaperone-like activity of HSP-1/2, a major protein of horse seminal plasma by anionic and cationic surfactants.* Int J Biol Macromol, 2017. **96**: p. 524-531.
95. Kumar, C.S. and M.J. Swamy, *A pH Switch Regulates the Inverse Relationship between Membranolytic and Chaperone-like Activities of HSP-1/2, a Major Protein of Horse Seminal Plasma.* Biochemistry, 2016. **55**(26): p. 3650-7.
96. Ekhlasi-Hundrieser, M., et al., *Expression of spermadhesin genes in porcine male and female reproductive tracts.* Mol Reprod Dev, 2002. **61**(1): p. 32-41.
97. Sinowatz, F., et al., *Immunohistochemical localization of spermadhesin AWN in the porcine male genital tract.* Cell Tissue Res, 1995. **282**(1): p. 175-9.
98. Dostalova, Z., et al., *Boar spermadhesin AWN-1. Oligosaccharide and zona pellucida binding characteristics.* Eur J Biochem, 1995. **230**(1): p. 329-36.
99. Dostalova, Z., et al., *Immunolocalization and quantitation of acidic seminal fluid protein (aSFP) in ejaculated, swim-up, and capacitated bull spermatozoa.* Biol Chem Hoppe Seyler, 1994. **375**(7): p. 457-61.

100. Reinert, M., et al., *Primary structure of stallion seminal plasma protein HSP-7, a zona-pellucida-binding protein of the spermadhesin family.* Eur J Biochem, 1996. **242**(3): p. 636-40.
101. Hoshiba, H. and F. Sinowatz, *Immunohistochemical localization of the spermadhesin AWN-1 in the equine male genital tract.* Anat Histol Embryol, 1998. **27**(5): p. 351-3.
102. Gibbs, G.M., K. Roelants, and M.K. O'Bryan, *The CAP superfamily: cysteine-rich secretory proteins, antigen 5, and pathogenesis-related 1 proteins--roles in reproduction, cancer, and immune defense.* Endocr Rev, 2008. **29**(7): p. 865-97.
103. Mochca-Morales, J., B.M. Martin, and L.D. Possani, *Isolation and characterization of helothermine, a novel toxin from Heloderma horridum horridum (Mexican beaded lizard) venom.* Toxicon, 1990. **28**(3): p. 299-309.
104. Giese, A., et al., *Molecular characterization of the equine testis-specific protein 1 (TPX1) and acidic epididymal glycoprotein 2 (AEG2) genes encoding members of the cysteine-rich secretory protein (CRISP) family.* Gene, 2002. **299**(1-2): p. 101-9.
105. Schambony, A., et al., *Equine CRISP-3: primary structure and expression in the male genital tract.* Biochim Biophys Acta, 1998. **1387**(1-2): p. 206-16.
106. Roberts, K.P., et al., *Association of the protein D and protein E forms of rat CRISP1 with epididymal sperm.* Biol Reprod, 2008. **79**(6): p. 1046-53.
107. Rochwerger, L., D.J. Cohen, and P.S. Cuasnicu, *Mammalian sperm-egg fusion: the rat egg has complementary sites for a sperm protein that mediates gamete fusion.* Dev Biol, 1992. **153**(1): p. 83-90.
108. Cohen, D.J., et al., *Evidence that human epididymal protein ARP plays a role in gamete fusion through complementary sites on the surface of the human egg.* Biol Reprod, 2001. **65**(4): p. 1000-5.
109. Cohen, D.J., D.A. Ellerman, and P.S. Cuasnicu, *Mammalian sperm-egg fusion: evidence that epididymal protein DE plays a role in mouse gamete fusion.* Biol Reprod, 2000. **63**(2): p. 462-8.
110. Cohen, D.J., et al., *Relationship between the association of rat epididymal protein "DE" with spermatozoa and the behavior and function of the protein.* Mol Reprod Dev, 2000. **56**(2): p. 180-8.
111. Busso, D., et al., *Evidence for the involvement of testicular protein CRISP2 in mouse sperm-egg fusion.* Biol Reprod, 2007. **76**(4): p. 701-8.
112. Udby, L., et al., *An ELISA for SGP28/CRISP-3, a cysteine-rich secretory protein in human neutrophils, plasma, and exocrine secretions.* J Immunol Methods, 2002. **263**(1-2): p. 43-55.
113. Koppers, A.J., T. Reddy, and M.K. O'Bryan, *The role of cysteine-rich secretory proteins in male fertility.* Asian J Androl, 2011. **13**(1): p. 111-7.
114. Kratzschmar, J., et al., *The human cysteine-rich secretory protein (CRISP) family. Primary structure and tissue distribution of CRISP-1, CRISP-2 and CRISP-3.* Eur J Biochem, 1996. **236**(3): p. 827-36.
115. Magdaleno, L., et al., *Biochemical and conformational characterisation of HSP-3, a stallion seminal plasma protein of the cysteine-rich secretory protein (CRISP) family.* FEBS Lett, 1997. **420**(2-3): p. 179-85.
116. Brooks, D.E., et al., *Molecular cloning of the cDNA for androgen-dependent sperm-coating glycoproteins secreted by the rat epididymis.* Eur J Biochem, 1986. **161**(1): p. 13-8.
117. Eberspaecher, U., et al., *Mouse androgen-dependent epididymal glycoprotein CRISP-1 (DE/AEG): isolation, biochemical characterization, and expression in recombinant form.* Mol Reprod Dev, 1995. **42**(2): p. 157-72.

