

**Surface Architectural Anatomy of The  
Penile and Preputial Epithelium of Bulls**

by

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## Abstract

It has been a long held assumption that older bulls develop deeper folds or crypts in the epithelium covering the penis and prepuce than are found in their younger counterparts. These deeper crypts reportedly facilitate the carrier state of bovine venereal disease by providing a protected environment suitable for long term maintenance of infection. No published reports have been found to support this assumption. This study was conducted to characterize the surface architectural anatomy of the epithelium and epithelial crypts in younger and older bulls.

This survey included Angus bulls sampled between December 2008 and March 2009. Bulls were placed into two groups of six according to age. Group 1 consisted of bulls 2 years of age, and group 2 consisted of bulls  $\geq 5$  years of age. Penile epithelium was collected from three anatomical locations (Proximal, Middle, and Distal) and tissue samples were prepared for examination by light microscopy and scanning electron microscopy. Three parameters were examined; 1) area of the epithelium per unit of linear measurement, 2) area encompassed by the folds, and 3) total number of folds per unit of linear measurement. Findings were then compared within and between the age groups. Results indicated that there are no significant differences in the area of the epithelium, area encompassed by the epithelial folds or total number of epithelial folds per unit of linear measurement between the two age groups.

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## Chapter 1

### Introduction

The gross anatomy of the bovine penis and prepuce has been described in detail, and the intricate mechanisms leading to erection and ejaculation defined.<sup>(1)</sup> Compounded wrinkles and folds of the prepuce epithelium and creases on the surface of the glans penis are present and thought to allow for the mechanics of extension during erection (Personal communication Dr. David Bartlett). The surface architecture of the genital epithelium has been speculatively discussed for decades but qualitative study of the microscopic anatomy and the effects of ageing are lacking. Pervasive opinion suggests that the epithelial microstructure of the penis and prepuce undergoes significant age-associated changes and that these changes are important in establishment of a state of persistent infection with *Trichomonas foetus* (*T. foetus*) or *Campylobacter fetus* subsp. *venerealis* (*C. fetus*).<sup>(2)</sup>

Trichomoniasis and campylobacteriosis, the two most commonly recognized bovine venereal diseases, results in substantial economic loss in cow herds. Bulls may be asymptomatic carriers for either the protozoan *Trichomonas foetus* (*T. foetus*) or the bacterium *Campylobacter fetus* subsp. *venerealis* (*C. fetus*). Infection in females may result in early embryonic death, abortion, pyometra, fetal maceration, or infertility, all of which negatively influence the profitability of a cattle operation. Either venereal disease may persist without detection in endemic herds.<sup>(3-6)</sup> Herd infection with either organism significantly reduces a cattle producer's profits.

Persistence of infection with these organisms is thought to be related to deeper epithelial folds in the preputial epithelium of mature bulls, and these folds are popularly designated as crypts. Little information has been published on the micro-architecture of the penile and preputial epithelium. Epithelial crypts are poorly defined on the penis and prepuce of the bull. In contrast, Baillière's *Comprehensive Veterinary Dictionary* defines a crypt as a blind pit or tube-like structure on a free surface.<sup>(7)</sup> The Crypts of Liberkühn are defined as a lumen of intestinal glands on the surface of the intestinal mucus membrane that serve to secrete or are absorptive in nature.<sup>(8)</sup>

The hypotheses of this study are: 1) important differences exist between the surface architectural anatomy of the penile and preputial epithelium of bulls of different ages, 2) epithelial coverings of the penis and prepuce become thicker as bulls mature, 3) the epithelium develops an increased number of infoldings with age, and 4) the area contained within these infoldings increases with age. This study will compare the above parameters in bulls 2 years of age and bulls  $\geq 5$  years of age.

## **Chapter 2**

### **Literature Review**

#### **Penile Anatomy of the Bull**

The skin covers the body and protects it against injury while providing temperature regulation through heat dissipation or by surface evaporation. The skin consists of two layers, the outer epidermis and the inner dermis. The epidermis is composed of stratified squamous epithelium and the dermis is mostly connective tissue fibers. The surface of the penis and prepuce within the preputial cavity are among the very few non-haired areas of skin present on the bovine.

The penis of the bull is classified as fibro-elastic and it functions as the shared outlet for the excretion of urine and the deposition of semen into the female reproductive tract during copulation.<sup>(8)</sup> The penis is cylindrical and extends forward from the ischiatic arch to the umbilical region of the abdominal wall. It is supported by the penile fascia and skin and is situated in the sheath.<sup>(9)</sup> The penis of the adult bull is almost 1 meter long with approximately a quarter of its length incorporated in a sigmoid flexure. Paired retractor penis muscles insert on the ventrum of the distal bend of the sigmoid flexure and serve to hold the penis within the prepuce during the resting state. These muscles also serve to retract the penis and prepuce into the sheath following detumescence.<sup>(10)</sup> The free portion of the non-erect penis is approximately 12cm long and lies within the caudal portion of the preputial cavity when the

penis is non erect.<sup>(11)</sup> Because the penis serves as the organ of copulation and ejaculation of spermatozoa, much of the penis consists of erectile tissue. The erectile tissues are composed of the corpus cavernosum penis, corpus spongiosum penis, and the glans penis.

The corpus cavernosum of the penis (CCP) consists of a paired dorsal column of erectile tissue surrounded by dense connective tissue, the tunica albuginea.<sup>(8)</sup> The corpus cavernosum penis is spongy and divided into many spaces. Erection of the penis occurs with sexual stimulation which results in dilatation of the arteries that supply blood to the penis. Blood flow increases from the deep artery of the penis into the crus penis and eventually the CCP. As sexual stimulation is continued the ischiocavernosus muscles begin rhythmic contractions and occlude arterial and venous outflow of the penis creating a closed hydraulic system. Blood is then forced into the CCP from the crus penis and significantly increases the CCP pressure. Ventral to the corpus cavernosum, and partially surrounded by it, is the corpus spongiosum penis (CSP) which is a smaller column of erectile tissue that surrounds the urethra.

The free portion of the penis is distal to the attachment of the prepuce and lies within the caudal portion of the preputial cavity when non-erect. The free portion is capped by a small cushion of asymmetrical, ventrally directed, slightly spiraled tissue which comprises the glans penis.<sup>(11)</sup> Sensation from the glans is necessary for intromission and ejaculation.<sup>(12)</sup>

The prepuce of the bull is 35 to 40 cm long and approximately 4 cm in diameter with wide variations among breeds.<sup>(13)</sup> The prepuce receives its blood supply from branches of the internal iliac artery and is composed of an external, parietal, and visceral layer. The external portion, the sheath, is haired skin which is continuous with the abdominal skin. The external layer turns inward at the preputial opening to become the parietal prepuce. This, in turn, reflects at the fornix and continues onto the preputial attachment to the free portion of the penis as the

visceral prepuce.<sup>(14,15)</sup> The parietal and visceral layers of the prepuce are covered with stratified squamous epithelium, and nerve innervation is from branches of the ventral abdominal (T13, L1-4) and pudendal (S2-4) nerve trunks.<sup>(11)</sup>

As bulls age there is an increase in plasma cells in the preputial and penile epithelium that is considered to be a response from repeated infectious challenge. Bulls naturally infected with or vaccinated against antigens derived from *Campylobacter fetus* subsp. *venerealis* have an increase of specific immunoglobulins IgG<sub>1</sub>, IgG<sub>2</sub>, IgA, and IgM in the preputial and penile epithelium. These immunoglobulins are directed against antigens from *C. fetus* subsp. *venerealis*.<sup>(16)</sup> Natural infection with *T. foetus* also results in deposition of specific IgG<sub>1</sub>, IgG<sub>2</sub>, IgA and IgM antibodies in the preputial cavity.<sup>(17)</sup> Immunoglobulins IgG<sub>1</sub>, IgG<sub>2</sub>, IgE, and IgA directed against *T. foetus* are elevated in the preputial secretions post vaccination with *T. foetus* antigens.<sup>(18)</sup>

The free portion of the penis is covered with stratified squamous epithelium which is very tightly adhered over the apex of the distal end. This epithelium transitions caudally to become more loosely attached at the junction of the free portion and prepuce allowing the epithelium to change its orientation when the penis is extended or withdrawn.<sup>(11)</sup> The most distal portion of the glans penis is encapsulated by the smooth cap-like galea glandis, while the remainder of the glans has a rough uneven epithelial surface. The epithelium of this area is characterized by a network of fine infoldings which vary from slight irregular depressions to relatively deep, closely-arranged crevices that produce a papillate appearance.<sup>(19)</sup> It has been alleged that these infoldings or crypts become deeper as a bull matures, and that this infolding of the epithelium provides a suitable protected environment for the development of a chronic carrier state of venereal diseases.<sup>(20)</sup>

## Venereal Diseases

*Tritrichomonas foetus*, an obligate protozoal parasite of the bovine reproductive tract, is a pyriform protozoan with a rounded anterior end and a pointed posterior end. It varies in size from 10 to 25  $\mu\text{m}$  in length and 5 to 10  $\mu\text{m}$  in width.<sup>(21)</sup> The preliminary identification of the organism is based on: 1) size and single nucleus, 2) presence of three anterior flagella and one posterior flagella, 3) a distinct undulating membrane along one side of the protozoa, and 4) characteristic motility. Ancillary testing by polymerase chain reaction is necessary for positive identification.

The male is an asymptomatic carrier of *T. foetus* while the female suffers identifiable consequences of infection. *T. foetus* in bulls is found in the smegma (secretions) of the epithelial lining of the penis, prepuce, and distal urethra.<sup>(22)</sup> The organism does not invade the epithelium and fails to invoke a protective immune response by the bull.<sup>(23)</sup> *T. foetus* infection causes no penile or preputial lesions and does not affect libido.<sup>(22,24)</sup> Infected bulls have no observable changes in semen quality.<sup>(22)</sup> Conversely, *In-vitro* exposure of spermatozoa to *T. foetus* for one hour resulted in decreased spermatozoa motility, agglutination of sperm cells, and eventual phagocytosis of spermatozoa by the trichomonads.<sup>(25)</sup>

Infection in young bulls (less than 3-4 years of age) is most often transient, with disease transmission only occurring if sexual contact with a noninfected female occurs within minutes to days following breeding of an infected female. Clearance of the organism by a young bull may be possible within 20 minutes following breeding an infected cow.<sup>(23,26)</sup> Bull to bull transmission has been theorized in a case study in which young virgin bulls were housed with infected bulls. The assumption is that the penis of uninfected bulls became infected during homosexual mounting of another animal recently mounted by an infected bull.<sup>(27)</sup> This is considered a rare

event, but possible if “clean” and “dirty” bulls are kept in close confinement. Trichomonads morphologically similar to *T. foetus* have been described in the feces of cattle.<sup>(28)</sup> Bulls housed together often mount and sodomize each other making fecal contamination of the preputial cavity with a non-*T. foetus* trichomonads possible. Differentiating non-pathogenic fecal trichomonads from *T. foetus* becomes an important diagnostic challenge.<sup>(29)</sup>

Infection of the cow or heifer with *T. foetus* occurs during coitus with an infected bull. Following vaginal deposition, the organism transverse the cervix and colonizes the entire reproductive tract within 1-2 weeks.<sup>(20)</sup> Pregnancy may be established in the face of infection, as fertilization and early development of the embryo are not necessarily compromised.<sup>(30)</sup> The viability of the conceptus may be maintained until after maternal recognition of pregnancy, which occurs from days 14 to 18 after a fertile breeding, resulting in an affected cow with a prolonged interestrus interval.<sup>(31)</sup> The cause of pregnancy loss has is not been defined, but prevalent theories include: 1) overgrowth of the organism resulting in separation of the fetomaternal interface, 2) antigens from the parasite’s surface triggering a destructive immune response, and 3) a cytotoxic effect on fetal and maternal tissues.<sup>(20)</sup> Pyometra and abortion are sometimes the first signs of trichomoniasis noticed in herds, but these occur in less than 5 % of infected animals.<sup>(24)</sup> A small number of cows will abort in the second or even third trimester, and an even smaller number of cows (less than 1%) will maintain an infection through a normal gestation.<sup>(32)</sup> The few cows capable of maintaining a *T. foetus* infection throughout gestation represent a source of reinfection for the herd during the following breeding season.

Bovine genital campylobacteriosis is also a sexually transmitted disease caused by the gram negative microaerophilic rod *Campylobacter fetus* subsp. *veneralis*. Similar to *Tritrichomonas foetus*, this organism is an obligate parasite of the bovine reproductive tract with

bulls serving as asymptomatic carriers. Bulls younger than three years of age tend to be resistant to persistent infection and clear the organism, whereas mature bulls become chronically infected. It has been suggested that age related changes in the penile and preputial epithelium provide favorable habitat for *C. fetus* subsp. *venerealis* survival.<sup>(33)</sup>

Clinical signs of infection with *C. fetus* subsp. *venerealis* in the cow are similar to those of trichomoniasis. Campylobacteriosis is associated with vaginitis, cervicitis, and endometritis. Infection leads to an early embryonic death as a result of the inflammatory response in the uterus and uterine tubes.<sup>(34)</sup> In closely monitored herds, an increased number of repeat breeders will be identified. The hallmark of infection is irregular and delayed return to estrus. Herd pregnancy rates are decreased, and a wide range of gestational ages may be found at the time of pregnancy examination.

The asymptomatic chronic carrier state associated with either *T. foetus* or *C. fetus* subsp. *venerealis* infections of bulls older than 4 years of age rarely spontaneously resolves.<sup>(35)</sup> The carrier state of both organisms has been apocryphally related to the depth of the preputial and penile epithelial crypts for decades.<sup>(2,20,21)</sup> Reports of the location of *T. foetus* along the penis and within prepuce have varied. Hammond and Bartlett reported that trichomonads were most often found on the glans penis.<sup>(19)</sup> Rhyan *et al* reported trichomonads more commonly in the crypts along the midshaft of the penis.<sup>(17)</sup> Peter, in a review, states that most trichomonads are located in preputial crypts.<sup>(36)</sup>

### **Therapy**

Because both of these diseases are venereally transmitted, artificial insemination of the entire herd maybe utilized to reduce or eliminate these venereal diseases from a producer's



operation. Annual testing for disease and culling of positive animals, as well as using younger test negative bulls are additional tools that can be useful in reduction of disease.

