COMPARISON OF INFLAMMATORY CELL POPULATIONS IN YOUNG AND MATURE BEEF BULLS; A PRELIMINARY STUDY

by

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ABSTRACT

Bovine venereal diseases, such as *Tritrichomonas foetus (T. foetus)* infection, can be economically devastating to the cattle industry. Decreases in calf crops due to abortion and infertility, premature culling, and loss of animals with valuable genetics are among the costliest effects of venereal diseases. Unidentified *T. foetus* carrier bulls spread disease throughout a herd very efficiently. There is evidence supporting clearance of this disease state in young bulls, but infected mature bulls are often chronic, lifelong carriers of *T. foetus* and serve as the major reservoir for the disease (Clark et al., 1974).

The reasons mature bulls fail to eliminate the *T. foetus* and become chronic carriers as opposed to the apparent resolution of venereal infections in young bulls have not been illuminated. It has been hypothesized that there may be variation in the inflammatory response of the reproductive tract as a bull ages. At the present time, little is known about the normal inflammatory cell populations in bovine penile and preputial epithelium. Hence, the first objective of this study was to evaluate the cell population of the normal bovine penile and preputial epithelium. The second objective was to examine any differences in cell populations between young (n=6) and mature (n=6) age groups. Our hypothesis was that there is a significant difference in immune cell types between young bulls and mature bulls.

Two penile and one preputial biopsies were obtained from each bull. Slides were prepared with hematoxylin and eosin stain and immunohistochemistry markers for MUM1, IBA1, CD79a and CD3 were applied for identification of plasma cells,

macrophages, T lymphocytes, and B lymphocytes, respectively. Histologic evaluation was performed by a board-certified anatomic pathologist.

Inflammation scores, marginated neutrophil infiltration scores, CD3 positive T cell numbers, CD 3 positive T cells numbers around vessels, CD79a positive B cell infiltration scores within lymphoid nodules, IBA1 positive cell numbers in the epidermis, IBA1 positive cells numbers in the superficial dermis, epidermal dermal junction basement membrane disruption scores, and epidermal junction cellular hyperplasia scores were all found to be statistically different ($p<0.05$) when comparing Group A versus Group B (bull type). Location was found to be significant ($p<0.05$) for CD3 positive T cells numbers in the epidermal-dermal interface and CD79a positive B cell infiltration score within the lymphoid nodules between the three sampling sites (distal, proximal, and preputial). Additionally, the interaction between group (bull type) and location was found to be significantly different ($p<0.05$) when comparing the numbers of IBA1 positive macrophages within the superficial dermis between groups and locations.

The results support the hypothesis that there are changes in the cellular populations in the penile and preputial epithelium as bulls age. Hence, the null hypothesis was rejected and the alternative hypothesis was accepted. There seems to be an increase in the amount of inflammation seen in the young bulls which may be integral for clearance of *T. foetus* from the penis and prepuce. The increase in the other cell types, such as macrophages in the mature group is understandable due to exposure of these bulls to numerous cows and/or heifers. This study provides a starting point for further

investigations to more fully elucidate the variation in cell types present in the preputial and penile skin between young and mature bulls.

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ABBREVIATIONS

Tritrichomonas foetus	*T. foetus*
Corpus cavernosum penis	CCP
Corpus spongiosum penis	CSP
Tunica albuginea	TA
Antigen presenting cell	APC
Toll-like receptors	TLR
Polymerase light chain reaction	PCR
Immunohistochemistry	IHC
Hematoxylin and eosin	H&E
Monoclonal antibodies	mAb
Cluster of differentiation 3	CD3
Cluster of differentiation 8	CD8
Cluster of differentiation 79a	CD79a
Multiple myeloma 1	MUM1
Interferon Regulatory Factor 4	IRF4

CHAPTER I: INTRODUCTION

Tritrichomonas foetus (T. foetus) is the causative agent of the venereal disease trichomoniasis in cattle. This organism is an obligate, microaerophilic, protozoal parasite of the bovid reproductive tract (Roberts, 1986b). Transmission of the organism occurs during coitus from an infected animal to a susceptible naïve animal. Approximately, 80 to 90 percent of females exposed to the organism becoming infected (Ondrak, 2016). Infected females often conceive but lose the fetus 15 to 80 days into gestation (Rae, 1989). Cows infected at breeding may incur early embryonic death or abortion of the conceptus, vaginitis, cervicitis, and rarely pyometra. On the other hand, the bull commonly becomes an asymptomatic persistent carrier (Roberts, 1986a; Skirrow and BonDurant, 1990). Most females clear the infection within four months with only a small percentage (<1%) developing a chronic carrier state (Skirrow, 1987). However, in a study by Skirrow et al. two of 40 infected cows from two separate herds where found to have carried the infection through gestation and as far as nine weeks postpartum (1987). Trichomoniasis has a huge economic impact on cattle producers. Economic losses result from the cost of maintaining open females, decreased size of calf crop, decreased weaning weights of calves due to being born later in the calving season, and cost of replacement animals. There is also a loss in genetics as positive animals are culled due to a lack of a currently available legal treatment. Additionally, there is the potential for immature bulls, less than 36 months of age, to clear the disease, although the risk of becoming a chronic carrier increases with age (Clark et al., 1974).

It is unknown if young bulls are more capable of mounting an immune response to the organism to clear the parasite or if they are more resistant to infection. The ability of some young bulls to clear *T. foetus,* compared to the frequent development of the carrier state that occurs in mature bulls, suggests there may be differences in the penile and preputial environment of the bulls as they reach maturity. Historically, it was suggested that an increase in the depth of the penile and preputial epithelial "crypts" occurs as bulls age and that these structures provide a more advantageous environment for survival of *T. foetus* on the penile and preputial surfaces. A study by Strickland et al. demonstrated no observable difference in the degree of epithelial infolding of the preputial and penile skin between the young and mature bulls (2014). Also, Strickland et al. found that the thickness of the penile and preputial epithelium did not differ between the young and mature bulls evaluated in the study (2014). Additionally, there were no structures that could be identified as "crypts" in either group. (Strickland et al., 2014).

Currently, there is little known about the etiology of the chronic carrier state in bulls. Cobo et al. reported variable immunological responses in bulls vaccinated for *T. foetus* (2009). In a study evaluating systemic and preputial immunologic aspects of *T. foetus* vaccinations, they stated that the chronic/persistent carrier states in bulls results when the animal lacks the ability to respond to vaccination with no evidence of antibody response (Cobo et al., 2009). Therefore, it is believed that there are differences in the immune system and cellular environment of the penile and preputial epithelium between young and mature bulls.

The hypothesis of this study was that the presence of specific cell populations and their densities within the penile and preputial epithelium change as a bull ages. Hence,

this evidence may provide an explanation as to the reason young bulls can potentially clear a *T. foetus* infection.

CHAPTER II: LITERATURE REVIEW

Anatomy of the Bovine Penis and Prepuce

The penis is the male copulatory organ, and in the bull it is a fibroelastic, cylindrical structure consisting of three parts; base, shaft, and glans (Nabors and Linford, 2015; Senger, 2012). The base consists of paired crura, one originating at each ischial arch and merging together as one corpus cavernosum penis, which continues as the shaft of the penis to the distal penis terminating near the glans penis just caudal to the umbilicus. In prepubertal bulls, the penis is shorter and lacks a sigmoid flexure (Chenoweth, 2007). The formation of the S-shaped flexure and the breakdown of the penile-preputial attachment is androgen dependent and begins developing as the bull enters puberty (Chenoweth, 2007). The proximal curve opens caudally while the distal curve opens proximally. Paired retractor penis muscles originate at the first and second coccygeal vertebrae (Roberts, 1986a) and insert on the ventral surface of the distal sigmoid flexure of the penis. During the non-aroused state, these muscles are contracted to maintain the sigmoid flexure and keep the penis within the preputial cavity (Senger, 2012). During erection, the retractor penis muscles relax to allow the penis to protrude from the preputial sheath to achieve intromission of the vagina (Beckett, 1999).

The bovine penis contains two erectile tissues; the corpus cavernosum penis (CCP) and corpus spongiosum penis (CSP). The CSP forms from the bulb of the penis which originates at the midline of the ischial arch and surrounds the urethra in the ventrum of the penis. Blood to fill the CSP is supplied by the artery of the bulb of the penis which is a branch of the internal pudendal artery (Nabors and Linford, 2015). Venous drainage of the CSP occurs through paired exhaust veins, which exit at the distal end of the penis.

The CCP is formed by paired crura, which come together as the structures move ventrally to form the body of the penis (Roberts, 1986a). The CCP is filled by blood supplied by a branch of the internal pudenal artery, namely, the deep artery of the penis. There is no terminal drainage for the CCP (Roberts, 1986a). Venous drainage for the CCP is located at the ischial arch (Beckett et al., 1999). There are dorsal penetrating veins, which are also occluded during erection. The CCP is surrounded by a rigid connective tissue covering called the tunica albuginea (TA), which contains elastic fibers with few or no smooth muscle cells. The fibers of the TA are continuous with the trabeculae, which make up the internal structure of the cavernous spaces of the CCP (Samuelson, 2007d; Wrobel and Bergmann, 2006). The cavernous spaces are lined with endothelial cells. The blood supply to the cavernous spaces is supplied by helicine arteries, which contain epithelial smooth muscles cells in the tunica intima (Bacha and Bacha, 2012; Wrobel and Bergmann, 2006). When these muscle cells relax, dilation of the vessel occurs to allow increased influx of blood necessary for erection. The glans penis is the asymmetrical semi-spiraled distal tip of the penis and consists of dense connective tissue, numerous veins, and smooth muscle strands (Bloom and Fawcett, 1968). Sensory stimulation of the glans is necessary for intromission and ejaculation during copulation (Beckett, 1999). This surface of the glans is heavily populated with nerves for sensing tactile stimuli to aid the bull's search for the vagina. In the bull, the glans contains mucous connective tissue, adipose tissue, and intercellular spaces. The glans penis of male ruminants possesses a central strand of connective tissue with intersecting trabeculae (Dellman and Brown, 1987; Nabors and Linford, 2015; Roberts, 1986a).

The skin is the largest organ of the body (Samuelson, 2007c) providing protection and temperature regulation through heat dispersal. It is composed of two layers; epidermis and dermis (Samuelson, 2007c). The outer layer of epidermis is composed of stratified squamous epithelium while the dermis is mostly connective tissue (Samuelson, 2007c). The skin of the penile and preputial epithelium is one of the few hairless areas of the body of the male bovine. Superficially, the skin close to the glans penis grossly resembles a mucous membrane (Wrobel and Bergmann, 2006). The dermis of the glans penis is weaved with the underlying connective tissue and has sebaceous glands (Bloom and Fawcett, 1968), which are not associated with hair follicles in this region. These glands are called glands of Tyson (Bloom and Fawcett, 1968). Although once thought to produce smegma, Hyman and Brownstein indicate that the glands of Tyson do not contribute to the formation of smegma. (1969).

The prepuce is a double invagination of epithelium covering the free portion of the non-erect penis (Wrobel and Bergmann, 2006). The external portion, also known as the preputial sheath, is a continuum with the abdominal skin and reflects inward at the preputial orifice to join the internal portion of the prepuce (Wrobel and Bergmann, 2006). The sheath consists of haired skin containing both sebaceous and sweat glands (Samuelson, 2007d). Some of the sebaceous glands may not be associated with hair follicles. The area of the external prepuce adjacent to the preputial orifice possesses long bristle-like hairs. (Roberts, 1986a; Wrobel and Bergmann, 2006)

The internal portion of prepuce consists of parietal and visceral portions. The parietal portion extends from the preputial opening to the preputial fornix, at which point the visceral prepuce begins and extends to the penile-preputial attachment. The most

proximal portion of the parietal prepuce, the portion closest to haired skin on the sheath, contains a variable number of fine hairs and sebaceous glands (Bacha and Bacha, 2012; Dellman and Brown, 1987; Wrobel and Bergmann, 2006).

Previous dogma was that the preputial skin contains crypts which deepened with advancing age of the animal (Bondurant, 2005). The previous theory that the epithelium thickens with age and crypts deepen was hypothesized to provide a microaerophilic environment to establish the carrier state of *T. foetus* in mature bulls (Strickland et al., 2014). Strickland et al. demonstrated an absence of crypts or crypt like structures in the epithelium of the bovine penis and prepuce (2014). In that study, there were two groups, each containing six bulls. The bulls were grouped by age, and three samples were collected from each bull; distal penis, distal prepuce, and proximal prepuce. Tissues were formalin fixed and examined histologically. The results documented epithelial infoldings on the surface epithelium of penile and preputial skin but did not demonstrate any structures which could be described as "crypts". There was no significant difference in the number, density or depth of the epithelial infoldings between the two groups. The proximal and distal biopsy samples possessed higher numbers of infoldings as compared to the middle sample. However, the density of these infoldings did not differ between the two groups at any location evaluated. Additionally, there were no detectable differences in thickness of the preputial and penile epithelium as the bulls aged contrary to the frequently stated dogma that the epithelium of the bovine preputial and penile epithelium increases in thickness and depth as bulls' age (Strickland et al., 2014). The etiology of the establishment of chronic infections in mature bulls is still unknown.

Keratinized Stratified Squamous Epithelium

Epithelium is classified based on the shape of the cells present and number of layers of cells. The epithelium of the male external genitalia is of the semi-keratinized stratified squamous type (Samuelson and Winter, 1966). Stratified epithelium is composed of two or more layers of cells overlying a basement membrane. The squamous cells are flat and irregularly shaped. In stratified squamous epithelium, only the more external layers are squamous, with cells becoming more cuboidal in the deeper layers closer to the basement membrane (Samuelson, 2007b). The layer in contact with the basement membrane consists of a single layer of simple cuboidal to columnar cells and is known as the stratum basale. The cells of the stratum basale are mitotically active, providing a continuous supply of cells for the other layers (Samuelson, 2007c). The nucleus in these cells is large, occupying most of the space within the cell. The layer next to the stratum basale is the stratum spinosum. In this layer, cells become more irregular in shape, are more polyhedral, and are connected by desmosomes (Frappier, 2006). During processing of this tissue for histopathology, the cytoplasm of cells in this layer shrinks, leaving small processes where the desmosomes connect adjacent cells, resulting in a "spiny" appearance between the cells, hence the name stratum spinosum (Frappier, 2006). The next layer just above stratum spinosum is the stratum granulosum. The cells of this layer are flatter and contain keratohyalin and lamellar granules (Monteiro-Riviere, 2006). In non-haired regions, the next layer is stratum lucidum, which is characterized by several layers of flattened keratinized cells (Frappier, 2006). These cells become translucent due to the presence of eleidin, a clear intracellular protein (Frappier, 2006).

As the cells move outward from the stratum basale to the stratum corneum, they undergo the process of keratinization in which there is a continuous loss of the nuclei and organelles (Frappier, 2006). As cells progress to the superficial layers, there is loss of lysosomal activity and gradual buildup of tonofilaments (Frappier, 2006). Cytokeratin is produced by the cells of the stratum basale. As the cells formed in this layer move to the stratum spinosum layer, tonofilaments condense and later attach to the desmosomes. The flattened cells of the stratum granulosum begin to accumulate keratohyalin (Monteiro-Riviere, 2006). These cells also contain laminar granules of Golgi origin at the periphery. As cells progress outward to the stratum lucidum, cellular shape becomes more ovoid and flattened and intracellular organelles are lost (Monteiro-Riviere, 2006; Samuelson, 2007b, c). When cells reach the outermost layer, the stratum corneum, the laminar granules have been exocytosed, creating a protective barrier between the cells (Monteiro-Riviere, 2006; Samuelson, 2007c). After the cells are present in this layer for some time, separation between the cells develops which is sometimes referred to as the stratum disjunctum (Monteiro-Riviere, 2006). The tissue in this area is keratinized, meaning that the cells on the surface are anuclear and contain a proteinaceous compound known as keratin. Keratins provide structural integrity to the epithelium so that these cells can withstand mechanical stressors (Coulombe and Omary, 2002; Vaidya and Kanojia, 2007). Keratin has water resistant properties and provides protection to the outer layers of the tissue (Frappier, 2006; Monteiro-Riviere, 2006; Samuelson, 2007b, c).

Immune Response of the Skin

The skin serves as a protective physical barrier between the rest of the body and environment. This organ faces a plethora of toxins, pathogens, and physical traumatic elements (Salmon et al., 1994). The cells involved in cutaneous immunity are Langerhans' cells, keratinocytes, and lymphocytes. (Streilein, 1983). The lymphoid tissue of the skin is sometimes referred to as Skin Associated Lymphoid Tissue or SALT (Streilein, 1983). The skin associated lymphoid tissue includes a microenvironment within the skin that is capable of antigen presentation and processing, peripheral lymph nodes with the ability to receive immunological indicators from the skin, and T lymphocytes with an affinity for the skin and associated lymph nodes. (Streilein, 1983).

The immune response of the skin includes a coordination of events between the epidermis and dermis and a web of cytokines (Salmon et al., 1994). Blood and lymphatic vessels are contained within the dermis and most of the lymphocytes in skin are within the dermis. Even though the epidermis does not possess vessels, it is adequately equipped by the cell populations including Langerhans' cells, antigen presenting cells, T lymphocytes, B lymphocytes, and dendritic cells (Salmon et al., 1994). Langerhans' cells are the predominant antigen presenting cell of skin (Salmon et al., 1994). Although macrophages primarily have phagocytosis ability, Langerhans' cells do as well but to a lesser extent (Salmon et al., 1994).

Keratinocytes are the first line of innate immunity within the epidermis to fight against pathogens. These cells have the ability to recognize pathogens utilizing Toll-like receptors (TLR) and initiate an inflammatory response (Velykoredko and Bohdanowicz,

2017). Keratinocytes also interact with the immune system through cytokines, antimicrobial proteins, and chemokines (Salmon et al., 1994; Velykoredko and Bohdanowicz, 2017). Keratinocytes possess the ability to activate T lymphocytes and natural killer cells (Salmon et al., 1994; Velykoredko and Bohdanowicz, 2017).