118. Kjeldsen, L., et al., *SGP28, a novel matrix glycoprotein in specific granules of human neutrophils with similarity to a human testis-specific gene product and a rodent sperm-coating glycoprotein.* FEBS Lett, 1996. **380**(3): p. 246-50.
119. Brooks, D.E., *The major androgen-regulated secretory proteins of the rat epididymis bear sequence homology with members of the alpha 2u-globulin superfamily.* Biochem Int, 1987. **14**(2): p. 235-40.
120. Hamann, H., et al., *A polymorphism within the equine CRISP3 gene is associated with stallion fertility in Hanoverian warmblood horses.* Anim Genet, 2007. **38**(3): p. 259-64.
121. Novak, S., et al., *Biomarkers of in vivo fertility in sperm and seminal plasma of fertile stallions.* Theriogenology, 2010. **74**(6): p. 956-67.
122. Jobim, M.I., et al., *Two-dimensional polyacrylamide gel electrophoresis of equine seminal plasma proteins and their relation with semen freezability.* Theriogenology, 2011. **76**(4): p. 765-71.
123. Troedsson, M.E.-V., A; Scoggin, KE; Woodward, EM; Squires, EL; Ball, BA; Maxwell,H *Equine seminal plasma derived lactoferrin regulates binding of polymorphonuclear neutrophils (PMNs) to spermatozoa.* Journal of Equine Veterinary Science, 2013.
124. Alghamdi, A., Scoggin, K., Woodward, E., Davolli, G., Fedorka, C., Claes, A., Troedsson, MH., *Localization of lactoferrin binding sites on equine spermatozoa.* ISER Proceedings, 2014. **11**(156).
125. Inagaki, M., et al., *Purification and quantification of lactoferrin in equine seminal plasma.* J Vet Med Sci, 2002. **64**(1): p. 75-7.
126. Jin, Y.Z., et al., *Direct evidence for the secretion of lactoferrin and its binding to sperm in the porcine epididymis.* Mol Reprod Dev, 1997. **47**(4): p. 490-6.
127. Metz-Boutigue, M.H., et al., *Human lactotransferrin: amino acid sequence and structural comparisons with other transferrins.* Eur J Biochem, 1984. **145**(3): p. 659-76.
128. Rado, T.A., et al., *Lactoferrin biosynthesis during granulocytopoiesis.* Blood, 1984. **64**(5): p. 1103-9.
129. Arnold, R.R., M. Brewer, and J.J. Gauthier, *Bactericidal activity of human lactoferrin: sensitivity of a variety of microorganisms.* Infect Immun, 1980. **28**(3): p. 893-8.
130. Bezault, J., et al., *Human lactoferrin inhibits growth of solid tumors and development of experimental metastases in mice.* Cancer Res, 1994. **54**(9): p. 2310-2.
131. Wakabayashi, H., et al., *Lactoferrin given in food facilitates dermatophytosis cure in guinea pig models.* J Antimicrob Chemother, 2000. **46**(4): p. 595-602.
132. Roseanu, A., et al., *Inhibition of binding of lactoferrin to the human promonocyte cell line THP-1 by heparin: the role of cell surface sulphated molecules.* Biochim Biophys Acta, 2000. **1475**(1): p. 35-8.
133. Slater, K. and J. Fletcher, *Lactoferrin derived from neutrophils inhibits the mixed lymphocyte reaction.* Blood, 1987. **69**(5): p. 1328-33.
134. Crouch, S.P., K.J. Slater, and J. Fletcher, *Regulation of cytokine release from mononuclear cells by the iron-binding protein lactoferrin.* Blood, 1992. **80**(1): p. 235-40.
135. Machnicki, M., M. Zimecki, and T. Zagulski, *Lactoferrin regulates the release of tumour necrosis factor alpha and interleukin 6 in vivo.* Int J Exp Pathol, 1993. **74**(5): p. 433-9.
136. Haversen, L., et al., *Lactoferrin down-regulates the LPS-induced cytokine production in monocytic cells via NF-kappa B.* Cell Immunol, 2002. **220**(2): p. 83-95.

