CORTISOL AND THE TEAR FILM: THE RELATIONSHIP BETWEEN STRESS, ADRENAL DISEASE AND TEAR CORTISOL LEVELS

by

BRITTANY MARIE WYNNE

(Under the Direction of KATHERN MYRNA)

ABSTRACT

The endocrine system and the lacrimal functional unit are linked in ways that are not currently well understood. The included study investigated concentrations of the endogenous glucocorticoid cortisol in the healthy canine tear film at rest and after intravenous stimulation with adrenocorticotropic hormone (ACTH). Endogenous cortisol was detected in the canine tear film at rest and was found to increase significantly in concert with serum cortisol levels following simulated stress via ACTH administration. The presence of elevated endogenous cortisol levels in the tear film has the potential to adversely affect the outcome of ophthalmic disease. A proposed study would investigate endogenous cortisol concentrations in horses with naturally occurring fungal keratitis.

INDEX WORDS: CORTISOL, TEAR FILM, CORTICOSTEROID, CANINE HYPERADRENOCORTICISM, HYPOTHALAMIC-PITUITARY AXIS, EQUINE FUNGAL KERATITIS, EQUINE PITUITARY PARS INTERMEDIA DYSFUNCTION

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DEDICATION

For my husband, greatest supporter, and perpetual study buddy.

I love you very much.

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I would like to thank my major professor, Dr. Kate Myrna, for all the faith you've had in me over the past two years and for always challenging me to do better. Your mentorship has made a huge difference in my career and I will be forever grateful. I would also like to thank my committee members, Dr. Kathryn Diehl and Dr. Kelsey Hart, for all of your help and encouragement. I feel so lucky to have had the opportunity to learn from you all, it really has been a dream come true.

Thank you to Dr. Sarah James, for your continued support in my career aspirations. You are an inspiring doctor (in fact, you inspired my interest in ophthalmology) and I am so thankful to have you as a friend.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

The hypothalamic-pituitary-adrenal (HPA) axis and the lacrimal functional unit are linked in ways that are not currently well understood. Cortisol, a corticosteroid hormone regulated by the HPA axis, has vast systemic metabolic and immune effects ranging from energy mobilization to inflammatory pathway effects. [1] Hyperadrenocorticism (canine Cushing's syndrome) is a relatively common disease in middle aged to older dogs wherein affected dogs experience hypercortisolism. Canine hyperadrenocorticism is usually either ACTH dependent and caused by a pituitary adenoma, or ACTH independent and caused by an adrenocortical adenoma or adenocarcinoma. [2] Horses experience a similar syndrome with dissimilar etiology. Pituitary Pars Intermedia Dysfunction (PPID, equine Cushing's syndrome) is primarily a disease of older horses that is characterized by a loss of the dopaminergic inhibition of the pars intermedia of the pituitary by the hypothalamus. PPID results in the overproduction of ACTH by the pituitary but does not necessarily result in hypercortisolism. [1] Interestingly, a recent study documented elevated tear film cortisol concentrations, but not serum cortisol concentrations, in horses diagnosed with PPID when compared to a control group comprised of age-matched healthy horses. [3] Therefore, dogs suffering from hyperadrenocorticism may experience similarly elevated tear cortisol concentrations.

Elevated endogenous concentrations of cortisol in the tear film may have negative implications in animals with ophthalmic disease. The use of topical corticosteroids as a treatment

for ophthalmic disease has well documented negative side effects, which include an increased susceptibility to infection and impaired corneal wound healing. [4-7] Increased systemic levels of cortisol in dogs with hyperadrenocorticism have also been documented to increase risk of ocular infection and delay corneal healing, both of which increase the risk for ocular disease progression [8, 9] This thesis presents data confirming the presence of cortisol in the normal canine tear film and explores the importance of further study of cortisol concentrations in the tear film of dogs and horses suffering from endocrine or ophthalmic disease. Plans for a future study investigating cortisol concentrations in the tear film of horses diagnosed with naturally occurring fungal keratitis, a condition for which topical corticosteroid use is a predisposing factor, are also discussed. [10]

CHAPTER 2

THE HYPOTHALAMIC-PITUITARY-ADRENAL AXIS

The hypothalamic-pituitary-adrenal (HPA) axis is a major neuroendocrine system responsible for the "fight or flight" response to physical or emotional stress. In conjunction with the sympathetic nervous system, the HPA axis responds to stressful stimuli by increasing the availability of energy to the heart, muscles, and brain, increasing heart rate, breathing rate, and blood pressure. The HPA axis also plays a crucial role in homeostasis, with effects ranging from the metabolic system to the immune system, affecting every tissue in the body. Regulation of the HPA axis is very complex and results from both neural and endocrine signals with various positive and negative feedback effects. [1]

The HPA axis is activated by the release of corticotropin-releasing hormone (CRH) into the localized hypothalamic–hypophyseal portal system by CRH neurons located in the parvicellular division of the paraventricular nucleus of the hypothalamus. CRH is then carried through this portal system to receptors in the anterior pituitary gland. CRH then stimulates the biosynthesis and release of adrenocorticotropic hormone (ACTH) by corticotrope cells in the anterior pituitary. ACTH is derived from pro-opiomelanocortin (POMC), a large precursor protein, and is released by the anterior pituitary into the general circulation. [1, 11] ACTH both stimulates adrenal cortical cells to synthesize and release cortisol and provides negative feedback to the hypothalamus, acting to reduce CRH levels. Cortisol also acts via a negative feedback mechanism on the hypothalamus to inhibit CRH secretion in addition to providing negative

feedback to the pituitary to inhibit ACTH secretion. (Figure 1) ACTH, and therefore cortisol, is important in maintaining the circadian rhythm. ACTH and cortisol levels are generally highest in the early morning hours (04:00-07:00 h) and lowest in the middle of the night (23:00-03:00h), except in dogs and cats. [1]

The adrenal gland is comprised of the adrenal medulla and the adrenal cortex. The adrenal cortex includes three zones: the zona glomerulosa, the zona fasciculata, and the zona reticularis. (Figure 2) The zona fasciculata makes up approximately 80% of the cortex and is responsible for the production of glucocorticoids, including cortisol. [12] Cortisol is the primary glucocorticoid produced by the zona fasciculata of the adrenal cortex and is synthesized from cholesterol via steroidogenesis in mitochondria. All synthesized cortisol is immediately secreted. The effect of ACTH on adrenal cortex steroidogenesis has both immediate and long-term effects. Immediately, cholesterol delivery to steroidogenic enzymes is increased. Over the following 24 hours, the synthesis of steroidogenic enzymes, HDL receptors, and LDL receptors increases through transcriptional control. Cortisol binds to the ligand-binding domain of the intracellular ligand receptor and the DNA binding domain of the receptor binds to the hormone response element of the target gene in the nucleus, stimulating transcription. (Figure 3) Cortisol affects many systemic processes by mobilizing energy stores to increase blood glucose levels, stimulating hepatic gluconeogenesis, glycolysis, and protein synthesis, and decreasing lipogenesis and glucose uptake by adipose tissues. Cortisol also affects inflammatory pathways by decreasing levels of inflammatory cytokines, neutrophil migration into tissues, and lymphocyte activity while stabilizing lysosomal membranes. [1]

Many peripheral tissues, including liver and adipose, have the ability to convert cortisol to the relatively less biologically active glucocorticoid cortisone, and vice versa, via the bidirectional enzyme 11βHS1. Cortisol is hydrophobic and is therefore transported in the blood by cortisol binding globulin (CBG), rendering the hormone soluble and inactive. Approximately 90-95% of cortisol in the circulation is bound to CBG. Therefore in principal, only unbound free cortisol has access to enter cells via diffusion through the plasma membrane and is biologically active. [1, 13] CBG is also biologically active in ways that are not yet well understood, but may have implications in inflammatory processes. [13]

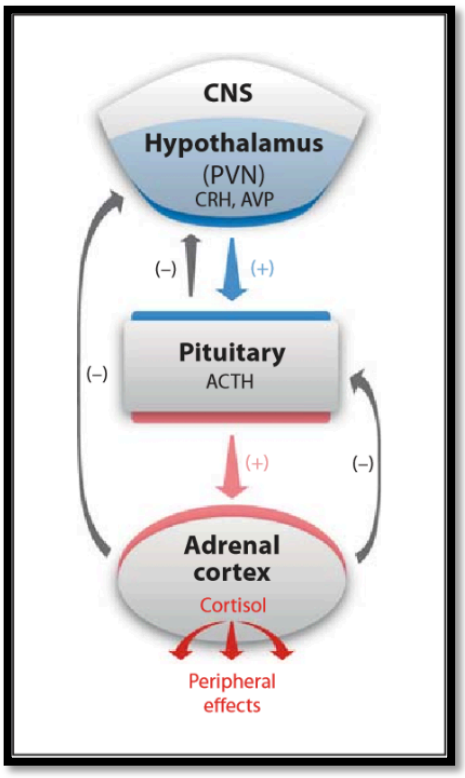

Figure 1: Depiction of positive and negative feedback effects within the hypothalamic-pituitary axis [1]

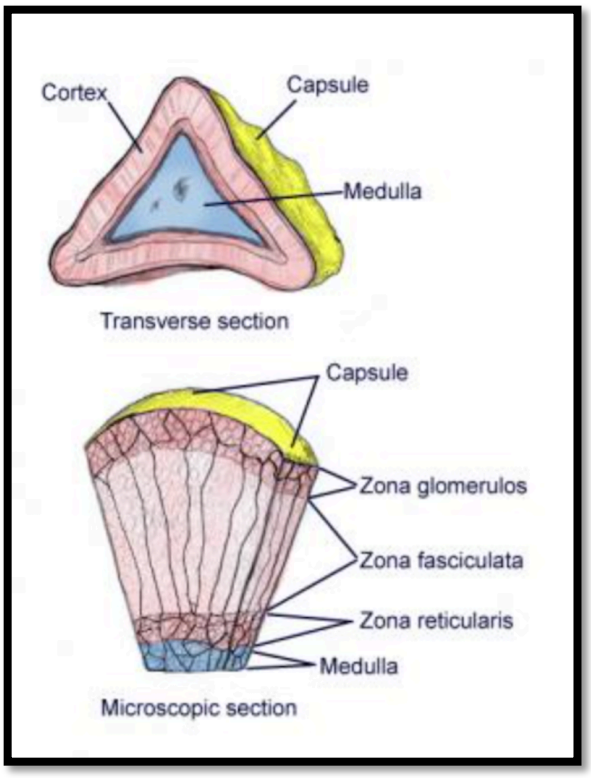

Figure 2: Depiction of a microscopic transverse section of an adrenal gland. [14]