T lymphocytes are unable to directly recognize pathogens. The T cell receptor binds to the receptor on an antigen presenting cell which allows for immature T cells to develop into effector T cells (Velykoredko and Bohdanowicz, 2017). B lymphocytes establish memory from a previously encountered pathogen allowing for a rapid immune response to future infections with the same pathogen and persistent immunity. B cells produce antibodies (immunoglobulins) which bind to specific antigens (Hidde, 1955; Velykoredko and Bohdanowicz, 2017).

All of these cells function together to protect the integument, providing a physical barrier from toxins, pathogens, and trauma from the environment.

Immunoglobulins in the Bull Reproductive Tract

The immunologic response of the male bovine reproductive tract is poorly understood. The development of chronic carrier states following venereal exposure to *T. foetus* suggests that the immune response in the tissues of the penis and prepuce are unable to eliminate the infective organism from the penile and preputial epithelial surface (Cobo et al., 2009). Natural infection with *T. foetus* induces the deposition of IgG1, IgG2, IgA, and IgM in the preputial cavity(Flower et al., 1983). The predominant immunoglobulin type found in the male bovine reproductive tract is IgG2 (Bier et al., 1977; Cobo et al., 2009; Flower et al., 1983; Rhyan et al., 1999). The IgG level is twice

as great as the IgA level and seven times greater than the IgM level. Flowers *et al.* showed no significant correlations between intrapreputial immunoglobulin concentration, age, and presence of infection (1983). Vaccination with *T. foetus* antigens induces a rise in IgG1, IgG2, IgA, and IgE in preputial secretions (Cobo et al., 2009). *T. foetus* releases surface antigens, which are capable of binding to antibodies, therefore, preventing the organism from being identified and cleared by antibody mediated immunity (Singh et al., 1991).

Cell Types

The mononuclear cells, which are found in the penile and preputial epithelium of bulls includes plasma cells, large and small lymphocytes, macrophages, neutrophils, and eosinophils. In cattle, lymphocytes are the predominant leukocyte. Lymphocytes are responsible for establishing an acquired immune system (Tizard, 2004c) and are initially produced in lymphoid tissues such as bone marrow and thymus. In mature animals, they can be derived from secondary lymphoid tissues such as tonsil, lymph nodes, spleen, bronchial-associated lymphoid tissue, and gut-associated lymphoid tissue (Latimer et al., 2003). Lymphocytes are round cells with a large, round, non-segmented nucleus which may have an indented appearance. Lymphocytes can be categorized based on size or function (Latimer et al., 2003). Ruminants tend to have primarily large lymphocytes. Large lymphocytes often possess multiple nucleoli within the nucleus and have a greater amount of cytoplasm than the small lymphocytes. Small lymphocytes, more common in carnivores, have less abundant cytoplasm than large lymphocytes and stain blue with

most commonly employed laboratory stains (Giemsa, H&E, DiffQuick) (Latimer et al., 2003; Samuelson, 2007a).

Lymphocytes perform critical functions in the immune system and are classified into three types based on function; B cells, T cells, and natural killer cells. The most common type in the body are T cells, which are produced in the thymus and are part of cellular immune response (Samuelson, 2007a). The less numerous B cells are produced in the bone marrow and act in humoral immunity by producing antibodies and generate plasma cells in response to antigenic stimulation (Latimer et al., 2003; Samuelson, 2007a). This commonly occurs in lymph nodes. Both T cells and B cells can become memory cells to provide immunity against an agent in the future. Natural killer cells are few but powerful and provide innate immunity. These cells can remove foreign cells by inducing cell-mediated cytotoxicity (Latimer et al., 2003; Samuelson, 2007a).

Lymphocytes possess surface antigen receptors, which contain proteins that either bind to antigens or act as a signal transducer. Both B cells and T cell can be divided into categories based on the peptide chains in the receptors. T cells have either α and β or γ and δ peptide chains. B cells differ as they can use five different peptide chains; γ, μ, α, ε, and δ. B cells are able to release the B cell antigen receptor into tissue fluid or blood as antibodies (Tizard, 2004c).

The skin possesses a population of resident T cells and employs circulating T cells. Immature T cells do not have the ability to recognize pathogens. The T cell surface receptor must bind to the MCH complex on the surface of an antigen presenting cell (APC) thus inducing the maturation of the T cell to an effector T cell. After binding to the MCH complex, the effector T cell becomes either a cytotoxic T cell or a helper T cell

(Velykoredko and Bohdanowicz, 2017). Cytotoxic T cells directly kill the host cells by binding to a surface protein, Fas death receptor, which activates an enzyme pathway and cytokine pathway to induce apoptosis. Helper T cells can activate B cells to induce the production of specific immunoglobulins. When re-exposed to the same pathogen, memory T cells can react and quickly mount an immune response. Type 1 helper T cells function in cell-mediated immunity by killing intracellular pathogens through production of interferon gamma and through activation of macrophages and natural killer cells to phagocytize infected cells. When type 2 helper T cells are activated, B cells are stimulated and immunoglobulins are produced (Velykoredko and Bohdanowicz, 2017).

Cluster of differentiation 3 (CD3) proteins in the T cell antigen receptors act as signal transducers. The antigen binds to the receptor, and the CD3 proteins are able to pass to the T cell (Pernick, 2013; Tizard, 2004c). The CD3 receptors are found on all types of T cells, in contrast to CD8, which is only found on cytotoxic T cells and is a receptor for the major histocompatibility complex class I molecules. The cluster of differentiation 8 (CD8) group of proteins is necessary for identification of processed endogenous antigen (Tizard, 2004c). Currently, CD3 is the most specific monoclonal antibody for identification of T cells (Pernick, 2013).

B cells are concentrated in the lymph nodes, spleen, bone marrow, and Peyer's patches. All antigen receptors on an individual B cell are identical and generated randomly during formation of the cell (Tizard, 2004a). This means each B cell can only bind to and be signaled by one specific antigen. If a B cell encounters an antigen to which it is capable of binding, the cell responds by releasing antibodies (Tizard, 2004a). The antigen binding component of the antigen receptor is composed of two heavy and two

light chains. The light chains are linked to the heavy chains by disulfide bonds forming a Y-shaped component (Tizard, 2004a). The tail of the Y is known as the Fc region and is formed by the pair of heavy chains. This is the portion which attaches to the surface of the B cell in the lipid bilayer (Tizard, 2004a). The two arms of the Y-shape are composed of the two light chains which bind antigens. This area is known as the Fab region (Figure 1). Light chains are composed of two domains; the constant and variable domains, each containing 110 amino acids (Tizard, 2004a). The constant domain is at the C-terminal, and the variable domain is located at the N-terminal. The constant domain has the same identical sequence of amino acids. The sequence of amino acids in the variable domain differs in each light chain. In mammals, the light chains are either kappa (κ) or lambda (λ); cattle contain 95% κ. Heavy chains consist of 400 to 500 amino acids (Tizard, 2004a) with four to five domains comprised of 110 amino acids each. There are five classes of heavy chains with varying structures and sequences of amino acids in B cells which correspond to an immunoglobulin class; α for Immunoglobulin A (IgA), γ for IgG, μ for IgM, δ for IgD, and ε for IgE (Tizard, 2004a).

The variable regions of both light and heavy chains are composed of three small hypervariable regions consisting of six to ten amino acids. The shape of these regions determines the specificity of the binding antigen. Since the antigen binding location corresponds to the shape of the antigenic determinant, the hypervariable region is also known as the complementarity determining region. The variable domains are folded so that all three complementarity determining regions are in close proximity to the antigen (Tizard, 2004a).

The constant regions differ between the Ig. The α, δ, and γ chains have a similar structure containing three constant domains; CH1, CH2, and CH3. The μ and ε chains have a fourth domain known as CH4. These heavy chains are paired and provide a structure for the antigens to exert their effects. (Tizard, 2004c)

The B cell receptor immunoglobulins are not able to signal their B cell directly due to the cytoplasmic domains only containing three amino acids. Hence, there is a need for a signal transducing agent, cluster of differentiation 79 (CD79), to aid in signaling of the B cells. The protein CD79 is an essential membrane bound protein composed of two heterodimers. These are expressed early in B cell maturation and remain until the final maturation to a plasma cell. The CD79b chains are the same in all B cell receptors. However, CD79a chains are different because of the type of heavy chain present and stimulation of different signaling pathways. Antigen binding initiates signaling of the B cell receptor inducing phosphorylation of the immunoreceptor and tyrosine based activation motifs within the cytoplasmic domain of CD79a and CD79b. This action modulates the gene expression of the B-cell (Luger et al., 2013; Mason et al., 1995; Tizard, 2004a)

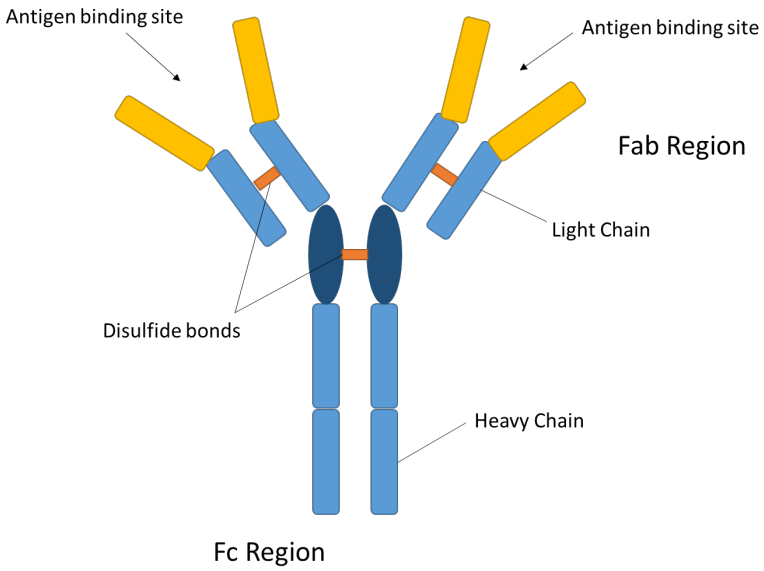

Illustration 1. Structure of immunoglobulin molecule. Modified from Tizard (Tizard, 2004a)

Plasma cells form from B cells stimulated by antigens and are distributed throughout the body with greatest concentrations in the spleen, bone marrow, and lymph nodes. These cells are ovoid and have a large nucleus that is pushed to one side of the cell and stains intensely blue with hematoxylin. Plasma cells possess the ability to produce immunoglobulins that are closely modulated after the receptor of the original B cell precursor (Latimer et al., 2003; Samuelson, 2007a; Tizard, 2004a).

Multiple Myeloma 1 (MUM1), also known as Interferon Regulatory Factor 4 (IRF4) is a transcription factor expressed by B cells capable of developing into plasma cells (Falini et al., 2000; Gualco et al., 2010; Pernick, 2018). This protein is highly regulated during B cell transition to plasma cells and continues to be expressed in neoplastic cells (Gaidano and Carbone, 2000). The MUM1 staining is primarily localized to the cell nucleus but can be present in the cytoplasm providing easy identification of marked cells (Natkunam et al., 2001).

Macrophages develop from monocytes and contain blue staining enzymatic granules in the cytoplasm and a large nonsegmented nucleus. These cells remove foreign material and viruses by antibody-dependent cytotoxicity. Their response is slower than neutrophils (Latimer et al., 2003). Ionized calcium binding adaptor molecule 1 (IBA1) is a calcium binding protein commonly used as a specific marker for microglia and macrophages (Ohsawa et al., 2004). It is involved in the reorganization of actin cytoskeleton during cell movement and phagocytosis (Pierezan et al., 2014).

Neutrophils are produced in the bone marrow, and the nucleus becomes progressively more segmented as the cell matures. Neutrophils are involved in removal of bacterial agents via phagocytosis in tissues. Like neutrophils, eosinophils are produced in the bone marrow. Eosinophils have a segmented nucleus which stain blue with eosin stained granules in the cytoplasm. These cells are involved in immunity, removal of parasites, promotion of inflammation, and may diminish hypersensitivity reactions (Latimer et al., 2003; Samuelson, 2007a).

Dendritic cells are differentiated from bone marrow stem cells. The cell was named due to its' structure of having a small cell body and extensive cytoplasmic processes (Tizard, 2004b). In the immature stage, these cells migrate throughout the body developing into a lattice framework in almost all tissues except for the brain and eye. Dendritic cells concentrate in lymphoid tissue and on mucosal surfaces as these are sites where antigens are likely to be encountered (Tizard, 2004b). Dendritic cells function as very powerful APCs and can present a variety of different antigens. These cells are attracted to inflammation and are involved in innate immunity (Tizard, 2004b). Any dendritic cells that do not contact an antigen within 2-4 days of formation dies and is

removed. These are the only APCs able to activate naïve T cells, making dendritic cells crucial in initiation of primary immunity. (Tizard, 2004b). It has been suggested that dendritic cells play a major role in immunity of the human preputial tissues (Qin et al., 2009b; Wang and Duan, 2016).

Langerhans' cells, a subset of immature dendritic cells, have been seen in human preputial tissue predominately in the superficial epithelium (Wang and Duan, 2016). Human males with a history of infectious disease have a higher concentration of Langerhans' cells in the preputial epithelium as compared to males free of disease (Qin et al., 2009a). These cells are typically one of the first cells in the preputial epithelium to interact with pathogens due to their location within the tissue (Hladik and McElrath, 2008; Wang and Duan, 2016), thus potentially making them essential to cellular immunity in the epithelium.

Previous studies have shown that neutrophils and lymphocytes, including CD3 positive and CD8 positive T cells, are present in the preputial epithelium of healthy bulls (Cobo et al., 2009). The CD3 positive T cells are reported to congregate close to the basement membrane and the surrounding lymphoid nodules of the subepithelium. The CD8 positive T cells are also present in the epithelium close to the basement membrane, although in lesser numbers than the CD3 positive cells (Cobo et al., 2009; Flower et al., 1983). The CD79a positive B cells are found both in the epithelium and in the center of the lymphoid nodules (Cobo et al., 2009). These cells are present to a greater extent in bulls vaccinated for *T. foetus* than in carrier bulls (Cobo et al., 2009; Flower et al., 1983). The lowest numbers of the CD79a B cells are present in non-infected, non-vaccinated

bulls (Cobo et al., 2009). This suggests the possibility of the induction of an immune response resulting from vaccination or infection.

Plasma cells and lymphoid nodules were a common finding in the preputial sub-epithelium (Cobo et al., 2009; Flower et al., 1983). In a previous study, plasma cells were the most predominant cell type, increasing with age, presence of venereal disease, and history of vaccination. Plasma cells were localized to the epidermis of the tissue samples (Flower et al., 1983). This suggests antibody production and release stimulated by venereal infection in bulls, but it is insufficient to clear a *T. foetus* infection. The frequency of lymphoid nodules within the subepithelium of the preputial dermis was increased in bulls vaccinated against *T. foetus* (Flower et al., 1983). In the previous studies, samples were from *Bos indicus* and *Bos taurus* breeds and did not define age groups for comparison. To date, there is not a comparative histological study of the penile and preputial epithelium in *T. foetus* negative bulls between age groups.

Description of *Tritrichomonas foetus*

Tritrichomonas foetus is a member of the phylum Parabasalia. Protozoa in this phylum are anaerobic flagellates that do not contain mitochondria and are obligate parasites of the gastrointestinal and urogenital tracts of vertebrates (Brugerolle and Lee, 2001).

Tritrichomonas foetus is pleomorphic and can be found in trophozoite and pseudocyst forms. The trophozoite, the predominate form, is pyriform in shape with a rounded anterior end and pointed posterior end and has three posterior and one anterior flagella with an undulating membrane (Benchimol, 2004; Parker et al., 2003a; Rae and

Crews, 2006). The cytoskeleton of *T. foetus* is formed by an axostyle and a pelta (Benchimol, 2004), both of which are composed of microtubules. The axostyle is a ribbon-like structure spanning longitudinally across the protozoa (Benchimol, 2004).

The protozoan is 10-25μm long and 5-10μm in width with a single nucleus (Rae and Crews, 2006) (Parker et al., 2003a). In wet mounts, *T. foetus* moves with a characteristic herky-jerky motion (Benchimol, 2004). During stressful conditions such as exposure to medications, sudden changes in temperatures, or a decline in available nutrients, *T. foetus* transforms into a pseudocyst state with internalized flagella and absence of cell wall (Benchimol and Engelke, 2003; Pereira-Neves et al., 2011).

T. foetus organisms, like other members of the phylum Parabasalia, do not contain mitochondria. Instead, ATP is formed in a unique organelle called the hydrogenosome. (Benchimol and Engelke, 2003).

Bovine Trichomoniasis in the Cow

Exposure during coitus with a chronically infected bull is the most common route of transmission, although it is possible to have passive transfer from a previously negative bull that recently breed an infected cow to the subsequent naïve cow (Rae and Crews, 2006). In either case, the organism is deposited into the vagina during copulation. Transmission has been reported following artificial insemination with contamination semen, as the parasite can survive cryopreservation procedures, or by the use of contaminated gynecologic instruments (BonDurant, 1997; Roberts, 1986b).

Infection following coitus induces genital inflammation causing vaginitis, cervicitis, and endometritis. Most infected females develop a detectable humoral immune

response within two to three weeks following infection and clear the infection within two to four months (BonDurant, 1997; Michi et al., 2016; Skirrow and BonDurant, 1990). The induced immunity is not lifelong, and previously infected cows can become infected again following coitus with an infected bull (Clark et al., 1983; Parsonson et al., 1974).

Following deposition into the vagina, *T. foetus* organisms can transverse an open cervix during estrus and populate the entire uterus within one to two weeks of initial infection (BonDurant, 1997). Fertilization is not disrupted, and the cow can conceive in the face of *T. foetus* infection (Abbitt, 1980). Most reproductive loss following infection with *T. foetus* is due to embryonic death after entry of the conceptus into the hostile environment of the inflamed uterus. Embryo viability may sometimes be maintained past the point of maternal recognition of pregnancy, 14-18 days post coitum, with embryonic or fetal loss occurring beyond that point resulting in a prolonged inter-estrus interval (Bazer et al., 1991; BonDurant, 1997).