137. Damiens, E., et al., *Effects of human lactoferrin on NK cell cytotoxicity against haematopoietic and epithelial tumour cells.* Biochim Biophys Acta, 1998. **1402**(3): p. 277-87.
138. Dashper, S.G., et al., *Lactoferrin inhibits Porphyromonas gingivalis proteinases and has sustained biofilm inhibitory activity.* Antimicrob Agents Chemother, 2012. **56**(3): p. 1548-56.
139. Kanwar, J.R., et al., *Multifunctional iron bound lactoferrin and nanomedicinal approaches to enhance its bioactive functions.* Molecules, 2015. **20**(6): p. 9703-31.
140. Wang, Z., et al., *Characterization of an eppin protein complex from human semen and spermatozoa.* Biol Reprod, 2007. **77**(3): p. 476-84.
141. Buckett, W.M., et al., *Seminal plasma lactoferrin concentrations in normal and abnormal semen samples.* J Androl, 1997. **18**(3): p. 302-4.
142. Zalata, A., et al., *The possible meaning of transferrin and its soluble receptors in seminal plasma as markers of the seminiferous epithelium.* Hum Reprod, 1996. **11**(4): p. 761-4.
143. Ber, A., et al., *Transferrin in seminal plasma and in serum of men: its correlation with sperm quality and hormonal status.* Hum Reprod, 1990. **5**(3): p. 294-7.
144. Kolm, G., et al., *Lactoferrin expression in the horse endometrium: relevance in persisting mating-induced endometritis.* Vet Immunol Immunopathol, 2006. **114**(1-2): p. 159-67.
145. Pearl, C.A. and J.F. Roser, *Lactoferrin expression and secretion in the stallion epididymis.* Reprod Biol, 2014. **14**(2): p. 148-54.
146. Fraser, L.R. and O.O. Osiguwa, *Human sperm responses to calcitonin, angiotensin II and fertilization-promoting peptide in prepared semen samples from normal donors and infertility patients.* Hum Reprod, 2004. **19**(3): p. 596-606.
147. Jonsson, M., et al., *Semenogelins I and II bind zinc and regulate the activity of prostate-specific antigen.* Biochem J, 2005. **387**(Pt 2): p. 447-53.
148. Miller, L.M., et al., *Distribution pattern(s) of sperm protein at 22 kDa (SP22) on fresh, cooled and frozen/thawed equine spermatozoa and expression of SP22 in tissues from the testes and epididymides of normal stallions.* Reprod Domest Anim, 2015. **50**(2): p. 275-82.
149. Kaydos, E.H., et al., *Haloacid induced alterations in fertility and the sperm biomarker SP22 in the rat are additive: validation of an ELISA.* Toxicol Sci, 2004. **81**(2): p. 430-42.
150. Parlevliet, J.M., et al., *Immunolocalization of estrogen and androgen receptors and steroid concentrations in the stallion epididymis.* Theriogenology, 2006. **66**(4): p. 755-65.
151. Roser, J.F., *Endocrine basis for testicular function in the stallion.* Theriogenology, 1997. **48**(5): p. 883-92.
152. Stewart, B.L. and J.F. Roser, *Effects of age, season, and fertility status on plasma and intratesticular immunoreactive (IR) inhibin concentrations in stallions.* Domest Anim Endocrinol, 1998. **15**(2): p. 129-39.
153. McDonnell, S., *Reproductive behavior of the stallion.* Vet Clin North Am Equine Pract, 1986. **2**(3): p. 535-55.
154. McDowell, K.J., et al., *Characterisation of proteins in the seminal plasma of stallions, geldings and supplemented with testosterone.* Res Vet Sci, 1996. **61**(1): p. 33-7.
155. Milardi, D., et al., *Novel biomarkers of androgen deficiency from seminal plasma profiling using high-resolution mass spectrometry.* J Clin Endocrinol Metab, 2014. **99**(8): p. 2813-20.