Vaccination against *C. fetus* subsp. *venerealis* is reported as protective and curative for the bull and cow.<sup>(33)</sup> Vaccination against *T. foetus* is reported to be effective in the cow<sup>(37)</sup>. In the bull, most reports state that vaccination is of limited to no value.<sup>(2,37,38)</sup> However, some reports indicate that vaccination against *T. foetus* may be protective in the bull.<sup>(39,40)</sup> Cobo *et al* noted that immunoglobulins IgG<sub>1</sub>, IgG<sub>2</sub>, IgA, and IgE directed against *T. foetus* appeared in the preputial secretions of bulls vaccinated with *T. foetus* antigens. Immunoglobulins IgG<sub>1</sub> and IgG<sub>2</sub> directed against *T. foetus* were elevated in the serum. Vaccinated bulls demonstrated resistance to *T. foetus* colonization.<sup>(18)</sup>

One complicating factor with bovine trichomoniasis in the United States is the lack of effective treatments with U.S. Food and Drug Administration approval.<sup>(20)</sup> Historically, the most successful treatment for bulls with trichomoniasis was systemic treatment with nitromidazole derivatives.<sup>(41)</sup> Currently, the use of nitromidazole derivatives is illegal in food-producing animals in the U.S., and no effective alternative treatments are available. The lack of effective, approved therapies for bovine trichomoniasis emphasizes the need for appropriate preventive and control measures.

### **Prevalence**

Several estimates are available regarding the prevalence of trichomoniasis in different regions of North America. In 1964, Johnson reported a 7.5 % prevalence in western range bulls.<sup>(42)</sup> Later studies from Florida, Oklahoma and California found prevalence rates of 7.3, 7.8 and 4.1 %, respectively.<sup>(43-45)</sup> The Florida and Oklahoma studies sampled bulls from sale barns or abattoirs, while the California study sampled bulls from randomly selected herds. Rae *et al*

reported a 6% prevalence of *T. foetus* in randomly selected natural service beef herds in Florida between 1997 and 1999.<sup>(46)</sup> In a 2005-2006 study conducted by Rodning *et al*, the prevalence of trichomoniasis in a random subset of southern Alabama beef herds was reported to be < 1%.<sup>(47)</sup> Few or no reports exist regarding the prevalence of bovine genital campylobacteriosis in the United States. Swai *et al* reported a 5.1% prevalence in bulls in Tanzania, and Pefanis *et al* reported a 28.7% in The Republic of Transkei.<sup>(48,49)</sup>

### **Economic Impact**

Economic losses due to venereal disease result from increased culling, increased requirement for herd replacements, lower percentage of cows calving, and lower weaning weight secondary to late calving.<sup>(3)</sup> Fitzgerald *et al* in 1958 estimated an annual loss of \$800 per bull infected with *T. foetus* in large herds.<sup>(50)</sup> In 1979, Wilson *et al* estimated a \$2.5 million annual calf loss due to *T. foetus* in Oklahoma replacement heifers.<sup>(44)</sup> In 1986, Fitzgerald estimated that the total economic impact of *T. foetus* in the USA was \$65 million annually.<sup>(4)</sup> A 1991 study by Speer *et al* estimated that annual losses due to *T. foetus* could approach \$650 million in the United States.<sup>(51)</sup> A report in 2000 indicated the prevalence and economic impact of *T. foetus* may be greater than previously reported.<sup>(52)</sup>

## Chapter 3

### Methods and Materials

#### Study of Penile and Preputial Epithelium in Beef Bulls

The research design (Illustration 1) for the study of penile and preputial epithelium in beef bulls focuses on sampling bulls two years of age and bulls greater than or equal to five years of age.

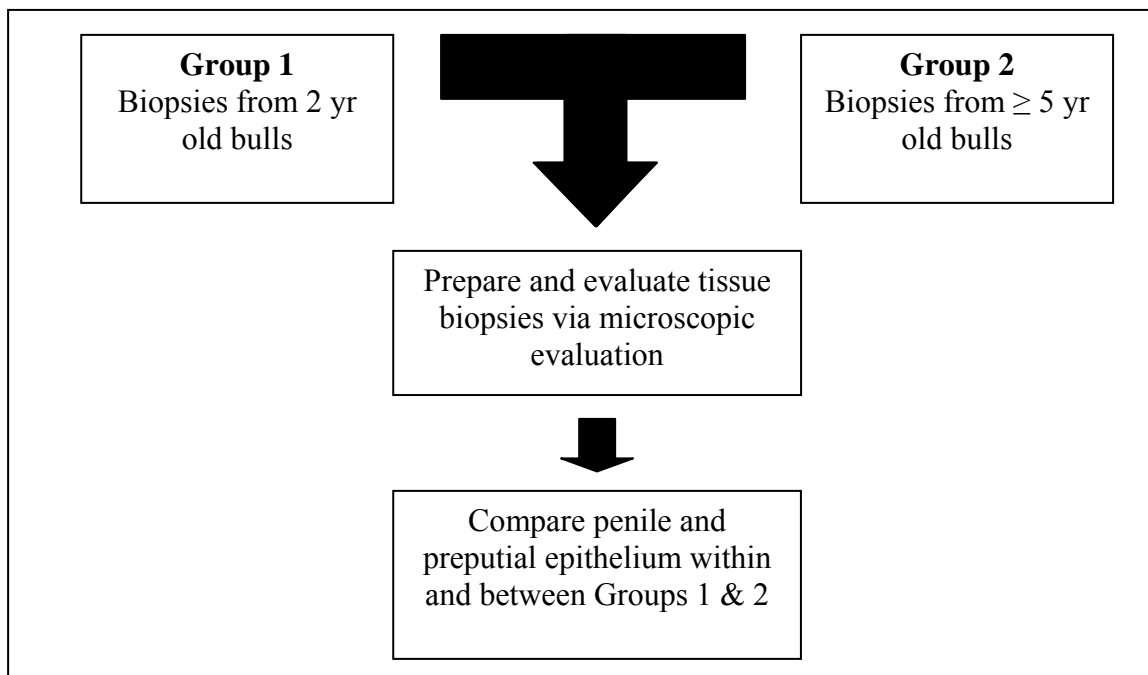


Illustration 1. Research Design Schematic.

## **Animals**

Bulls were placed into two groups of six each according to age. Group 1 (N=6) consisted of bulls two years of age ( $\pm 3$  mo). Group 2 (N=6) consisted of bulls five years of age and older. Ages of both groups were determined by dentition and owner records. These age groups were selected because most bulls enter the breeding herd at 2 years of age and most are removed from the herd at 5 years of age. It is speculated that age-related penile and preputial epithelial differences will be apparent by 5 years of age. All tissue samples were collected from bulls that presented to the John Thomas Vaughn Large Animal Teaching Hospital, Auburn University College of Veterinary Medicine for routine breeding soundness exams. Written consent was obtained from cattle producers prior to tissue collection. Each bull was sampled for presence of *T. foetus* and *C. fetus* subsp. *venerealis* and none were found to be positive.

### **Sampling for *Tritrichomonas foetus***

The external preputial area was cleaned with disposable paper towels without soap or disinfectants. A new pair of exam gloves was used for each bull, and a sterile, dry, plastic infusion pipette (Equine AI pipette Butler<sup>a</sup>) with a 12 mL syringe attached to one end was placed into the preputial fornix. The pipette tip was vigorously scraped across the penile and preputial epithelium prior to the application of negative pressure with the syringe to aspirate approximately 3 mls of the preputial smegma. The negative pressure was released before removing the pipette from the sheath to minimize unnecessary aspiration of urine or other contaminants. After removal of the pipette from the sheath, the sample was immediately placed into modified Diamond's media (Alabama State Diagnostic Lab<sup>b</sup>) and held at ambient

temperature for no more than two hours prior to the transport to the laboratory. A new sterile syringe and pipette was used for each bull. The culture media was incubated for 24 hours at 37° C before examination for trichomonads. Each sample was examined once daily for 5 days by direct light microscopy at 20x and 40x for presence of the trichomonad. Any suspect cultures were submitted for polymerase chain reaction for confirmation of *T. foetus*.

### **Polymerase Chain Reaction (PCR) Verification**

Microscopic identification of *T. foetus* may be complicated by the presence of other trichomonad protozoa.<sup>(53-57)</sup> Contamination of the preputial orifice or cavity with feces likely explains the presence of these opportunistic contaminants. None of the contaminating trichomonads result in reproductive pathology in cows or bulls.<sup>(58)</sup> To avoid false-positives, all suspect samples were submitted to the Alabama State Diagnostic Laboratory for PCR verification (Appendix A). Polymerase chain reaction accurately differentiates *T. foetus* from non-pathogenic fecal contaminants.

### **Sampling for *Campylobacter fetus* subsp. *venerealis***

Samples for *C. fetus* subsp. *venerealis* culture were collected using sterile swabs in the preputial fornix in a similar manner to the pipette method used for *T. foetus* sampling. Swabs were immersed in 1 ml physiologic saline. The resulting fluid was placed on Clark's media (Alabama State Diagnostic Lab<sup>c</sup>) and held at 37° C for transport to the laboratory. Transported samples were inoculated onto blood agar plates and incubated under microaerophilic conditions for 5-7 days at 35-37° C.

### **Biopsy technique**

Each bull was appropriately restrained in a livestock chute to prevent injury to the bulls and personnel. Three separate locations were chosen for biopsy specimens: 1) the distal penis 1cm proximal to the glans, 2) 1 cm distal to the attachment of the prepuce to the free portion of the penis, and 3) the proximal prepuce 6 cm distal to the preputial orifice when the penis is extended. The penis was manually extended, held by sterile surgical gauze, and cleaned with water prior to aseptic surgical preparation. The bilateral dorsal penile nerves were anesthetized with 7 to 12 mls of 2% lidocaine hydrochloride. A #22 scalpel blade was used to excise an approximately 1cm diameter sample of tissue from each target area. Each excision area was closed with #1-0 chromic gut suture material in a cruciate pattern. Anesthesia and collection of all samples were conducted by the same investigator to minimize differences of collection methods. Tissue samples were pressed onto sections of tongue depressors to reduce artifact folding during fixation (Dr. Joe Newton personal communication). Each tissue sample was immersion fixed and stored in a mixture of 4% paraformalin, 1% glutaraldehyde (Electron Microscopy Services, Hatfield, PA<sup>d</sup>), and 150 mM phosphate buffered saline (Sigma Chemical, St Louis, MO<sup>e</sup>) prior to processing.

### **Light Microscopy**

All steps of the tissue preparation were performed under a ventilated hood in compliance with OSHA guidelines for handling potentially toxic chemicals. Tissues for light microscopy were prepared by rinsing in phosphate buffered saline (PBS) in 3 separate rinses of 20 min each to remove fixative agents. Fixative-free tissues were dehydrated with ethyl alcohol (ETOH) in graded strengths to displace water from the tissues. The following steps were performed: 1) each sample was placed in 30% ETOH and agitated at room temperature for 30 min, 2) the samples

were placed in a series of ascending concentrations of ETOH (50%, 70%, 85%, 100%) for 30 min each, 3) samples were washed two times in hexamethyldisilazane (HMDS, Electron Microscopy Services Hatfield, PA<sup>d</sup>) for 30 min each to clear residual ETOH, and 4) tissue samples were placed under a fume hood overnight to allow remaining chemicals to be removed.

Next, tissue samples were placed in molds for embedding in paraffin. Samples were processed in a Tissue-Tek VIP E300™ (Ames Co., Inc., Elkhart, IN<sup>f</sup>) for 2-3 hrs. Each sample was cut into 7 micrometer sections using a Reichert-Jung 2040 Autocut Microtome™,g, mounted on a glass microscope slide with resin, and allowed to dry for 20 minutes. Each section was stained with hematoxylin and eosin stain (Sigma-Chemical, St Louis, MO<sup>e</sup>).

Each biopsy sample was evaluated at 40 X magnification to describe and evaluate the morphology of the penile and preputial surface epithelium. The initial evaluation was performed at this magnification to ensure that all morphological structures could be identified. Evaluation was performed by a veterinary pathologist who was blinded as to the group and location from which the biopsy specimen was collected. Image J software (NIH.gov<sup>h</sup>) was used by the author to determine epithelial surface area, area of infoldings, and total number of infoldings. Measurements of each parameter from each location were compared within and between groups.

### **Scanning Electron Microscopy**

Tissues for scanning electron microscopy (SEM) were collected and stored in a mixture of 4% paraformalin, 1% glutaraldehyde (Electron Microscopy Service, Hatfield, PA<sup>d</sup>), and phosphate buffered saline (Sigma Chemical, St Louis, MO<sup>e</sup>) at 4 °C for up to 4 weeks. The tissues were rinsed in a phosphate buffered saline (PBS) bath for 20 min with agitation. Tissues were fixed in 4% osmium tetroxide/ PBS (Electron Microscopy Services<sup>d</sup>) for 2 hours, rinsed with PBS for 3 changes of 20 min each, and rinsed with distilled water for 20 min.

Tissues were dehydrated in the manner as previously described for light microscopy. All samples were stored under a vacuum to ensure complete dehydration. The samples were mounted on an aluminum specimen stud (Electron Microscopy Services, Hatfield, PA<sup>d</sup>) and sputter coated with colloidal gold (Electron Microscopy Services, Hatfield, PA<sup>d</sup>) prior to viewing with an EVO 50 SEM<sup>TM</sup> (Zeiss XVP, USA<sup>i</sup>). Images were captured with the EVO 50<sup>TM</sup> (Zeiss XVP<sup>i</sup>) computer and stored on removable file.

Similar to light microscopy samples, surface epithelium of each sample was evaluated by a veterinary pathologist who was blinded to group and location from which biopsy specimens were collected. Differences in the surface epithelium at each anatomical location were compared within and between groups by subjective evaluation.

### **Areas Measured**

The following parameters were measured for each biopsy site: 1) area of the epithelium from the basement membrane to the luminal surface, 2) area of infoldings under a tangent line drawn from the pinnacles of the surface epithelium at the edges of the infolding, and 3) total number of infoldings per linear unit of tissue. These parameters were chosen as they could be consistently measured with the Image J software. Direct measurements of epithelial thickness and depth of infolding were not repeatable.