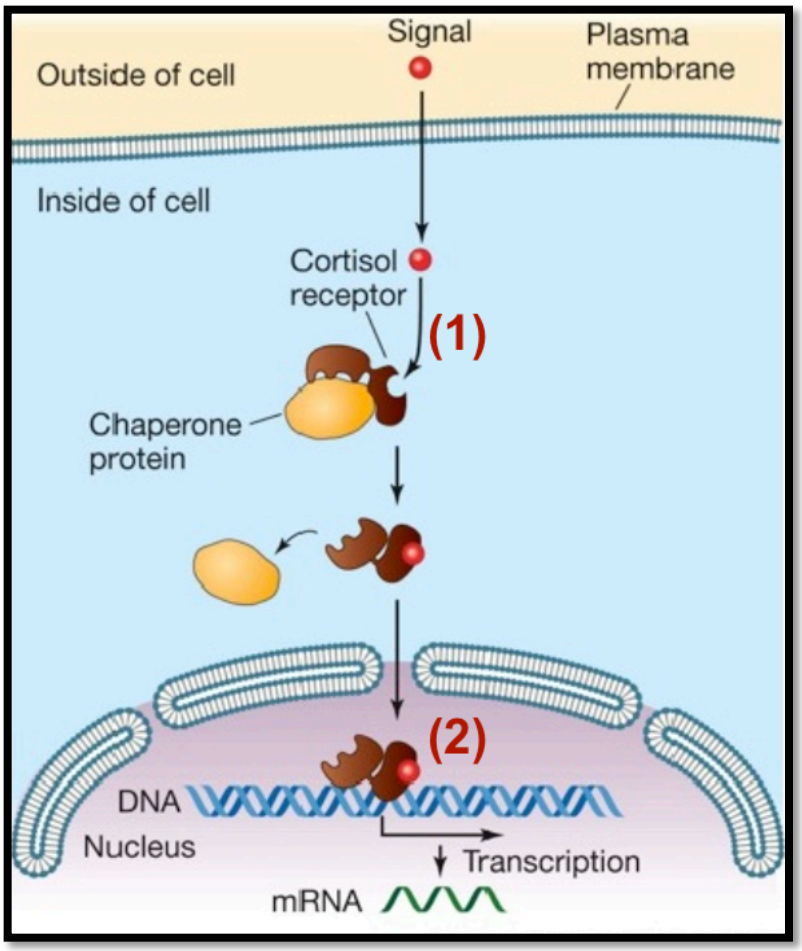

Figure 3: (1) Cortisol binds to the ligand-binding domain of the intracellular ligand receptor and (2) the DNA binding domain of the receptor binds to the hormone response element of the target gene in the nucleus, stimulating transcription. [1, 15]

CHAPTER 3

CANINE HYPERADRENOCORTICISM AND EQUINE PITUITARY PARS INTERMEDIA DYSFUNCTION

Canine Hyperadrenocorticism

Canine hyperadrenocorticism, also known as canine Cushing's syndrome, is a relatively common disease diagnosed primarily in middle aged to older dogs, with a reported incidence of 1-2 cases per 1000 dogs. [2, 16] Symptoms of hyperadrenocorticism are related to the utilization of protein for gluconeogenesis and lipogenesis and other biochemical effects of hypercortisolism. [2] (Figure 4) Common clinical observations include polyuria, polydipsia, alopecia, hepatomegaly, weight gain, muscle atrophy and skin changes consistent with endocrine disease. [2, 17] In about 80% of cases the syndrome is caused by hypersecretion of ACTH by a pituitary corticotroph adenoma that may be located in the anterior lobe or the pars intermedia. The excess ACTH upregulates the HPA axis and stimulates the adrenal cortex to synthesize and release excess glucocorticoids. The remaining cases are ACTH independent and caused by hypersecretion of glucocorticoids by an adrenocortical adenoma or adenocarcinoma. [2] Occasionally, the syndrome can be iatrogenic, resulting from the administration of glucocorticoids as a treatment for disease, and very rarely the syndrome has been attributed to ectopic ACTH secretion. [2, 18, 19]

Equine Pituitary Pars Intermedia Dysfunction

Equine Pituitary Pars Intermedia Dysfunction (PPID), also known as equine Cushing's syndrome, is a an endocrine disease mostly attributed to aged horses [20] The most important risk factor for the development of PPID is advanced age and some horses, namely ponies, may have a genetic predisposition for the development of PPID. Clinical signs of PPID include hirsutism, hyperhidrosis, weight loss, muscle loss, and chronic or recurrent laminitis. Approximately 30% of cases experience polyuria and polydipsia. [3, 20] Decreased immunocompetency may be evident, with opportunistic pathogens establishing infections in approximately 35% of horses diagnosed with PPID versus 11% of normal horses of advanced age. [20]

The equine pituitary gland is comprised of 4 lobes: pars intermedia, pars distalis, pars tuberalis and pars nervosa. The pars intermedia is directly innervated by the dopaminergic neurons of the paraventricular nucleus of the hypothalamus and consists of a single cell type, the melanotrope. Melanotropes produce pro-opiomelanocortin (POMC), a precursor protein for a diverse range of products, including ACTH. PPID is characterized by the loss of inhibition from the dopaminergic neurons of the paraventricular nucleus of the hypothalamus to the pars intermedia of the pituitary gland. The cause of the loss of dopaminergic inhibition is unknown but there is evidence that oxidative stress may contribute to neuronal damage and cell death. This loss of inhibition to the pars intermedia leads to the overproduction of POMC-peptides by melanotropes. The prohormone convertases in the pars intermedia and pars distalis that normally work together to first cleave POMC into ACTH and then cleave ACTH into the smaller peptide products a-MSH and CLIP are overwhelmed, and the result is an overabundance of ACTH being

produced in the pars intermedia. [20] (Figure 5) However, recent evidence suggests that some of this overproduction may be comprised of a less bioactive isoform of ACTH. [3, 21] This fits with the findings of several studies that did not detect elevated serum cortisol levels in horses with PPID. [3, 20, 22] A recent ophthalmic study detected significantly elevated cortisol concentrations in the tear film of horses diagnosed with PPID that were not accompanied by significantly increased serum cortisol concentrations, with cortisol concentrations being 1.5-17 fold higher in tears than in serum. These results are evidence that increased tear cortisol concentrations in horses with PPID are not solely dependent on the overproduction of ACTH by the pars intermedia and that increased local ocular production of cortisol or altered tissue cortisol metabolism may play a role. [3]

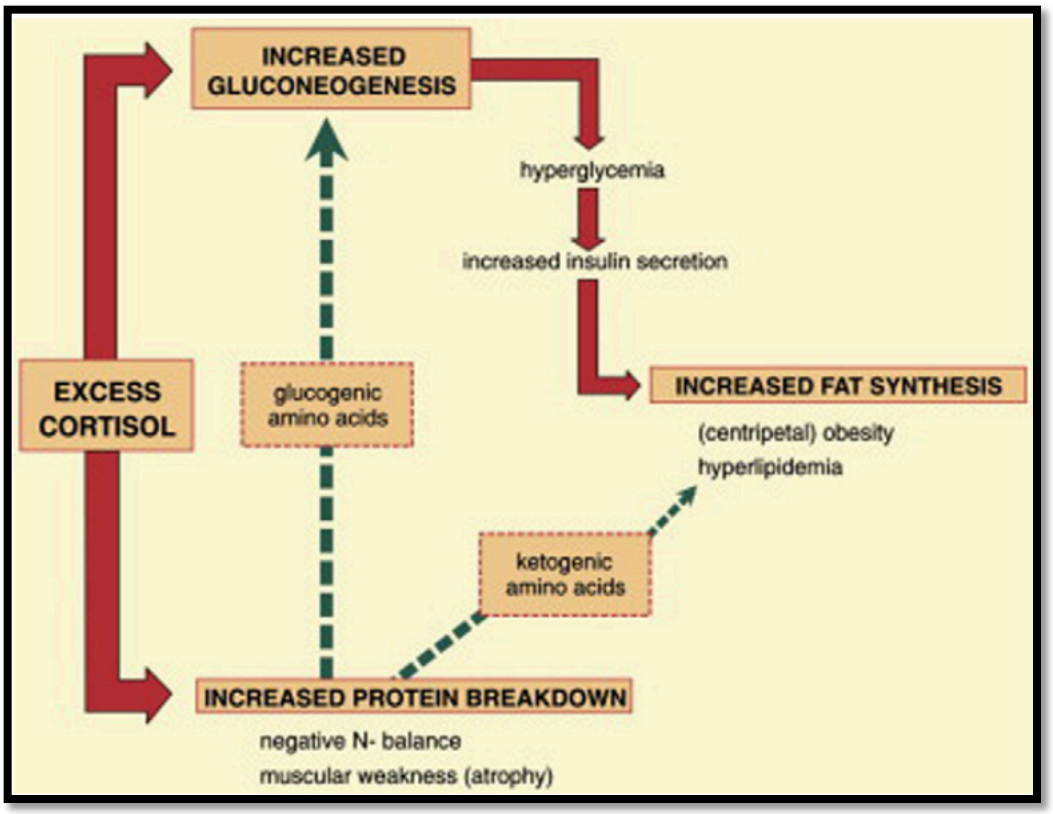

Figure 4: Biochemical effects of hypercortisolism include the utilization of protein for gluconeogenesis and lipogenesis. [2]

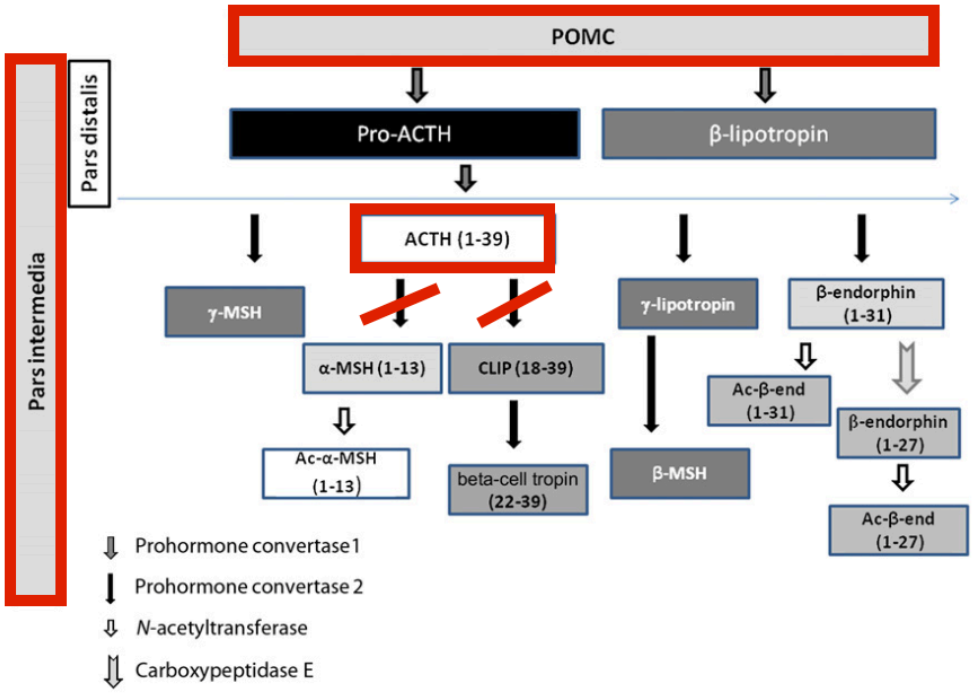

Figure 5: In horses with PPID the system of prohormone convertases in the pars intermedia and pars distalis that normally work together to cleave POMC into smaller peptides is overwhelmed, resulting in an overabundance of ACTH in the pars intermedia. [20]