Proposed mechanisms leading to embryonic loss include (1) disruption of the feto-maternal unit by overgrowth of *T. foetus*, (2) a uterine immune response to the surface antigens of *T. foetus,* (3) direct cytotoxic effects of *T. foetus* on the embryo and maternal tissues, or (4) the production of enzymes by *T. foetus* which break down the connections between the fetal and maternal units of the placentome (BonDurant, 1997). A small number of cows maintain pregnancy beyond the embryonic and early fetal stages and abort in the second or third trimester, but this is regarded as uncommon (Rhyan et al., 1988). Fetuses aborted due to *T. foetus* infection commonly have bronchopneumonia with trichomonads present in the bronchi. The organism can be cultured from the abomasal fluid of aborted fetuses (Rhyan et al., 1988; Rhyan et al., 1999).

In infected herds with relatively long calving seasons, some cows may become infected and successfully mount an immune response before the end of the breeding season. In these animals, pregnancy may be established. These successful pregnancies occur in the latter half of the breeding season and calves born in the subsequent calving season are younger at weaning, resulting in lower weaning weights.

A small number of infected cows, usually less than five percent, develop pyometra following breeding. The diagnosis of "post service pyometra" should always raise suspicion of herd infection with *T. foetus.*

Although most cows clear the infection within three estrous cycles following introduction of the organism, a small population of cows (less than one percent) may develop a persistent infection (Skirrow, 1987). Persistently infected cows can maintain the infection through gestation and into the following breeding season. These carriers can then serve as a reservoir for infection in the herd. Although a producer may test and cull positive bulls, these relatively uncommon carrier females can potentially infect the new or previously negative bulls in subsequent breeding seasons (Mancebo et al., 1995; Rae and Crews, 2006; Skirrow, 1987).

Bovine Trichomoniasis in the Bull

In bulls, natural infection with *T. foetus* occurs when the bull achieves intromission during the breeding act with an infected female. The organism colonizes and establishes a long term infection on the epithelium of the free portion of the penis, the prepuce and rarely in the lumen of the distal urethra, without invading deeper tissues (Bartlett, 1947; Parsonson et al., 1974; Roberts, 1986a). Unlike the cow, no immune

response sufficient to eliminate the infection is stimulated in the bull. Hence, the bull is unable to clear the infestation on the surface epithelium resulting in a persistent carrier state (Cobo et al., 2011; Parsonson et al., 1974).

Reports dating back decades indicate that young bulls, typically less than 24 months of age, are not at risk of long term infection, while mature bulls, usually three or more years of age, consistently develop a chronic asymptomatic carrier state following infection (Clark et al., 1977). Carrier bulls provide a continuous reservoir of infection, but neither libido nor spermatogenesis are affected. Sperm morphology is reportedly unaffected by a *T. foetus* infection. However, the organism produces cytotoxic substances which reduce progressive motility of the spermatozoa (Ribeiro et al., 2010).

Disease transmission by transiently infected young bulls is thought to be inefficient compared to transmission following coitus with persistently infected carrier bulls (Clark et al., 1977). The efficiency of transmission by immature bulls increases if the bull had coitus with an infected cow within minutes to hours prior to breeding a naïve cow (Clark et al., 1977).

In a study examining *T. foetus* infection in bulls one to two years of age, infection could not be established in 16 of the 18 bulls (Clark et al., 1974). The two remaining bulls developed a temporary infection which persisted for less than four months (Clark et al., 1974). Similarly, prevalence studies have shown differences in infection rates in young and mature bulls. In a California surveillance study, 6.7% of mature bulls, four years or older, were infected with *T. foetus*, whereas only 2% of bulls three years of age or younger were infected (Bondurant, 2005). A report from Florida indicated that bulls five years of age or older were 2.2 times more likely to be infected with *T.* foetus than

bulls less than five years of age (Rae et al., 2004). It is unclear whether the apparent resistance to chronic infection by young bulls is due to the ability to mount an effective immune response to *T. foetus* or if unknown factors unrelated to the immune system make the prepuce of younger animals a less hospitable environment.

Diagnostic Testing Techniques

Historically, diagnosis of bovine venereal trichomoniasis has been challenging for veterinarians due to compromised sample collection methods and improper transport of the samples to the diagnostic lab. Some available diagnostic tests lack sensitivity and false negatives are possible. Traditional culture involves growing the organism in a specialized media that allows for proliferation of *T. foetus* while the antimicrobial agents inhibit growth of bacterial contaminants. A large number of bacteria in the sample can interfere with the identification of *T. foetus*. The bacteria deplete the antimicrobial compounds in the media resulting in depletion of the nutrients causing buildup of metabolic by-products. Additionally, *T. foetus* is slower to replicate than the bacteria and cannot survive in the altered media (Clothier et al., 2015). Temperature can have detrimental effects on the accuracy of the test as well. The ideal temperature for transport after collection is 37°C. The sample should be protected from extreme temperatures (Bryan et al., 1999; Davidson et al., 2011).

Common samples collected for testing include preputial smegma from bulls, and cervicovaginal mucus and uterine discharge from cows. Sampling technique may play a role in accurate diagnosis. Preputial scrapings with a pipette, preputial swabs and preputial lavage are the most common sample collection methods in the bull. Each of

these methods have been used with varying rates of success (Rae and Crews, 2006; Schonmann et al., 1994). Although there is not a statistical difference in the accuracy of diagnosis between the two methods, the douching or lavage method is more common in Europe while preputial scrapes with a pipette are preferred in the United States (Schonmann et al., 1994). There is evidence to support that collection from the right side of the chute will increase the sensitivity of detecting a positive bull. This might possibly be due to the counterclockwise rotation of the distal free portion of the penis and glans penis resulting in the urethral groove and median raphe to be slightly to the right, possibly providing an area with a larger number of *T. foetus* in that location (Parker et al., 2003b).

For a bull to be considered truly negative for *T. foetus,* many states' regulations require, serial testing by culture once weekly for three weeks or a single PCR. However, current standards for bulls at artificial insemination centers require serial testing, either culture or PCR, which must be performed once weekly for six weeks. This level of testing is considered the current "Gold Standard". This multiple sampling is uncommon in production operations due to cost and logistics. Although rarely done, allowing two weeks of sexual rest prior to testing will increase the sensitivity of testing because it will maximize the number of organisms present in the prepuce at the time of sampling (Peter, 1997). Since infected bulls can intermittently test negative when checked during the breeding season, (Clark et al., 1983; Clark et al., 1971) any bull which tests negative during the breeding season should have a test repeated two weeks after the end of the season.

Many states now have implemented regulatory testing for trichomoniasis prior to sale or entry into the respective state. The official tests vary from state to state and most commonly include culture and quantitative polymerase light chain reaction (PCR).

Economic Impact of Bovine Trichomoniasis

There is substantial economic loss due to the reduction in size of calf crop due to embryonic loss and abortion, lowered weaning weights of calves due to delayed conception, and costs associated with the necessity to cull and replace infected cattle. Fitzgerald et al. calculated an annual loss of $800 per infected bull (1958). Wilson et al. showed a $2.5 million loss in the calf crop of replacement heifers in Oklahoma (1979). In 1986, Fitzgerald et al. estimated the annual loss due to *T. foetus* infections to be $65 million (1986). The most recent study, by Speer et al., suggests the annual loss due to *T. foetus* in the United States to be $650 million (1991).

The prevalence of trichomoniasis varies across the United States. In many western states, trichomoniasis is a reportable disease and all non-virgin bulls must be tested before transport or turnout on public lands. In Texas, test results must be reported to the Texas Animal Health Commission within 48 hours. Many states are implementing a mandatory slaughter policy for bulls that are confirmed to be positive (Cima, 2009). At the time of this thesis, at least 25 states have rules requiring testing of bulls for infection with this organism.

Venereal Trichomoniasis Infections in Humans

Trichomonas vaginalis is a protozoal organism causing the venereal disease trichomoniasis in humans. This organism possesses five flagella, four anterior and one located within the undulating membrane (Petrin et al., 1998). Trichomoniasis is one of the most common infections of the human urogenital tract and has a significant public health impact worldwide (Cogne et al., 1985; Conrad et al., 2013). Like *T. foetus,* this organism also requires iron as an essential nutrient (Sehgal et al., 2012). The organism populates the preputial tissue and urethra of men. In women, *T. vaginalis* inhabits the vagina, cervix, and uterus.

Men develop one of three disease states. Asymptomatic carrier status is the most common infection stage of men. The second is acute infection with profuse purulent urethritis. The third presentation is mild, symptomatic disease characterized by nongonococcal urethritis (Petrin et al., 1998). Since males are typically asymptomatic carriers, the condition often goes undiagnosed in men. However, men can exhibit urethral inflammation, urethral discharge, dysuria, and urethral pruritus. The disease state is short lived in males, but this is not the case in females where there is a much longer infective state (Petrin et al., 1998). Approximately 25-50% of women develop a carrier state of infection, with only 50% of this group developing clinical signs (Petrin et al., 1998). Common clinical signs in women are a frothy, malodorous vaginal discharge, vulvar irritation and inflammation, cervicitis, and cervical microhemorrhages. Other adverse effects of trichomoniasis include infertility, pelvic pain, abortion, and a two-fold higher risk of HIV-1 transmission when both diseases are present as *T. vaginalis* appears to increase the transmission of HIV-1 (Conrad et al., 2013; Sorvillo et al., 2001).

Additionally, serum IgG1 levels are higher in females than males (Cogne et al., 1985). Nitroimidazoles are the drug of choice for treatment of trichomoniasis infections in humans (Conrad et al., 2013).

Diagnosis cannot solely be made by clinical signs. Typically culture is performed as it is the gold standard for diagnosis (Petrin et al., 1998). The organism can sometimes be detected in a wet mount preparation of the vaginal or urethral discharge material but is not consistent (Petrin et al., 1998).

Human Preputial Tissue and Venereal Disease

In men, the rate of some venereal infections is greater in adults than in younger men. Keratin provides a barrier of protection against HIV in humans (de Vincenzi and Mertens, 1994). Qin et al. showed a greater degree of keratinization in the inner prepuce as compared to the outer prepuce of males with a history of infectious disease (2009b). The degree of keratinization is less in young as compared to mature men. Langerhans' cells are the primary antigen presenting cell of the skin and are present in higher densities in the inner prepuce of young males with a history of infectious disease as compared to those who are healthy. These cells occur in higher densities in young men as compared to adult (Qin et al., 2009b). With these changes evident in human preputial tissues as males' age, it is possible that the same changes could be seen in other species and should be examined in bovine preputial epithelium to aid in the understanding of venereal disease transmission.

Several studies have demonstrated that circumcision provides some protection against the transmission of many sexually transmitted diseases including, HIV,

Chlamydia trachomastic, and *Trichomonas vaginalis.* (Bailey et al., 2007; Gray et al., 2004; Gray et al., 2009; Sobngwi-Tambekou et al., 2009; Tobian et al., 2010; Weiss, 2007). Studies reported circumcision could reduce the incidence of HIV by 53-60% and *T. vaginalis* by 48% in human males (Tobian et al., 2010). Additional limited studies show a lower prevalence of *Chlamydia trachomastic* in women with a circumcised partner (Weiss, 2007). From these studies, it can be concluded that the absence of excess prepuce could reduce the transmission of disease and self-reinfection of the male (Gray et al., 2009). Based on these human studies, it is possible that circumcision could reduce the transmission of venereal diseases in cattle. To date, there is not a definitive study to prove that cattle breeds with more preputial tissue (*Bos indicus* influenced) are more susceptible to venereal disease infection or more prone to transmitting the diseases; however, human studies indicate that this might be possible.

Immunohistochemistry

Immunohistochemistry (IHC) was first described in the 1930s and is the process of labeling cells by performing specific antigen-antibody reactions on fixed tissues embedded in paraffin. It is used in disease diagnosis, staging of cancer, drug development, and research (Duraiyan et al., 2012). Once the paraffin embedded tissues have been cut with a microtome to 4-5µm, the sample is adhered to a glass slide. Paraffin must be removed (deparaffinization) prior to IHC staining as it is hydrophobic in nature and will repel the aqueous IHC stains. Historically, this has been performed by using xylene, a toxic, volatile solution. Although, today, there are safer alternatives. Many tissues are fixed in formaldehyde which forms methylene bridges to crosslink proteins.

These bridges can decrease antibody binding; thus, formaldehyde fixed tissues may be subjected to an antigen retrieval process, but this step is not always required. Typically, antigen retrieval involved heating the sample in a buffer solution causing an enzymatic reaction to change the pH. The prepared sample is then ready for IHC staining and undergoes a series of incubations with primary and secondary antibodies and washings. The primary antibody binds to the antigen of interest (Pernick, 2015). Washing removes the unbound antibodies and antibodies bound to nonspecific sites. The next step is to add a counter stain to provide contrast. Hematoxylin, eosin, methyl green, and Hoechst fluorescent stains are commonly used as counter stains with IHC. Finally, a coverslip is added. (Pernick, 2015)

CHAPTER III: JOURNAL ARTICLE

ABSTRACT

Bovine venereal diseases, such as *Tritrichomonas foetus (T. foetus)* infection, can be economically devastating to the cattle industry. Decreases in calf crops due to abortion and infertility, premature culling, and loss of animals with valuable genetics are among the costliest effects of venereal diseases. Unidentified *T. foetus* carrier bulls spread disease throughout a herd very efficiently. There is evidence supporting clearance of this disease state in young bulls, but infected mature bulls are often chronic, lifelong carriers of *T. foetus* and serve as the major reservoir for the disease (Clark et al., 1974).

The reasons mature bulls fail to eliminate the *T. foetus* and become chronic carriers as opposed to the apparent resolution of venereal infections in young bulls have not been illuminated. It has been hypothesized that there may be variation in the inflammatory response of the reproductive tract as a bull ages. At the present time, little is known about the normal inflammatory cell populations in bovine penile and preputial epithelium. Hence, the first objective of this study was to evaluate the cell population of the normal bovine penile and preputial epithelium. The second objective was to examine any differences in cell populations between young (n=6) and mature (n=6) age groups. Our hypothesis was that there is a significant difference in immune cell types between young bulls and mature bulls.

Two penile and one preputial biopsies were obtained from each bull. Slides were prepared with hematoxylin and eosin stain and immunohistochemistry markers for MUM1, IBA1, CD79a and CD3 were applied for identification of plasma cells, macrophages, T lymphocytes, and B lymphocytes, respectively. Histologic evaluation was performed by a board-certified anatomic pathologist.

Inflammation scores, marginated neutrophil infiltration scores, CD3 positive T cell numbers, CD 3 positive T cells numbers around vessels, CD79a positive B cell infiltration scores within lymphoid nodules, IBA1 positive cell numbers in the epidermis, IBA1 positive cells numbers in the superficial dermis, epidermal dermal junction basement membrane disruption scores, and epidermal junction cellular hyperplasia scores were all found to be statistically different ($p<0.05$) when comparing Group A versus Group B (bull type). Location was found to be significant ($p<0.05$) for CD3 positive T cells numbers in the epidermal-dermal interface and CD79a positive B cell infiltration score within the lymphoid nodules between the three sampling sites (distal, proximal, and preputial). Additionally, the interaction between group (bull type) and location was found to be significantly different ($p<0.05$) when comparing the numbers of IBA1 positive macrophages within the superficial dermis between groups and locations.

The results support the hypothesis that there are changes in the cellular populations in the penile and preputial epithelium as bulls age. Hence, the null hypothesis was rejected and the alternative hypothesis was accepted. There seems to be an increase in the amount of inflammation seen in the young bulls which may be integral for clearance of *T. foetus* from the penis and prepuce. The increase in the other cell types, such as macrophages in the mature group is understandable due to exposure of these bulls to numerous cows and/or heifers. This study provides a starting point for further investigations to more fully elucidate the variation in cell types present in the preputial and penile skin between young and mature bulls.

INTRODUCTION

Tritrichomonas foetus is an obligate, microaerophilic, protozoal parasite of the bovine reproductive tract and is the causative agent of bovine trichomoniasis. In cattle herds, infection with *T. foetus* decreases reproductive efficiency. Transmission occurs during coitus between an infected animal with a naïve animal. Infection of the female reproductive tract can be followed by vaginitis, metritis, early embryonic death, fetal death, and, occasionally, pyometra (Roberts, 1986b). Infected cows mount an immune response following infection and usually clear the organism from the reproductive tract. The impact of the infection in the cow herd is fewer calves per year, with many calves being born late in the calving season, resulting in small calf crops and lower weaning weights. Additional expenses are incurred with the cost of replacing open cows and infected cattle. The economic impact can be devastating to producers. Speers and White estimated an annual loss of $650 million in the United States due to trichomoniasis (1991).

In bulls, the parasite resides on the surface of penile and preputial epithelium, and the distal urethral (Bartlett, 1947; Bondurant, 2005). Infected bulls typically become asymptomatic carriers of the parasite with no negative effects on libido or spermatogenesis (Anderson et al., 1994; Parsonson et al., 1974). However, the cytotoxic properties of *T. foetus* have been reported to negatively affect sperm motility (Ribeiro et al., 2010).

Following natural infection, bulls do not mount an immune response sufficient to eliminate *T. foetus* from the penis and prepuce. Instead, the organism commonly colonizes the penis and prepuce, persistently, resulting in a long-term carrier state. There

is evidence that immature bulls, less than three years of age, are either more resistant to infection or can clear the infection (Clark et al., 1974). A widely stated but unsupported explanation for this phenomenon has been that young bulls lack "crypts" or deep invaginations of the preputial skin (Bondurant, 2005; Roberts, 1986a). These preputial skin invaginations have been thought to deepen with age and provide the microaerophilic environment necessary for establishment of long term colonization by *T. foetus* (Roberts, 1986a). However, a study by Strickland et al. does not support this theory (2014). Strickland et al. found that the number and depth of these epithelial invaginations present in the preputial skin of young and mature bulls did not differ. Additionally, it was found that there were no structures that could be accurately described as "crypts" in either age group (Strickland et al., 2014). Thus, an explanation for resistance to long-term colonization or infection with *T. foetus* in younger bulls has yet to be elucidated.

The focus of this study was to further exploration, evaluation, and comparison of the penile and preputial epithelium of young and mature bulls. Ultimately, the goal of this study was to better understand the changes that might occur in the immune cell populations present in the penile and preputial epithelium as the bull ages and potentially how this might impact the development of persistent infections of *T. foetus* in the bull to prevent or cure disease states.

STATEMENT OF RESEARCH OBJECTIVES

The purpose of this study was to (1) determine the cell populations and densities within the penile and preputial epithelium and (2) assess differences in these parameters between young and mature bulls. It was hypothesized that as the bull matures there are significant changes in the numbers of the specific cell types within the penis and preputial epithelium of the bull.