### **Area of Epithelium**

Each sample of epithelium was labeled according to the anatomical location from which it was collected. Distal refers to the penis 1 cm proximal to the galea. Middle refers to the area 1cm distal to the attachment of the prepuce to the free portion of the penis. Proximal refers to the prepuce 6 cm distal to the preputial orifice when the penis is extended. From each specimen, color images of 4080 x 3072 pixel resolution (40X magnification) were acquired with a light



microscope (Olympus<sup>®</sup> BH-2<sup>j</sup>) and digital camera (Olympus<sup>®</sup> DP-71<sup>j</sup>) with the Image J analysis program (Image J NIH.gov<sup>h</sup>). To determine the area of the epithelium, the following steps were taken: 1) a line was drawn over the scale given in the image [analyze → set scale to the known distance], 2) the scanned color image was converted to grayscale [image → type → 8-bit], 3) the automated mode was used to threshold the image [process → binary → make binary], 4) the edges of the epithelium were highlighted with the tracing tool, 5) the area of the epithelium was calculated [analyze → analyze particles], and 6) minimum particle size was entered as 500 pixels [toggle show outlines, check display results, and click OK]. (Illustrations 2-8)

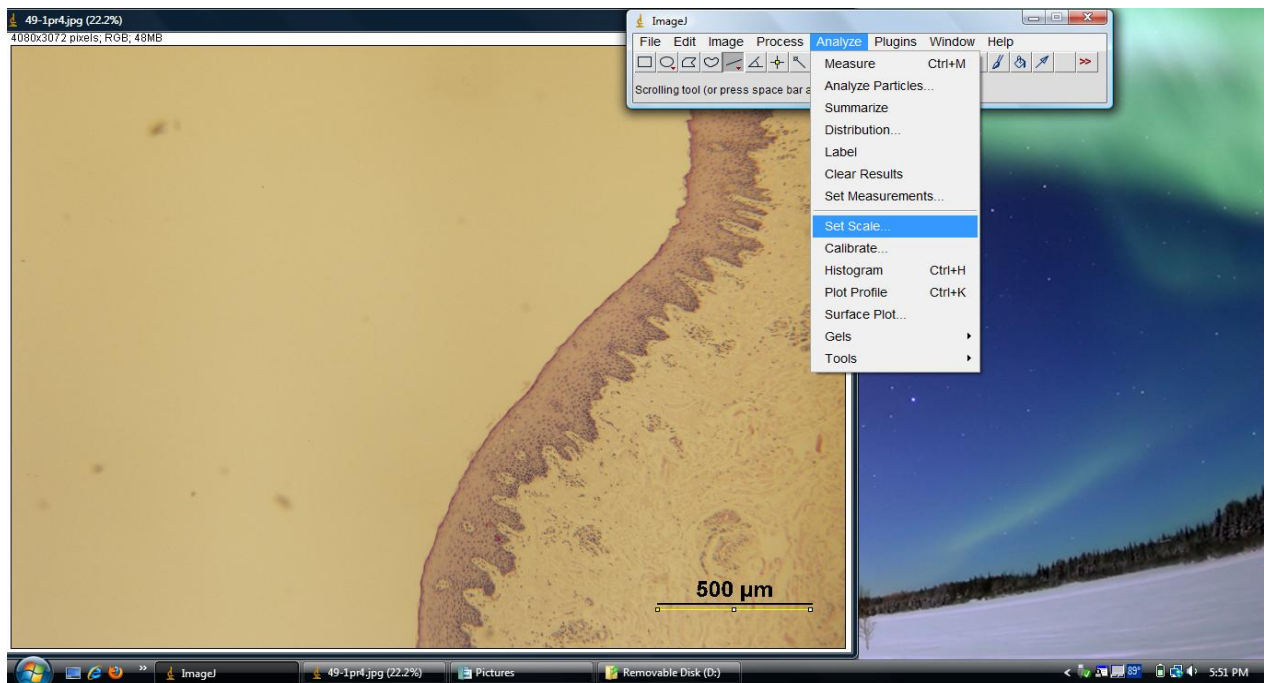


Illustration 2. Set Scale to known distance. Image at 40X magnification

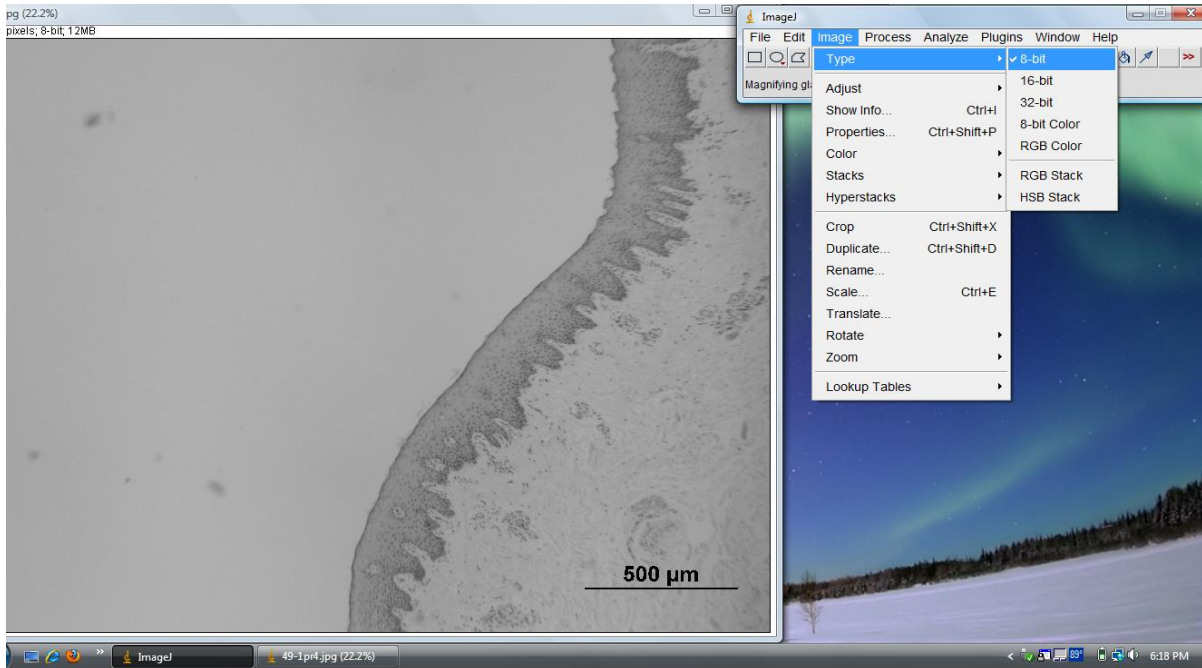


Illustration 3. Convert to grayscale.

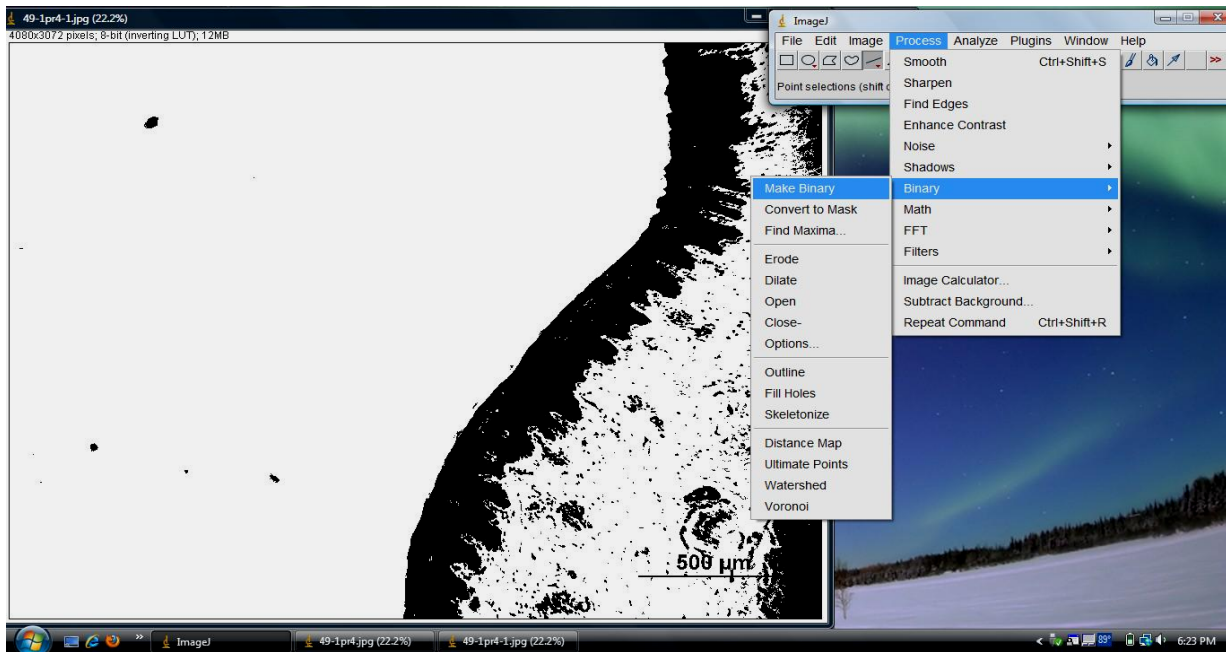


Illustration 4. Convert to binary image.

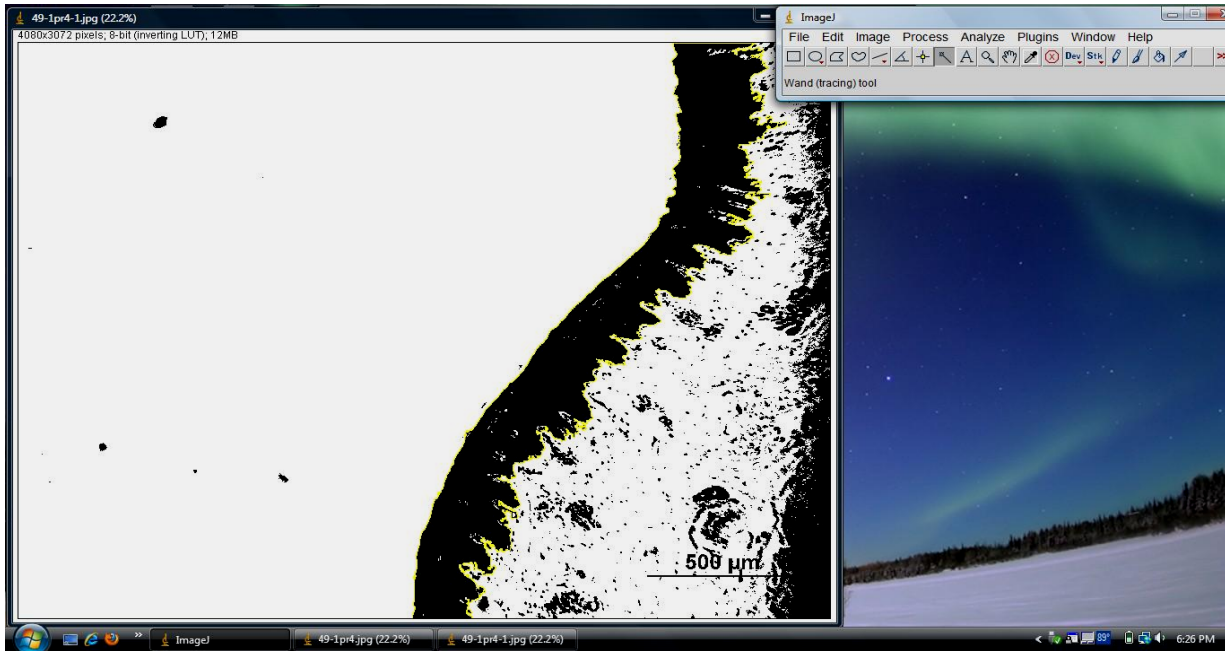


Illustration 5. Tracing tool to locate edges of the epithelium.

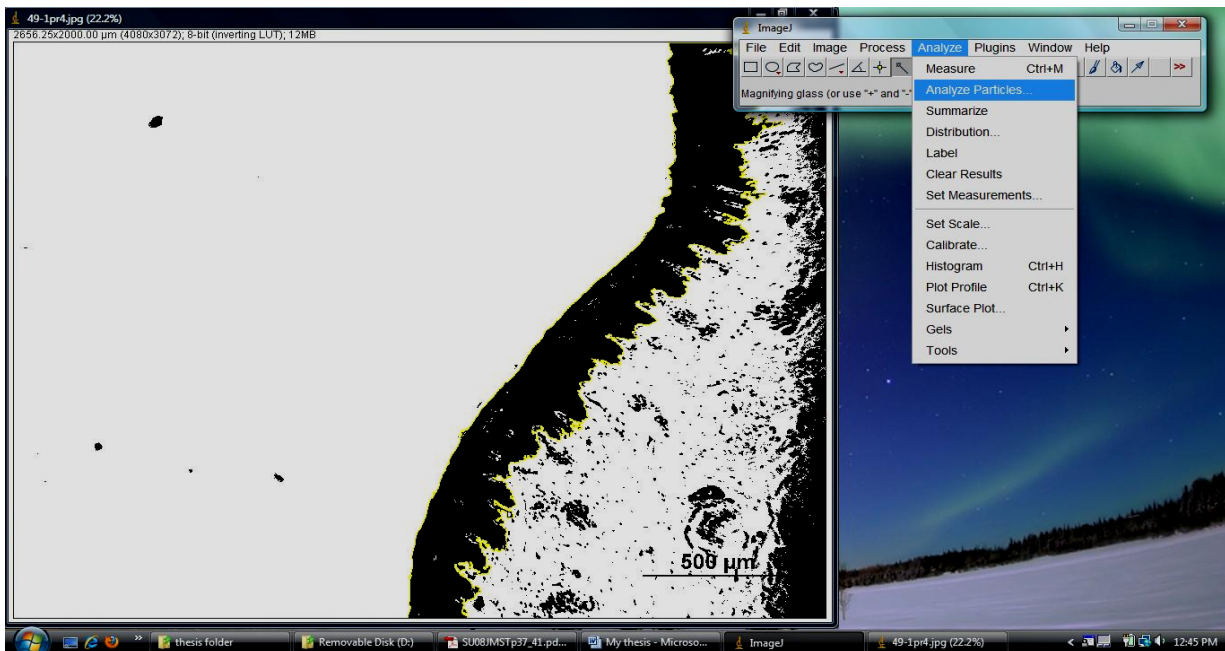


Illustration 6. Analyze particles

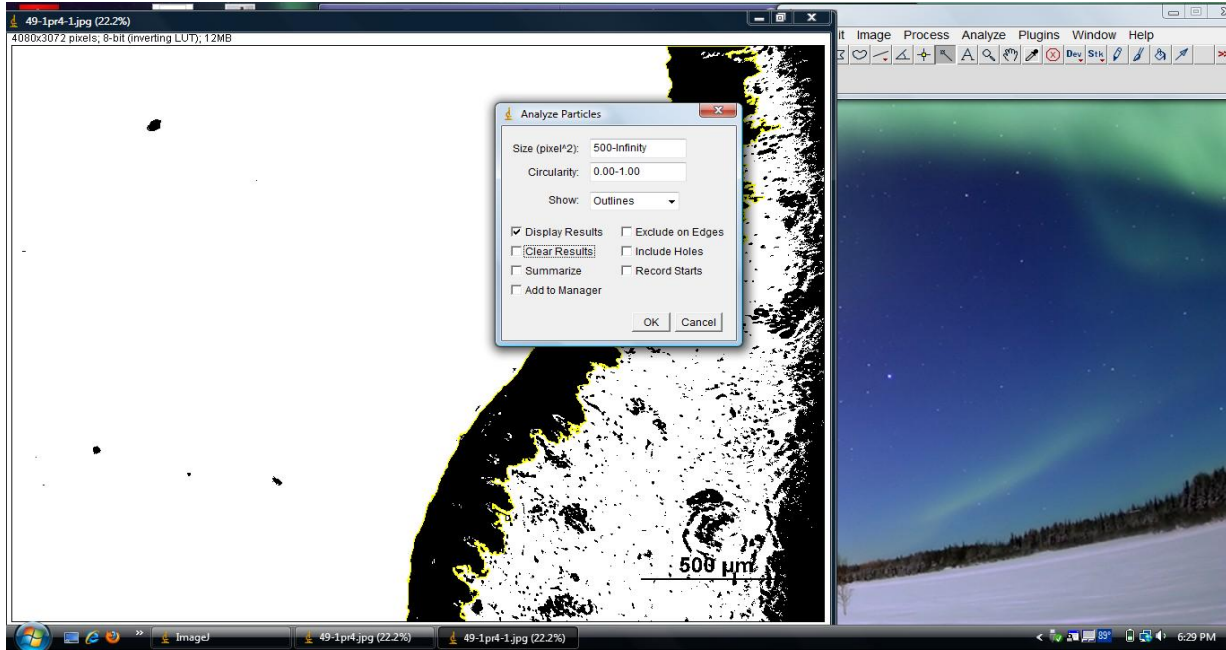


Illustration 7. Set particle size, display outlines and results.