156. Kelver, M.E., et al., *Estrogen regulation of lactoferrin expression in human endometrium.* Am J Reprod Immunol, 1996. **36**(5): p. 243-7.
157. Yu, L.C. and Y.H. Chen, *The developmental profile of lactoferrin in mouse epididymis.* Biochem J, 1993. **296 (Pt 1)**: p. 107-11.
158. Teng, C.T., C. Beard, and W. Gladwell, *Differential expression and estrogen response of lactoferrin gene in the female reproductive tract of mouse, rat, and hamster.* Biol Reprod, 2002. **67**(5): p. 1439-49.
159. Teng, C.T., et al., *Lactoferrin gene expression is estrogen responsive in human and rhesus monkey endometrium.* Mol Hum Reprod, 2002. **8**(1): p. 58-67.
160. Haendler, B., et al., *Transcripts for cysteine-rich secretory protein-1 (CRISP-1; DE/AEG) and the novel related CRISP-3 are expressed under androgen control in the mouse salivary gland.* Endocrinology, 1993. **133**(1): p. 192-8.
161. Haendler, B., et al., *Differential androgen regulation of the murine genes for cysteine-rich secretory proteins (CRISP).* Eur J Biochem, 1997. **250**(2): p. 440-6.
162. Vitali, C., et al., *Classification criteria for Sjogren's syndrome: a revised version of the European criteria proposed by the American-European Consensus Group.* Ann Rheum Dis, 2002. **61**(6): p. 554-8.
163. Porola, P., et al., *Androgen deficiency and defective intracrine processing of dehydroepiandrosterone in salivary glands in Sjogren's syndrome.* J Rheumatol, 2008. **35**(11): p. 2229-35.
164. Manjunath, P. and I. Therien, *Role of seminal plasma phospholipid-binding proteins in sperm membrane lipid modification that occurs during capacitation.* J Reprod Immunol, 2002. **53**(1-2): p. 109-19.
165. Fedorka, C.E., et al., *The effect of cysteine-rich secretory protein-3 (CRISP-3) and lactoferrin on endometrial cytokine expression after breeding in the horse.* JEVS, 2016. **In Press**.
166. Klein, C., J. Rutllant, and M.H. Troedsson, *Expression stability of putative reference genes in equine endometrial, testicular, and conceptus tissues.* BMC Res Notes, 2011. **4**: p. 120.
167. Livak, K.J. and T.D. Schmittgen, *Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method.* Methods, 2001. **25**(4): p. 402-8.
168. Klein, C., M. Troedsson, and J. Rutllant, *Expression of Aquaporin Water Channels in Equine Endometrium is Differentially Regulated During the Oestrous Cycle and Early Pregnancy.* Reprod Domest Anim, 2012.
169. Laine, M., et al., *Low salivary dehydroepiandrosterone and androgen-regulated cysteine-rich secretory protein 3 levels in Sjogren's syndrome.* Arthritis Rheum, 2007. **56**(8): p. 2575-84.
170. Pathak, B.R., et al., *Growth inhibition mediated by PSP94 or CRISP-3 is prostate cancer cell line specific.* Asian J Androl, 2010. **12**(5): p. 677-89.
171. Christoffersen, M., et al., *Effect of immunomodulatory therapy on the endometrial inflammatory response to induced infectious endometritis in susceptible mares.* Theriogenology, 2012. **78**(5): p. 991-1004.
172. Reghini, M.F., et al., *Inflammatory response in chronic degenerative endometritis mares treated with platelet-rich plasma.* Theriogenology, 2016.
173. Woodward, E.M., et al., *The effect of treatment with immune modulators on endometrial cytokine expression in mares susceptible to persistent breeding-induced endometritis.* Equine Vet J, 2015. **47**(2): p. 235-9.
174. Troedsson, M.H.T., et al., *The role of seminal plasma in post-breeding uterine inflammation.* J Reprod Fertil Suppl, 2000(56): p. 341-9.

175. Troedsson, M.H., et al., *Interaction between equine semen and the endometrium: the inflammatory response to semen.* Anim Reprod Sci, 2001. **68**(3-4): p. 273-8.
176. Katila, T., *Neutrophils in uterine fluid after insemination with fresh live-spermatozoa or with killed spermatozoa.* Pferdeheilkunde, 1997. **13**: p. 540.
177. Ricketts, S.W. and P.D. Rossdale, *Endometrial biopsy findings in mares with contagious equine metritis.* J Reprod Fertil Suppl, 1979(27): p. 355-9.
178. Cyktor, J.C. and J. Turner, *Interleukin-10 and immunity against prokaryotic and eukaryotic intracellular pathogens.* Infect Immun, 2011. **79**(8): p. 2964-73.
179. Troedsson, M.H., *Breeding-induced endometritis in mares.* Vet Clin North Am Equine Pract, 2006. **22**(3): p. 705-12.
180. Betteridge, K.J., M.D. Eaglesome, and P.F. Flood, *Embryo transport through the mare's oviduct depends upon cleavage and is independent of the ipsilateral corpus luteum.* J Reprod Fertil Suppl, 1979(27): p. 387-94.
181. Ricketts, S. and S. Alonso, *Assessment of the breeding prognosis of mares using paired endometrial biopsy techniques.* Equine Vet J, 1991. **23**(3): p. 185-8.
182. Troedsson, M.A., AS; Mattisen, J., *Equine seminal plasma protects the fertility of spermatozoa in an inflamed uterine environment.* Theriogenology, 2002. **58**: p. 453-456.
183. O'Leary, S., S.A. Robertson, and D.T. Armstrong, *The influence of seminal plasma on ovarian function in pigs--a novel inflammatory mechanism?* J Reprod Immunol, 2002. **57**(1-2): p. 225-38.
184. Troedsson, M.H.T., R.K. Franklin, and B.G. Crabo, *Suppression of PMN-chemotaxis by different molecular weight fractions of equine seminal plasma.* Pferdeheilkunde, 1999. **15**(6): p. 568-+.
185. Kenney, R. and P. Doig, *Equine endometrial biopsy*. 2nd ed. Current Therapy in Theriogenology: Diagnosis, Treatment, and Prevention of Reproductive Diseases in Small and Large Animals. 1986: WB Saunders.
186. Otsuki, K., et al., *Lactoferrin and interleukin-6 interaction in amniotic infection.* Adv Exp Med Biol, 1998. **443**: p. 267-71.
187. Zimecki, M., et al., *Lactoferrin increases the output of neutrophil precursors and attenuates the spontaneous production of TNF-alpha and IL-6 by peripheral blood cells.* Arch Immunol Ther Exp (Warsz), 1999. **47**(2): p. 113-8.
188. Jesus, A.A. and R. Goldbach-Mansky, *IL-1 blockade in autoinflammatory syndromes.* Annu Rev Med, 2014. **65**: p. 223-44.
189. Gerard, N., et al., *The interleukin-1 system and female reproduction.* J Endocrinol, 2004. **180**(2): p. 203-12.
190. Martoriati, A. and N. Gerard, *Interleukin-1 (IL-1) system gene expression in granulosa cells: kinetics during terminal preovulatory follicle maturation in the mare.* Reprod Biol Endocrinol, 2003. **1**: p. 42.
191. Schambony, A., et al., *Expression of CRISP proteins in the male equine genital tract.* J Reprod Fertil Suppl, 1998. **53**: p. 67-72.
192. Katila, T., *Onset and duration of uterine inflammatory response of mares after insemination with fresh semen.* Biol Reprod Mono, 1995. **1**: p. 515–517.
193. Woodward, E.M., et al., *An investigation of uterine nitric oxide production in mares susceptible and resistant to persistent breeding-induced endometritis and the effects of immunomodulation.* Reprod Domest Anim, 2013. **48**(4): p. 554-61.
194. Kikuchi, M., et al., *Relationship between seminal plasma lactoferrin and gonadal function in horses.* J Vet Med Sci, 2003. **65**(11): p. 1273-4.