MATERIALS AND METHODS

Animals

The animals used in this study were either from the Auburn University research and teaching herds or privately-owned bulls. Written consent was obtained for each privately-owned bull prior to sampling. All procedures were approved by the Auburn University Institutional Care and Use Committee (PRN 2017-3022). Bulls were placed into two groups each containing six bulls. The first group (N=6) consisted of bulls 14-24 months of age, and the second group (N=6) consisted of bulls 5 years of age or older. Age was determined by dentition and records. Bulls included in the study were *Bos taurus.* One PCR test for *T. foetus* was performed at Thompson Bishop Sparks Alabama State Diagnostic Lab on all bulls enrolled in this study and were found to be negative. Bulls were randomly assigned a number 1-12 by a random number generator (www.random.org) as samples were collected and evaluators were blinded to the age of the bull or site of the tissue sample.

Sampling for *T. foetus*

Clean latex examination gloves were worn for each bull. A new equine AI pipette (Sterile Pipette 21" with adapter, Jorgensen Laboratories Inc, Loveland CO) was used for each bull. The external preputial orifice was held open, and the pipette was placed into the preputial cavity. A 5 mL syringe was attached to the pipette and negative pressure applied while scraping the end of the pipette along the preputial epithelium while gentle massage was performed. The sample was transferred to modified Diamond's media (TF Transit Tube, BIOMED Diagnostics, Inc, White City, OR) and held at ambient temperature for no more than twenty to thirty minutes before placing in an incubator at 37 degrees Celsius or being submitted to the Thompson Bishop Sparks Alabama State Diagnostic Lab for PCR analysis.

Polymerase Light Chain Reaction (PCR)

Samples were sent to the Thompson Bishop Sparks Alabama State Diagnostic Lab for quantitative PCR testing [Trich-kit, Life-Tech (Cat#4483869; VetMax Gold Trich Detection Kit)]. The PCR test can differentiate between *T. foetus* and other trichomonad protozoa.

Tissue Sampling and Processing

The penis was manually extended and held with a surgical gauze pad. A towel clamp was placed under the dorsal apical ligament to keep the penis extended. Local anesthesia was provided by injection of 10 milliliters of 2% lidocaine hydrochloride subcutaneously in the dorsal aspect of the penis to block the dorsal penile nerves. After

aseptic preparation with betadine scrub, a 1 cm by 1 cm by 0.5 cm in depth tissue biopsy was collected with a #22 scalpel blade (Becton Dickinson AcuteCare, Franklin Lakes, NJ) from the following three locations: (1) the distal penis near the glans, (2) 1 centimeter distal to the attachment of the prepuce to the free portion of the penis, and (3) proximal prepuce 6 cm distal to the preputial orifice. Biopsy areas were then sutured with #1-0 chromic gut absorbable suture material. The tissue samples from the first two bulls were placed in 10% buffered formalin (VMR International, LLC, Radnor, PA) and had mild tissue distortion. Thereafter, the remaining tissues were pressed to a small piece of wooden tongue depressor prior to being stored in 10% buffered formalin (VMR International, LLC, Radnor, PA) until tissues were processed. Adherence of the tissues to the tongue depressor prevented distortion of the tissue when placed in formalin.

Hematoxylin and Eosin

Penile and preputial tissues were fixed in 10% formalin, embedded in paraffin, cut (4µm thickness), mounted on slides, and stained with H&E. The H&E staining was performed with a Leica Autostainer XL (Leica Biosystems Inc., Buffalo Grove, IL). Heat was applied for 10 minutes at 60°C. Hemo-De was applied for eight minutes followed by two more at five minute increments. Next, 100% ethanol (Koptec, King of Prussia, PA) was applied twice at two-minute increments followed by 95% ethanol twice at two minute increments. A wash was then performed. Hematoxylin (Gill's II, Newcomer Supply, Middleton, WI) was applied for five minutes followed by a second wash. Scott's solution (Newcomer Supply, Middleton, WI) was then applied for two minutes. Then, 80% ethanol was applied for two minutes. Eosin stain was applied for 2 minutes. Ethanol

was applied at 95% concentration twice at two-minute increments followed by 100% twice for two minutes. Xylene (Azer Scientific, Morgantown, PA) was then applied three times at two minutes increments and cover slipped.

Immunohistochemistry

Immunohistochemistry markers CD3, CD79, Iba-1, and MUM1 were used to identify T cells, B cells, macrophages, and plasma cell respectively. The Dako Envision™ FLEX detection system (DAKO Corporation, Carpinteria, CA) was used for IHC staining of bovine penile and preputial epithelium with specific monoclonal antibodies (Table 2). Mature B and T cells were identified by mAb to surface mouse CD79a and mouse CD3, respectfully. Plasma cells and macrophages were identified by mAb to surface mouse MUM1 protein and rabbit IBA1 protein. All IHC markers have successfully been used in bovine tissues in the laboratory. Sections (4μm) of penile and preputial epithelium were mounted on a glass slides and then the coated paraffin embedded samples were deparaffinized and hydrated followed by heat-induced epitope retrieval using the EnVision™ FLEX Target high pH (9) Solution Retrieval method. The FLEX peroxidase block was added to the slides and then rinsed with a buffer. For CD3, sections were incubated with the primary mAb for 15 minutes twice followed by a buffer rinse. A labeled polymer (FLEX/HRP) was applied for 15 minutes. The slide was then rinsed twice with a buffer. The substrate-chromogen (FLEX DAB + Sub-Chromo) was applied for 10 minutes. The slide was rinsed again with a buffer. FLEX hematoxylin was used as a counterstain and applied for 5 minutes. The slide was rinsed with buffer followed by distilled water and allowed to dry before a glass cover slip was applied. For

MUM1, CD79a, and IBA1, sections were incubated with the primary mAb for 15 minutes followed by a buffer rinse. A labeled polymer (FLEX/HRP) was applied for 15 minutes. The slide was then rinsed twice with a buffer. The substrate-chromogen (FLEX DAB + Sub-Chromo) was applied for 10 minutes. The slide was rinsed again with a buffer. FLEX hematoxylin was used as a counterstain and applied for 5 minutes. The slide was rinsed with buffer followed by distilled water and allowed to dry before a glass cover slip was applied.

Table 1. Antibodies used for identifying immune cell phenotypes by immunohistochemistry

Antibody	Target Cell	Clone
Anti-human CD3	T cell	F7.2.38, DAKO Corporation (Carpinteria, CA)
Anti-mouse CD79a	B cell	HM47/A9, Biocare Medical (Concord, CA)
Anti-human MUM1 protein	Plasma cell	MUM1p, DAKO Corporation (Carpinteria, CA)
Anti-rabbit polyclonal antibody	Macrophages	N/A, Biocare Medical (Concord, CA)

This study was conducted from January through December 2017. The two groups were compared (table 2). A total of 36 samples were collected; three from each of the twelve bulls.

Table 2. Age and Breed of Bulls

Group A	Group B
Angus, 14 months	Angus, 5 years
Angus, 18 months	Angus, 7 years
Angus, 18 months	Angus, 7 years
Angus, 18 months	Angus, 7 years
Angus, 18 months	Simmental, 5 years
Angus, 18 months	Simmental-Angus, 8 years

Histological Examination

All slides were analyzed by a board-certified pathologist blinded to the age of the bull and the location of biopsy site. A subjectively graded (scale of 0-3) for general inflammation and marginated neutrophils in the superficial dermis, epidermal-dermal interface characteristics and dermal lymphoid nodules. In general, 0 = represents no cells observed, 1 = mild, 2 = moderate, 3 = severe based on this population of bulls.

Each slide was photographed at 20x objective (200x magnification) field. The absolute number of immune positive cells was counted on each slide for each marker at each location. The CD3 positive lymphocytes were counted in the epithelium and the epidermal-dermal junction for each slide. The number of CD3 lymphocytes around superficial vessels were counted and divided by the number of vessels. The absolute number of CD79 positive lymphocytes was counted in the superficial dermis to a depth equal to approximately the thickness of the epidermis. The deeper lymphocytes were not

counted. The IBA1 positive macrophages were counted within the epidermal-dermal junctions and the superficial dermis.

Integrity of the epithelial-dermal interface was evaluated for crisp to blurred definition of the basement membrane, separation of basal cells and scalloped basement membrane and assigned a grade of 0 to 3 (Table 3). Grade 0 showed no disruption. Grade 1 showed mild disruption which included mild scalloped basal cells, and intermittent loss of basement membrane integrity Grades 2-3 had disruption of basement membrane with 2 having moderate disruption and 3 having severe disruption. Epidermal hyperplasia was assigned grades 0 to 3 (Table 4). Grades 0 had single layers of basal cells. Grade 1 had multiple layers of epithelial cells and mild hyperplasia. Grade 2 had multiple layers of epithelial cells and moderate hyperplasia. Grades 3 had severe hyperplasia of the epithelial cells.

Table 3. Subjective grading score for the disruption of the basement membrane

Grade	Basement Membrane Disruption
0	None
1	Mild
2	Moderate
3	Severe

Table 4. Subjective grading score for epithelial hyperplasia

Grade	Epithelial Hyperplasia
0	One layer of basal cells and no hyperplasia
1	Multiple layers of epithelial cells and mild hyperplasia
2	Moderate Multiple layers of epithelial cells and moderate hyperplasia
3	Multiple layers of epithelial cells and severe hyperplasia

RESULTS

The IHC staining was found to be variable between bulls, this could be accounted for by variable handling of the tissue at variable pH, formalin concentration and temperature. The MUM1 immunohistochemistry marker did not stain cells in any of the samples. A positive control (bovine colon) was run and stained successfully.

Statistical Analysis

Evaluation was performed using SAS software (PROC MIXED, SAS, version 9.3, SAS Institute Inc, Cary NC). The Mixed Linear Model was used to discover which variable, bull type (young or mature), location sampled, and the interaction of the bull type and location (Bull Type*Location), was significant for the defined measures. This method was chosen for the following reasons: a) the experiment did not have the full design of possible experiments, b) the sample size was small and, c) there were several missing values. The alpha or significant level was considered as 0.05. Therefore, any p-value less than or equal to 0.05, represents the significance of the corresponding variable and any $p<0.1$ was indicative of a trend.

1.1 Inflammatory Cell Infiltration Score

There was a wide variety in the degree of inflammatory cell infiltration detected in the population of bulls evaluated. The comparison of the degree of inflammatory cell infiltration detected between bulls in Group A and bulls in Group B was statistically significant ($p=0.0001$) (Figure 2). There was a greater degree of inflammatory cell infiltration in bulls in Group A than that of Group B. The biopsy location was not

statistically significant (p=0.2112) and the interaction of the location sampled and bull type (young vs. old) was also not found to be statistically significant (p=0.0926). The presence of submucosal lymphoid nodules varied among samples with some samples having large nodules and others having a complete absence. The distribution of the lymphoid nodules in these tissues varied between and among groups with more nodules present in the mature group. Inflammatory cell infiltration was determined by cellular counts. The number of neutrophils and lymphocytes was higher in the young group as compared to the mature group, thus indicating a higher presence of inflammatory cell infiltration in the young group. It is unknown if the young group had greater exposure of pathogens to induce a greater immune response or if the ability of the young group to mount an immune response is greater as compared to the mature group.

Table 5. Statistical significance evaluation of bull type, location, and the interaction between bull type and location for the subjective observation of inflammation

	F-value	P-value
Bull Type	87.9488	<0.0001
Location	1.7276	0.2112
Bull Type*Location	2.7998	0.0926

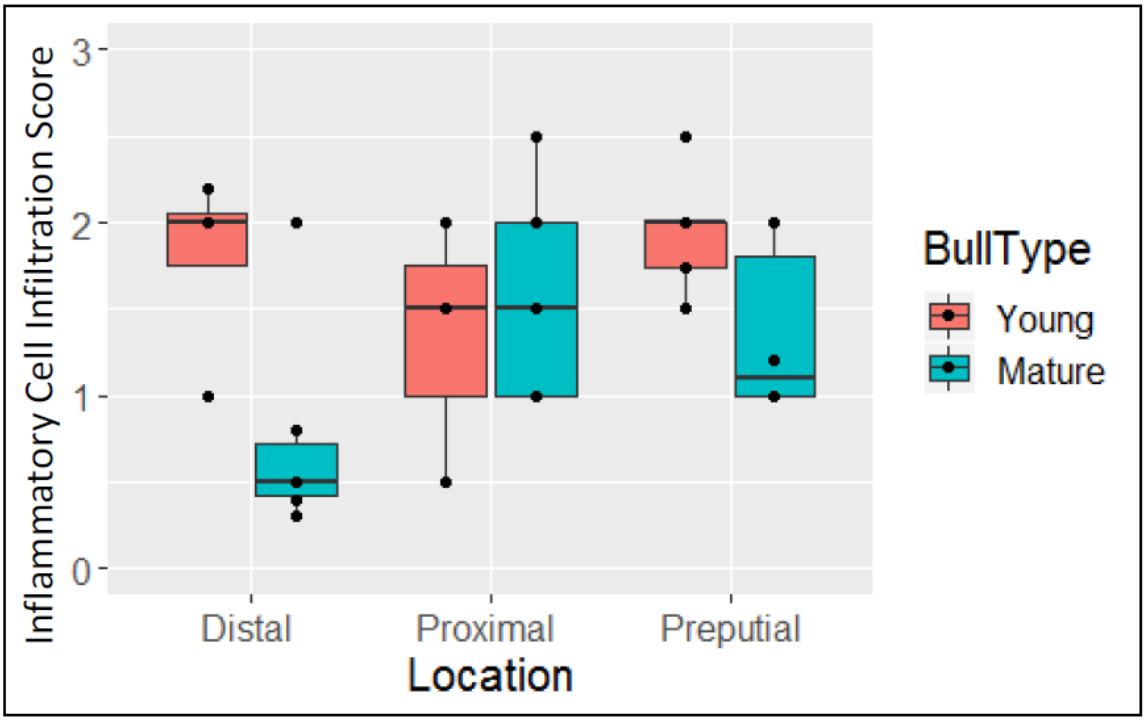

Figure 2. Graph illustrating the amount of inflammatory cell infiltration found in each group of bulls at each of the three sampling locations for each group of bulls, young (Group A) and mature (Groups B)

1.2 Marginated Neutrophils

The scores for the presence of marginated neutrophils between bulls in Group A and bulls in Group B were statistically significant (p=0.0007) (Figure 3). There was a greater degree of marginated neutrophils detected in bulls in Group B than that of Group A. Differences in marginated neutrophil scores by location were not statistically significant (p=0.4906), and the interaction of the location sampled and bull type (young vs. old) was also not found to be statistically significant (p=0.4114).

Table 6. Statistical significance evaluation of bull type, location, and the interaction between bull type and location for the subjective observation of marginated neutrophils

	F-value	P-value
Bull Type	17.9066	0.0007
Location	0.7471	0.4906
Bull Type*Location	0.9430	0.4114

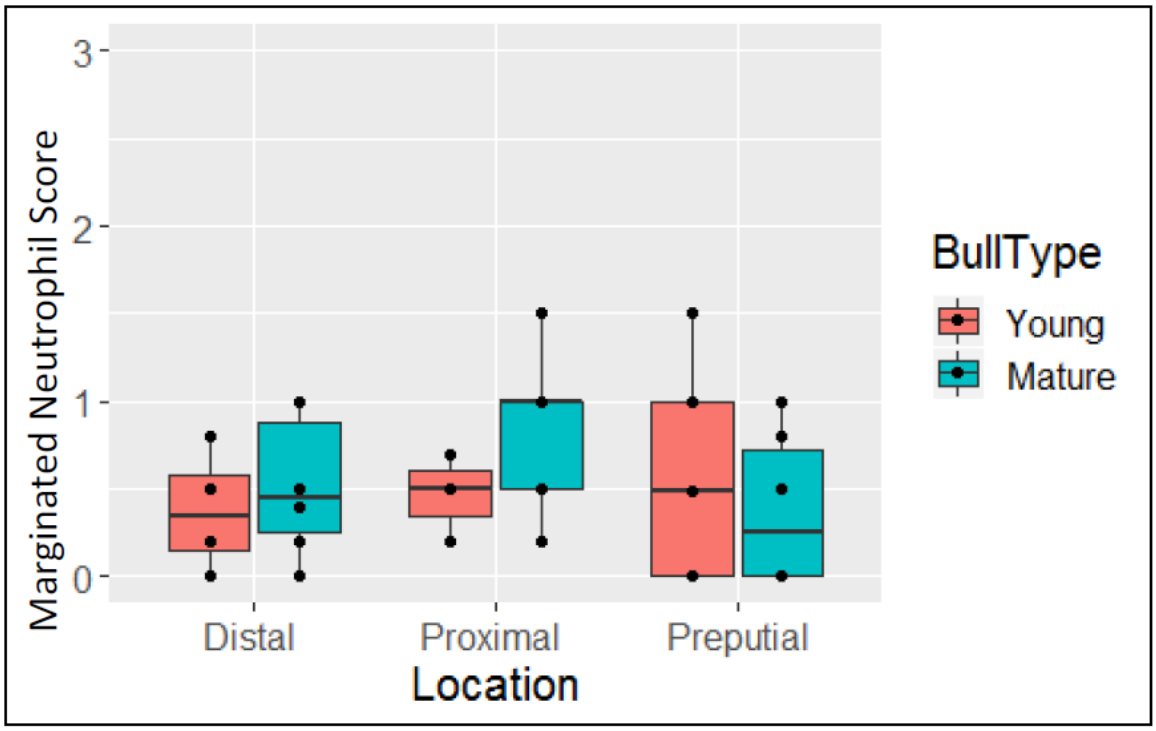

Figure 3. Graph illustrating objective grading of marginated neutrophils found in each group of bulls at each of the three sampling locations for each group of bulls, young (Group A) and mature (Groups B)