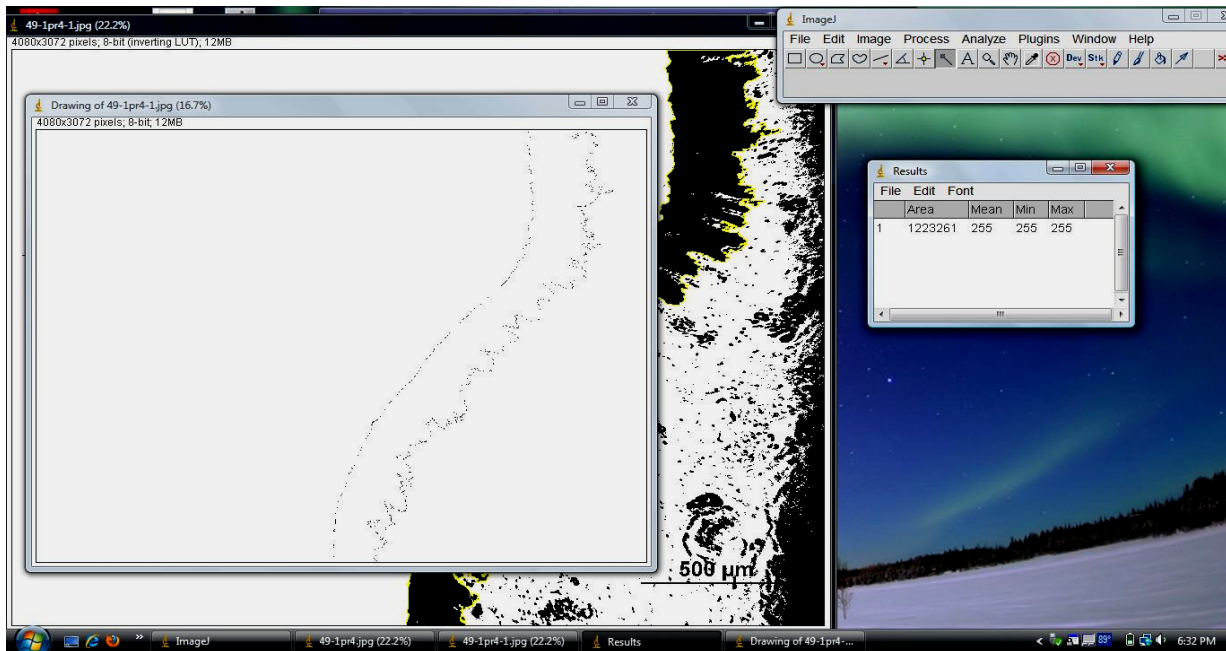


Illustration 8. Outline of area and results of area calculated.

## Area of infoldings

Each of the three samples (Proximal, Middle, Distal) were evaluated for area of infoldings to determine differences within and between groups. To determine the area of the infoldings, the following steps were taken: 1) a line was drawn over the scale given in the image [analyze → set scale to the known distance], 2) the arrow tool was selected, and a tangent line was drawn that touches the tissue at exactly two points determined by visual observation to be the pinnacles of the tissue on either side of the fold. The tangent line touched the pinnacles but did not intersect the tissue, 3) the polygon tool was selected and used to trace the borders of the fold, 4) the threshold area was outlined [image → adjust → threshold], 5) the color image was converted to a binary image [process → binary → make binary], 6) the outlined area was calculated [analyze → analyze particles], and 7) minimum particle size was entered as 500 pixels [toggle show outlines, check display results, and click OK]. (Illustrations 9- 14)

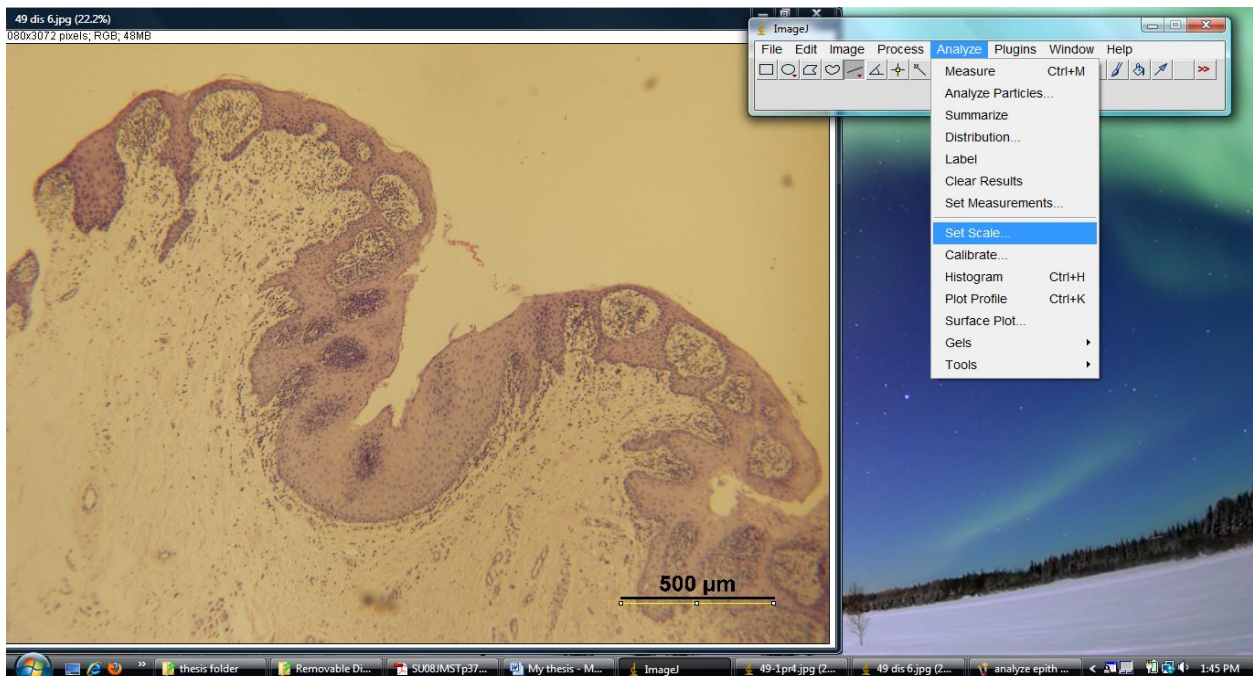


Illustration 9. Set scale to known distance

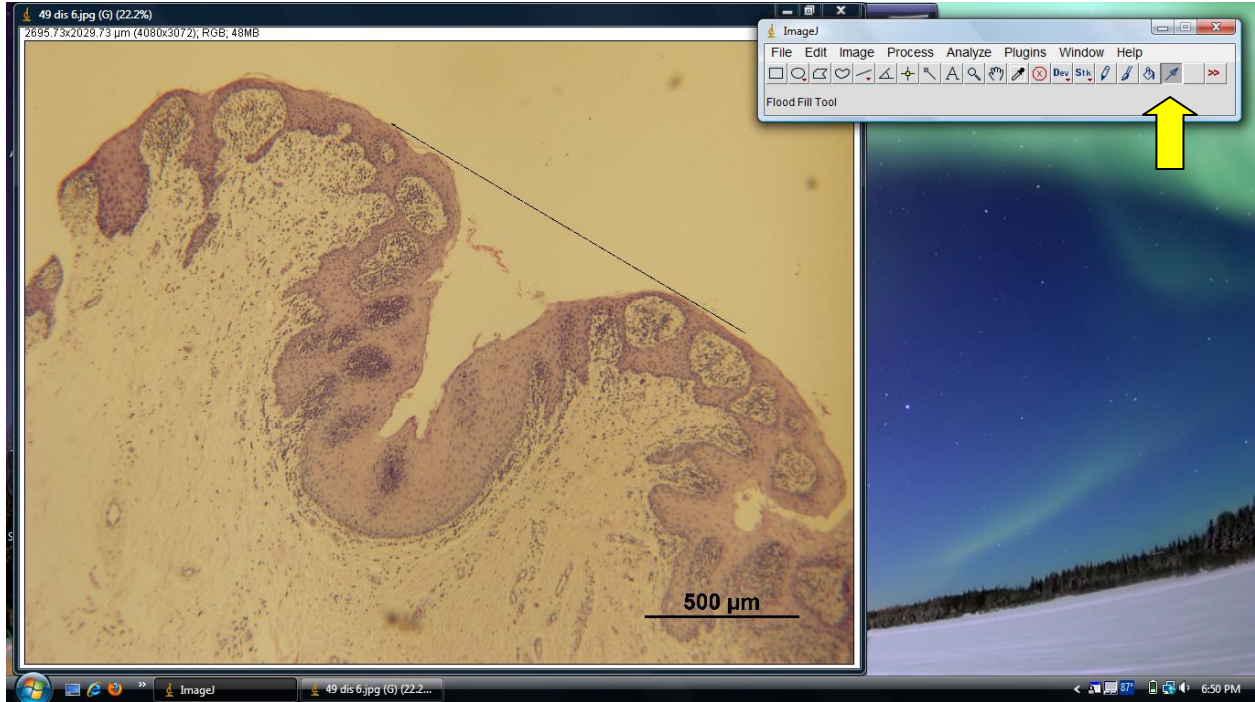


Illustration 10. Using arrow tool to draw tangent line.



Illustration 11. Tracing the outline of the fold

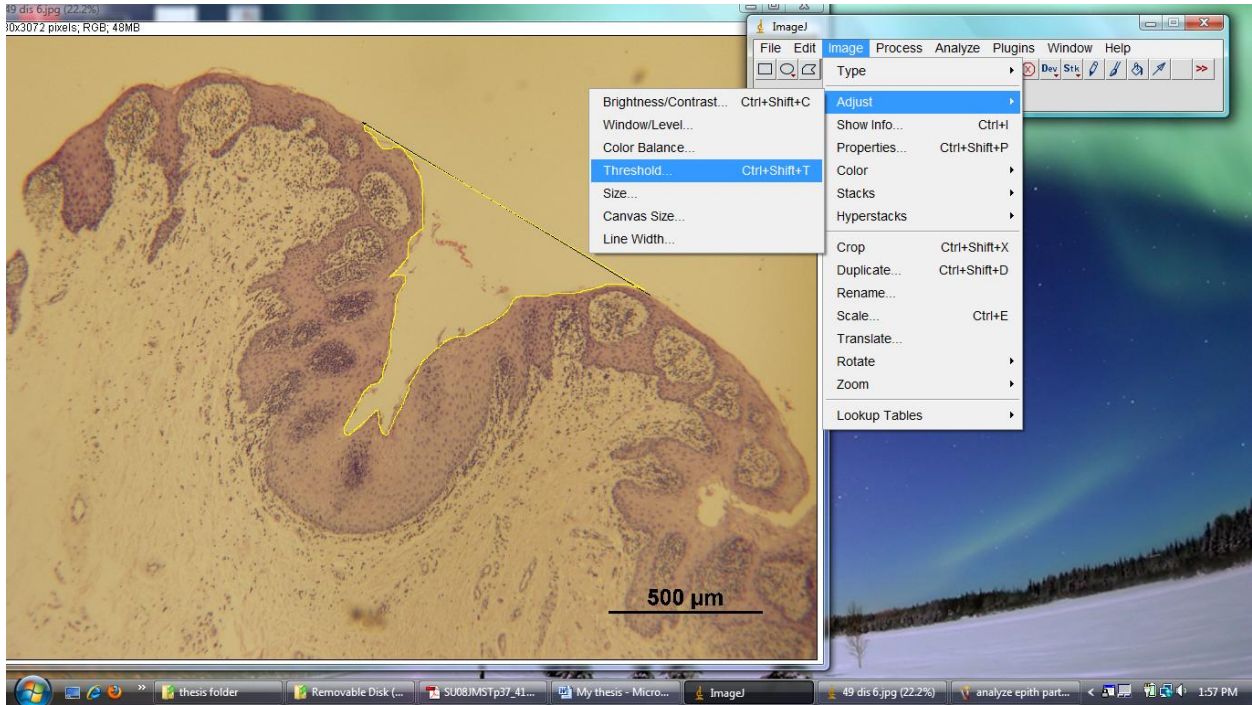


Illustration 12. Adjusting image to threshold.