195. Kolm, G., et al., *Lactoferrin, a glycoprotein with immunomodulatory and mast cell stabilising properties, in skin of horses suffering from Culicoides hypersensitivity.* Res Vet Sci, 2007. **83**(2): p. 165-70.
196. Ammons, M.C. and V. Copie, *Mini-review: Lactoferrin: a bioinspired, anti-biofilm therapeutic.* Biofouling, 2013. **29**(4): p. 443-55.
197. Bertuccini, L., et al., *Lactoferrin prevents invasion and inflammatory response following E. coli strain LF82 infection in experimental model of Crohn's disease.* Dig Liver Dis, 2014. **46**(6): p. 496-504.
198. Zhang, G., et al., *Anti-infective proteins in breast milk and asthma-associated phenotypes during early childhood.* Pediatr Allergy Immunol, 2014. **25**(6): p. 544-51.
199. Zhang, Y., C.F. Lima, and L.R. Rodrigues, *Anticancer effects of lactoferrin: underlying mechanisms and future trends in cancer therapy.* Nutr Rev, 2014. **72**(12): p. 763-73.
200. Zhang, Y., et al., *Bovine lactoferrin induces cell cycle arrest and inhibits mTOR signaling in breast cancer cells.* Nutr Cancer, 2014. **66**(8): p. 1371-85.
201. Fedorka, C.E., et al., *The effect of cysteine-rich secretory protein-3 (CRISP-3) and lactoferrin on endometrial cytokine expression after breeding in the horse.* Journal of Equine Veterinary Science, 2016. **Accepted**.
202. Kenney, R.M. and P.A. Doig, *Equine Endometrial Biopsy*, in *Current Therapy in Theriogenology: Diagnosis, Treatment, and Prevention of Reproductive Diseases in Small and Large Animals*, E.D. Marrow, Editor. 1986, WB Saunders Philadelphia, PA. p. 723-729.
203. Brook, D., *Uterine culture in mares.* Mod Vet Pract, 1984. **65**(5): p. A3-8.
204. Kruse, R., P.C. Dutta, and J.M. Morrell, *Colloid centrifugation removes seminal plasma and cholesterol from boar spermatozoa.* Reprod Fertil Dev, 2011. **23**(7): p. 858-65.
205. Morrell, J.M., et al., *Effect of heterologous and homologous seminal plasma on stallion sperm quality.* Theriogenology, 2014. **82**(1): p. 176-83.
206. Jasko, D.J., et al., *Effect of seminal plasma dilution or removal on spermatozoal motion characteristics of cooled stallion semen.* Theriogenology, 1991. **35**(6): p. 1059-1067.
207. Dinarello, C.A., *Interleukin-1.* Rev Infect Dis, 1984. **6**(1): p. 51-95.
208. Robertson, S.A., et al., *Transforming growth factor beta--a mediator of immune deviation in seminal plasma.* J Reprod Immunol, 2002. **57**(1-2): p. 109-28.
209. Robertson, S.A., *Seminal plasma and male factor signalling in the female reproductive tract.* Cell Tissue Res, 2005. **322**(1): p. 43-52.
210. Watson, E.D. and S.R. Thomson, *Lymphocyte subsets in the endometrium of genitally normal mares and mares susceptible to endometritis.* Equine Vet J, 1996. **28**(2): p. 106-10.
211. Teng, C.T., et al., *Lactotransferrin gene expression in the mouse uterus and mammary gland.* Endocrinology, 1989. **124**(2): p. 992-9.
212. Reiter, B., J.H. Brock, and E.D. Steel, *Inhibition of Escherichia coli by bovine colostrum and post-colostral milk. II. The bacteriostatic effect of lactoferrin on a serum susceptible and serum resistant strain of E. coli.* Immunology, 1975. **28**(1): p. 83-95.
213. Bellamy, W., et al., *Antibacterial spectrum of lactoferricin B, a potent bactericidal peptide derived from the N-terminal region of bovine lactoferrin.* J Appl Bacteriol, 1992. **73**(6): p. 472-9.
214. Papanicolaou, P., et al., *Increased TNF-alpha, IL-6 and decreased IL-1beta immunohistochemical expression by the stromal spindle-shaped cells in the central giant cell granuloma of the jaws.* Med Oral Patol Oral Cir Bucal, 2012. **17**(1): p. e56-62.
215. Szostek, A.Z., et al., *Interleukins affect equine endometrial cell function: modulatory action of ovarian steroids.* Mediators Inflamm, 2014. **2014**: p. 208103.