CHAPTER 4

THE LACRIMAL FUNCTIONAL UNIT AND TEAR DYNAMICS

The lacrimal functional unit (LFU) includes the lacrimal glands, ocular surface and interconnecting innervation. In dogs, the orbital gland and the gland of the nictitating membrane produce most of the tear film, which reaches the ocular surface through multiple small ductules. [23, 24] (Figure 6) The eyelids and the nictitating membrane distribute the tear film over the corneal surface. The tear film then exits the ocular surface through the nasolacrimal system or absorption into ocular tissues. [23, 24] Sympathetic innervation of the lacrimal gland is derived from the superior cervical sympathetic ganglion, which branches into the trigeminal nerve, the ophthalmic nerve, and then the lacrimal nerve. Parasympathetic innervation of the lacrimal gland is derived from the lacrimal nucleus of the facial nerve, which eventually branches into the zygomatic temporal nerve that reaches the lacrimal gland. [24]

The precorneal tear film is comprised of three layers: the mucin layer, the aqueous layer and the lipid layer. A fourth innermost glycocalyx layer extending from the ocular epithelia to the mucin layer is also sometimes described. (Figure 7) Classically, the three layers of the tear film are thought of separately, but emerging research describes the tear film as intermixed. [24] The mucin layer is produced by conjunctival goblet cells and is the layer of the tear film that interfaces with the hydrophobic cornea. It facilitates the adherence of the aqueous layer to the corneal and conjunctival epithelial cells and provides an immunological barrier, immobilizing more than 30% of IgA in the tear film. The aqueous layer is the intermediate tear film layer

produced by the orbital gland and the gland of the nictitating membrane. It functions to provide the cornea with nutrition and is a mixture of water, ions, glucose, proteins, and glycoproteins including immunoglobulins and enzymes. The aqueous layer also functions to remove waste, such as carbon dioxide, lactic acid, and other debris from the ocular surface. [24, 25] The lipid layer is produced by the meibomian glands of the eyelids and is comprised of triglycerides, free fatty acids, waxes, and esterified cholesterol. It is the most superficial layer of the tear film and functions to stabilize and prevent evaporation of the aqueous layer. The three layers together also function to prevent desiccation of the cornea, lubricate the eyelids, maintain the refractive power of the cornea, and provide protection against infections. The tear film is rich in lysozyme, betalysin, lactoferrin, antibodies, and IgA and IgG secreted by the lacrimal glands. A small amount (1%) of serum protein is also present in the tear film, which includes additional immunoglobulins. [25, 26] A recent proteome study identified 125 proteins in the canine tear film, with serum albumin as one of the most abundant. [27] Abnormalities in either the quantity or quality of any of the tear components can compromise tear function. [25]

The conjunctiva is a vascular mucous membrane that covers the anterior surface of the globe, the posterior surface of the eyelids, and the anterior and posterior surface of the nictitating membrane. The conjunctiva secretes mucus and immunocompetent cells, which initiate and mediate inflammatory reactions and synthesize immunoglobulin. Microvilli and enzymatic activity also confer protection against foreign particles. The conjunctiva is composed of the epithelium, the basement membrane zone and the chorion or conjuctival stroma. The chorion is abundantly vascularized and contains immunocompetent cells capable of rapid inflammatory reactions. Both serous and mucous glands are present in the conjunctiva, along with goblet cells

in the conjunctival epithelium. The mucin layer of the tear film attaches to the glycocalyx of the cornea and conjunctiva. The glycocalyx includes glycoproteins and glycolipids that cover the microvilli and microplicae of the corneal and conjunctival epithelium. The thickness of the glycocalyx is approximately 300 nm and it helps to protect the epithelium by causing the shear forces of blinking to break up the mucous layer farther from the cell surface. Mucus attachment to the glycocalyx helps the aqueous layer of the tear film to spread evenly over the corneal and conjunctival epithelium. [24]

Glands are also positioned on the eyelids that secrete components of the tear film. The meibomian glands that produce the lipid layer of the tear film are located on the leading edge of the eyelid and are not associated with the cilia. They are holocrine glands and secretion may be partially under neural or hormonal control. [24]

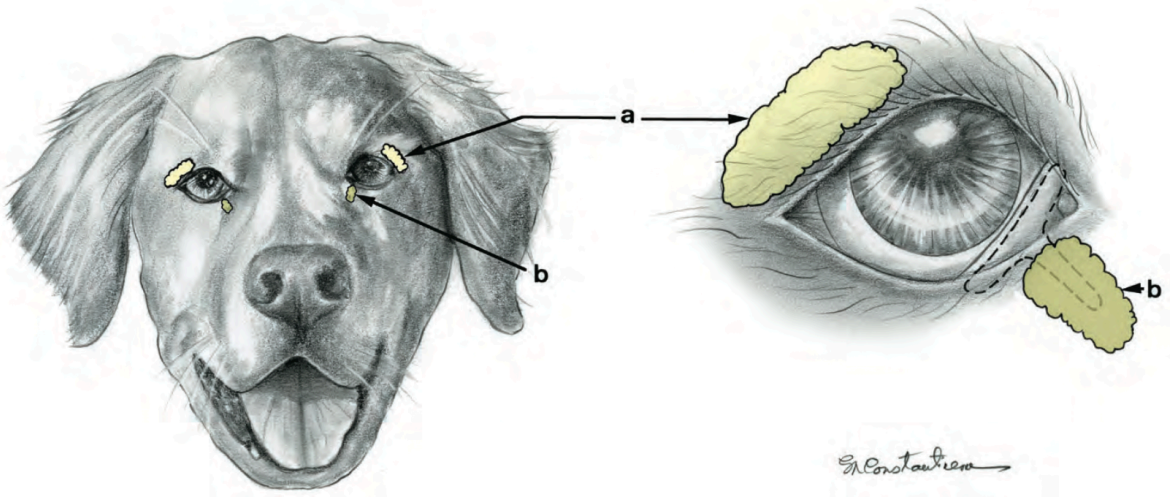

Figure 6: A topographical depiction of a) the orbital gland and b) the gland of the nictitating membrane. [24]

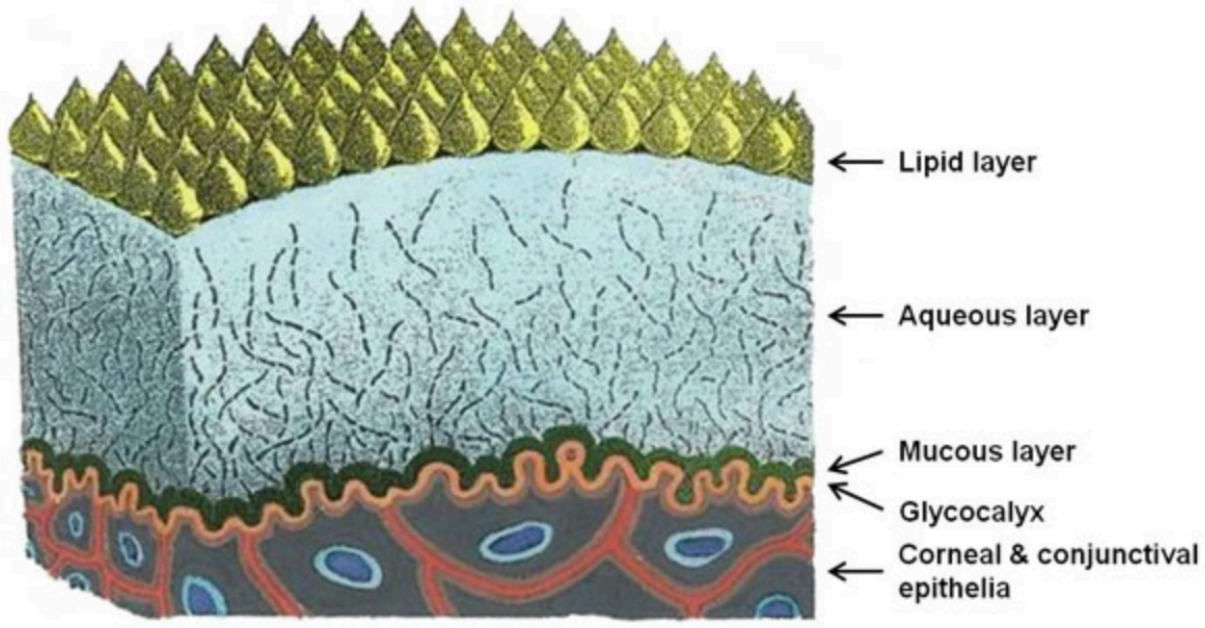

Figure 7: The layers of the precorneal tear film. [28]

CHAPTER 5

ENDOGENOUS CORTISOL CONCENTRATION IN CANINE TEARS AND SERUM AT REST AND AFTER A SIMULATED STRESS EVENT[1]

[1] Wynne, B.M., Hart, K.A., Norton, N.A., Diehl, K.A., Myrna, K.E. To be submitted to *Veterinary Ophthalmology*.

Abstract

Purpose. The concentration of cortisol, an endogenous glucocorticoid, in canine tears may have implications for corneal healing in the event of ophthalmic disease. The relationship between canine tear and serum cortisol concentrations is unknown. This study evaluates levels of endogenous total cortisol in normal canine tears and serum at rest and after a simulated stress event. **Methods.** Paired tear and serum samples were collected from 11 normal, adult dogs once daily for 3 consecutive days. Paired tear and serum samples were then collected from 4 normal, adult dogs once before and at 1 and 2 hours post intravenous injection with ACTH. (Cortrosyn®, 5µg/kg IV) Tear and serum free cortisol concentrations were determined using a competitive ELISA and an IMMULITE® immunoassay respectively. Tears were collected by placing a glass capillary tube at the medial conjunctival fornix and blood was collected via jugular venipuncture. Tear and serum cortisol concentrations were compared using one-tailed t-tests. **Results.** Cortisol was present in resting canine tears and serum at concentrations between 0-3.305 ug/dL and 0.57-16.8 ug/dL respectively. The concentration of cortisol in both tears and serum was significantly elevated one hour after ACTH stimulation (p-value < 0.05) and remained significantly elevated two hours after ACTH stimulation ((p-value < 0.05). **Conclusions.** Cortisol is present in canine tears and serum at rest. Cortisol concentrations in canine tears and serum increase significantly following stress simulated by intravenous administration of ACTH. The presence of cortisol in canine tears has the potential to adversely affect the outcome of canine ophthalmic disease.
Supported by the University of Georgia College of Veterinary Medicine Ocular Research Fund.