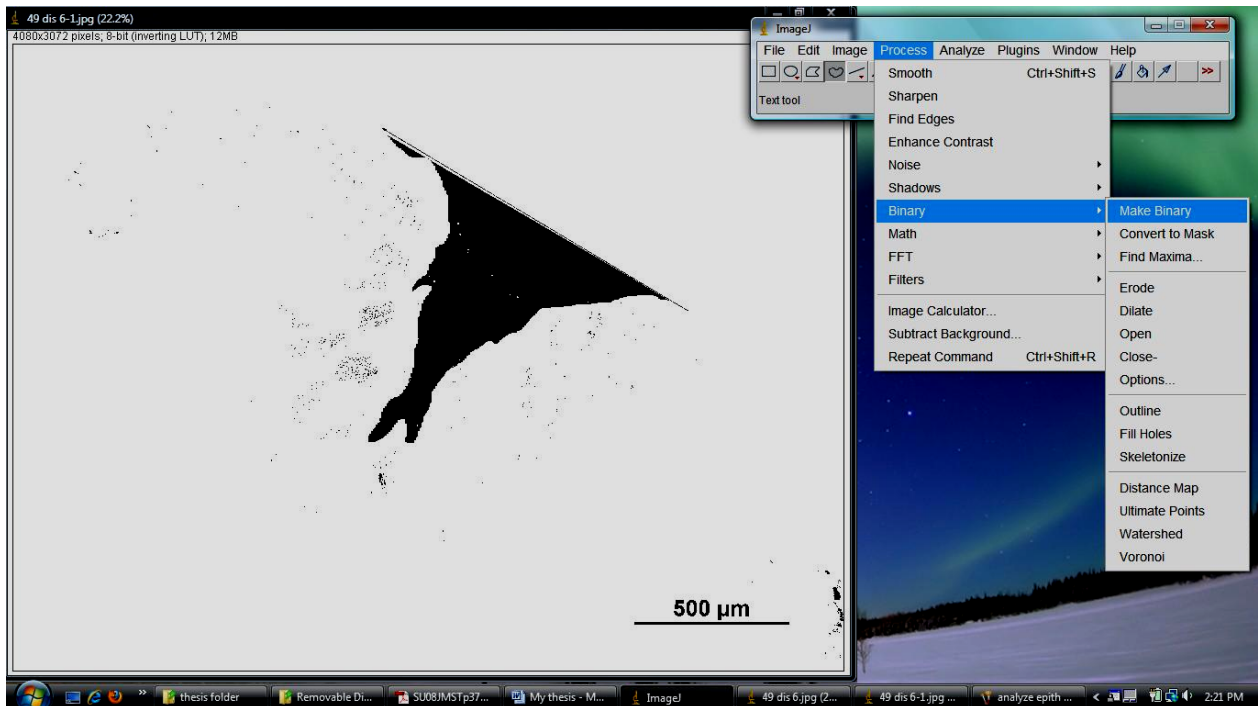


Illustration 13. Converting to binary image

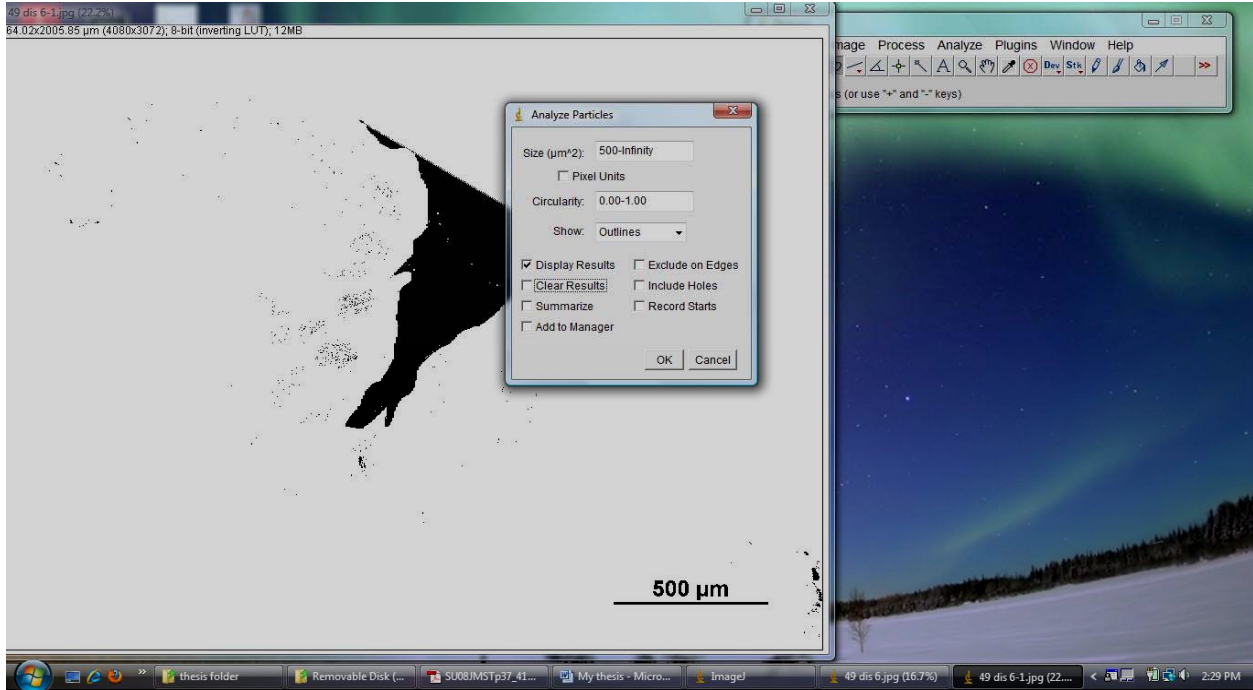


Illustration 14. Analyzing particles

### **Total Number of Infoldings**

The total number of infoldings per linear unit was recorded by Image J software (NIH.gov<sup>h</sup>). Results were compared within and between groups.

### **Statistical Analysis**

All measurements of light microscopy slides were collected with Image J software (NIH.gov<sup>h</sup>). Results of the area of epithelium, area of folds, and total number of infoldings per linear unit were entered into a Microsoft Excel<sup>®k</sup> spread sheet and analyzed with the SAS analytical system (SAS software, SAS Institute<sup>l</sup>) using an ANOVA procedure. The ANOVA procedure compared results within and between age groups. A value of  $p \leq 0.05$  indicated a significant difference.



### Examination for Crypts

Samples from each biopsy site were examined with electron microscopy for presence of structures that met the criteria for crypts as previously described.<sup>(7)</sup>(Illustration 18) Examinations were performed with a scanning electron microscope at 31-170X magnification level. The surface epithelium of all three segments from each bull was evaluated. Because software for measurement was not available, electron microscopy samples were subjectively evaluated (Illustrations 15-17).

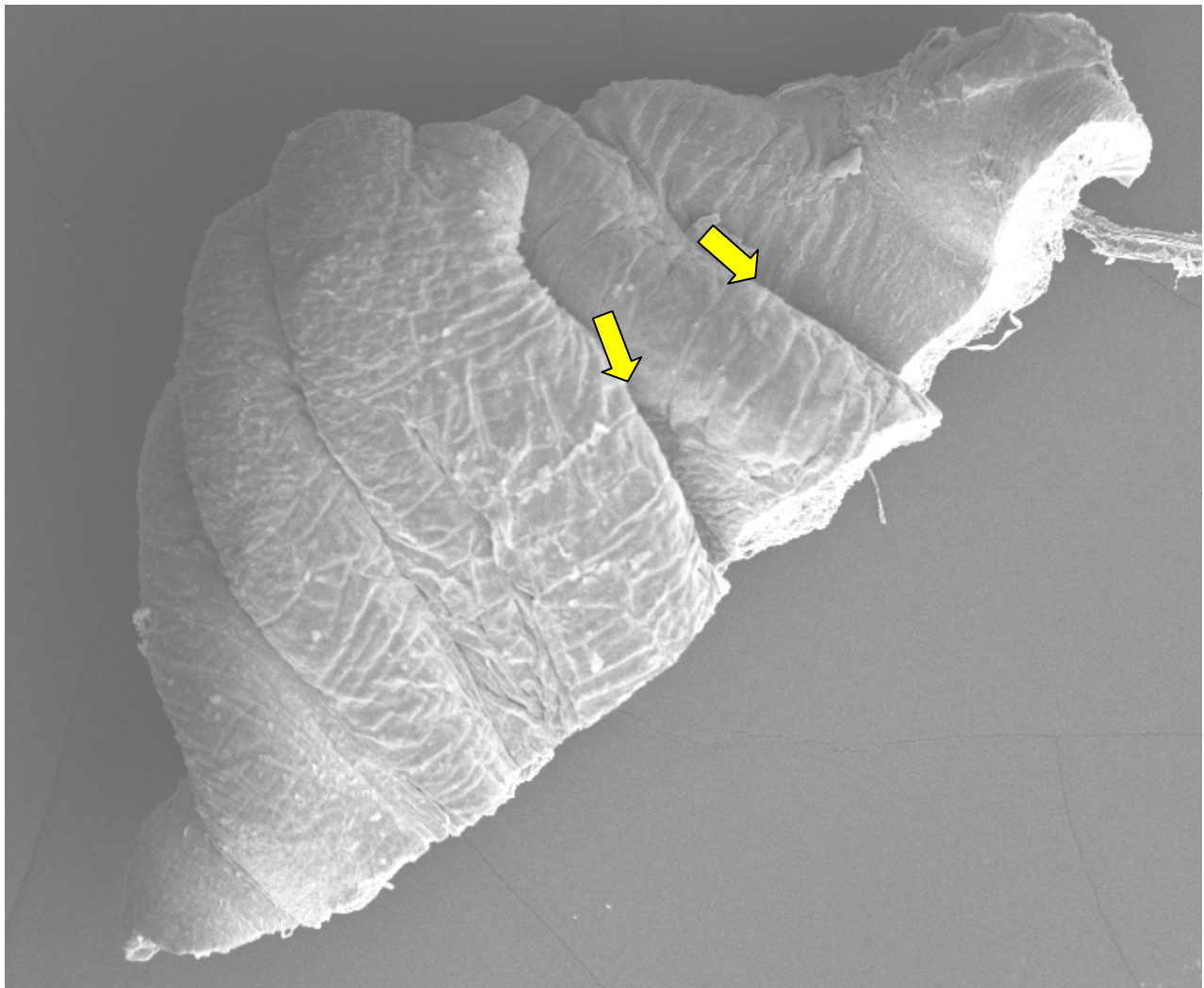


Illustration 15. Scanning electron image proximal sample 2yr old (31X magnification). Infoldings are indicated by arrows.

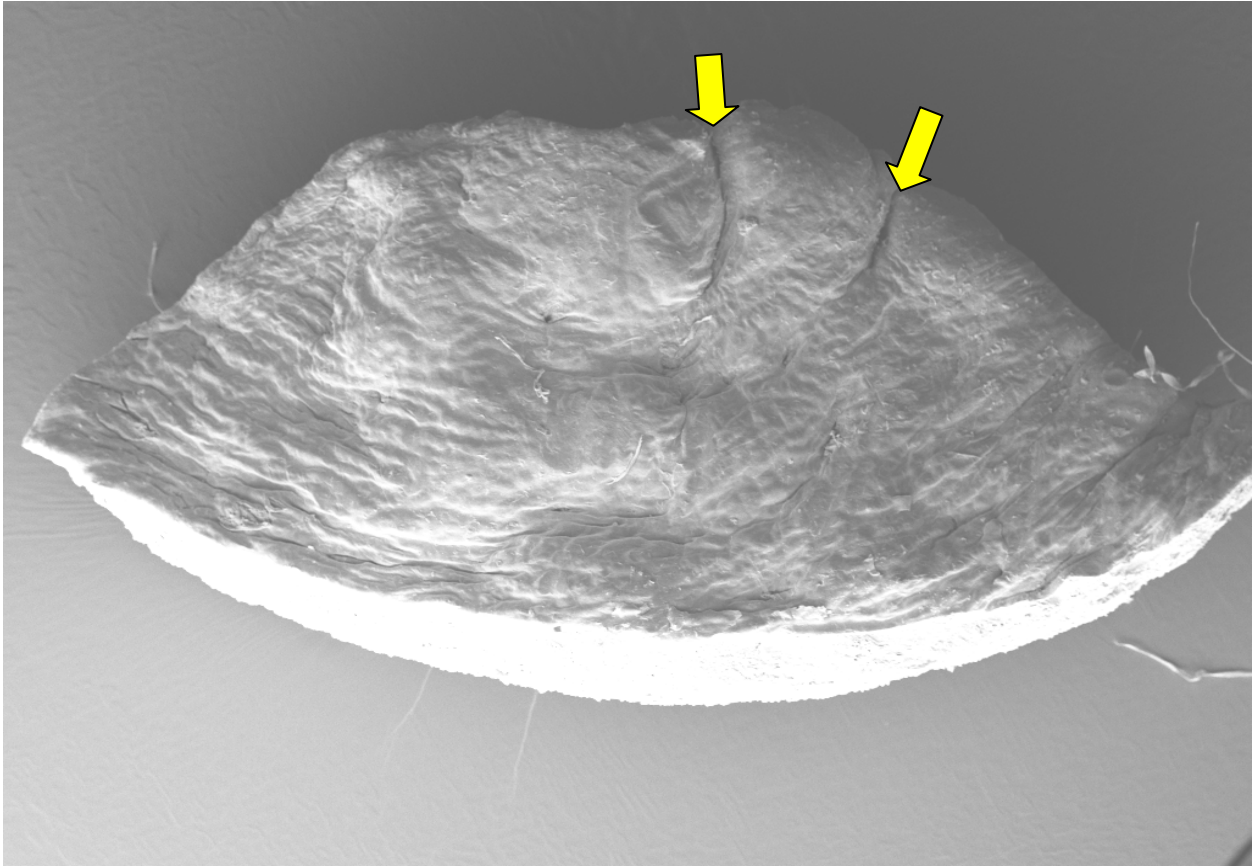


Illustration 16. Scanning electron image middle sample 6yr old (31X magnification). Infoldings are indicated by arrows.

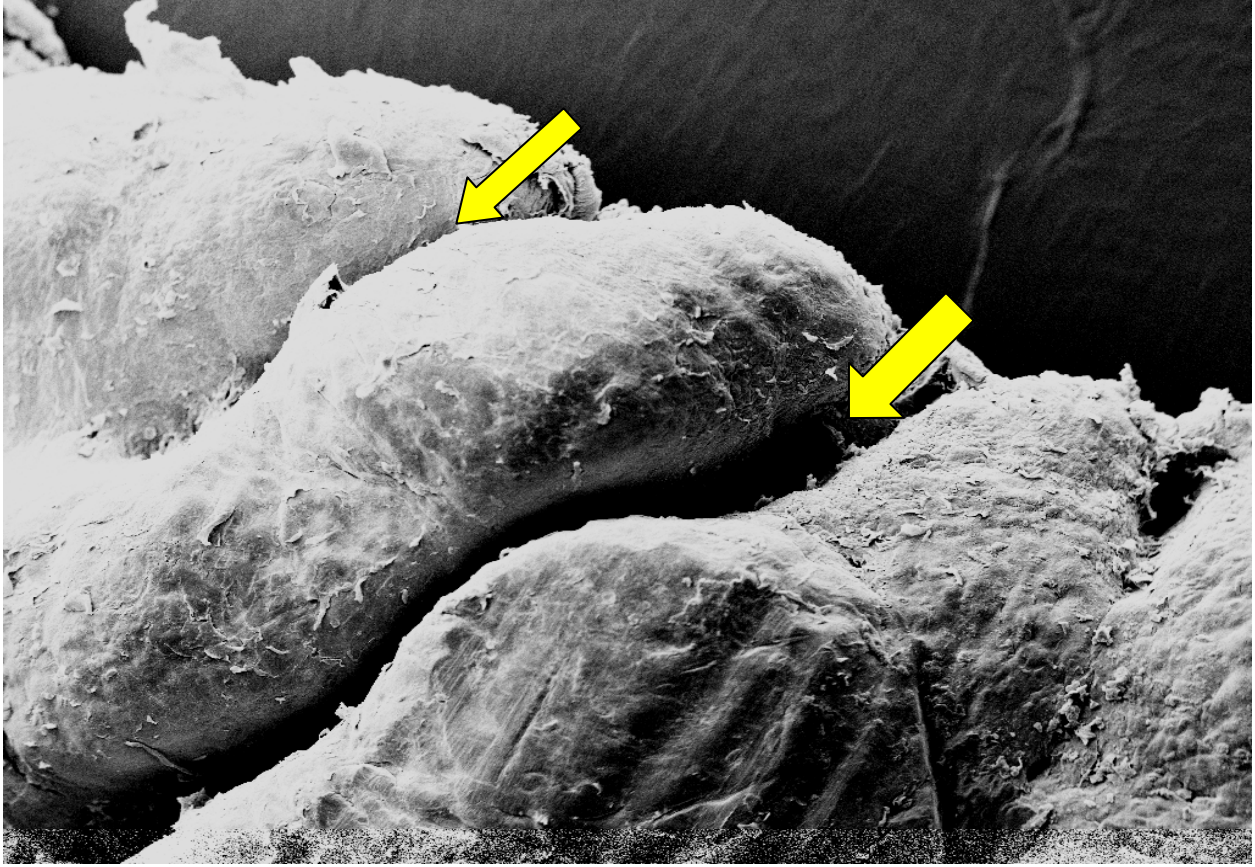


Illustration 17. Scanning electron image distal sample 5yr old (90 X magnification). Infoldings are indicated by arrows.