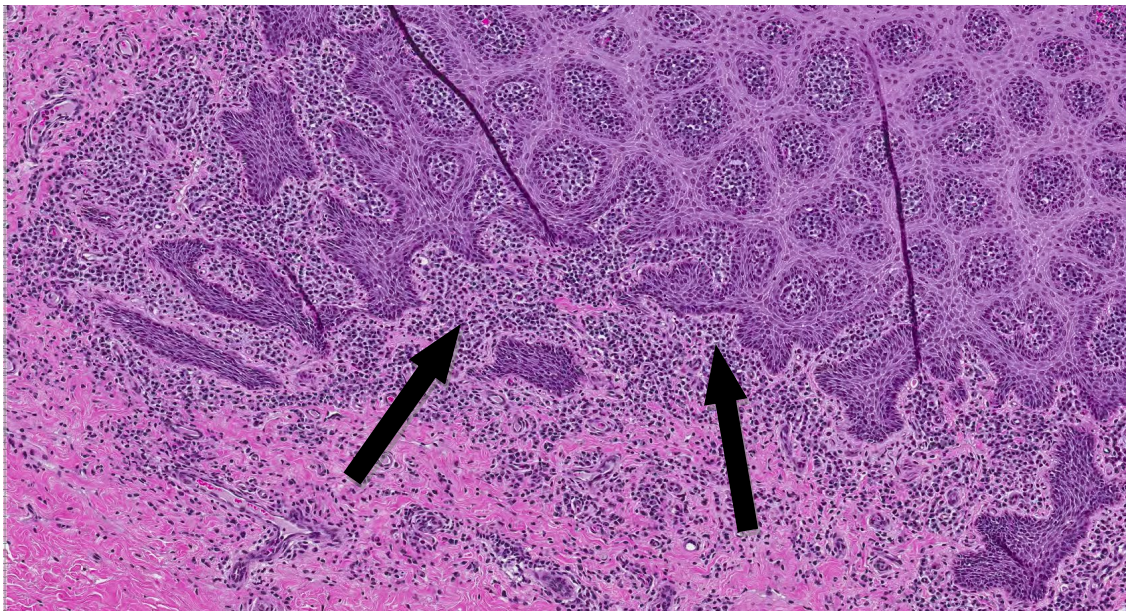

Figure 4. Hematoxylin and eosin stained section showing moderate inflammatory cell infiltration due to the presence of numerous neutrophils and lymphocytes (black arrows) in the dermis of the preputial epithelium from an eighteen-month-old bull

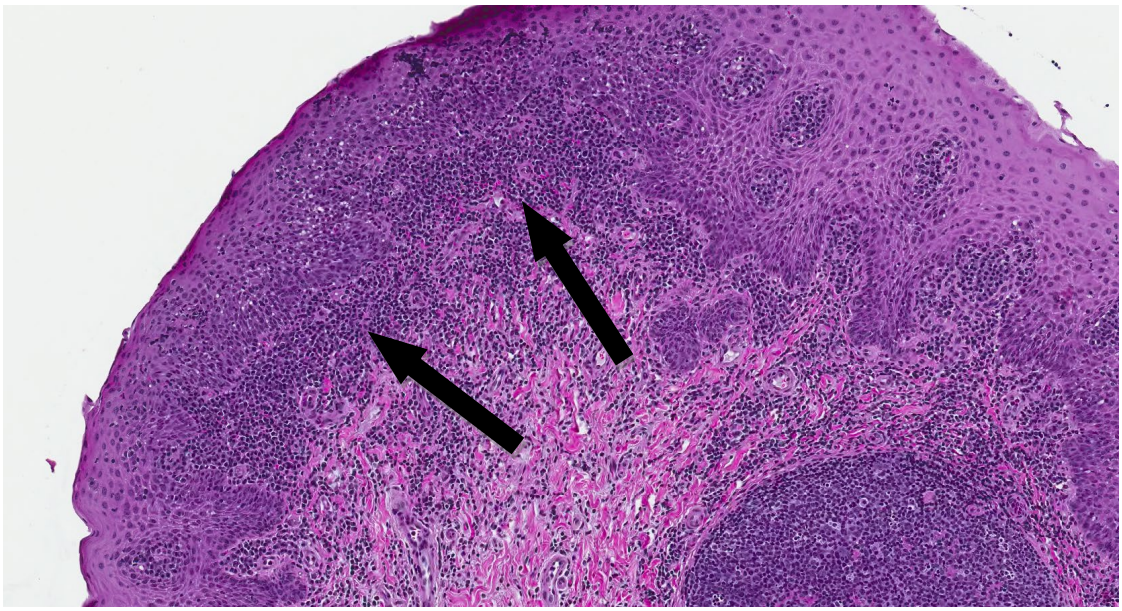

Figure 5. Hematoxylin and eosin stained section showing moderate inflammation (black arrows) in the dermis of the preputial epithelium from a seven-year-old bull

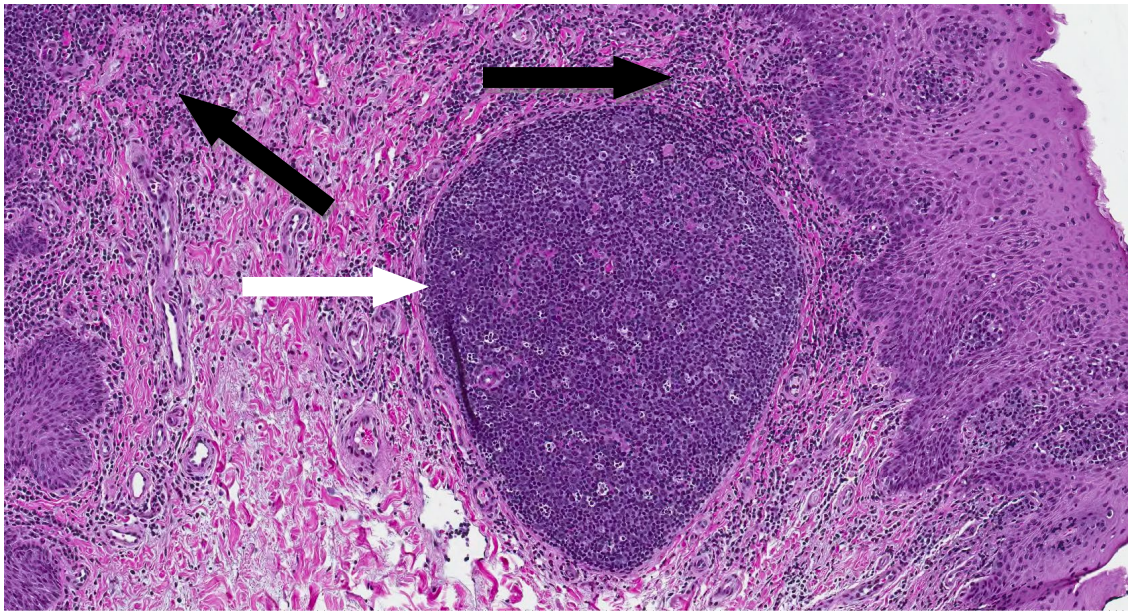

Figure 6. Hematoxylin and eosin stained dermal lymphoid nodule (white arrow) with moderate inflammatory cell infiltration (black arrows) in the dermis of the preputial epithelium of a seven-year-old bull

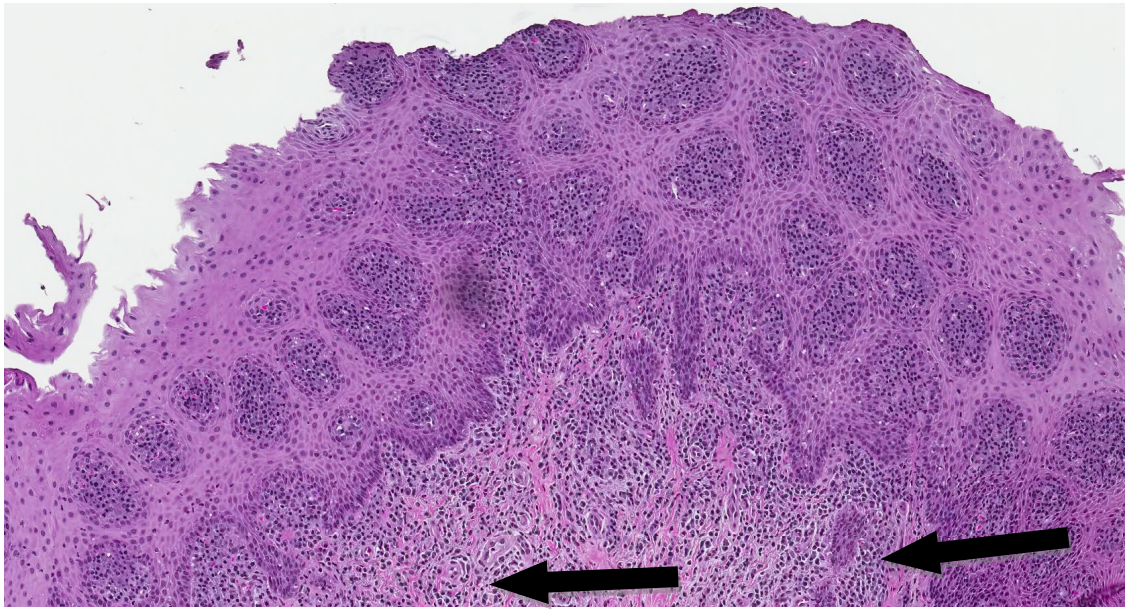

Figure 7. Hematoxylin and eosin stained section of preputial epithelium from an eighteen-month-old bull with moderate inflammatory cell infiltration in the dermis and around dermal vessels

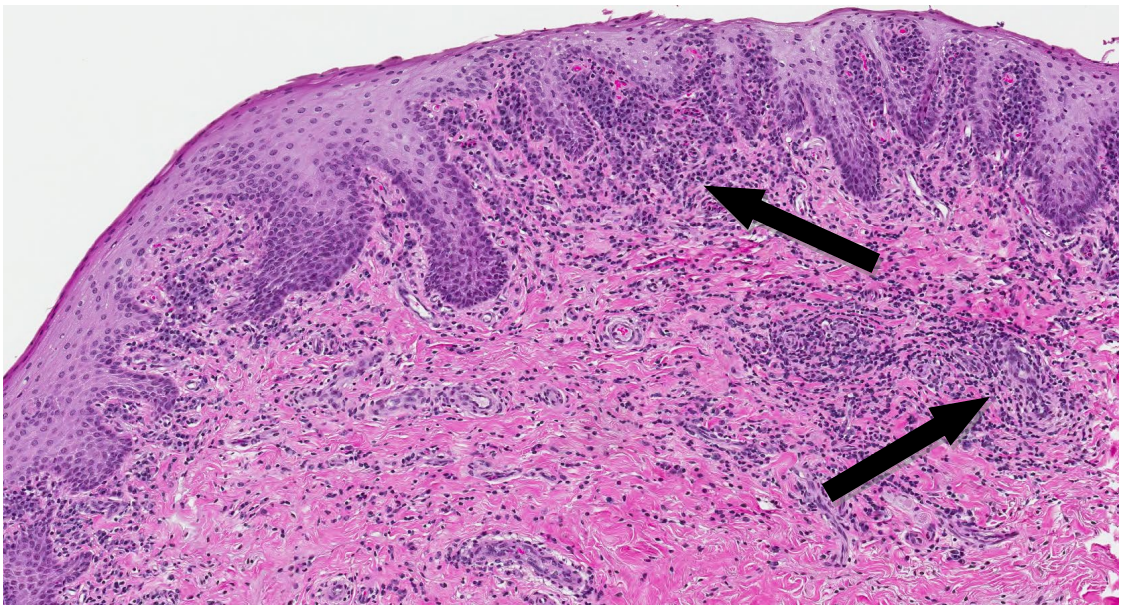

Figure 8. Hematoxylin and eosin stained section of distal penile epithelium from an eighteen-month-old bull with mild inflammatory cell infiltration in the dermis and around dermal vessels (black arrow)

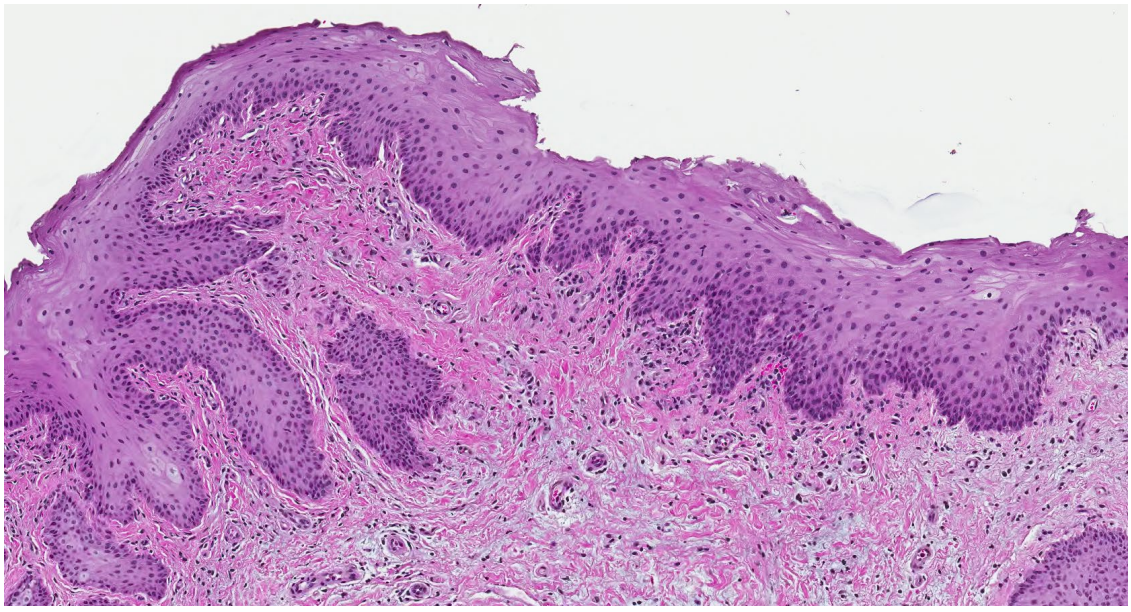

Figure 9. Hematoxylin and eosin stained section of distal penile epithelium from a five-year-old bull with minimal inflammatory cell infiltration in the dermis and around dermal vessels

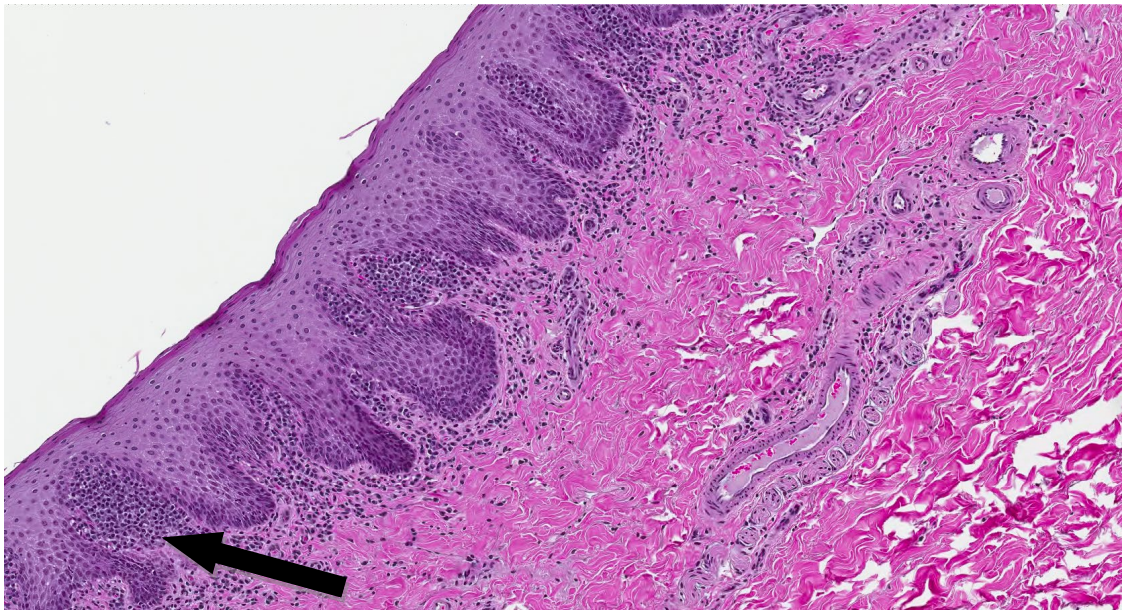

Figure 10. Hematoxylin and eosin stained section of proximal penile epithelium from an eighteen-month-old bull with mild inflammatory cell infiltration in the dermis (black arrow)

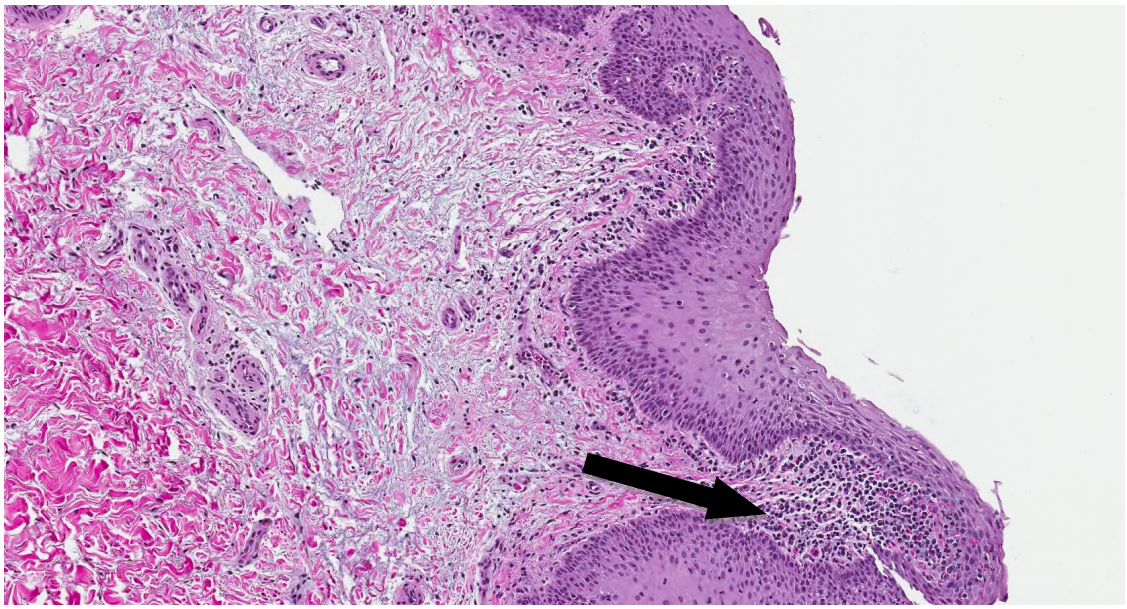

Figure 11. Hematoxylin and eosin stained section of proximal penile epithelium from seven-year-old bull with mild inflammatory cell infiltration in the dermis

1.2 CD3 Positive T Lymphocytes

CD3 positive T lymphocytes were found at the epidermal-dermal interface, in the epidermis, and cuffing the vessels in the superficial dermis. There was a statistically significant (p=0.0001) difference in the number of CD3 positive T lymphocytes in bulls in Group A versus that of Group B (Figure 12) located at the epidermal-dermal interface. Location was found to be significant (p=0.0075) in regards to the number of CD3 positive T lymphocytes found at each site as seen in the figure below (Figure 12). However, the interaction of the bull type and location was not found to be statistically significant (p=0.1505).