Introduction

Exogenous corticosteroids are commonly employed in the treatment of canine ocular disease. [29] Treatment with topical corticosteroids is indicated in a variety of ocular inflammatory conditions and is an effective method of reducing corneal scarring. However, the side effects of topical corticosteroid use require careful consideration when used on the corneal surface. Use of topical corticosteroids has been associated with impaired corneal healing, a loss of corneal immunocompetency, and loss of stromal cell density. [4, 5, 30] Additionally, treatment with topical corticosteroid has been shown to predispose the cornea to infection. [5-7] In a recent case series of 11 dogs diagnosed with keratomycosis, 5 of the dogs were receiving long-term topical corticosteroid therapy following cataract surgery. [5] A recently published study investigating susceptibility patterns of bacterial keratitis reported that 31% of cases had been previously treated with a topical corticosteroid. [7] Another recent retrospective equine study of 47 horses diagnosed with equine keratomycosis reported that almost 50% of presented cases had received treatment with topical corticosteroids for conditions such as uveitis, eosinophilic keratitis and conjunctivitis. [6] When corneal infection is established, matrix metalloproteinase (MMP) activity is increased up to 15-fold by corticosteroids. MMP's possess collagenolytic properties that may play a key role in the progression of keratitis and potential loss of the eye. [31, 32]

Hyperadrenocorticism (HAC) is a common disease of middle-aged to older dogs, with a reported incidence of 1-2 cases per 1000 dogs per year. [2, 16] Approximately 85% of cases of HAC are ACTH-dependent, arising from a hypersecretory pituitary corticotroph adenoma. The remaining percentage of HAC cases are ACTH-independent and result from the hypersecretion

of glucocorticoids by an adrenocortical adenoma or adenocarcinoma. [2] Elevated endogenous cortisol levels have also been detected in dogs undergoing psychological stress, such as those exposed to an animal shelter environment. [33] Like topical treatment with corticosteroids, increased systemic cortisol levels due to hyperadrenocorticism also increases the risk of infection of the eye by pathogens. [8] Approximately 80% of HAC dogs suffer from lymphopenia and dogs diagnosed with pituitary-dependent HAC have abnormal lymphocyte subsets, both of which increase susceptibility to common infectious diseases. [34, 35] Additionally, dogs suffering from hyperadrenocorticism exhibit delayed corneal healing, which increases risk of ocular disease progression. [8, 9] Calcific band keratopathy has also been associated with elevated systemic cortisol concentrations, with 11/14 cases in one study having suspected hyperadrenocorticism. [9]

This pilot study begins to investigate the theory that increased endogenous cortisol concentrations in canine tears associated with ACTH-dependent hyperadrenocorticism may exist. Endogenous cortisol concentrations have been evaluated in canine serum and saliva, but have not been characterized in canine tears. A 2014 study from our research group evaluated free and total cortisol concentrations in both tears and serum in horses at rest and following simulated stress via intravenous ACTH administration. Cortisol concentrations in equine serum and tears were found to be significantly elevated following ACTH administration. [36] The purpose of the current study was to evaluate tear and serum total cortisol concentrations in healthy dogs at rest and after ACTH administration. We hypothesized that cortisol would be present in canine tears at rest and that concentrations would increase in concert with serum total cortisol concentrations following simulated stress via ACTH stimulation.

Methods

The following protocol was approved by the University of Georgia's clinical research committee and was preformed in accordance with the ACVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Eleven client-owned dogs were recruited for the study and admitted to the University of Georgia College of Veterinary Medicine. Breeds included one Doberman Pinscher, one Labrador Retriever, two small mixed breeds, three large mixed breeds, and four Rottweilers, Four dogs were male and seven female, all were neutered or spayed. Mean age was 5.1 +/- 1.8 years (range 1-7 years). All dogs were determined to be ophthalmically and systemically normal before inclusion in the study. A complete ophthalmic examination was performed by a veterinary ophthalmologist. (KM) Ophthalmic screening included a Schirmer tear test (Schering-Plough Animal Health, Union, NJ), fluorescein stain (Fluorets, Chauvin Pharmaceuticals, Essex, England), slit-lamp biomicroscopy (SL-14 Portable Slit Lamp, Kowa, Torrance, CA), and indirect ophthalmoscopy with a 20 diopter lens (Volk, Mentor, OH) and transilluminator (3.5V Finnoff Transilluminator, Welch Allyn, Skaneateles Falls, NY). Intraocular pressure was assessed using an applanation tonometer (TonoPen XL, Mentor O and O, Norwell, MA) after instillation of topical 0.5% proparacaine (Alcon, Fort Worth, TX). Dogs were also screened for adrenal dysfunction before inclusion via complete blood count, serum chemistry and urinalysis. Patients were excluded due to the presence of ocular disease or evidence of systemic disease on physical exam or clinicopathologic analysis.

Phase 1: Canine tear and serum cortisol concentrations at rest:

Paired tear and serum samples were collected from 11 client owned dogs once daily between 8:00 a.m. – 10:00 a.m. for a total of 3 days. An average of 62 uL (range 12.5-100 uL) of tears were collected from both eyes via a glass capillary tube gently placed in the medial conjuctival fornix as previously described. [36] Manual restraint was used as needed, with all but one dog needing only mild restraint. Tears were transferred to plastic Eppindorf tubes for storage. Blood was collected from the jugular vein or cephalic vein using the technique deemed less stressful to the participant. 2 mL of whole blood were collected in a red top tube and centrifuged to separate the serum. All serum samples were then transferred to plastic Eppindof tubes for storage. All tear and serum samples were centrifuged within 60 minutes of collection and stored in an -80 F freezer until analysis.

Phase 2: Tear and serum cortisol concentrations after intravenous ACTH stimulation:

Paired tear and serum samples were collected from 4 dogs immediately before (T0, included in the baseline measurements,) 1 hour after (T1) and 2 hours after (T2) intravenous stimulation with cosyntropin (Cortrosyn® 5ug/kg, Amphastar Pharmaceuticals Inc, Rancho Cucamonga, CA.) Dogs were allowed to acclimate to the hospital environment for 72 hours prior to collection. Tears and serum were collected as previously described for the baseline collections. Samples were centrifuged within 180 minutes of collection. All samples were stored in an -80 F freezer until analysis. Sample analysis proceeded in the same fashion as phase 1.

Sample Analysis:

Total cortisol concentrations were determined for both the tear and serum samples. Serum total cortisol concentrations were analyzed at the University of Georgia College of Veterinary Medicine Diagnostic Laboratory using an Immulite® 2000 immunoassay (Siemens Healthcare, Munich Germany). Tear total cortisol levels were determined using a commercially available Salimetrics® salivary cortisol ELISA previously validated for use in mammalian tears.

Because use of this ELISA for measurement of canine tear cortisol was not previously described, preliminary validation of the assay was performed. Two hundred µl of canine tears were collected from healthy dogs for another study, pooled and stored at -80 degrees. The tears were divided into 25 µl equal aliquots diluted 1:2 with assay buffer. Assay standard was added to the aliquots to reach final added cortisol concentrations of 0.0 ng/ml, 0.04 ng/ml, 0.1 ng/ml, 0.2 ng/ml, 0.4 ng/ml, 1 ng/ml, and 2 ng/ml. Cortisol was measured with the ELISA in each spiked tear sample in duplicate and percent recovery of expected cortisol concentration calculated for each sample as follows: (ELISA cortisol value/expected cortisol value) x 100. Average ± SD percent recovery was acceptable at 86.28 ± 16.52%.

Bland-Altman analysis was used to compare observed vs. expected cortisol concentrations to determine preliminary assay accuracy (Figure 8). Mean bias ± standard deviation (95% limits of agreement) was also acceptable at 0.24 ± 0.37 (-0.49 – 0.97). Intra- and inter-assay coefficients of variation for use in tears has been previously reported. [3] Average percent recovery for use in canine tears was 86.28% +/- 16.52%.

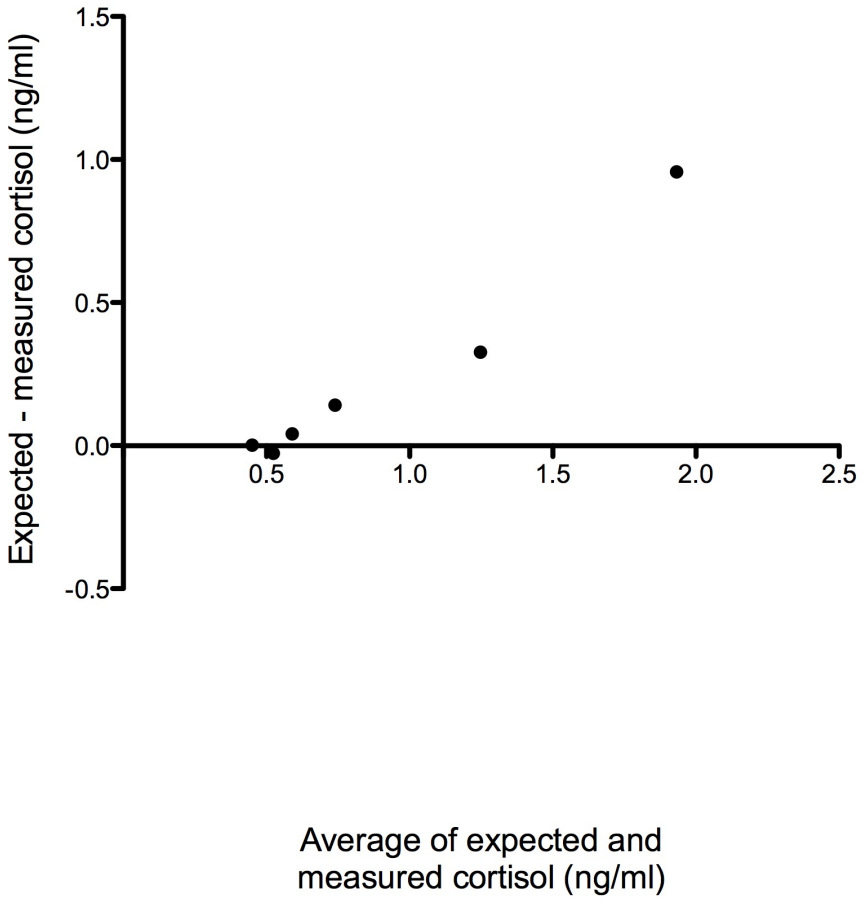

Figure 8: Bland-Altman Analysis of observed vs. expected cortisol concentrations in canine tears

Statistical Analysis:

The following statistical analysis has been previously described by Monk et al. (Monk, 2014) Serum and tear ELISA results were analyzed using Bland-Altman analysis, with bias expressed as mean ± standard deviation (95% limits of agreement). All tear and serum cortisol concentrations are indicated as mean ± standard deviation.

The effect of sampling day on resting serum and tear cortisol concentrations in phase 1 was analyzed using repeated measures analysis of variance (ANOVA). Repeated measures ANOVA was also used to analyze serum and tear cortisol concentrations before and 1 and 2 hours after intravenous administration of cosyntropin in Phase II. Tukey-Kramer tests were used for all post-hoc analyses.

Multiple regression analysis of tear total cortisol concentrations with dog and serum total cortisol concentrations as predictors was performed for phase 2. A scatter plot with a line of best fit for each dog determined using linear regression was generated to illustrate the relationship between serum and tear total cortisol for each dog. $P < 0.05$ was considered significant for all analyses. Analyses were performed using commercial statistical software (GraphPad Prism V. 5, GraphPad Software, Inc. La Jolla, CA; Stata, StataCorp LP, College Station, TX).

Results

Phase 1: Canine tear and serum cortisol concentrations at rest:

Cortisol was detected in all tear and serum samples. Tear and serum total cortisol concentrations are shown in Table 1. There was no significant difference in the resting tear total cortisol concentration, serum total cortisol concentration, or tear:serum total cortisol concentration ratio over the three collection days (Figure 9). Mean resting tear total cortisol concentration was 0.57 ± 0.72 µg/dL. Mean resting serum total cortisol concentration was 3.13 ± 3.81 µg/dL.

Phase 2: Canine tear and serum cortisol concentrations before and after ACTH stimulation:

Cortisol was detected in all tear and serum samples. Tear and serum total concentrations are shown in Table 2. There was no significant difference in the tear:serum total cortisol concentration ratio at any time during phase 2. There was a significant increase in both tear and serum total cortisol concentrations at T1 vs. T0 ($P < 0.05$). Tear and serum total cortisol concentrations were not significantly different at T1 vs. T2 or T0 vs. T2 ($P > 0.05$). A strong, positive correlation between tear and serum total cortisol concentrations existed for all time points during phase 2 ($P<0.05$, $R=0.975$)(Figure 10). Peak tear and serum total cortisol concentrations occurred at T1 for all dogs. Average tear and serum total cortisol concentrations for each dog are depicted in Figure 11.