216. Loyi, T., et al., *Differential expression of pro-inflammatory cytokines in endometrial tissue of buffaloes with clinical and sub-clinical endometritis.* Res Vet Sci, 2013. **94**(2): p. 336-40.
217. Bader, H., *An investigation of sperm migration into the oviducts of the mare.* J Reprod Fertil Suppl, 1982. **32**: p. 59-64.
218. Scott, M.A., *A glimpse at sperm function in vivo: sperm transport and epithelial interaction in the female reproductive tract.* Anim Reprod Sci, 2000. **60-61**: p. 337-48.
219. Troedsson, M.H. and E.M. Woodward, *Our current understanding of the pathophysiology of equine endometritis with an emphasis on breeding-induced endometritis.* Reprod Biol, 2016. **16**(1): p. 8-12.
220. Portus, B.J., T. Reilas, and T. Katila, *Effect of seminal plasma on uterine inflammation, contractility and pregnancy rates in mares.* Equine Vet J, 2005. **37**(6): p. 515-9.
221. Zent, W., M.H. Troedsson, and J. Xue. *Postbreeding uterine fluid accumulation in a normal population of Thoroughbred mares: a field study*. in *Annul Convention of the AAEP*. 1998.
222. Ferris, R.A., et al., *In Vitro Efficacy of Nonantibiotic Treatments on Biofilm Disruption of Gram-Negative Pathogens and an In Vivo Model of Infectious Endometritis Utilizing Isolates from the Equine Uterus.* J Clin Microbiol, 2016. **54**(3): p. 631-9.
223. Fedorka, C.E., et al., *Expression and localization of cysteine-rich secretory protein-3 (CRISP-3) in the prepubertal and postpubertal male horse.* Theriogenology, 2016.
224. Luo, B., et al., *Novel lactoferrin-conjugated amphiphilic poly(aminoethyl ethylene phosphate)/poly(L-lactide) copolymer nanobubbles for tumor-targeting ultrasonic imaging.* Int J Nanomedicine, 2015. **10**: p. 5805-17.
225. Troedsson, M.H., et al., *Equine seminal plasma derived lactoferrin regulates binding of polymorphonuclear neutrophils (PMNs) to spermatozoa.* JEVS, 2014. **34**(1): p. 1.
226. Fedorka, C.E., et al., *The effect of select seminal plasma proteins on endometrial mRNA cytokine expression in mares susceptible to persistent mating-induced endometritis.* Reprod Domest Anim, 2016.
227. Kenney, R.M., *Prognostic value of endometrial biopsy of the mare.* J Reprod Fertil Suppl, 1975(23): p. 347-8.
228. Kenney, R.M., *Cyclic and pathologic changes of the mare endometrium as detected by biopsy, with a note on early embryonic death.* J Am Vet Med Assoc, 1978. **172**(3): p. 241-62.
229. Ricketts, S.W., *Endometrial biopsy as a guide to diagnosis of endometrial pathology in the mare.* J Reprod Fertil Suppl, 1975(23): p. 341-5.
230. Fedorka, C.E.W., E.M.; Scoggin, K.E.; Esteller-Vico, A.; Squires, E.L.; Ball, B.A.; Troedsson, M.H.T., *The effect of cysteine-rich secretory protein-3 (CRISP-3) and lactoferrin on endometrial cytokine expression after breeding in the horse.* Journal of Equine Veterinary Science, 2016. **Accepted**.
231. LeBlanc, M.M. and R.C. Causey, *Clinical and subclinical endometritis in the mare: both threats to fertility.* Reprod Domest Anim, 2009. **44 Suppl 3**: p. 10-22.
232. Williamson, P., et al., *Dynamics of the acute uterine response to infection, endotoxin infusion and physical manipulation of the reproductive tract in the mare.* J Reprod Fertil Suppl, 1987. **35**: p. 317-25.
233. Cocchia, N., et al., *Comparison of the cytobrush, cottonswab, and low-volume uterine flush techniques to evaluate endometrial cytology for diagnosing endometritis in chronically infertile mares.* Theriogenology, 2012. **77**(1): p. 89-98.
234. Feghali, C.A. and T.M. Wright, *Cytokines in acute and chronic inflammation.* Front Biosci, 1997. **2**: p. d12-26.