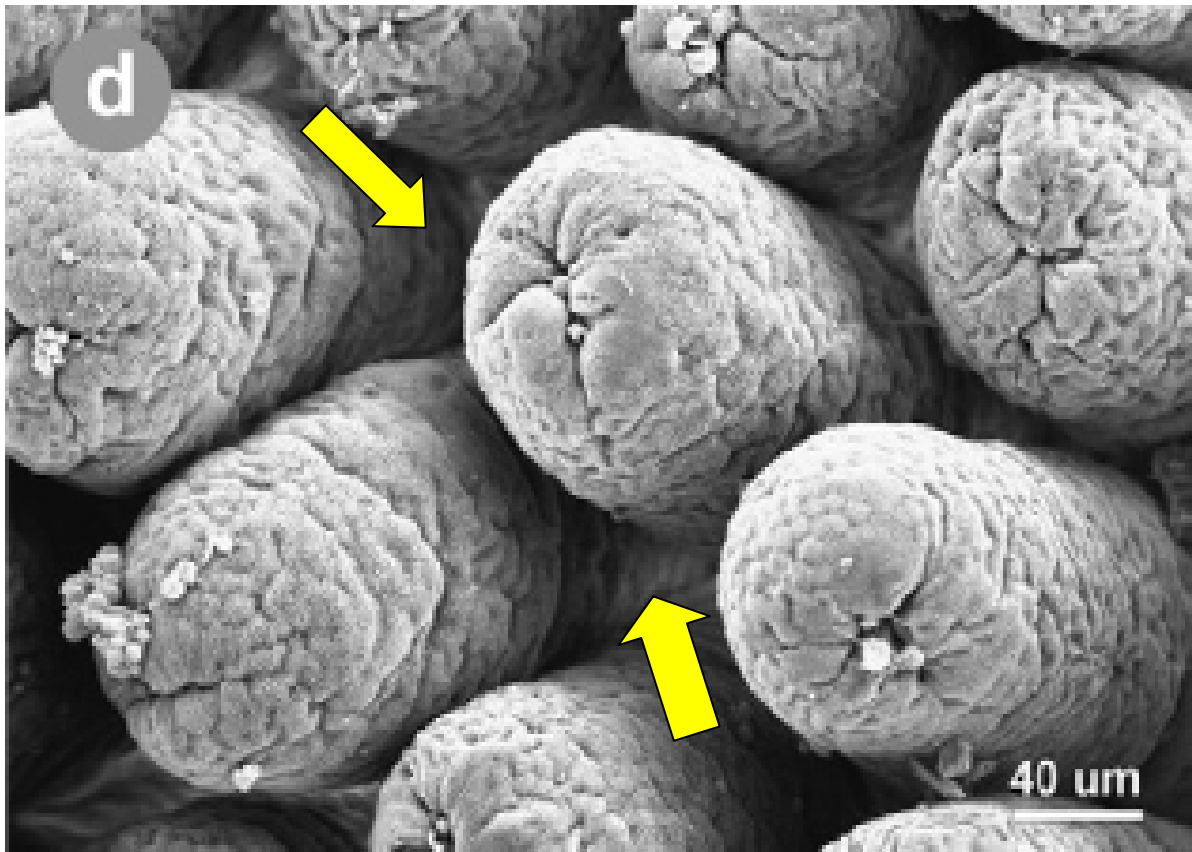


Illustration 18. Scanning electron image of intestinal crypts (Skrzypek *et al*<sup>59</sup>). Crypts are indicated by arrows. (170 X magnification)

## Chapter 4

### Results

This study was conducted from December 2008 to March 2009. Two age groups were compared (Table 1), and a total of 36 samples were collected from 12 bulls in each age group. Samples were evaluated using Image J software (NIH.gov<sup>h</sup>). These results were analyzed by ANOVA.

Group # 1	Group # 2
Angus 25 mo	Angus 6 yrs
Angus 26 mo	Angus 5 yrs
Angus 25 mo	Angus 12 yrs
Brahman 24 mo	Angus 5 yrs
Angus 27 mo	Angus 7 yrs
Angus 26 mo	Angus 6 yrs

Table 1. Age and breed of bulls

Three parameters were evaluated for each sample. The area of the epithelium was measured and compared within and between groups. No significant difference in related epithelial area was detected between groups ( $p = 2.96$ , Illustration 19). The ranges of the epithelial area in  $\mu\text{M}^2$  for group 1 are: distal  $0.4 \times 10^6 - 1.3 \times 10^6$ , middle  $0.3 \times 10^6 - 1.3 \times 10^6$ , and proximal  $0.2 \times 10^6 - 1.8 \times 10^6$ . The ranges of the epithelial area in  $\mu\text{M}^2$  for group 2 are: distal  $0.3 \times 10^6 - 1.9 \times 10^6$ , middle  $0.3 \times 10^6 - 1.6 \times 10^6$ , and proximal  $0.4 \times 10^6 - 1.7 \times 10^6$  (Table 2). The mean (means  $\pm$  standard deviation) epithelial areas in  $\mu\text{M}^2$  for group 1 are: distal  $0.8 \times 10^6 \pm 0.3 \times 10^6$ , middle  $0.7 \times 10^6 \pm 0.2 \times 10^6$ , and distal  $1.0 \times 10^6 \pm 0.3 \times 10^6$ . The mean (means  $\pm$  standard deviation) epithelial areas in  $\mu\text{M}^2$  for group 2 are: distal  $0.8 \times 10^6 \pm 0.3 \times 10^6$ , middle  $0.7 \times 10^6 \pm 0.3 \times 10^6$ , and proximal  $0.8 \times 10^6 \pm 0.3 \times 10^6$  (Illustration 19).

Group	Range ( $\mu\text{M}^2$ )
1. 2yr old ( $\pm 3$ mo)	distal $0.4 \times 10^6 - 1.3 \times 10^6$ middle $0.3 \times 10^6 - 1.3 \times 10^6$ proximal $0.2 \times 10^6 - 1.8 \times 10^6$
2. $\geq 5$ yr old	distal $0.3 \times 10^6 - 1.9 \times 10^6$ middle $0.3 \times 10^6 - 1.6 \times 10^6$ proximal $0.4 \times 10^6 - 1.7 \times 10^6$

Table 2. Range of epithelial area.

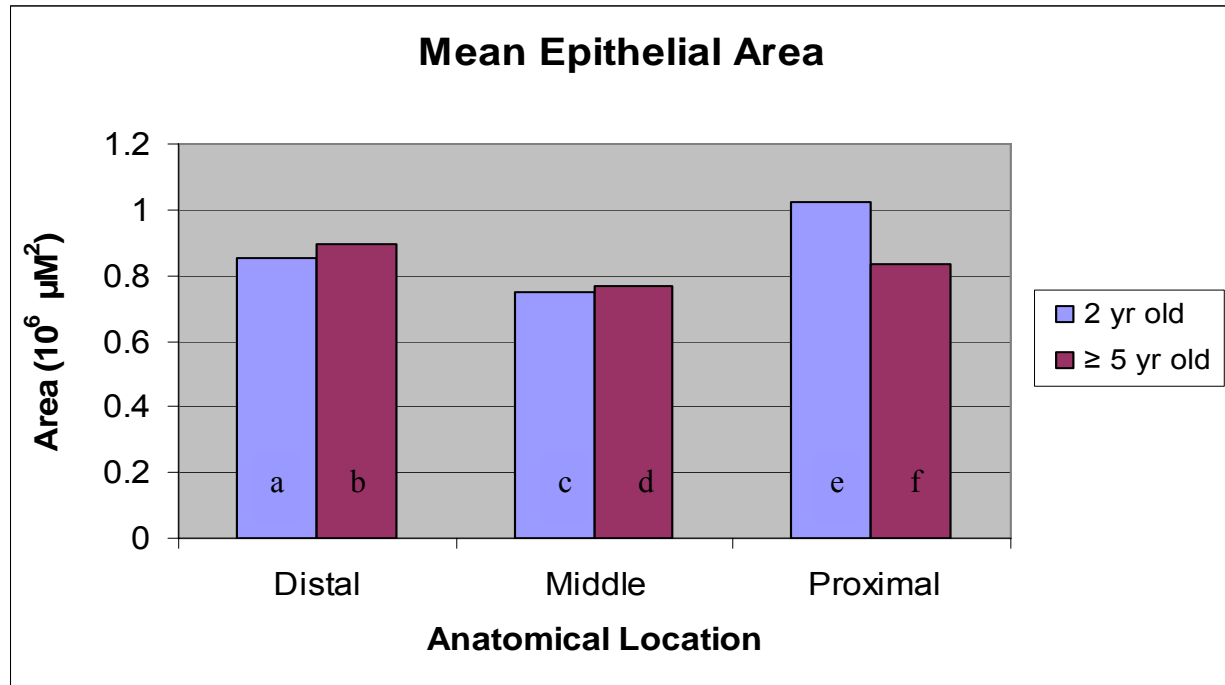


Illustration 19. Mean epithelial area and standard deviation (a-f) per site. Area between groups is not different ( $p=2.96$ ) a)  $sd \pm 0.3 \times 10^6$  b)  $sd \pm 0.3 \times 10^6$ , c)  $sd \pm 0.2 \times 10^6$ , d)  $sd \pm 0.3 \times 10^6$ , e)  $sd \pm 0.3 \times 10^6$ , f)  $sd \pm 0.3 \times 10^6$

The area encompassed by the infoldings was measured and compared within and between groups. No significant difference was detected between groups ( $p = 2.67$ , Illustration 20). The ranges of the area encompassed by the infoldings in  $\mu M^2$  for group 1 are: distal  $0.009 \times 10^6 - 0.7 \times 10^6$ , middle  $0.002 \times 10^6 - 0.3 \times 10^6$ , and proximal  $0.006 \times 10^6 - 0.8 \times 10^6$ . The ranges of the area encompassed by the infoldings in  $\mu M^2$  for group 2 are: distal  $0.006 \times 10^6 - 0.8 \times 10^6$ , middle  $0.003 \times 10^6 - 0.3 \times 10^6$ , and proximal  $0.004 \times 10^6 - 1.0 \times 10^6$  (Table 3). The mean (means  $\pm$  standard deviation) of the areas encompassed by the infoldings in  $\mu M^2$  of group 1 are: distal  $0.1 \times 10^6 \pm 0.1 \times 10^6$ , middle  $0.1 \times 10^6 \pm 0.03 \times 10^6$ , and proximal  $0.2 \times 10^6 \pm 0.1 \times 10^6$ . The mean (means  $\pm$  standard deviation) of the areas encompassed by the infoldings in  $\mu M^2$  of group 2 are: distal  $0.1 \times 10^6 \pm 0.09 \times 10^6$ , middle  $0.07 \times 10^6 \pm 0.05 \times 10^6$ , and proximal  $0.1 \times 10^6 \pm 0.1 \times 10^6$  (Illustration 20).

Group	Range $\mu\text{M}^2$
1. 2 yr old ( $\pm 3$ mo)	distal $0.9 \times 10^4 - 0.8 \times 10^6$ middle $0.003 \times 10^6 - 0.3 \times 10^6$ proximal $0.007 \times 10^6 - 0.9 \times 10^6$
2. $\geq 5$ yr old	distal $0.007 \times 10^6 - 0.8 \times 10^6$ middle $0.003 \times 10^6 - 0.3 \times 10^6$ proximal $0.005 \times 10^6 - 1.0 \times 10^6$

Table 3. Range of epithelial fold area.