Table 1: The average tear and serum total cortisol concentrations during phase 1 for each dog.

Dog	Average Tear Total Cortisol Concentration (ug/dL)	Average Serum Total Cortisol Concentration (ug/dL)
1	0.856 +/- 1.061	1.093 +/- 0.179
2	0.813 +/- 0.869	0.943 +/- 0.223
3	0.400 +/- 0.347	1.070 +/- 0.246
4	0.175 +/- 0.040	0.797 +/- 0.350
5	0.409 +/- 0.432	0.703 +/- 0.112
6	0.886 +/- 0.680	3.500 +/- 0.985
7	0.120 +/- 0.128	4.133 +/- 1.721
8	1.782 +/- 1.397	13.867 +/- 2.730
9	0.293 +/- 0.244	2.433 +/- 0.404
10	0.256 +/- 0.221	1.733 +/- 0.321
11	0.318 +/-0.153	4.133 +/- 1.850
ALL	0.573 +/- 0.720	3.128 +/- 3.807

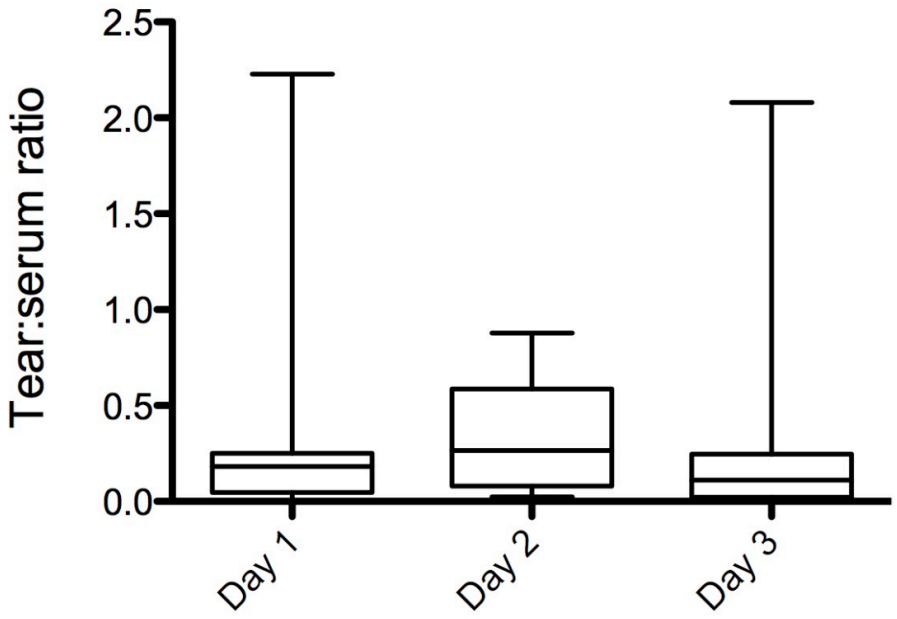

Figure 9: Resting tear:serum total cortisol concentration ratio over time. No significant difference in this ratio over time was observed.

Table 2: The average tear and serum total cortisol concentrations at each time point during phase 2. Four dogs total were stimulated during phase 2.

Time (minutes)	Average Tear Total Cortisol Concentration ug/dL	Average Serum Total Cortisol Concentration (ug/dL)
0	0.510 +/- 0.659	5.750 +/- 5.301
60	2.235 +/- 0.706	17.175 +/- 4.225
120	2.026 +/- 1.838	11.575 +/- 5.031

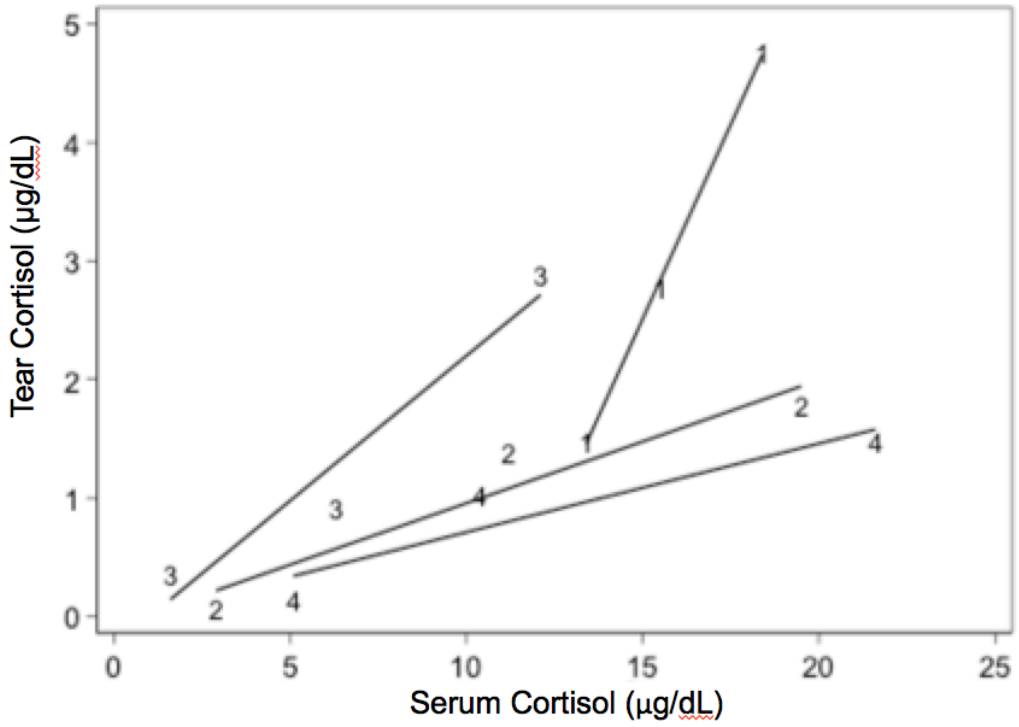

Figure 10: Regression analysis of tear total cortisol concentrations with dog and serum total cortisol concentrations as predictors. A strong, positive correlation between tear and serum total cortisol concentrations existed for all time points during phase 2.

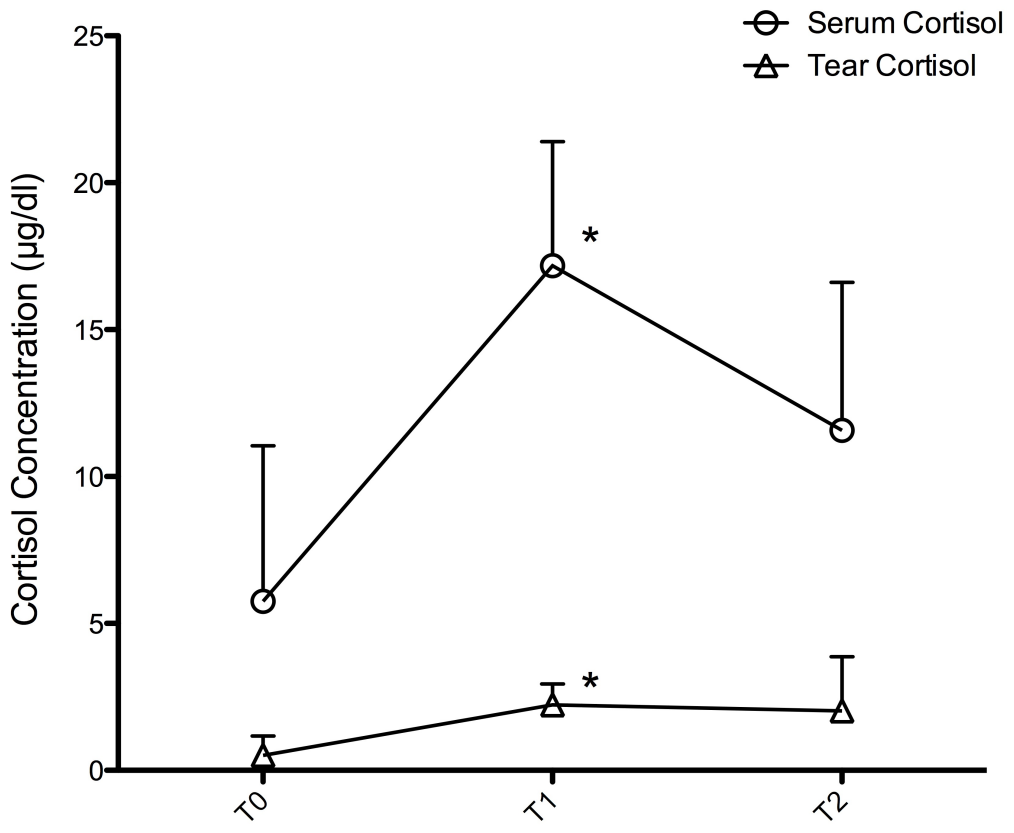

Figure 11: Average tear and serum total cortisol concentrations before ACTH stimulation (T0), 1 hour after (T1), and 2 hours after (T2).

Discussion

Endogenous cortisol was detected in the normal canine tear film at rest. Total cortisol concentrations in the tear film increased significantly following intravenous ACTH administration. The ratio of tear:serum total cortisol concentrations remained consistent over time whether at rest or following ACTH administration and a strongly positive correlation was found to exist between tear and serum total cortisol concentrations both at rest and after the administration of ACTH. These results parallel a recent similar study wherein baseline tear cortisol concentrations at rest and tear cortisol concentrations after ACTH stimulation were established in normal horses. [36] One dog included in the current study was under a noticeable amount of stress during the duration of its stay in the hospital and required more restraint than the other dogs included in this study. Under resting conditions, this dog showed tear cortisol levels 6.2 times higher and serum cortisol levels 5 times higher than the other three dogs under identical conditions that did not appear stressed. The stressed dog, like the others, showed a significant increase in tear and serum cortisol levels following ACTH stimulation. This also echoes the equine study wherein a particular horse exhibiting behavioral signs of stress provided similar results. Both situations demonstrate the potential of stressed animals with high baseline cortisol concentrations to experience an additional increase in cortisol upon exposure to excess ACTH. [36]

It is unknown how cortisol enters the tear film. In normal adult dogs, approximately 90% of plasma cortisol in exists in protein-bound form and approximately 10% as free cortisol. [37] Generally, it is the free cortisol fraction that is able to diffuse across cell membranes and is therefore considered biologically active. [3] Free cortisol enters the saliva via diffusion across

the salivary gland from the circulation. Therefore, saliva free cortisol concentrations directly correlate with serum free cortisol concentrations. [38, 39] In the current study, the ratio of tear:serum total cortisol concentrations did not vary over time when the dogs were at rest, or following stimulation with ACTH. However, because tear volumes were not sufficient to analyze both free cortisol and total cortisol levels in the current study, only total cortisol levels were measured in both tears and serum. In equine tears, free cortisol concentrations were found to be lower in serum than total cortisol concentrations in tears. Therefore, it is suspected that cortisol does not enter the tears via diffusion of free cortisol alone. It is likely that protein-bound cortisol also exists the tear film, and it is possible that canine corneal epithelial cells may contribute to the tear film cortisol concentration through local production, as has been observed of human corneal epithelial cells in culture. [36, 40]

A recent study demonstrated that the tear film of horses and ponies diagnosed with pituitary pars intermedia dysfunction (PPID) contained significantly increased concentrations of cortisol when compared to healthy horses of all age groups. Interestingly, the serum cortisol concentrations of the PPID animals were not significantly increased when compared to the healthy animals. These results are at odds with results obtained from normal horses stimulated with ACTH, which did show a significant increase in serum cortisol concentration in concert with a significant increase in tear cortisol concentration. These results strengthen the argument that tear cortisol concentrations may not be solely dependent on systemic HPA axis upregulation. [3] Although elevated serum cortisol concentrations are generally observed in dogs suffering from HAC, as was observed in the current study after stimulation with ACTH, investigating tear vs. serum cortisol concentrations of HAC dogs may provide useful information about the state of

the dogs' ocular environment and indicate the level of possible risk of adverse effects related to elevated tear film cortisol concentrations.