Table 7. Statistical significance evaluation of bull type, location, and the interaction between bull type and location for the number of CD3 positive T lymphocytes found at the epidermal-dermal interface

	F-value	P-value
Bull Type	29.2931	0.0001
Location	6.9031	0.0075
Bull Type*Location	2.1540	0.1505

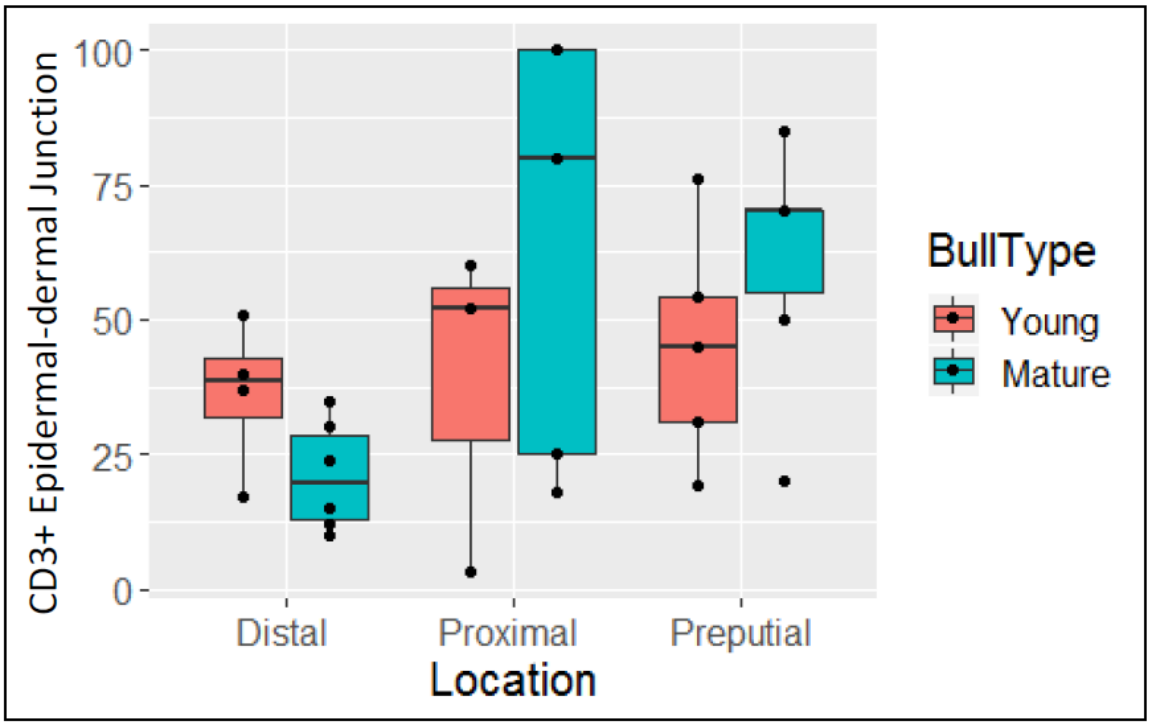

Figure 12. Graph illustrating the number of CD3 positive T lymphocytes found at the epidermal-dermal junction at each of the three sampling locations for each group of bulls, young (Group A) and mature (Groups B)

The number of CD3 positive T lymphocytes around superficial vessels in the epidermis, and cuffing the vessels in the superficial dermis were found to be a statistically significant (p=0.0006) when comparing bulls in Group A versus that of Group B (Figure 13). Location was not found to be significant (p=0.5202) regarding the number of CD3 positive T lymphocytes found at each site as seen in the figure below (Figure 13). The interaction of the bull type and location was not found to be statistically significant (p=0.1512).

Table 8. Statistical significance evaluation of bull type, location, and the interaction between bull type and location for the number of CD3 positive T lymphocytes found around superficial vessels

	F-value	P-value
Bull Type	18.9922	0.0006
Location	0.6829	0.5202
Bull Type*Location	2.1484	0.1512

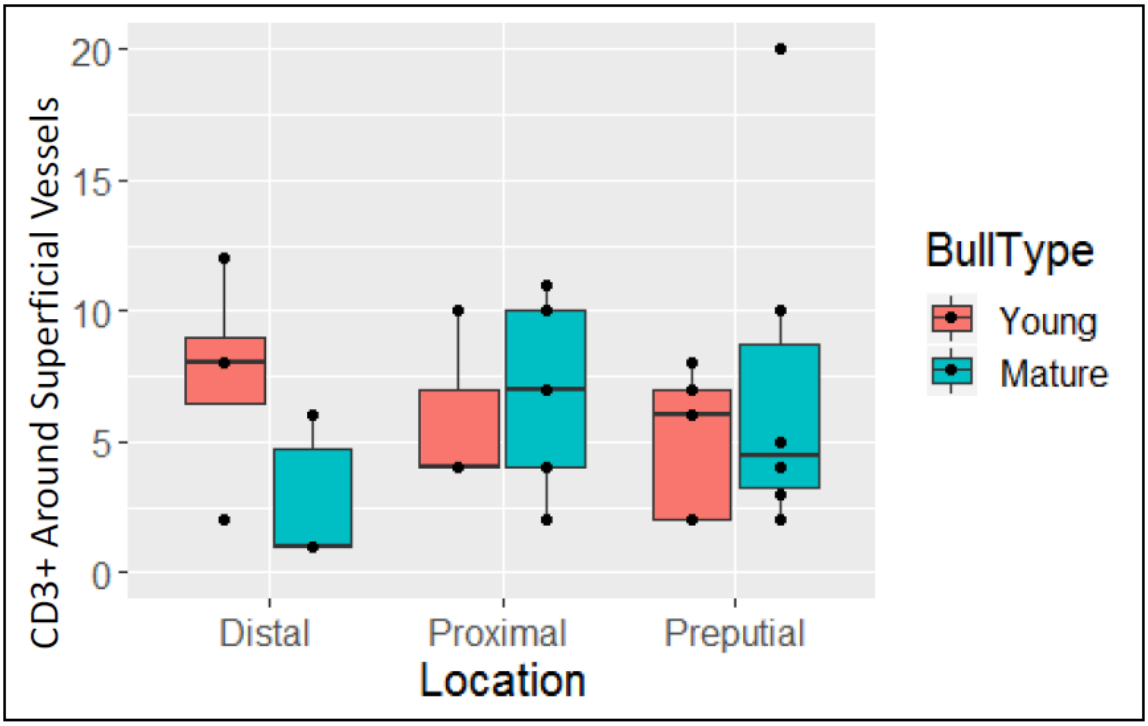

Figure 13. Graph illustrating the number of CD3 positive T lymphocytes found around superficial vessels at each of the three sampling locations for each group of bulls, young (Group A) and mature (Groups B)

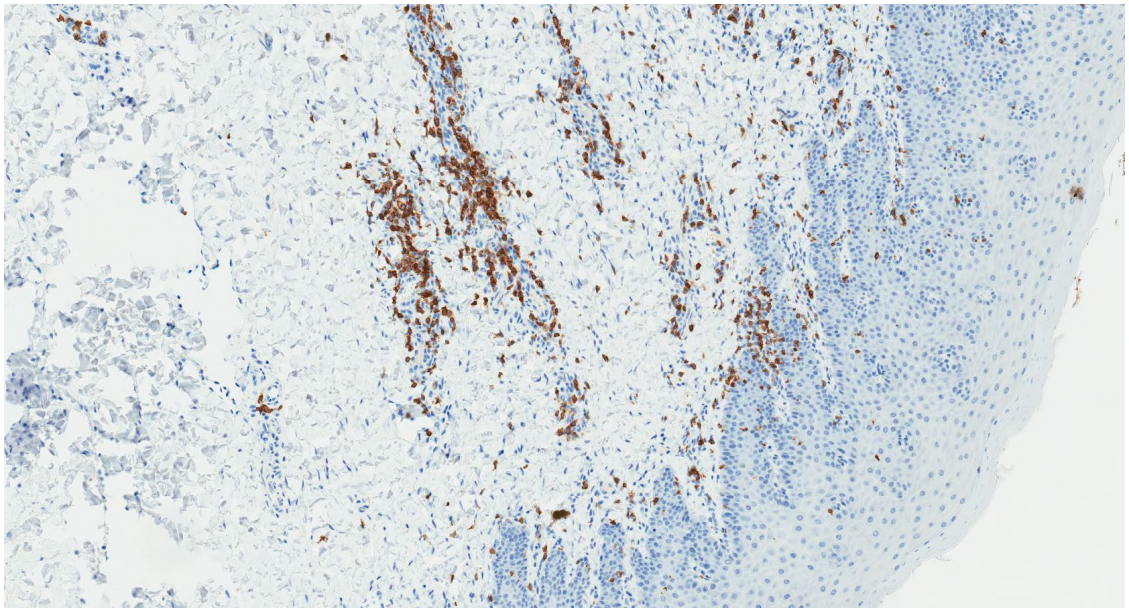

Figure 14. IIHC CD3 stained section of proximal penile epithelium from eighteen-month-old bull with moderate CD3 positive T lymphocytes at epidermal-dermal interface, in the epidermis, and cuffing the vessels in the superficial dermis

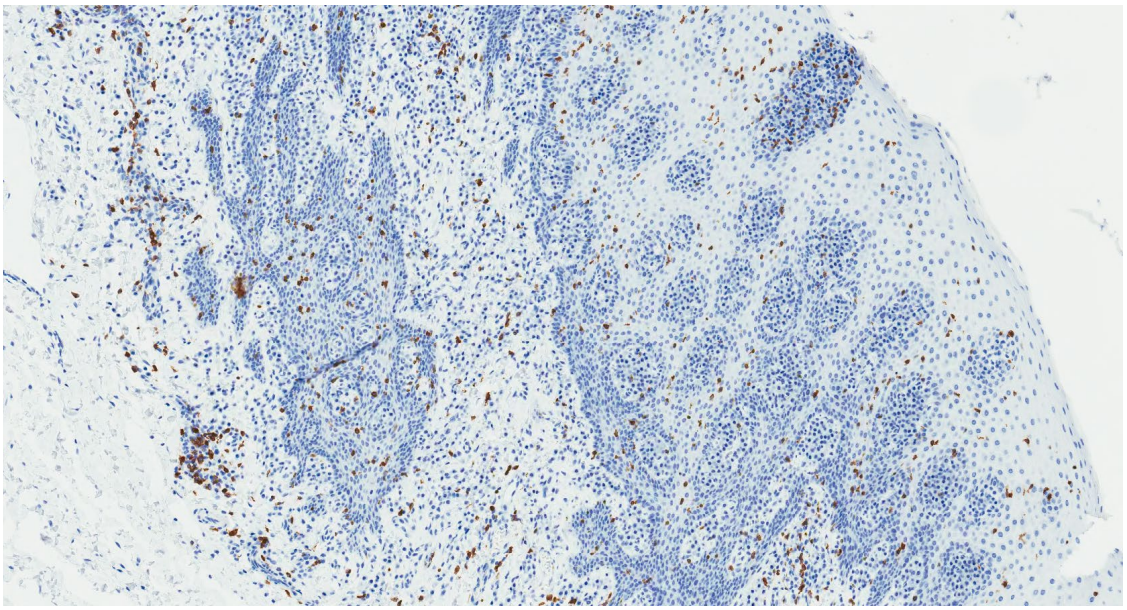

Figure 15. IHC CD3 stained section of proximal penile epithelium from seven-year-old bull with mild CD3 positive T lymphocytes at epidermal-dermal interface, in the epidermis, and cuffing the vessels in the superficial dermis

1.3 CD79a Positive B Lymphocytes

The CD79a positive B lymphocytes prominently cuff submucosal vessels. These lymphocytes were found in the epidermis and around the dermal vessels but not in the epidermal-dermal interface area of the tissues examined. There was a significant difference ($p=0.0233$) found in the scores evaluating cellular infiltrate of CD79a positive B lymphocytes located in the lymphoid nodules between bulls in Group A versus bulls in Group B. Additionally, there was a significant difference ($p=0.0108$) in the number of CD79a positive B lymphocytes found between the different locations sampled. In the distal sampling site, it was found that there was a larger density in the population of CD79a positive B lymphocytes in Group A than Group B (Figure 16). However, in the other two sampling sites there was a greater density in the population of CD79a positive B lymphocytes in bulls in Group B (Figure 16). However, the interaction of the bull type and location was not found to be statistically significant but there was a trend ($p=0.0901$).

Table 9. Statistical significance evaluation of bull type, location, and the interaction between bull type and location for the number of CD79a positive B lymphocytes

	F-value	P-value
Bull Type	5.8719	0.0233
Location	6.2135	0.0108
Bull Type*Location	2.8379	0.0901

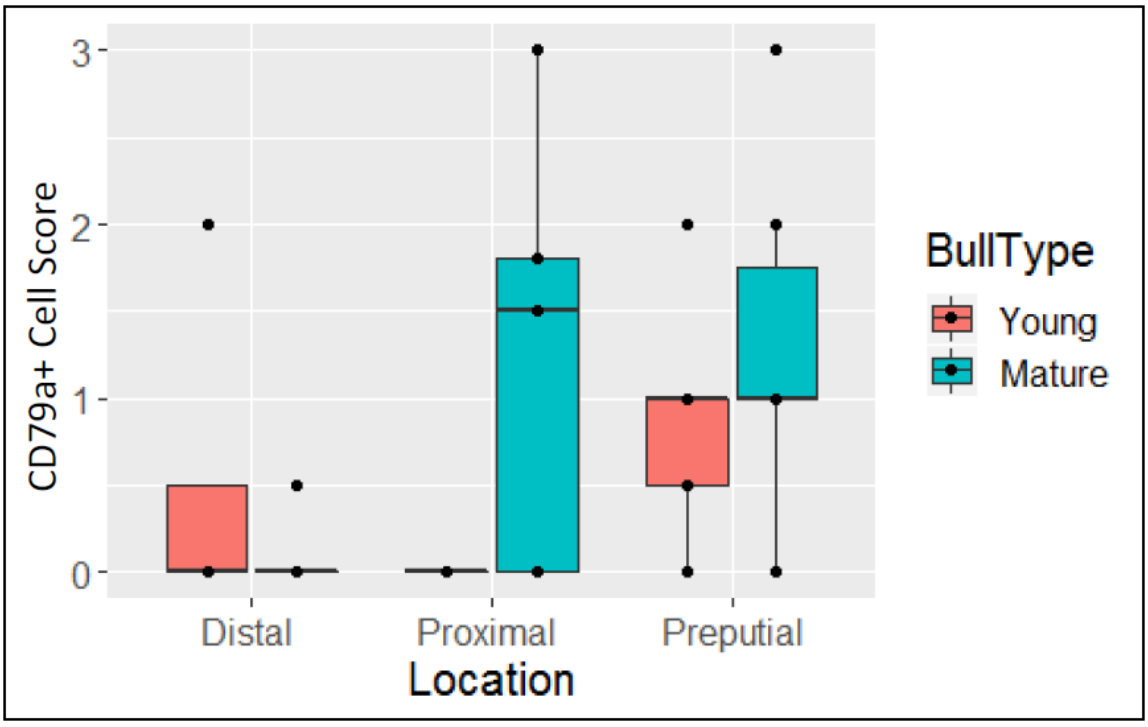

Figure 16. Graph illustrating the difference in the number of CD79a positive B lymphocytes at each of the three locations for each group of bulls, young (Group A) and mature (Groups B)

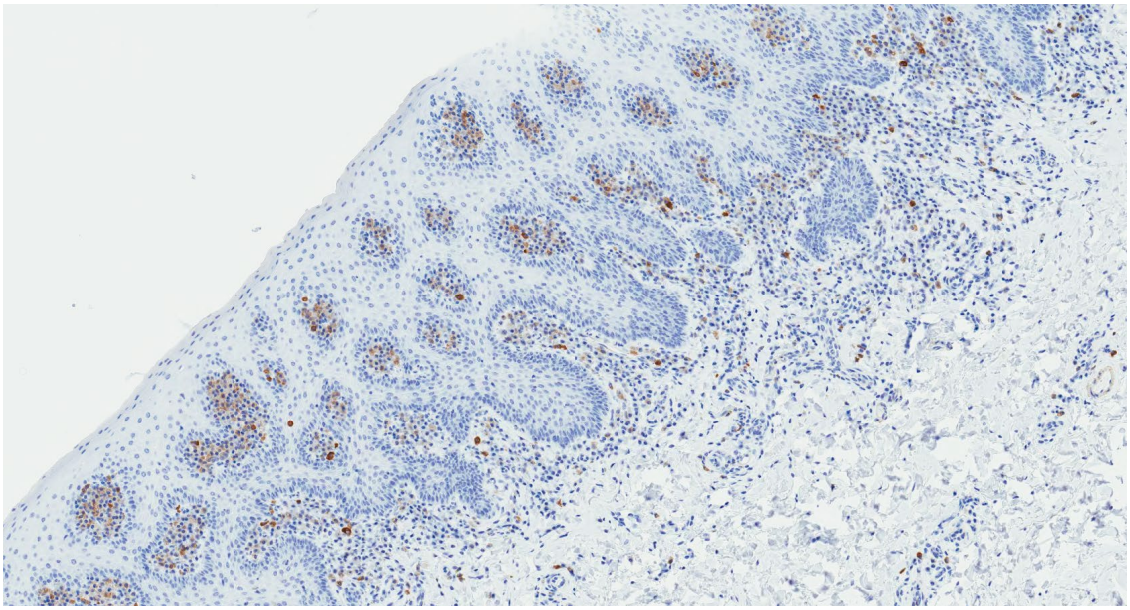

Figure 17. IHC CD79a stained section of proximal penile epithelium from an eighteen-month-old bull with moderate CD79a positive B lymphocytes