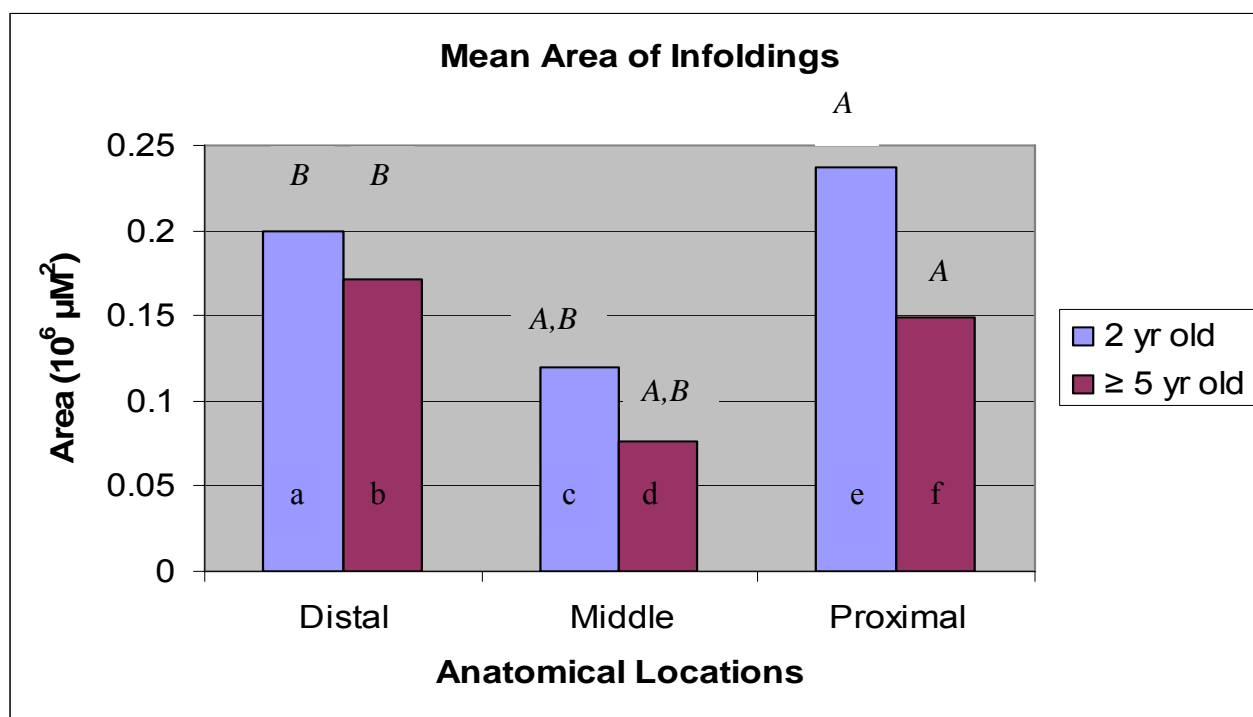


Illustration 20. Mean area of infoldings and standard deviations (a-f). Area of infoldings between age groups are not different ( $p = 2.67$ ) a)  $\text{sd} \pm 0.1 \times 10^6$  b)  $\text{sd} \pm 0.09 \times 10^6$ , c)  $\text{sd} \pm 0.03 \times 10^6$ , d)  $\text{sd} \pm 0.05 \times 10^6$ , e)  $\text{sd} \pm 0.1 \times 10^6$ , f)  $\text{sd} \pm 0.1 \times 10^6$

A) Mean area of infoldings within each group was greater in proximal sites than in middle sites ( $p = 0.025$ )

B) Mean area of infoldings within each group was greater in distal sites than in middle sites ( $p = 0.028$ )



The number of infoldings per 3256  $\mu\text{M}$  of epithelium was compared between age groups. These numbers were compared to related portions. No significant difference in total fold numbers was detected between age groups ( $p = 0.69$ ). The number of infoldings per 3256  $\mu\text{M}$  for each site within group 1 is: distal 74, middle 55, and proximal 76. The number of infoldings per 3256  $\mu\text{M}$  site within group 2 is: distal 69, middle 64, and proximal 85. The mean number (mean  $\pm$  standard deviation) of infoldings per site for group 1 is: distal  $12.3 \pm 3.8$ , middle  $9.2 \pm 3.4$ , and proximal  $12.7 \pm 5.8$ . The mean number (mean  $\pm$  standard deviation) of infoldings per site for group 2 is: distal  $12.3 \pm 6.7$ , middle  $10.6 \pm 5.3$ , and proximal  $14.1 \pm 11.4$  (Illustration 21).

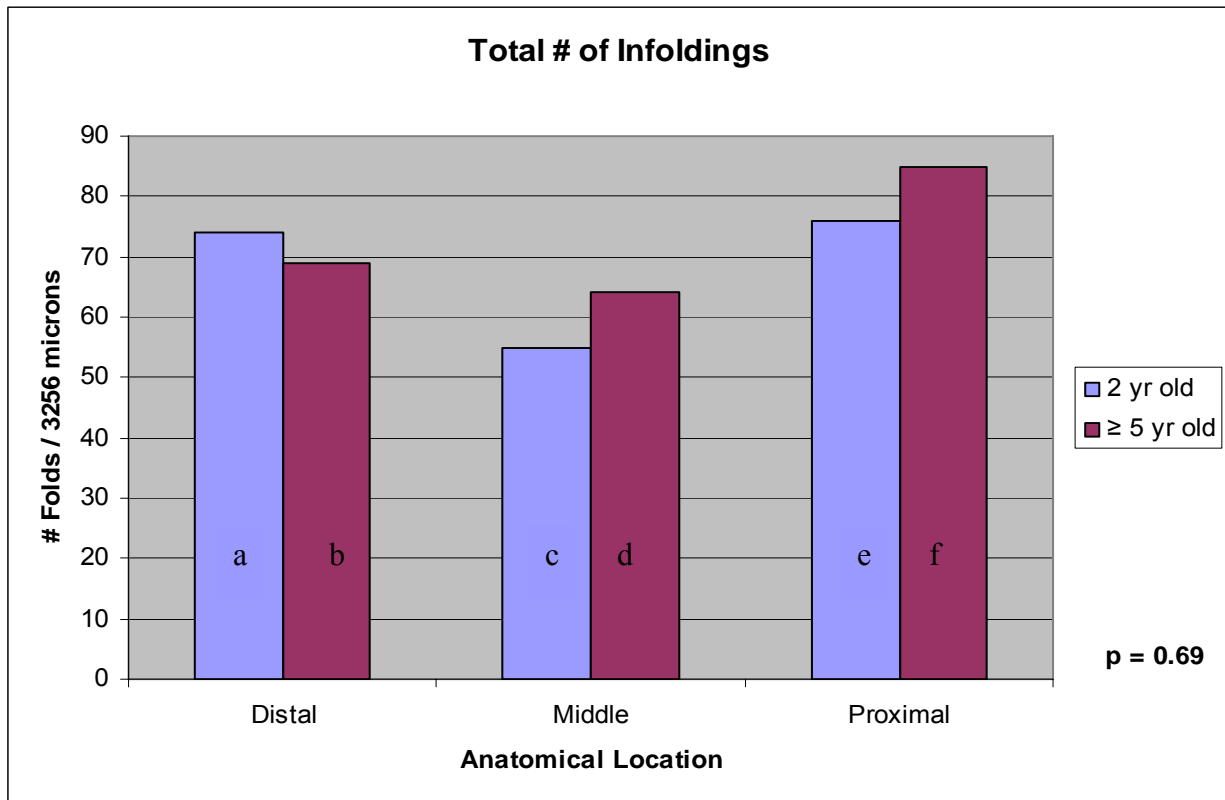


Illustration 21. Total number of folds / 3256 $\mu\text{M}$  and standard deviations (a-f).  
 a) Mean 12.3 sd  $\pm$  3.8, b) Mean 12.3 sd  $\pm$  6.7, c) Mean 9.2 sd  $\pm$  3.4, d) Mean 10.6 sd  $\pm$  5.3  
 e) Mean 12.6 sd  $\pm$  5.8 f) Mean 14.2 sd  $\pm$  11.4

### **Electron microscopic evaluation**

Tissue samples were examined by electron microscopy for presence of epithelial crypts at 31 – 170X magnification. Several images of intestinal crypts have been published.<sup>(59)</sup>

(Illustration 18) Infoldings of epithelium were present on all sections that were viewed, but no structures comparable to intestinal crypts were located in any sections examined.

## Chapter 5

### Discussion

Shortly after the initial description of *Tritrichomonas foetus*, a report in 1941 by Abelein noted that there were numerous infoldings present on the surface of the penis.<sup>(60)</sup> The author suggested that *Tritrichomonas foetus* established long term infection in these crevices.<sup>(19)</sup> It has been stated that presence of deeper crypts in older bulls allows the development of chronic infection with *C. fetus* subsp. *venerealis*.<sup>(2)</sup> For decades it has been assumed that older bulls have deeper crypts than younger bulls, but little documentation exists to support this conclusion.

Chronic infections can be established in older bulls.<sup>(35)</sup> Christensen *et al*, indicated that the herd prevalence of trichomoniasis could be reduced by exclusive use of younger bulls, suggesting that chronic trichomoniasis is primarily a problem of older bulls.<sup>(61)</sup> Other studies have shown that young bulls (< 2yrs) may be susceptible to chronic infections with *T. foetus*.<sup>(41)</sup>

This study was undertaken to evaluate the differences in the penile and preputial epithelium between different aged bulls at three different anatomical locations. The study was not intended to prove or disprove that folds of the epithelium exist.

An early hypothesis of this study was that the epithelial layer of the penis and prepuce becomes thicker as the bull matures, which results in deeper infoldings. The area of the surface of the epithelium was not different between groups (p = 2.96, Illustration 19).

The second objective of this study was to determine the area encompassed by the epithelial folds and to compare differences within and between groups. There was no statistical difference in area contained within the infoldings between groups. This was an unexpected

finding contrary to the widely held belief that older bulls would likely have increased areas of infolding. ( $p= 2.67$ , Illustration 20). In both groups the area contained within the infoldings was greater at the proximal and distal sites as compared to the middle. ( $p = 0.02$ )

The final assumption tested was that the total number of infoldings was different between age groups. The results of this study do not support this assumption. The total number of folds did not differ between the two groups ( $p=0.69$ , Illustration 21).

Previous studies compared breed prevalence of trichomoniasis and campylobacterosis.<sup>(48,49,62)</sup> The bulls in the current study were predominantly Angus, consistent with the population of beef bulls currently used in Alabama. A recent retrospective review of breeding soundness evaluations revealed that 72% of the 1,076 bulls presented to the Auburn University Food Animal Theriogenology service for breeding soundness evaluation from 2006 to 2008 were Angus.<sup>(63)</sup>

Based upon visual examination of sections prepared for electron microscopy, there is an absence of structures that can be classified as crypts. Due to the absence of the crypt or crypt like structures, the author proposes that the term epithelial folds or infoldings be used as the preferred descriptive term.

Collectively, these findings indicate that chronic venereal infection of the bull is not likely due to the presence of increased epithelial area, increased area within epithelial folds, or increased density of epithelial folds of the prepuce and free portion of the penis. Other differences in the penile and preputial environment must exist to allow establishment of persistent infection in mature bulls. A review by Felleisen suggests that carbohydrate receptors and lectin molecules play a role in the development of venereal infection.<sup>(65)</sup> It is possible that the penile and preputial epithelium of older bulls have a higher concentration of these receptors,

creating a favorable environment for chronic infection. Early studies by Hammond *et al* noted that distribution of *T. foetus* in the preputial cavity was correlated with the distribution of smegma.<sup>(19)</sup> The quantity and consistency of smegma may favor the development of venereal disease in older bulls. Architectural characterization and electron microscopic evaluation of penile and preputial epithelium may enhance future studies of the pathophysiology and immunology of venereal infections in bulls.

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## Appendix A

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Thompson Bishop Sparks Diagnostic Lab  
Alabama Department of Agriculture and Industries

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 Thompson Bishop Sparks State Diagnostic Laboratory Policy or Operating Procedure			
SOP Title: Procedure for Polymerase Chain Reaction (PCR) for the detection of <i>Trichomonas foetus</i> DNA.			
Author: Alicia Wise			SOP#: MD.023
Area: Mol. Det.	Date: 1-13-09	Rev Date:	Rev Level:

**Purpose and application of procedure:** This standard operating procedure (SOP) applies to the testing of cultures provided by BAC for *Trichomonas foetus* DNA.

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Approved:  Date: 1-15-09  
QM Approved:  Date: 4/24/07  
CF-1.1 SOP template K.L.F. September 2008

			
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<b>Author: Alicia Wise</b>			<b>SOP#: MD.023</b>
<b>Area: Mol. Det.</b>	<b>Date: 1-13-09</b>	<b>Rev Date:</b>	<b>Rev Level:</b>

**1. Theory/Scope**

1.1. Theory/Principles of the Method:

1.1.1. *Tritrichomonas foetus* is the causative agent of bovine tritrichomonosis, a sexually transmitted disease leading to infertility and abortion. PCR is based upon primers TFR3 and TFR4 directed to rRNA gene units of *T. foetus*.

1.2. Scope and Application

1.2.1. The PCR is designed to detect the presence of low levels of *Tritrichomonas foetus* in cultures provided by BAC.

1.2.2. This SOP is used in TBSSDL of the Alabama Department of Agriculture and Industries.

**2. Definitions**

- 2.1. TRI= *Tritrichomonas foetus*
- 2.2. NVSL=National Veterinary Services Laboratory
- 2.3. SOP=Standard Operating Procedure
- 2.4. TBSSDL=Thompson Bishop Sparks State Diagnostic Laboratory
- 2.5. UVIS= Universal Veterinary Information Services
- 2.6. BAC=Bacteriology Department, TBSSDL
- 2.7. DEPC= deionized, diethylpyrocarbonate

**3. Safety Precautions**

3.1. Health and Safety

3.1.1. Handling *Tritrichomonas foetus* samples derived from cattle must be carried out according to Bio-Safety Level 2 practices in TBSSDL. All unused biological components are autoclaved (see BAC.502).

3.2. Cautions

- 3.2.1. If reagent Taq-polymerase is allowed to stay at room temperature (21<sup>o</sup>C-25<sup>o</sup>C) more than one hour, the quality of the test may be affected.
- 3.2.2. Wear gloves and change them as often as needed.

3.3. Environmental Conditions

3.3.1. Invitrogen DNA Kit components are kept at room temperature or 4<sup>o</sup>C according to manufacturer's instruction.



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The following components should be stored at 4<sup>0</sup>C:

Resuspension buffer, Precipitation Salt, and RNase A

- 3.3.2. Invitrogen PCR reagents are stored at -20<sup>0</sup>C, thaw and gently mix before use.
- 3.3.3. All enzymes are kept at -20<sup>0</sup>C (not auto-defrost) or in a bench-top cooler for a short period of time when setting the PCR reaction.
- 3.3.4. Extracted DNA is stored at 4<sup>0</sup>C for up 24 hours and at -20<sup>0</sup>C for long term storage.
- 3.3.5. Preputial washings from bulls and cervicovaginal secretions from female cattle are provided Bacteriology Department at TBSSDL. BAC then provides Molecular Detection with a culture to extract and test for TRI using PCR. No other sample is acceptable for this test.

**4. Materials and Equipment**


**4.1. Materials, reagents, and Media**

- 4.1.1. S.N.A.P. Miniprep Kit (Invitrogen # K1900-05) contains: S.N.A.P. Miniprep columns, 2.0 ml collection tubes, resuspension buffer, RNase A, lyophilized, lysis buffer, precipitation salt, binding buffer, wash buffer, and 4X final wash
- 4.1.2. Absolute ethanol
- 4.1.3. Double distilled water.
- 4.1.4. 10mM-dNTP (Invitrogen)
- 4.1.5. 50mM-MgCl<sub>2</sub> (Invitrogen)
- 4.1.6. 10 X PCR buffer (Invitrogen)
- 4.1.7. Taq-polymerase (Invitrogen)
- 4.1.8. DMSO (Sigma)
- 4.1.9. Agarose
- 4.1.10. 1X TAE
- 4.1.11. Ethidium bromide ( see SOP MD.003)
- 4.1.12. 70% ethanol
- 4.1.13. 10% bleach
- 4.1.14. Sterile saline solution (see SOP MD.004)
- 4.1.15. Synergel Agarose Clarifier
- 4.1.16. 70% isopropyl alcohol
- 4.1.17. DEPC water
- 4.1.18. Reverse Osmosis H<sub>2</sub>O
- 4.1.19. *Tritrichomonas foetus*

**TFR3 5'-CGG GTC TTC CTA TAT GAG ACA GAA CC-3'**

**TFR4 5'-CCT GCC GTT GGA TCA GTT TCG TTA A-3'**

**Product 347 base pairs**

			
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- 4.2. Equipment, Instrumentation, and Associated software.
  - 4.2.1. Pipettors
  - 4.2.2. Microcentrifuge
  - 4.2.3. Thermal cycler (Applied Biosystems)
  - 4.2.4. Electrophoresis Gel system (Bio-Rad)
  - 4.2.5. Mini vortex
  - 4.2.6. Timers
  - 4.2.7. Refrigerator
  - 4.2.8. Freezers (-20<sup>0</sup>C and -70<sup>0</sup>C)
  - 4.2.9. 0.2 ml PCR tubes (Applied Biosystems)
  - 4.2.10. Biohazard bags
  - 4.2.11. Aerosol-resistant pipette tips
  - 4.2.12. Powder-free gloves
  - 4.2.13. Whirl-Pak bag (Koch Supplies)
  - 4.2.14. Disposable transfer pipettes
  - 4.2.15. Microwave
  - 4.2.16. Beaker
  - 4.2.17. 2.0 mL DNase free microcentrifuge tubes
  
- 4.3. Instrument and Method Calibration
  - 4.3.1. Pippettors are calibrated annually following SYS.19 (Procedure of acceptance, use, and maintenance of laboratory equipment).
  