Further limitations of this study include the small sample size and the low sensitivity of the Salimetrics® salivary cortisol ELISA used for the measurement of total cortisol concentrations in tears. Another limitation was the inability to perform dexamethasone suppression tests to further confirm the absence of adrenal dysfunction in the dogs due to budget constraints. However, for all study participants there existed a complete absence of clinical suspicion of adrenal dysfunction following physical examination, complete blood count, serum chemistry and urinalysis.

Cortisol is present in normal canine tears at rest and the total cortisol concentration in canine tears increases significantly in concert with serum total cortisol concentrations when stimulated with intravenous ACTH. The tear:serum total cortisol concentration ratio was found to be consistent over time in dogs at rest and following ACTH stimulation. Further study is needed to investigate the mechanism of cortisol entry into the tear film. The quantification of free cortisol levels in canine tears versus serum is a potential next step in the attempt to elucidate this mechanism. The present study lays the groundwork for future investigations into cortisol concentrations in the tear film of dogs with endocrine or ophthalmic disease. Gaining more information about cortisol concentrations in the tear film of dogs suffering from ophthalmic disease could potentially lead to the development of new prognostic information, management guidelines, or therapeutic strategies.

CHAPTER 6

PROPOSED STUDY: CORTISOL CONCENTRATION IN THE TEAR FILM OF HORSES DIAGNOSED WITH FUNGAL KERATITIS

Equine fungal keratitis is a challenging, vision threatening disease associated with painful management and negative outcomes. Infection is usually established by a fungal species present in the normal microflora following ocular trauma. The disease is characterized by ocular pain, tearing, corneal edema, corneal vascularization, miosis, chemosis, keratomalacia and superficial or deep ulcerative keratitis accompanying fungal plaques on the cornea. It is difficult to treat, and substantial scarring or progression of disease is likely even after lengthy and costly treatment. [6] Progression of fungal keratitis can lead to corneal and stromal degeneration by both fungal and native proteolytic enzymes, perforation of the globe, and potential loss of the eye. [41] Reported long-term visual prognosis is variable, but generally poor, with vision retention rates between 53-97%. [6, 41-43]

One recognized risk factor for the development of fungal keratitis is topical corticosteroid use. Delayed repair after exposure to corticosteroids has been reported for several tissue types. [44-46] Topical corticosteroid use has been reported to delay corneal epithelial wound healing, with effects being dose-dependent. [29, 47-49] There is also evidence that corticosteroid exposure can increase risk of the cornea to infection. In one study, over 50% of horses presenting with keratomycosis had been previously treated with corticosteroids. [10] Corticosteroid use has

also been indicated as a risk factor for increased fungal isolation in rabbits and the progression of infectious keratitis in humans. [50, 51] However, corticosteroid treatment of a healthy, non-ulcerated equine eye does not result in an increase of potential fungal pathogens in the native corneal microflora. [52] Knowledge of the relationship between corticosteroid use and corneal disease and wound healing requires further study, however documented severe negative side effects are severe enough to contraindicate their use in equine fungal keratitis.

Elevated endogenous cortisol concentrations in the tear film could potentially have similar side effects as topical treatment with corticosteroids. In theory, elevated tear cortisol levels could delay epithelialization, allow for bacterial or fungal growth, activate matrix metalloproteinases and result in corneal ulcer progression. [10, 29, 31, 44-51, 53] Recently, the presence of endogenous cortisol in the equine tear film has been documented. Cortisol levels in the equine tear film have been found to increase in concert with serum cortisol levels after stimulation with intravenous ACTH administration. [36] Another recent study documented elevated endogenous cortisol levels in the tear film of horses diagnosed with PPID when compared to aged-matched control horses, but serum cortisol levels in these horses were not found to be elevated. [3] This suggests that tear film cortisol levels are determined independently of serum cortisol levels.

A study has been proposed wherein the cortisol concentrations in the tear film of horses diagnosed with fungal keratitis will be compared to that of similarly stressed, ophthalmically healthy horses. The control group will consist of horses presenting to the University of Georgia College of Veterinary Medicine for lameness examinations. This control group has been selected

in order to attempt to differentiate the effects of hospitalization, pain, and treatment stress from baseline tear cortisol concentration differences. Control horses ideally will be experiencing similar situational stress levels. The data collected from this study will help to further illuminate the state of the ocular environment of diseased horses and potentially provide a new metric for prognosis. The data could also indicate the potential of endogenous cortisol in the tear film as a novel therapeutic target. Currently, topical antifungal medications are fungistatic, not fungicidal, have poor penetration through the cornea, and require long treatment durations and frequent dosing. [24] Therefore, the identification of new potential therapeutic agents for the treatment of fungal keratitis is of great interest. If elevated endogenous cortisol levels are present in the tears of horses with fungal keratitis, a cortisol-binding agent that acts to lower ocular cortisol levels may prove to be valuable in impeding the progression of the disease.

CHAPTER 7

CONCLUSION

The concentration of the endogenous glucocorticoid cortisol in the tear film may have negative implications in animals with ophthalmic disease. The included study investigated the presence of endogenous cortisol in canine tears and a 2014 study from our research group detected the presence of endogenous cortisol in equine tears. In both cases, tear film cortisol levels were found to increase in concert with serum cortisol levels following simulated stress via intravenous ACTH stimulation. [36] Endogenous total cortisol concentrations have also recently been found to be elevated in the tear film of horses diagnosed with PPID when compared to age-matched control horses. However, serum total cortisol levels in horses with PPID were not found to be elevated.[3]

The relationship between systemic cortisol levels and tear cortisol levels is currently not well understood and further study is needed on this topic. Future studies investigating free cortisol versus total cortisol levels in the canine tear film and serum may provide useful information. A prominent challenge when studying the canine tear film is tear collection. On average we were able to collect 62 uL (range 12.5-100 uL) of tears per dog using the glass capillary tube method. Although tear volumes were sufficient for the purposes of determining tear total cortisol concentrations, we were left with no excess and tear free cortisol concentrations could not be investigated.

These studies documenting the presence of endogenous cortisol in canine and equine tears lay the groundwork for future studies investigating tear cortisol levels in the tears of animals with endocrine and ophthalmic disease. Measuring endogenous cortisol concentrations in the tears of dog with hyperadrenocorticism would not only provide more information about the ocular environment of these dogs, but could also help indicate whether they are potentially susceptible to the same side effects of topical corticosteroid use due to elevated endogenous cortisol levels. The same concept applies to the proposed study that would investigate the endogenous cortisol concentrations in the tear film of horses diagnosed with fungal keratitis. If elevated endogenous cortisol levels are found in the tears of diseased animals, a novel therapeutic agent aimed at sequestering excess ocular cortisol may prove to be a useful future tool for preventing, or impeding the progression of, ophthalmic disease.

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APPENDIX A:

ENDOGENOUS CORTISOL IN THE TEARS OF HORSES WITH NATURALLY OCCURRING FUNGAL KERATITIS

MORRIS ANIMAL FOUNDATION PROPOSAL APPLICATION

Submitted July 7th, 2015

I. Title and Abstract:

1. Title: Endogenous Cortisol in the Tears of Horses with Naturally Occurring Fungal Keratitis

2. Rationale: Corneal ulceration is one of the most common ocular diseases seen in equine practice. Corneal ulcers are always painful, often difficult to treat, and can result in loss of vision or the eye. Secondary fungal infection is particularly challenging to heal and is associated with negative outcomes, prolonged animal suffering, and client frustration despite intensive management. Understanding the pathophysiology of fungal keratitis and its resistance to current treatments is *critical* for developing new disease management strategies that will help preserve vision and eliminate suffering in patients with corneal ulceration. It has long been known that treatment with topical corticosteroids enhances fungal growth and decreases the efficacy of antifungal drugs in horses with fungal keratitis. The endogenous corticosteroid cortisol has also been shown to adversely affect corneal cell behavior. Our lab has recently established that

cortisol is present in the tears of healthy horses and that tear cortisol concentration increases in response to ACTH stimulation. To our knowledge, there is currently no research on the tear cortisol concentration of horses with naturally occurring corneal disease. We suspect that high levels of tear cortisol may contribute to the establishment of secondary fungal infection in horses with corneal ulceration. This unexplored area of tear dynamics may provide vital information for the assessment and treatment of corneal ulceration, fitting with the Morris Animal Foundation's mission to advance animal healthcare by investigating new routes for disease prevention and treatment.

3. Hypothesis/Objectives:

Hypothesis: Cortisol in the tear film of horses with fungal keratitis impairs corneal healing. To begin to address this hypothesis we propose the following objective:

Objective 1: To determine and compare the tear cortisol concentrations of horses with naturally occurring fungal keratitis and ophthalmically normal horses under similar levels of stress.

4. Study Design: Objective 1

12 client-owned horses in good systemic health, admitted by a board-certified ophthalmologist for treatment of fungal keratitis, will be recruited for objective 1. 12 client-owned horses in good ocular health, presenting for a lameness evaluation and hospitalized for diagnostics, will be included as a similarly stressed control population. Screening bloodwork, serum cortisol levels, and serum ACTH levels will be assessed. Horses with underlying disease that will affect cortisol dynamics will be excluded. Fungal keratitis will be confirmed by the identification of fungal hyphae on corneal cytology or by a positive corneal culture as indicated by the case. Paired tear

and serum samples will be collected at time 0 (admission to the hospital) and on days 1, 5, 10 (or as long as the animal is hospitalized) and at discharge. Tear and serum cortisol levels will be evaluated using a previously validated ELISA assay. Consent for inclusion in the study will be obtained through a client consent and waiver form.

5. Expected Results: We expect that horses with fungal keratitis will have elevated tear cortisol levels independent of serum cortisol levels when compared to ocularly normal horses under similar stress.

6. Budget and Timeline: The project will cost $9252.98 and we anticipate data collection will be complete within 12 months of funding based on the lowest number of fungal keratitis patients seen by the ophthalmology service per year over the previous 5 years.

7. Potential Impact for Animal Health: This research addresses a critical knowledge gap and will further our understanding of corneal wound healing and the devastating disease of equine fungal keratitis. Not only could we uncover a link between cortisol and corneal disease but the potential exists to identify new drugable targets for corneal ulcer therapy. Improving clinical outcomes for ulcerative keratitis can reduce disease severity, vision loss, and help to prevent blindness in companion animals. This pilot study is a necessary first step in establishing the effect of tear cortisol dynamics on naturally occurring corneal disease.

II. Resubmission Summary:

This proposal is not a resubmission.