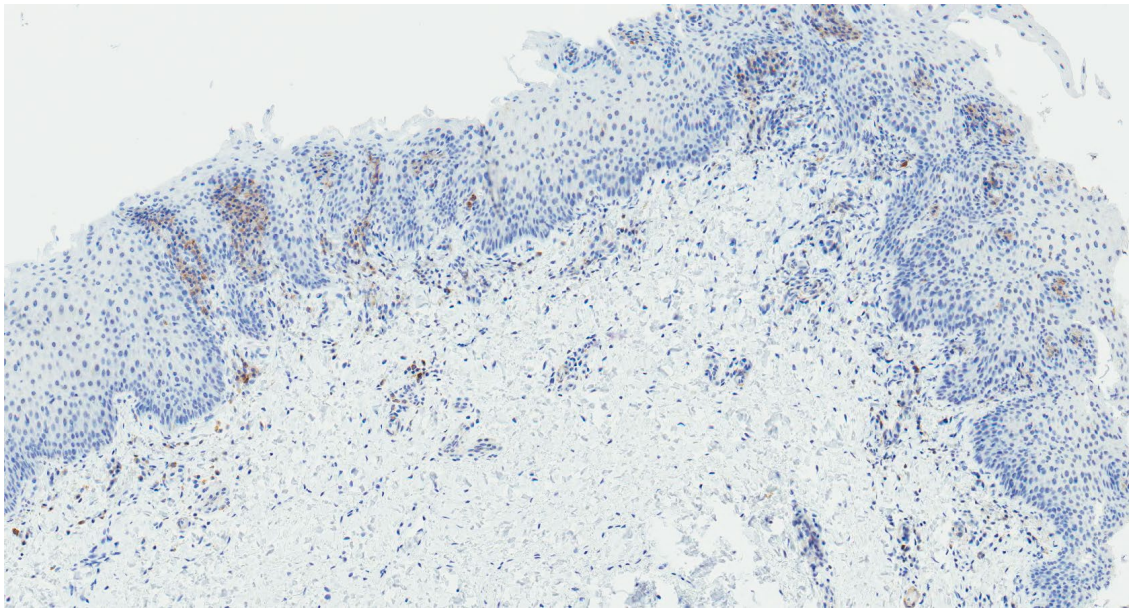

Figure 18. IHC CD79a stained section of preputial epithelium from five-year-old bull with mild CD79a positive B lymphocytes in the dermis

1.4 MUM1 Plasma Cells

The MUM1 immunohistochemistry marker did not stain cells in any of the samples. A positive control (bovine colon) was run for MUM1 and stained successfully. Hence, there seems to be a low prevalence of MUM1 Plasma cells in all of the bulls sampled. There was insufficient data to perform a statistical analysis on the presence of these cell types.

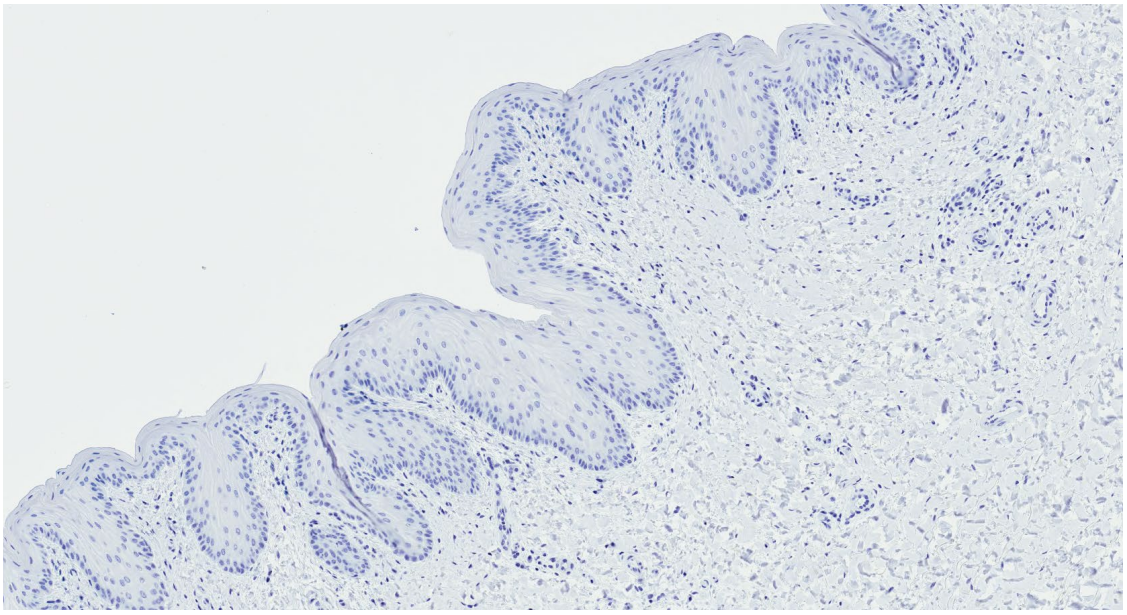

Figure 19. IHC MUM1 stained section of proximal penile epithelium from an eighteen-month-old bull revealing absence of staining for MUM1 plasma cells

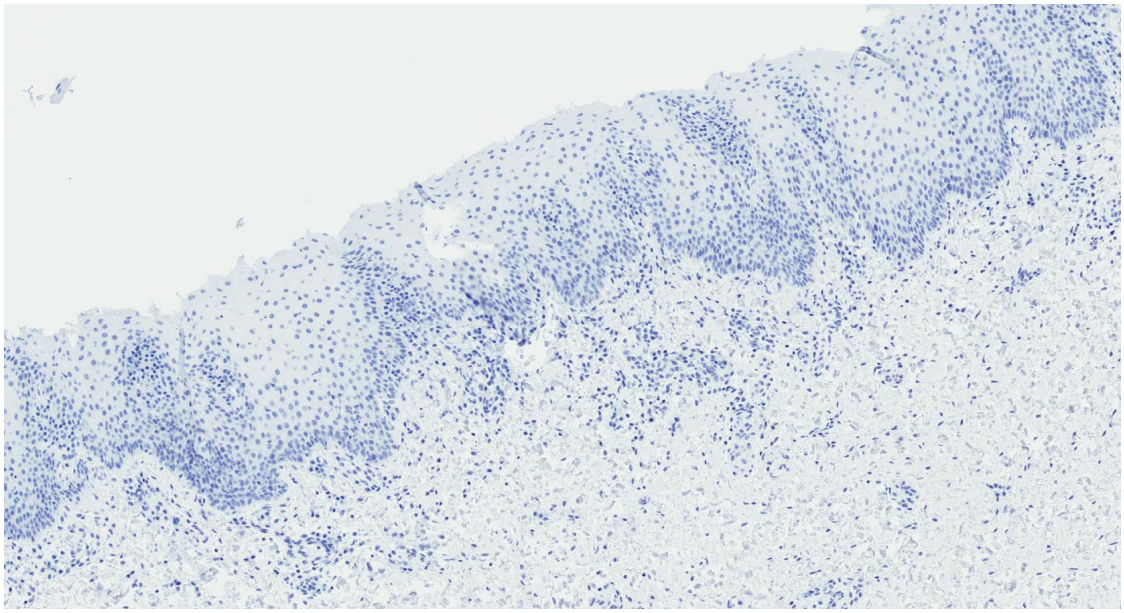

Figure 20. IHC MUM1 stained section of preputial epithelium from a five-year-old bull revealing absence of staining for MUM1 plasma cells

1.5 IBA1 Positive Macrophages

The IBA1 positive macrophages were located at both epidermal-dermal interface and around dermal vessels. There was a significant difference ($p<0.0001$) in the number of IBA1 positive macrophages in Group A versus what was found in Group B located in the epidermal-dermal interface (Figure 21) and in the dermis (Figure 22). There were a greater number of IBA1 positive macrophages found in bulls in Group B in the epidermal-dermal interface and in the dermis than in Group A. There was no significant difference in the number of IBA1 positive macrophages found in the epidermal-dermal interface ($p=0.7826$) and in the dermis ($p=0.4594$) between the locations sampled. However, there was significant difference was found for the interaction between bull type and location at the epidermal-dermal interface ($p=0.1919$) but there was a significant difference in the dermis ($p=0.0285$).

Table 10. Statistical significance evaluation of bull type, location, and the interaction between bull type and location for the number of IBA positive macrophages in the epidermis

	F-value	P-value
Bull Type	76.4347	<0.0001
Location	0.2492	0.7826
Bull Type*Location	1.8463	0.1919

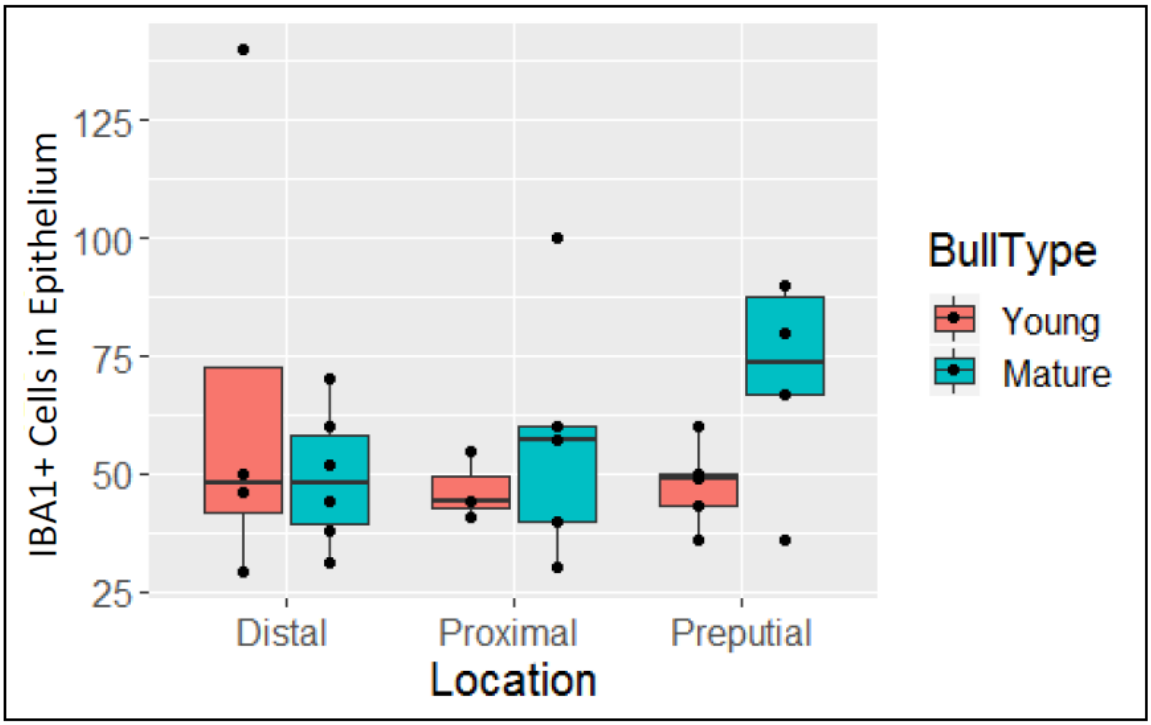

Figure 21. Graph illustrating the number of the IBA1 positive macrophages located at the epidermal-dermal interface at each of the three locations for each group of bulls, young (Group A) and mature (Groups B)

Table 11. Statistical significance evaluation of bull type, location, and the interaction between bull type and location for the number of IBA positive macrophages in the superficial dermis

	F-value	P-value
Bull Type	50.3755	<0.0001
Location	0.8197	0.4594
Bull Type*Location	4.5538	0.0285

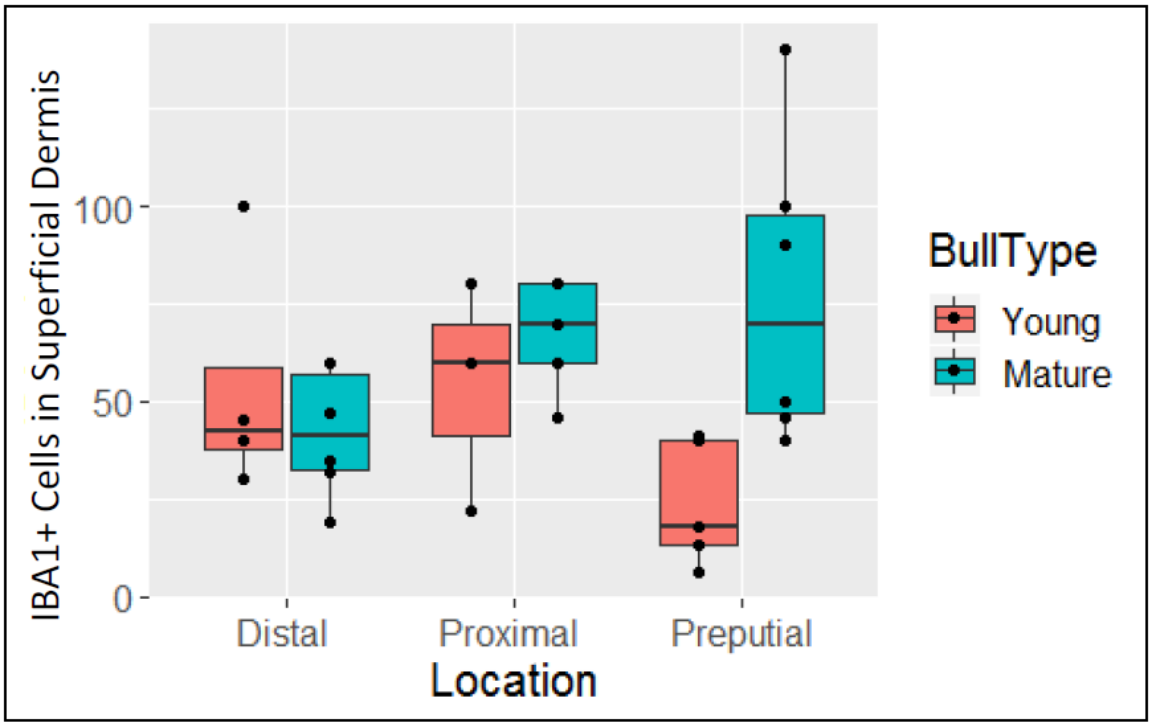

Figure 22. Graph illustrating the number of the IBA1 positive macrophages located in the dermis at each of the three locations for each group of bulls, young (Group A) and mature (Groups B)

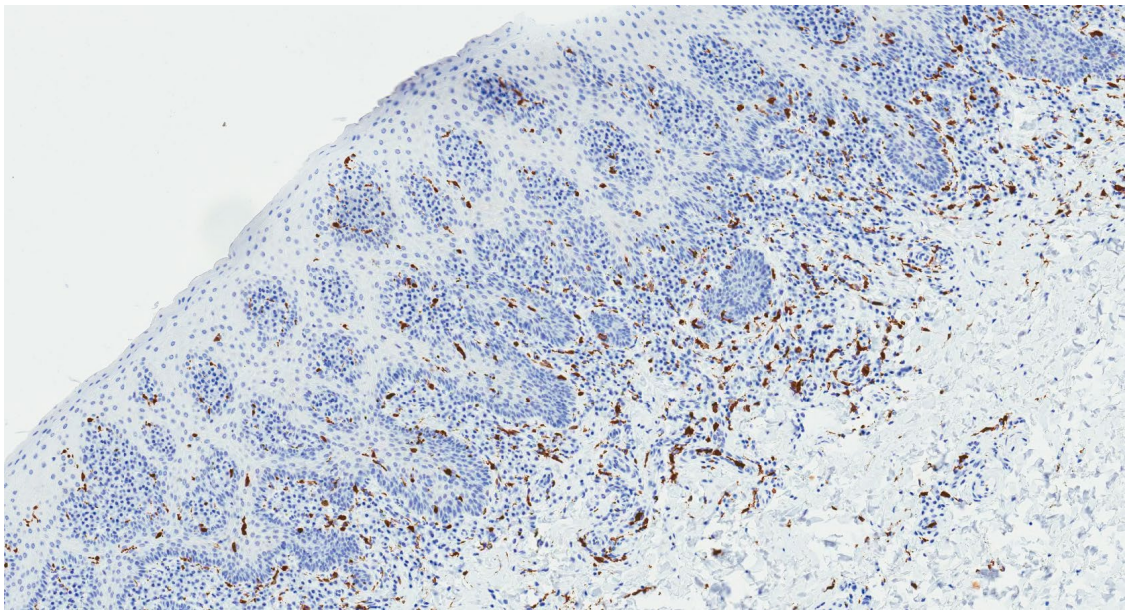

Figure 23. IHC IBA1 stained section of proximal penile epithelium from an eighteen-month-old bull with mild infiltration of IBA1 positive macrophages

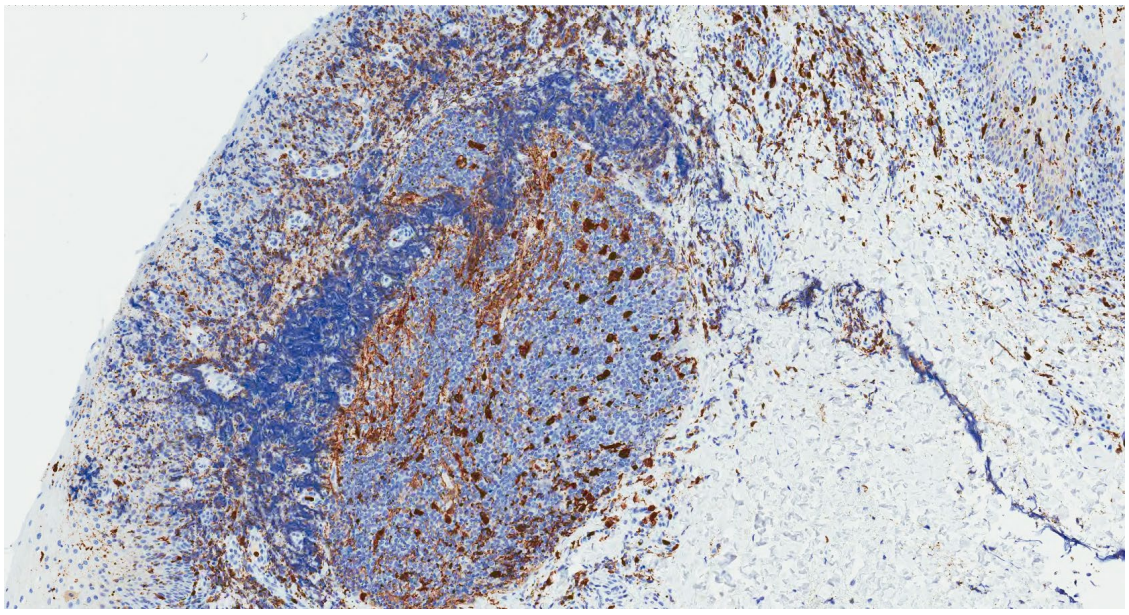

Figure 24. IHC IBA1 stained section of preputial epithelium from a seven-year-old bull with pronounces infiltration of IBA1positive macrophages in the dermis, epidermis, and within the dermal lymphoid follicle

Table 12. Averages of cell type, disruption of the basement membrane and basal cell hyperplasia by age and location on a 0-3 scale

	Inflammation cellular infiltration	Mononuclear cells	Epidermal dermal junction disruption of basement membrane	Epidermal dermal junction basal cell hyperplasia
Group A Distal Penis	1.60	0.300	1.720	1.200
Group A Proximal Penis	1.33	NA	1.566	0.900
Group A Prepuce	NA	1.333	1.540	1.320
Group B Distal Penis	0.750	0.516	0.933	1.167
Group B Proximal Penis	1.600	0.840	1.580	1.500
Group B Prepuce	1.360	0.383	1.700	1.633

*NA = not applicable; slide was able to be read

Table 13. Averages of the total number of CD3+, CD79a+, and IBA cells by age and location.