- 4.4. Sample Handling and Storage
  - 4.4.1. Upon receiving, the specimens are stored in a refrigerator at -4<sup>0</sup>C until testing. After the samples have been taken, the specimens are stored in a non-defrosting freezer (-20<sup>0</sup>C or lower) for a minimum of two months. The positive specimens are stored in a -70<sup>0</sup>C freezer.
  
- 5. Preparation for the Tests
  - 5.1. Personnel Qualification/training
    - 5.1.1 Personnel must be trained and qualified to perform the test. Training records are kept in the individual employee training record file.
  
  - 5.2. Disinfectants and Initial Preparation



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- 5.2.1. Biohazard hoods must be UV irradiated for at least 15 minutes before use. Hoods are cleaned out with 10% bleach, DNase Away, and then 70% ethanol or isopropyl alcohol before use.
- 5.2.2. Pipettors are cleaned with DNase Away and then with 70% ethanol or isopropyl alcohol.
- 5.2.3. Powder-free gloves must be worn and changed often.
- 5.2.4. Only aerosol-resistant pipette tips are used.
  
- 5.3. Preparation of reagents/controls
  - 5.3.1. Reference Standards and Reference Materials  
Positive control DNA for PCR is a NVSL strain extracted from TRI culture provided by BAC.
  - 5.3.2. Resuspend the entire contents of RNase A (1.55 mg) in 200 ul of Resuspension buffer and add to the remaining Resuspension buffer. Resuspension buffer should now be stored at 4°C.
  - 5.3.3. Add the 25 ml of 4X final Wash to 75 ml of (95% ethanol to make 100 ml of 1 X Final Wash).
  - 5.3.4. Prepare 70% ethanol with 100% (absolute) ethanol and RNase-free water.
  - 5.3.5. Check the Lysis buffer for a white precipitate. If present, place the buffer in a 37°C incubator for 5 minutes until the solution clears.
  - 5.3.6. Stock Primer (200 pmoles/ul) preparation  
Take the amount of nmoles for the primer, multiply the number by 5, and add this amount of RNase/DNase-free water in micro liters to the primer tube. This equals 0.2 nmoles/ul or 200 pmoles/ul.  
Example: Stock primer has 72.6 nmoles  
 $72.6 \times 5 = 363$   
Add 363 ul of RNase/DNase-free water to the primer tube, mix well.
  - 5.3.7. Working Primer (20pmoles/ul) preparation  
Add 1 part of stock primer solution plus 9 part of RNase/DNase-free water in a 1.5 ml microcentrifuge tube, mix well. The final concentration is 20 pmoles/ul
  
- 6. Procedure
  - 6.1. DNA Extraction S.N.A.P. Miniprep Kit
    - 6.1.1. Sample Preparation  
The TRI culture is centrifuged at 5000 rpm for 2 minutes at room temperature. Discard the supernatant, use the precipitating pellet for DNA extraction.

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- 6.1.2. Resuspend the pellet in **150 of Resuspension buffer** and mix well by vortexing or gently pipetting up and down.
- 6.1.3. Add **150 ul of lysis buffer** and mix gently by pipetting up and down. Incubate for 3 minutes at room temperature.
- 6.1.4. Add **150 ul of cold Precipitation salt** and vortex to ensure thorough mixing of all components.
- 6.1.5. Centrifuge in a microcentrifuge at room temperature at 14,000 x g for 5 minutes.
- 6.1.6. Place the S.N.A.P Mini-prep column inside the 2 ml collection tube.
- 6.1.7. Add **600 ul of binding** buffer into sterile microcentrifuge tube. Pipette the supernatant into binding buffer and mix well. Discard the gelatinous pellet.
- 6.1.8. Pipette or pour the entire solution onto the S.N.A.P Miniprep column/Collection tube.
- 6.1.9. Centrifuge the S.N.A.P. Miniprep Column/Collection tube at room temperature at 3,000x g for 30 seconds. Discard the column flow through.
- 6.1.10. Add 500 ul of Wash buffer.
- 6.1.11. Centrifuge the S.N.A.P. Miniprep column/collection tube at room temperature at 3,000 x g for 30 seconds. Discard the column flow through.
- 6.1.12. Add **900 ul of 1x Final Wash** and centrifuge at 3,000 x g for 1 minute. Discard the column flow through.
- 6.1.13. Centrifuge the S.N.A.P. Miniprep column/collection tube at room temperature at maximum speed for 1 minute to dry the resin.



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- 6.1.14. Transfer the S.N.A.P. Miniprep column to a sterile microcentrifuge tube, and add **60 ul sterile water** directly to the resin. Incubate for 3 minutes at room temperature.
- 6.1.15. Centrifuge the S.N.A.P. Miniprep column/collection tube at room Temperature at 8,000 x g for 30 seconds. The plasmid DNA is now eluted from the column. Remove and discard column. Label tube with case number and test to be performed.

6.2. PCR

- 6.2.1. In the "clean" hood, prepare a master mix of the following reagents sufficient for the number of samples being tested. The amount given in **table 1** is per sample. Prepare the reaction mix (everything but the template) by pipetting : DEPC H<sub>2</sub>O, 10X PCR buffer, 50mM MgCl<sub>2</sub>, primers, 10 mM dNTP, and Taq polymerase into a RNase/DNase –free microcentrifuge tube, mix completely. Distribute 20 ul of mix to a 0.2 mL PCR tube(if using 0.5 mL PCR tube then add 20 ul followed by two drops of mineral oil). Then add 5 ul of DNA template to the PCR tube. The positive control is *Tritrichomonas foetus* DNA, the negative control is DEPC water.
- 6.2.2. Information on setting-up and programming the thermal cycler can be found in the user's manual. The conditions for the RT-PCR on the PCR System 9700 or 2700 are shown in the **table 2**.
- 6.2.3. Record test sample and control sample results on a PCR test record sheet CF.MD-009.

**Table 1.** PCR reaction mix volumes for *Tritrichomonas foetus*

Items	Volume per reaction
DEPC H <sub>2</sub> O	14.875 ul
10 x PCR buffer	2.50 ul
50 mM MgCl <sub>2</sub>	1.00 ul
10 mM dNTP	0.50 ul
TFR3 primer (20pmol/ul)	0.25 ul
TFR4 primer (20pmol/ul)	0.25 ul
Taq polymerase (5 unit/ul)	0.125 ul
Mix per rxn	20.0 ul
DNA Template	5.0 ul
Total	25.0 ul



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Table 2. Thermal cycling conditions for *Tritrichomonas foetus* PCR

PCR	1 cycle	5 min	95 <sup>0</sup> C	
	40 cycles	denaturation	95 <sup>0</sup> C	30 sec
		annealing	67 <sup>0</sup> C	30 sec
		extension	72 <sup>0</sup> C	30 sec
	1 cycle	extension	72 <sup>0</sup> C	5 minutes
		soak	25 <sup>0</sup> C	

6.3. Data Analysis/Electrophoresis/Data Record

6.3.1. PCR product (347 bp) is electrophoresed (see MD.003)

6.3.2. Data Management

6.3.2.1. Record data on a separate PCR test record sheet for each test (CF.MD-009).

6.3.2.2. Data to be recorded includes date, accession number, thermal cycling program, and test results.

6.3.2.3. Tape the gel picture to the record page (CF.MD-009).

6.3.2.4. Give a Report to BAC.

7. Associated Forms or Files:


- 7.1. S.N.A.P Miniprep Kit laminated manual
- 7.2. CF.MD-009, Data Record Sheet
- 7.3. SOP SYS.19
- 7.4. UVIS VERSION 3.1.2
- 7.5. SOP MD.003
- 7.6. SOP MD.004
- 7.7. SOP BAC.502

8. Revisions:

N/A

9. References:

- 9.1. Felleisen, R.S., Lambelet, N., Bachmann, P., Nicolet, J., Muller, N., and Gottstein, B. Detection of *Tritrichomonas foetus* by PCR and DNA enzyme immunoassay based on rRNA gene unit sequences. *Journal of Clinical Microbiology*, 36: 513-519,1998.

			
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**10. Quality Control:**

- 10.1. PCR Positive Control: MpTb DNA is from an NVSL strain obtained from BAC.
- 10.2. PCR Negative Control: DEPC water.
- 10.3. Non-conformances and Corrective actions
  - 10.3.1. If the positive control result is negative, run the test again using the same reagents and new positive control or new reagents and new positive control.
  - 10.3.2. If the negative control result is positive, there is contamination either in the water, the reagents or the primer mix. Run the test again using new reagents, water, and primer mix.

**11. Policy Implementation steps:**

N/A

## Appendix B

### Individual Bull Epithelial Area Range and Mean

Bull # & Age	Distal		Middle		Proximal	
	Range $\mu\text{M}^2$	Mean $\mu\text{M}^2$	Range $\mu\text{M}^2$	Mean $\mu\text{M}^2$	Range $\mu\text{M}^2$	Mean $\mu\text{M}^2$
47 / 6yr old	882584 - 1936671	1340585	395088 - 833352	607728	634064 - 1133975	849104
48 / 5yr old	426944 - 1378165	734631	544824 - 974387	790637	650157 - 1799887	1104763
49 / 12yr old	384084 - 996036	747372	398172 - 847979	618233	470269 - 1150715	679985
50 / 5yr old	423180 - 1478482	1004658	752971 - 1634229	1132880	562485 - 1083634	821854
51 / 2yr old	572725 - 1042728	753338	389473 - 1046252	594487	251116 - 1832740	907015
52 / 2yr old	461594 - 936462	711866	502833 - 929470	718505	1058627 - 1522066	1190887
53 / 2yr old	545074 - 1291795	858087	708662 - 1145499	832032	629531 - 1100649	933549
54 / 7yr old	522978 - 915460	659214	708662 - 114549	832032	506954 - 686292	572387
55 / 6yr old	605503 - 1131074	872921	364399 - 796347	630623	605494	965749
101 / 2yr old	540479 - 1373499	943946	540338 - 968423	810046	1059679 - 1508778	1305229
102 / 2yr old	776480 - 1301775	1132321	358493 - 810975	574748	563783 - 1579383	1124585
103 / 2yr old	595344 - 982672	735344	598221 - 1354708	949096	401061 - 883053	677620

## Appendix C

### Individual Bull Infolding Area Range and Mean

Bull # & Age	Distal		Middle		Proximal	
	Range $\mu\text{M}^2$	Mean $\mu\text{M}^2$	Range $\mu\text{M}^2$	Mean $\mu\text{M}^2$	Range $\mu\text{M}^2$	Mean $\mu\text{M}^2$
47 / 6yr old	6544 - 810495	164436	3464 -52306	31563	4680 -730462	234342
48 / 5yr old	17360 - 402147	155535	28238 - 208921	854887	10004 -61802	27452
49 / 12yr old	87481 - 745203	52738	8903 -39163	25614	7142 -771173	214601
50 / 5yr old	13954 - 99886	52738	3359 - 298434	135512	7107 -59028	27711
51 / 2yr old	12629 - 181163	93192	42826 - 192462	141280	6509 -806099	157558
52 / 2yr old	9495 - 240145	118589	29767 - 235954	118404	111913 - 759245	366862
53 / 2yr old	38745 - 328114	157448	25708 - 219693	97294	34399 - 117680	76039
54 / 7yr old	32721 - 234905	113752	18420 - 53914	34145	73901 - 244595	159248
55 / 6yr old	73873 - 392790	241356	87302 - 307513	146425	24241 - 247292	79049
101 / 2yr old	69842 - 731661	315065	98350 - 349685	165393	86871 - 488983	205962
102 / 2yr old	125988 - 783692	398433	37791 - 126860	84069	21220 - 891456	494694
103 / 2yr old	9857 - 275985	116472	34567 - 209739	113652	15719 - 210566	122926

## Appendix D

### Endnotes

- a. Butler Schein Animal Health, Dublin, OH
- b. Diamond's Media Reagents
  - 1. Bacto Agar **1gram**
  - 2. Bacto Peptone (trypticase peptone) **4.0 grams**
  - 3. Bovine serum **20 ml**
  - 4. Distilled or deionized water **18ml**
  - 5. L-ascorbic acid **.04grams**
  - 6. L-cysteine hydrochloride **.2grams**
  - 7. Maltose **1.0 grams**
  - 8. Penicillin G solution (100,000 units per milliliter) **2ml**
  - 9. Potassium phosphate dibasic  $K_2HPO_4$  **.16 grams**
  - 10. Potassium phosphate monobasic  $KH_2PO_4$  **.16 grams**
  - 11. Yeast extract **2.0 grams**
  - 12. Streptomycin sulfate solution (0.2 gram per milliliter) **1ml**
- c. Clark's Media Reagents.
  - 1. 5-Fluorouracil ( 300 $\mu$ g/ml) **3.0ml**
  - 2. Brilliant Green (50 $\mu$ g/ml) **.1ml**
  - 3. Calf serum **100ml**
  - 4. Cycloheximide (100 $\mu$ g/ml) **1.0ml**
  - 5. Polymixin B sulphate (100 IU/ml) **.1ml**
- d. Electron Microscopy Services, Hatfield, PA
- e. Sigma Chemical, St Louis, MO
- f. Tissue-Tek VIP E300™ Ames Co, Elkart, IN
- g. Reichert-Jung 2040 Autocut microtome™ Depew, NY
- h. Image J software NIH.gov
- i. EVO 50 SEM™ Zeiss XVP, USA
- j. Olympus BH-2, DP-71 © Olympus America Inc
- k. Microsoft Excel®
- l. Statistical Analysis System, Cary, NC