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IV. Study Proposal:

1. Hypothesis and Objectives

Hypothesis 1: Cortisol in the tear film of horses with fungal keratitis impairs corneal healing.

Objective 1: To evaluate and compare tear cortisol levels of horses diagnosed with fungal keratitis and ophthalmically normal horses presenting for lameness evaluation.

2. Justification, Significance and Literature Review

Equine fungal keratitis or keratomycosis is a common, painful ocular disease that is vision-threatening. It is costly and difficult to treat and will often lead to substantial scarring which can impact a horse's performance and quality of life. The disease is characterized by pain, tearing, corneal edema, corneal vascularization, miosis, chemosis, keratomalacia and superficial or deep ulcerative keratitis accompanying fungal plaques on the cornea. Fungal keratitis usually results from the corneal stromal invasion of a fungal species found in the normal equine ocular microflora or the environment after a small surface trauma. (5) Disease progression leads to the degradation of the corneal stroma by native and fungal proteolytic enzymes and can result in eventual perforation of the cornea and loss of the eye. For this reason aggressive treatment is always warranted in diagnosed cases. (6)

Currently the long-term visual prognosis for horses with fungal keratitis varies widely but remains poor with vision retention rates between 53-97%. (5)(6-8) The primary difficulty with

current topical antifungal medications are three fold in that they are 1. primarily fungistatic rather than fungicidal; 2. typically have poor penetration through the cornea and 3. must be applied at high frequency for a long treatment duration. (9) Given the high potential for animal suffering and vision loss, investigation into new preventative and therapeutic agents in the treatment of fungal keratitis is vital.

One factor of critical significance in disease process of corneal ulceration, infection, healing and scarring is corticosteroid use. Corticosteroids have been associated with a delayed repair in several tissues. (10-12) Specific to ophthalmology, corticosteroids have been found to retard corneal epithelial defect closure in a dose-dependent manner. (13-16) Stimulation of corticosteroid receptors on human keratocytes induces apoptosis and necrosis in these cells. (14) Corticosteroids are also implicated in corneal infection. In one equine study over 50% of horses presenting with keratomycosis had been previously treated with topical corticosteroids. (17) Similar results were found in two additional studies in other species, where topical corticosteroids were a risk factor for progression of clinical infectious keratitis in human patients (18) and ocular fungal isolation in an experimental rabbit study. (19) Furthermore, once corneal infection is present, matrix metalloproteinase (MMP) activity – which plays a key role in the progression of keratomalacia by causing ulcer expansion through collagenolysis – is also increased up to 15-fold by corticosteroids. (4,5) Corticosteroids alone are insufficient to dramatically change the native flora, however. A 2005 study showed that the application of topical steroids in normal, non-ulcerated horses did not increase the degree of fungal organisms in the corneal environment. (6) Our understanding of the role of corticosteroids in corneal

infection and wound-healing is incomplete but the documented negative effects are severe enough that the use of topical corticosteroids is contraindicated for use in equine fungal keratitis.

Concentrations of endogenous cortisol in the tear film could create similar clinical effects to those seen with exogenous steroid eye drops. Conceivably, high concentrations of cortisol could delay epithelialization, (7-14) encourage growth of bacteria or fungus, (4-6) or activate MMPs (7) resulting in ulcer expansion in horses. With this in mind, our research group demonstrated the presence of endogenous cortisol in equine tears. The study also documented elevated tear cortisol levels in tandem with elevated serum cortisol levels following stress simulation via ACTH administration. (8) A recent study (under review) from our lab demonstrated that the tear cortisol concentration in horses with naturally occurring PPID (equine Cushing's disease) was higher than age-matched control horses despite the inverse being true of the serum cortisol concentration. This suggests that horses can exhibit tear cortisol concentrations independent of serum cortisol concentrations. To the authors' knowledge, there is no research on the effect of endogenous tear cortisol on corneal disease. Our study aims to determine if horses diagnosed with fungal keratitis have elevated levels of the endogenous steroid cortisol in their tears compared with horses in good ocular health under similar levels of stress.

By examining tear and serum cortisol concentrations, we hope to differentiate the effect of hospitalization, disease associated pain, and treatment stress from baseline differences in tear cortisol concentrations. For this reason we have chosen horses presenting for lameness as our control group as they will provide similar baseline levels of stress and circulating serum cortisol. The data obtained from this study will potentially open the door for a completely novel

therapeutic target in the treatment of equine keratomycosis. Cortisol binding agents could be developed to help adjust for high native cortisol concentrations in the tears. We hope that this research may also provide a metric for prognosis and shed light on the complicated interplay of corticosteroid in corneal wound healing.

3. Preliminary Data: NA

4. Experimental Methods and Design

Power calculation: Previous work out of our research lab established that the mean tear cortisol concentration in a healthy horse was 5.4 +/- 8.2 nmol/L.[54] That same study demonstrated a 3-fold increase in tear cortisol in response to ACTH stimulation. Believing that a 3-fold increase is a reasonable target point for this pilot study of ophthalmically diseased horses, an expected mean in a diseased horse would be at least 16.2 nmol/L. In order to detect a 3-fold difference as being significantly different from a mean of 5.4 nmol/L (assuming a SD of 8.2), with alpha set at 0.05 and at a statistical power of 80%, we have calculated that we would need 10 horses per group. Thus we hope to recruit 12 horses to allow for errors in data collection of precluding complications.

Patient recruitment and screening: 12 client-owned horses in good systemic health, admitted to the University of Georgia Large Animal Hospital for treatment of fungal keratitis will be recruited for objective 1. To provide a control group and to account for the stress incurred upon the animals during trailering and travel to the clinic, 12 client-owned horses in good systemic

and ocular health admitted to the University of Georgia Large Animal Hospital for a lameness evaluation and hospitalization for diagnostics or therapy will also be included in the study. Each horse will receive an ophthalmic examination by a board-certified ophthalmologist and a physical examination by a board-certified large animal internist prior to inclusion in the study. Screening bloodwork, urinalysis, serum cortisol (chemiluminescent immunoassay) and serum ACTH level will be assessed. Horses with underlying disease that will affect cortisol dynamics, such as Pituitary Pars Intermedia Dysfunction (PPID), will be excluded. Ophthalmic exam will include a Schirmer tear test, fluorescein stain, indirect ophthalmoscopy, slit lamp biomicroscopy and an intraocular pressure reading with a TonoVet. Diagnosis of fungal keratitis will be confirmed by the identification of fungal hyphae on corneal cytology or by a positive corneal culture as indicated by the case.

Sample collection: Tears will be obtained by placing a glass capillary tube in the fornix between the nictitating membrane conjunctiva and the inferior palpebral conjunctiva. Initial tear and serum collection for all horses will occur upon admission to the hospital (day 0,) prior to treatment. To obtain serum and plasma samples, a total of 12 mL of blood will be collected by jugular venipuncture. While the animal is hospitalized, tear and serum collection will also occur on days 1, 5 and 10 (if hospitalization lasts that long) and on the day of discharge from the hospital or the day of surgical intervention if necessary. All collections will occur at the same time of day. We anticipate that control animals will not be hospitalized as long as horses undergoing in-house treatment for fungal keratitis. Samples will be taken for as long as the control horse is hospitalized (typically 2-5 days) and will not be taken after a surgical intervention has occurred. Tear and serum cortisol levels will be evaluated using a previously

validated ELISA assay.[54] All tear and serum samples will be held in a -80°F freezer until evaluation. Client consent for inclusion in the study will be obtained through a client consent and waiver form.

Statistical Analysis: Serum and tear total cortisol concentrations between horses with fungal keratitis and control animals will be compared with Mann-Whitney U tests because small sample sizes preclude accurate determination of the distribution of the data. Spearman's correlational analysis will be used to determine correlations between serum and tear cortisol concentrations in both groups. Significance will be set at $P < 0.05$ for all analyses.

5. Timeline, Expected Outcomes and Potential Pitfalls:

Aim 1: The UGA ophthalmology service sees an average of 17 horses (12-22) with fungal keratitis during the calendar year. Tear collection from horses with fungal keratitis will be complete by fall 2016 based on the lowest number of fungal keratitis cases seen by the UGA Ophthalmology service in the past 5 years. Age-matched controls of horses presenting for lameness will be collected in the fall of 2016. We expect 2-7 potential control horses will be admitted to the hospital per week based on the existing caseload for lameness evaluation and workup. Patients presenting for complete lameness evaluation and treatment are often hospitalized for 2-4 days depending on the nature of the evaluation. We anticipate processing and evaluation of samples to be finished by the winter 2016.

We expect that the tear concentration of cortisol is higher in horses with fungal keratitis than a mere stress response should evoke. Even if initial tear concentrations of cortisol are elevated in

horses presenting for lameness, we predict that the concentration will decrease in these animals over time as the stress of trailering wears off. We predict that the tear cortisol concentration of horses with fungal keratitis will remain elevated.

Pitfalls include a possible decrease in fungal keratitis patients presenting to the hospital. This may result in the inability to enroll 12 horses during the calendar year for the study. If numbers are low, we will explore active recruitment with a financial incentive provided through other research dollars. Additionally, local practices have tentatively agreed to allow us to recruit and sample horses housed in their treatment facilities should that become necessary. It is also possible that horses presenting for lameness will not be properly age-matched or will not be hospitalized a sufficient length of time for comparison at all time points. In this case, we could consider sampling healthy blood donor horses after trailering and while hospitalized as they will be present for the full 10 days. We feel that this control group is less suited as the only stressor imposed upon them is the trailer ride. Samples from horses with lameness would be superior even if we cannot obtain an equal number of time points.

It is possible that horses with fungal keratitis will have less than a 3-fold difference in tear cortisol concentrations making the difference insignificant. In this case, we hope to have enough preliminary data to expand the sample size in a future study.

V. Animal Involvement Justification:

A. Does this study involve biological samples, tissues, etc.? **__yes__**

Tears and blood will be collected from 24 privately owned horses.

B. If this study involves live animals, succinctly address the following: (please restate the questions and directives)

1. What species will be studied? **Equine**

2. State the status of your IACUC application/approval. **Submitted, approval pending**

3. List the USDA category for pain and distress (B, C, D, E): **C**

4. Does this proposal involve client-owned animals? **Yes see client consent form below**

5. Explain how animals will be acquired: **client-owned via recruitment and consent**

6. How many animals will be used? **24**

a. Summarize numerical justification: **Patient numbers were determined using a power analysis as outlined in the proposal. 10 animals are necessary to show a 3 fold difference in cortisol concentrations based on previous mean cortisol levels in healthy horses before and after ACTH induced stress.**

7. Does this study induce disease, injury, pain or distress in animals? **Blood and tear sampling require manual restraint and may cause mild irritation.**

8. Explain the environment and housing conditions in which the animals will live: **patients will be housed in the teaching hospital in the healthy wards in the large animal facility.**

9. What will happen to the animals upon completion of the study? **Client-owned**

Client Consent Form:

UNIVERSITY OF GEORGIA

COLLEGE OF VETERINARY MEDICINE

OWNER CONSENT AND WAIVER OF LIABILITY

Endogenous Cortisol in the Tears of Horses with Naturally Occurring Fungal Keratitis

Principal Investigator: Kathern Myrna, DVM, MS, DACVO

READ CAREFULLY BEFORE SIGNING

I, the undersigned, acknowledge that I am the owner of _____ (horse's name.)