235. Schroder, K., et al., *Interferon-gamma: an overview of signals, mechanisms and functions.* J Leukoc Biol, 2004. **75**(2): p. 163-89.
236. Szekeres-Bartho, J. and G. Chaouat, *Lymphocyte-derived progesterone-induced blocking factor corrects resorption in a murine abortion system.* Am J Reprod Immunol, 1990. **23**(1): p. 26-8.
237. Tayade, C., et al., *Differential gene expression in endometrium, endometrial lymphocytes, and trophoblasts during successful and abortive embryo implantation.* J Immunol, 2006. **176**(1): p. 148-56.
238. Carnevale, E.M. and O.J. Ginther, *Relationships of age to uterine function and reproductive efficiency in mares.* Theriogenology, 1992. **37**(5): p. 1101-15.
239. Woodward, E.M., et al., *Susceptibility to persistent breeding-induced endometritis in the mare: relationship to endometrial biopsy score and age, and variations between seasons.* Theriogenology, 2012. **78**(3): p. 495-501.
240. Troedsson, M.H., M.J. deMoraes, and I.K. Liu, *Correlations between histologic endometrial lesions in mares and clinical response to intrauterine exposure with Streptococcus zooepidemicus.* Am J Vet Res, 1993. **54**(4): p. 570-2.
241. Salazar, J.L., Jr., et al., *Effect of cryopreservation protocol on postthaw characteristics of stallion sperm.* Theriogenology, 2011. **76**(3): p. 409-18.
242. Alghamdi, A.S., et al., *Species-specific interaction of seminal plasma on sperm-neutrophil binding.* Anim Reprod Sci, 2009. **114**(4): p. 331-44.
243. Gahr, M., et al., *Influence of lactoferrin on the function of human polymorphonuclear leukocytes and monocytes.* J Leukoc Biol, 1991. **49**(5): p. 427-33.
244. Garcia Romero, G., et al., *A single administration of the GnRH antagonist acyline inhibits basal and GnRH-stimulated serum testosterone concentrations in male dogs.* Reprod Domest Anim, 2012. **47**(3): p. e32-5.
245. Amory, J.K., et al., *Oral administration of the GnRH antagonist acyline, in a GIPET-enhanced tablet form, acutely suppresses serum testosterone in normal men: single-dose pharmacokinetics and pharmacodynamics.* Cancer Chemother Pharmacol, 2009. **64**(3): p. 641-5.
246. Valiente, C., et al., *Effect of the GnRH antagonist, acyline, on canine testicular characteristics.* Theriogenology, 2007. **68**(5): p. 687-92.
247. Davolli, G.M., et al., *Reversible downregulation of the hypothalamic-pituitary-gonadal axis in stallions with a novel GnRH antagonist.* Theriogenology, 2016.
248. Bradford, M.M., *A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding.* Anal Biochem, 1976. **72**: p. 248-54.
249. Connor, M.C., *Development of an ELISA to measure CRISP-3 in equine seminal plasma*, in *College of Veterinary Medicine*. 2009, University of Florida. p. 63.
250. Crowther, J., *The ELISA Guidebook*. Second ed, ed. J.M. Walker. Vol. 1. 2001, Hertfordshire, UK: Humana Press. 566.
251. Murphy, K., *Janeway's Immunobiology*. 8 ed. Janeway's Immunobiology, ed. K. Murphy. 2012, New York, NY: Garland Science.
252. Udby, L., et al., *Characterization and localization of cysteine-rich secretory protein 3 (CRISP-3) in the human male reproductive tract.* J Androl, 2005. **26**(3): p. 333-42.
253. Anklesaria, J.H., et al., *Purification and characterization of CRISP-3 from human seminal plasma and its real-time binding kinetics with PSP94.* J Chromatogr B Analyt Technol Biomed Life Sci, 2016. **1039**: p. 59-65.

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Education

2008 **St. Lawrence University**, Canton, NY
Bachelor of Science
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Publications

Abstracts

Fedorka, C.E., Woodward, E.M., Scoggin, K.E., Squires, E.L., Ball, B.A.; Troedsson, M.H.T. *The effect of cysteine-rich secretory protein-3 and lactoferrin on endometrial cytokine mRNA expression after breeding in the horse*. Proceedings for the 7th International Symposium on Stallion Reproduction. Champaign IL, 2016.

Fedorka, C.E., Woodward, E.M., Scoggin, K.E., Squires, E.L., Ball, B.A., Troedsson, M.H.T. *Exogenous lactoferrin suppresses the expression of tumor necrosis factor (TNF)-α in mares susceptible to persistent mating-induced endometritis.* Proceedings for the 11th Annual Meeting of the University of Kentucky's Center for Clinical and Translational Sciences. Lexington KY, 2016.

Fedorka, C.E., Scoggin, K.E., Ball, B.A., Squires, E.L., Troedsson, M.H.T. *Cysteine-rich Secretory Protein-3 expression in the prepubertal and mature stallion.* Proceedings for the 48th Annual Meeting of the Society for the Study of Reproduction. San Juan PR, 2015.