	CD3 cells in the epidermal-dermal junction	CD3 cells around superficial vessels	CD79a+ cells in submucosa and around vessels	IBA cells in the epidermis	IBA cells in superficial dermis
Group A Distal Penis	30	6.6	39	64	49.2
Group A Proximal Penis	38.3	6	21.67	46.67	54
Group A Prepuce	45	5	NA	47.6	NA
Group B Distal Penis	21	2.67	22.5	49.167	42.167
Group B Proximal Penis	64	6.8	31	57.4	67.2
Group B Prepuce	60.83	7.33	55.67	71.67	77.67

*NA = not applicable; slide was able to be read

1.7 Evaluation of the Disruption of the Basement Membrane

Bull type was found to have significant difference ($p<0.0001$). However, there was no significant difference ($p=0.3825$) between the locations sampled. There was no significant difference ($p=0.0570$) for the interaction of bull type and location but there was a trend.

Table 14. Statistical significance evaluation of bull type, location, and the interaction between bull type and location for the disruption of the basement membrane at each of the three locations for each group of bulls, young (Group A) and mature (Groups B)

	F-value	P-value
Bull Type	124.5398	<0.0001
Location	1.0252	0.3825
Bull Type*Location	3.4874	0.0570

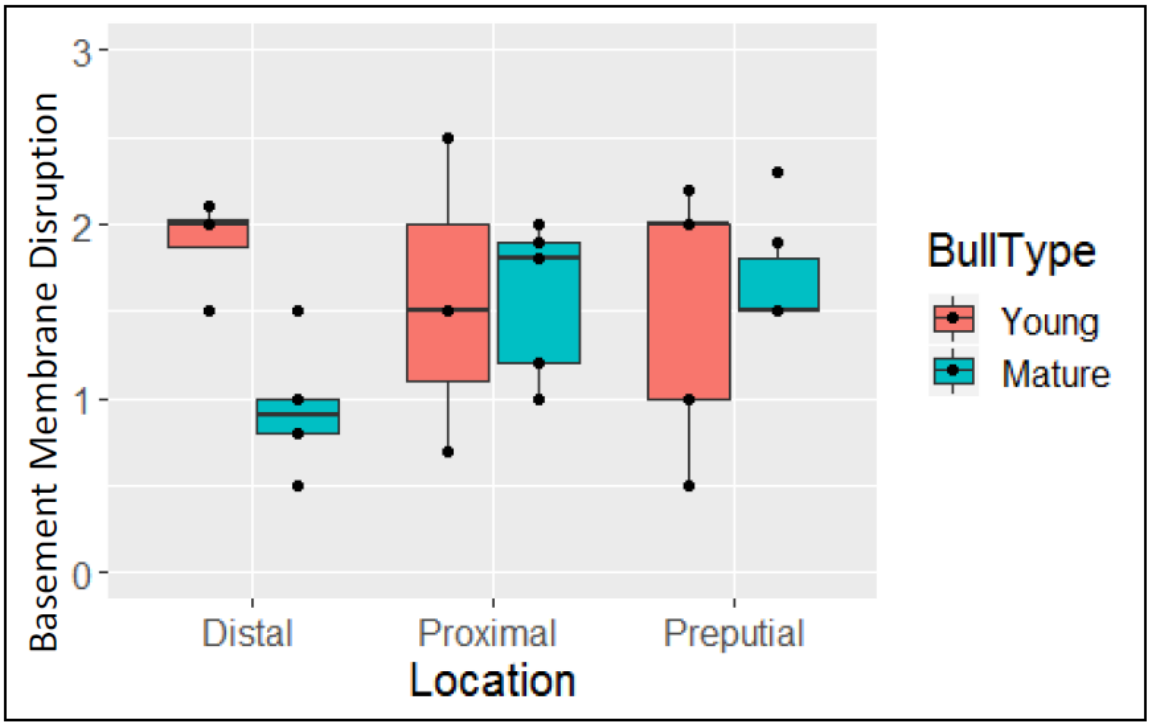

Figure 25. Graph illustrating the ranking of the epidermal-dermal interface disruption of the basement membrane at each of the three locations for each group of bulls, young (Group A) and mature (Groups B)

1.8 Evaluation of the Epithelial Hyperplasia

It was found that there was a significant difference ($p<0.0001$) for bull type. However, there was no significant difference ($p=0.3721$) between the locations sampled. There was no significant difference ($p=0.4522$) for the interaction between bull type and location.

Table 15. Statistical significance evaluation of bull type, location, and the interaction between bull type and location for the epidermal hyperplasia at each of the three locations for each group of bulls, young (Group A) and mature (Groups B)

	F-value	P-value
Bull Type	69.1444	<0.0001
Location	1.0566	0.3721
Bull Type*Location	0.8372	0.4522

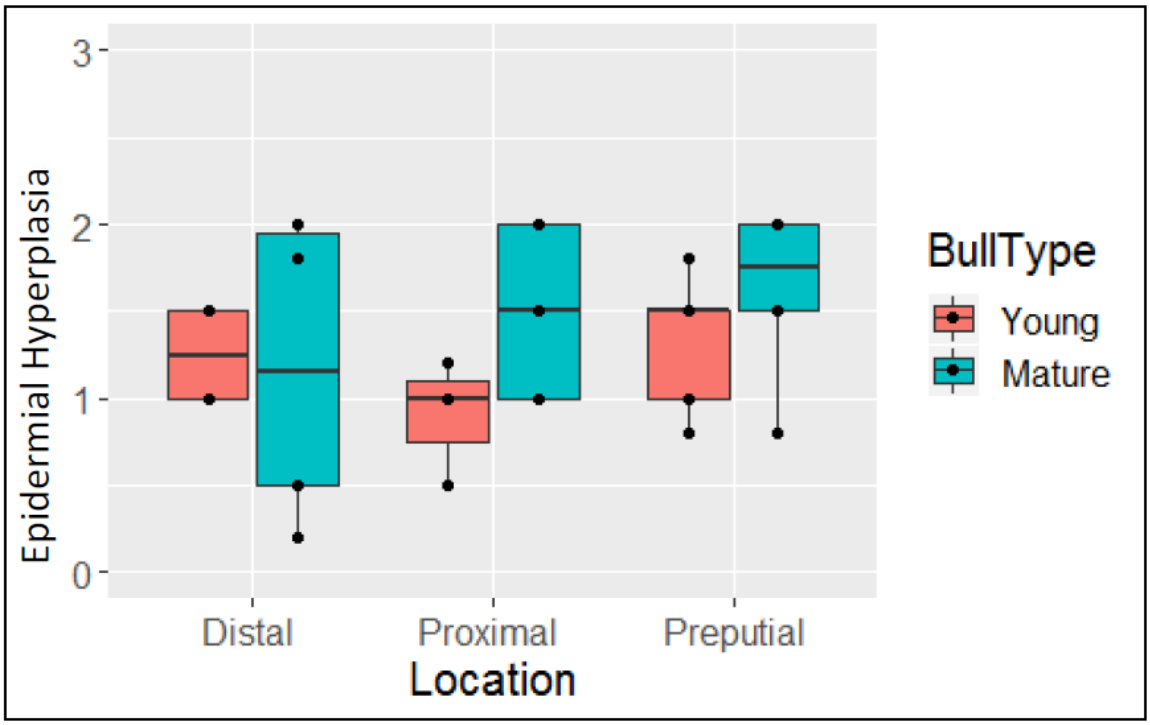

Figure 26. Graph illustrating the ranking of the epidermal-dermal junction basal cell hyperplasia of the basement membrane at each of the three locations for each group of bulls, young (Group A) and mature (Groups B)

DISCUSSION:

Very little is known about changes that occur in the bovine penile and preputial epithelium as the animal ages. The purpose of this study was to evaluate and compare changes in the penile and preputial epithelium of bulls in different age groups. The results support that there are differences in the cellular population present in the penile and preputial epithelium as bulls age, thus the null hypothesis is rejected.

Inflammatory cell infiltration and marginated neutrophil scores were generally greater in the immature group compared to the mature group. These results could be explained by how these animals were housed. Five of the six animals in this group came from the same farm and were kept in a pasture with other bulls approximately the same age. Homosexual mounting or riding behavior commonly occurs among young bulls housed together (Chenoweth, 1981; Hopkins, 2007). Bull with a strong libido exhibit a great incidence of mounting (Jezierski et al., 1989). The penis is protruded from the sheath during riding behavior and often contacts the rump of the standing bull, inducing irritation and abrasions. This activity can result in inflammation, facilitate the transmission of penile warts, and hair ring formation (Hopkins, 2007; Wolfe, 2015). Regardless of cause, higher numbers of inflammatory cells would potentially be beneficial to the protection from and potential clearance of trichomoniasis infections in bulls.

The CD3 positive T lymphocytes were found to be more prevalent in the young population (Group A) at the distal site in both the epidermal-dermal interface and around the superficial dermal vessels as compared to the mature population (Group B). Group B had greater numbers of CD3 positive T cells at the proximal site and the preputial site of

the epidermal-dermal interface and around the superficial dermal vessels as compared to Group B population. A previous study comparing preputial cellular and antibody response seen in vaccinated and *T. foetus* challenged bulls showed that bulls in the vaccinated group had higher numbers of CD3 positive T lymphocytes in the epidermis close to the basement membrane (Cobo et al., 2009). Cobo et al. did not compare the animals by age but did show that the vaccine promoted cellular immunity more than infection (2009). Thus, it might be possible for young vaccinated animals to mount a better CD3 positive T lymphocyte immune response as compared to mature animals. Age associated differences in the CD3 positive T lymphocyte response following vaccination against T. foetus warrants further investigation.

The location was found to be significant ($p=0.00108$) for CD79a positive B lymphocytes. The CD79a positive B lymphocytes were found to be more prevalent in the young population (Group A) at the distal penile biopsy site in the young group as compared to the mature population (Group B). At preputial and proximal penile biopsy sites the mature group possessed higher levels of CD79 positive B cells as compared to the young population. This suggests that the two population of animals may be capable of producing different local immune responses. Young bulls tend to masturbate more than older bulls which has the end of the penis coming in contact with the environment and potentially more abrasive objects. Mature bulls have bred more cows than young bulls, thus the proximal penile and preputial tissues have made more contact with vaginal mucosa providing for a greater potential for physical irritation, inflammatory response, and exposure to pathogens. Mature bulls typically receive yearly vaccinations to promote the immune system. These variances between groups could account for the different

levels of CD79a positive B cells at distinct locations. A previous study comparing preputial cellular and antibody response seen in vaccinated and *T. foetus* challenged bulls showed that bulls in both groups had CD79a positive B cells in the epidermal dermal junction and in the subepithelial lymphoid nodules with a higher level in the vaccinated animals (Cobo et al., 2009). Cobo et al.'s study documents cellular response in these tissues following vaccination or natural infection, but did not compare response by age (2009). The finding from the current study suggest that the younger animals may possess a heightened ability to mount a cellular immune response.

The absence of plasma cells in almost all samples cannot be fully explained. The positive control (bovine colon) run concurrently with the samples successfully identified MUM1, eliminating the possibility of staining failure. Previous studies have detected an increase in plasma cells in the genital skin as bulls age, associated with venereal infection, and following vaccination for *T. foetus* or *C. fetus venerealis* (Flower et al., 1983). The absence of plasma cells in samples included in the present study might be due by the population in this study as all bulls were negative for the presence of *T. foetus* and had not been vaccinated for *C. fetus venerealis*. Further investigation is needed.

IBA1 positive macrophages were found to be more prevalent in the mature population (Group B) at both the epidermal-dermal interface and around the superficial submucosal vessels as compared to the young population (Group A). Previous studies looking at bovine preputial tissue have not included macrophages in the analysis. A possible explanation for the higher level of macrophages in the mature population is that these animals have potentially be exposed to more infectious agents than the young population. Macrophages may reside in tissues for a prolong periods thus the mature

group could have higher levels. It is possible that macrophages may provide more protection in these tissues as the animal's age.

Evaluation of the basement membrane disruption has not been previously described. In the study, the distal penile location had more disruption than the mature group. This could be attributed to younger bulls having more homosexual mounting behavior, thus the distal penis would make repetitive contact with abrasive surfaces such as the rump of another bull.

Epithelial hyperplasia was seen to be greater in the mature group. This could be accounted for by the sexual rest the animals had received, as the bulls were sampled prior to the breeding season. Therefore, there was potentially less exfoliation of cells which might occur more during breeding in the mature group. In the current study, the evaluation was a subjective evaluation based on number of cellular layers present. The only other study to look at this was the Strickland et al. study, where they compared the thickness of the epithelium and no difference was found better the young and mature groups (2014).

This preliminary study demonstrates differences in the immune cell populations presented in the preputial and penile epithelium of bulls as they age. Higher lymphocyte scores in the younger population and greater macrophage scores in the mature population suggest that the cellular immune response of the skin of the external genitalia of male bovines is modified as the animals age. Additional research is necessary in this area, with larger numbers of animals and *T. foetus* positive animals included in future studies.

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APPENDIX

Sample	age	location	Inflamatio	MN 0-3	CD3 E nun	CD3V-nun	CD79 smn	CD79a-N	IBA epi nu	IBA sd nur	EDJ dis	EDJ bch	HE comment		
101	2	1	0.8	0.4	24	1	25	0	31	47	0.8	0.5	plasma cells in lymphatics		
102	2	2	1	1	100	10	120	3	57	80	1	1			
103	2	3	1.2	0.5	70	20	170	3	67	100	1.5	2	Nodules are not CD3 pos		
201	2	1	0.3	0.2	15	1	9	0	44	19	1	0.2			
202	2	2	2	1	100	11	8	1.8	60	70	1.2	1.5			
203	2	3	2	0.8	70	10	19	2	90	140	1.5	1.5			
301	1	1	2	0	51	12	25	0	29	100	1.5	1			
302	1	2	1.5	0.7	52	10	5	0	55	80	1.5	1.2			
303	1	3	2	0	76	6	30	1	36	13	2	1.5			
401	1	1	2.2	0.8	40	8	40	2	46	40	2	1.5			
403	1	3	2.5	1.5	54	8	bad stain	2	50	np	2	1.8			
502	1	2	0.5	0.5	3	4	np	0	44	22	0.7	0.5			
503	1	3	2	1	31	2	0	1	43	6	1	1.5			
601	1	1	2	0.5	37	2	50	0	140	30	2	1.5			
603	1	3	1.5	0	19	2	15	0.5	60	18	2.2	0.8			
701	1	1	1	0.2	17	8	80	0	50	45	2.1	1			
702	1	2	2	0.2	60	4	60	0	41	60	2.5	1			
703	1	3	missing		45	7	bad stain	0	49	40	0.5	1			
801	2	1	0.5	0	30	6	15	0.5	52	32	1.5	2			
802	2	2	1.5	1.5	18	7	10	1.5	30	46	1.8	1	Focal het dermatits		
803	2	3	2	0	50	2	40	1	90	40	2.3	0.8			
901	2	1	0.5	1	10	1	25	0	38	35	1	0.5			
903	2	3	1	1	70	3	55	1	80	46	1.9	1.5			
1001	2	1	0.4	0.5	35	6	1	0	60	60	0.8	1.8			
1002	2	2	1	0.5	80	4	5	0	100	60	2	2			
1003	2	3	1	0	85	5	30	1	67	50	1.5	2			
1101	2	1	2	1	12	1	60	0	70	60	0.5	2			
1102	2	2	2.5	0.2	25	2	12	0	40	80	1.9	2			
1103	2	3	1	0	20	4	20	0	36	90	1.5	2			
1201	1	1	0.8	0	9	3		0	55	31	1	1			

KEY

General inflammatir	Objective grading of overall inflamation
MN 0-3	Objective grading of marginated neutrophils
CD3E num	Number of CD3 cells in the epidermal-dermal juction
CD3 v num	Number of CD3 cells around superficial vessels
CD79 smnum	Number of CD79 cells in submucosa and around vessels
CD79a-N	CD79nod num = num cells obserfed in nodules within 20x field
IBAepi num	Number of IBA cells in the epidermis
IBA sd num	Number of IBA cells in superficial dermis
IBA-Nodule	Prominent 79a postive lymphatic nodules in dermis
EDJ dis	Epidermis dermal juction disruption of basement membrane
EDJ bch	Epidermis-dermal juction basal cell hyperplasia