Fedorka, C.E., Woodward, E.M., Esteller-Vico, A., Scoggin, K.E., Squires, E.L., Ball, B.A., Troedsson, M.H.T. *Seminal plasma proteins CRISP-3 and lactoferrin and their effect on the immune response of the equine uterus.* Proceedings for the 10th Annual Meeting of the University of Kentucky's Center for Clinical and Translational Sciences. Lexington KY, 2015.

Fedorka, C.E., Davolli, G.M., Ball, B.A., Squires, E.L., Troedsson, M.H.T. *Sperm motility and fertility of cooled preserved stallion semen extended in either INRA96 or Equipro CoolGuard extender.* Proceedings for the Society for Theriogenology. Portland OR, 2014.

Fedorka, C.E., Woodward, E.M., Scoggin, K.E., Squires, E.L., Ball, B.A., Troedsson, M.H.T. *Immune response of the equine uterus after insemination with live versus dead spermatozoa.* Proceedings for the 11th International Symposium on Equine Reproduction. Hamilton New Zealand, 2014.

Peer Reviewed Publications

Fedorka, C.E., Scoggin, K.E., Woodward, E.M., Squires, E.L., Ball, B.A., Troedsson, M.H.T. *The effect of select seminal plasma proteins on endometrial mRNA cytokine expression in mares susceptible to persistent breeding-induced endometritis*. Reproduction in Domestic Animals. Published February, 2017.

Fedorka, C.E., Scoggin, K.E., Squires, E.L., Ball, B.A., Troedsson, M.H.T. *Expression and localization of cysteine-rich secretory protein-3 (CRISP-3) in the prepubertal and postpubertal male horse.* Theriogenology. Published January, 2017.

Fedorka, C.E., Woodward, E.M., Scoggin, K.E., Esteller-Vico, A., Squires, E.L., Ball, B.A., Troedsson, M.H.T. *The effect of cysteine-rich secretory protein-3 (CRISP-3) and lactoferrin on endometrial cytokine expression after breeding in the horse.* Journal of Equine Veterinary Science. Published Online March, 2016.

Davolli G.M., Ball B.A., Esteller-Vico A., Claes A.N., Canisso I.F., **Fedorka C.E.**, Woodward E.M., Troedsson M.H., Squires E.L. *Reversible downregulation of the hypothalamic-pituitary-gonadal axis in stallions with a novel GnRH antagonist.* Theriogenology. Published December, 2016.

Invited Presentations

"*Puberty and its effect on the expression of CRISP-3*". Gluck Equine Research Center University of Kentucky's Departmental Seminar. Lexington, KY. April 30th, 2015.

"*The Seminal Plasma Protein CRISP-3*". Havemeyer Foundation meeting: RM Kenney Equine Reproduction Symposium II. Kennett Square, PA. September 26th, 2014.

"*Sperm motility and fertility of cooled preserved stallion semen extended in either INRA96 or Equipro CoolGuard extender"*. Society for Theriogenology. Portland, OR. August 8th, 2014.

"*Immune response of the equine uterus after insemination with live versus dead spermatozoa"*. International Symposium of Equine Reproduction. Hamilton, New Zealand. January 23th, 2014.

"Select Seminal Plasma Proteins and their Effect on the Immune Response of the Uterus." Gluck Equine Research Center University of Kentucky' Departmental Seminar. Lexington, KY. November 7th, 2013.

Other Publications

"*The effect of stallion age on fertility*." **C.E. Fedorka.** Stallion Spectator Ratings Midyear Publication, 2016.

"*Hormonal Manipulation of the Mare."* Maria R. Schnobrich and **Carleigh E. Fedorka**. Equine Internal Medicine, 4th Edition. Chapter 19, Section 5. Edited by S.M. Reed, W.M. Bayly, and D.C. Sellon. In press.

Applicable Work Experience:

- **Guest Lecturer: Lincoln Memorial University College of Veterinary Medicine,** Harrogate, TN — April 2017
- **Adjunct Professor: Midway University,** Midway, KY — August 2015-present
- **Writer: Paulick Report, Horse Illustrated, Chronicle of the Horse, Horse Network,** Lexington, KY. — August 2014-present
- **Assistant Farm Manager and Sales Director**: **Hinkle Farms**, Paris, KY — June 2010-September 2012
- **Assistant Farm Manager: Chesapeake Farm,** Lexington, KY — March 2009-June 2010

Professional Involvement:

- International Symposium on Stallion Reproduction Member — August 2016-present
- Society for the Study of Reproduction Trainee Member — June 2015-present

- Graduate Student Representative for the University of Kentucky's Equine Programs Council — September 2013-present
- Kentucky Thoroughbred Farm Managers Club Member — March 2009-present

Awards:

- University of Kentucky's Graduate School Three Minute Thesis Competition: 1st place and Peoples Choice Award
  - November, 2016
- Gluck Equine Research Center's Three Minute Thesis Competition: 2nd Place
  - March, 2016
- Animal & Food Science Graduate Student Association (AFSGA) Poster Competition: First place doctoral student
  - May, 2016