I consent to the inclusion of _____ in this study, which is conducted by Dr. Kathern Myrna as investigator and funded by the Morris Animal Foundation. Questions regarding the study were answered to my satisfaction. The benefits and risks of participation in this study have been explained to me.

Purpose of the Study

I understand that the purpose of this study is to characterize the expression of the stress hormone cortisol in the tears of horses with surface fungal eye infections as compared with similarly stressed horses that have no eye disease.

Duration of Participation

It has been explained to me that my horse will participate in this study on the first, second, fifth, tenth, and last day of his/her hospitalization at the hospital, or on as many of those days as my horse remains hospitalized. My horse will be housed in the teaching hospital for as long as needed for treatment and will not be held in the hospital solely for inclusion in this study.

Description of Procedures

I understand the procedures will be as follows: during the screening evaluation my horse will receive physical and ophthalmic exams including Schirmer tear testing, intraocular pressure reading, fluorescein staining, and cytology or culture if indicated. Additionally there will be collection of approximately 12 milliliters of blood for detection of any present underlying disease, including Cushing's disease. Tears and 3 milliliters of blood will be collected upon admission to the hospital and on the second, fifth, tenth and last day of hospitalization if the horse remains in the hospital for that period of time. I will notify the investigator of any suspected adverse events immediately.

Possible Benefits

It has been explained to me the benefits that my horse and I can expect from participation in this study are ophthalmic evaluations from a board-certified ophthalmologist and bloodwork.

Possible Discomforts and Risks

I understand participation in this study entails the following discomforts and risks: minimal discomfort after withdrawing blood including slight bruising or swelling of the vein and irritation

to the conjunctiva after tear sampling. I understand that there may be unforeseeable risks of participation in this study. In the event of unforeseen risks, the attending veterinarians will use their judgement to guide patient management.

Alternative Treatments

This study is non-therapeutic and does not require alternative treatments.

Confidentiality of Records

I understand that although information gained from this investigation may be published and used for educational or regulatory purposes, my identity and my animal's identify will remain confidential to the extent provided by law.

Financial Obligation, Withdrawal from Study

I understand that the costs of all evaluations relating to this study are paid by Morris Animal Foundation, Inc., including screening diagnostics (physical and ophthalmic exams, lab work.) I understand that I may withdraw my animal from this study at any time. If I withdraw my animal from the study I will be financially responsible for any further medical therapy that my horse receives from the University of Georgia College of Veterinary Medicine. I understand that the investigators may terminate my animal's participation in the study if continuation is not in the best interest of my animal or if I fail to comply with the procedural guidelines.

Waiver of Liability

For the sole consideration of the agreement of the University of Georgia College of Veterinary Medicine to sedate, withdraw blood, and collect tears, I hereby release, covenant not to sue, and forever discharge the University of Georgia, the Board of Regents of the University System of Georgia, their members individually, and their officers, agents, and employees, from any and all claims, demands, rights, and causes of action of whatever kind that I may have arising from or in any way connected with my animal's participation in this study.

Further Questions, Findings

The investigators will answer any further questions about the research during the course of the study. If I have questions I will contact Dr. Kate Myrna or Lisa Reno at 706/296-7818.

I am/am not an employee, spouse of an employee, or dependent of an employee of the University System of Georgia. I am at least 18 years of age and have read and understand the above.

_____ _____

Signature of Owner or Agent Date

_____ _____

Signature of Investigator Date

_____ _____

Witness Date

VI. Recombinant DNA/Biohazards: N/A

VII. Facilities and equipment:

University of Georgia College of Veterinary Medicine and Veterinary Teaching Hospital

The Veterinary Teaching Hospital is a part of the College of Veterinary Medicine. The VTH sees approximately 19,828 cases in house yearly. The Comparative Ophthalmology service has dedicated examination room space within the Veterinary Teaching Hospital where all study examinations and sample collections will be performed. Available ophthalmic equipment includes: slitlamp biomicroscopes, binocular indirect ophthalmoscopes, applanation and rebound tonometers, a phacoemulsification unit, anterior segment DSLR camera, diode laser, cryosurgical units, ultrasound and an operating microscope.

The College of Veterinary Medicine has designated laboratory space equipped with -80 °F freezers for sample storage as well as centrifuge for sample preparation. Designated bench space is available for ELISA and chemiluminescent assay preparation and appropriate pipetting tools and stock reagents are available.

VIII. Cited References:

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21. Monk, C.S., et al., Detection of endogenous cortisol in equine tears and blood at rest and after simulated stress. Vet Ophthalmol, 2014. 17 Suppl 1: p. 53-60.

IX. Budget:

Category	Year 1	Y 2	Y 3	Total
Personnel:				
1. Principal investigator: Kathern Myrna*	$0	$0	$0	$0
2. Co-investigator #1 Kelsey Hart	$0	$0	$0	$0
3. Co-investigator #2 Brittany Wynne	$0	$0	$0	$0
5. Student Assistant				
Salary ($10/hr x 10hr/wk x 30wk)	**$3000.00**			
Total Salaries & Wages	**$3000.00**			**$3000.00**
Supplies & Expenses:				
1. Tear Collection Supplies:				
Eppendorf Tubes x 120 samples	**$40.50**/500			
Uncoated Glass Capillary Tubes x 500	$3.15/vial 100 x 5 = **$15.75**			
2. Blood Collection Supplies				
Monoject 10mL Red Top Tubes x 144	$21.30/box 100 = **$30.67**			
Monoject 3mL Lavender Top Tubes x 24	$35.32/box 100 = **$8.47**			
12 cc disposable syringe x 144	$8.25/box 100 = **$11.88**			
18 g needle x 144	$20.25/box 80 = **$36.45**			
3. Ophthalmic Exam (control group)				
3 cc disposable syringe x 120	$8.25/100 = **$9.90**			
Xylazine 50mL	**$24.82**/1 bottle			
25 g needle x 48	$11.70/100 = **$5.62**			

Lidocaine 2% 150mL	**$24.82**/1 bottle			
Schirmer Tear Test Strip x 15	$49.60/box 50 = **$14.88**			
Fluorescein Sodium Strips x 12	$12.80/box 100 = **$1.53**			
TonoVet Probes x 12	$169.00 box 100 = **$20.28**			
4. Tear Evaluation:				
Salimetrics Cortisol ELISA 120 samples	$250 each x 4 = **$1000**			
5. Blood Tests:				
Complete Blood Count x 24	$24.00 x 24 = **$576.00**			
Serum Chemistry x 24	$32.00 x 24 = **$768.00**			
Plasma ACTH x 24	$27/test + 30 ship = **$678.00**			
Serum Cortisol 120 samples	$15/test +50ship = **$1850.00**			
6. Poster Printing	**$150.00**			
7. Statistical Consulting (3 hrs)	**$300.00**			
Total Supplies & Expenses:	**$5567.57**			**$5567.57**
Animal Use & Care: N/A	$0			$0
Subtotal of All Categories:	**$8567.57**			**$8567.57**
Maximum of 8% - Indirect Costs:**	**$685.41**			**$685.41**
Grand Total Requested from MAF:	**$9252.98**			**$9252.98**

X. Itemized Budget Justification:

Staff: Fungal keratitis patients arrive on an emergent basis often in the evening of on weekends. This will require staff on call to come in and collect the samples, process and store them. As subsequent samples must be taken at the same time of day, it is imperative that a designated worker be committed to performing these samples. Having a student worker to manage this process as well as assist in processing the samples and running the assays will greatly increase successful collection of the necessary samples. We believe that the hours listed would cover the hours necessary for all sample collection.

1. Tear Collection Supplies: 4 capillary tubes are necessary to collect the 50µl needed for each sample x 120 (5 time points x 24 subjects) and Eppendorf are necessary for storage of tear samples.
2. Blood Collection Supplies: $3.60/subject x 24 subjects – supplies needed for blood sampling at initial screening and during all paired tear and serum cortisol readings.
3. Ophthalmic Exam Supplies: x 12 – ophthalmic exams are routine for the patients presenting through the ophthalmology service, but the control population will require full ophthalmic exams covered by the study. This includes sedation with xylazine, administration of lidocaine auriculopalpebral nerve blocks to facilitate examination, a Schirmer tear test, fluorescein stain test, and tonometry using the TonoVet probes. All durable equipment (slit lamp biomicroscope, 20Diopter lens, TonoVet device, transilluminator) will be borrowed from the ophthalmology service at no direct charge.

4. Tear Evaluation: $250/kit x 4 kits to allow for 120 samples including space for positive and negative controls. This is the test for detection of the cortisol concentration in the tears.
5. Blood Tests: CBC/Chem: $56.00 x 24 patients – necessary for screening candidate patients for underlying disease; Serum ACTH: $27 x 24 patients + $30 shipping – necessary for screening candidate patients for subclinical pituitary or adrenal gland disease; Serum Cortisol Immulite Chemilluminescense: $15/sample x 120 samples – previously utilized method for assessment of cortisol in the serum. Plasma ACTH and serum cortisol are shipped to Cornell for evaluation at a lower cost than the UGA facility.
6. Data: statistical analysis support is provided based on preliminary consult with UGA statistician.
7. Poster printing for presentation of data at a national meeting as well as at the University veterinary research symposium.

XI. Other Support

<u>Dr. Kathern E Myrna (Primary Investigator)</u>

(1)

- *Source:* UGA Ophthalmology Research Fund and Martha Cannon Trust
- *Grant Title:* Levels of Endogenous Cortisol in the Tears of Horses with Naturally Occurring PPID

- *Major Goals:* To characterize the concentration of cortisol in the tears of horses with PPID and age-matched control horses
- *Annual Costs:* $3,000 (January 2013 – December 2016)
- *Overlap:* Although this study uses the same protocols, it does not directly overlap with the current proposal. PI.

(2)

- *Source:* New Faculty Research Fund
- *Grant Title:* Evaluation of corneal stromal wound healing the role of myofibroblast differentiation in $Pax6^{+/-}$ mice
- *Major Goals:* To characterize the molecular features of corneal stromal wound healing in the mouse model of human aniridia
- *Annual Costs:* $5,000 (January 2012 – December 2018)
- *Overlap:* None. PI.

Potential Overlap: There is no overlap with regards to scientific rationale, budget, or committed effort between these projects and the proposed study.

Dr. Kelsey A. Hart (Co-Investigator)

(1)

- *Source:* Morris Animal Foundation (D15-EQ303)
- *Grant Title:* Dendritic cell function in equine neonatal sepsis.

- *Major Goals:* To characterize effects of age, soluble plasma factors, and cortisol on dendritic cell function in horses and foals using an *ex vivo* bacterial sepsis model
- *Annual Costs:* $86,433 (January 2015 – December 2016)
- *Overlap:* None. PI.

(2)

- *Source:* Love of the Horse Competitive Intramural Equine Programs Research Fund, University of Georgia College of Veterinary Medicine.
- *Grant Title:* Effects of age on equine dendritic cell maturation, activation and function in foals
- *Major Goals:* To optimize cell isolation and function assessment methodology for foal DCs
- *Annual Costs:* $8,000 (2014-2015)
- *Overlap:* None. PI.

(3)

- *Source:* Veterinary Medical Experiment Station New Faculty Research Grant, University of Georgia College of Veterinary Medicine
- *Grant Title:* Immunologic effects of low-dose hydrocortisone in adult horses.
- *Major Goals:* To characterize the effect of low-dose hydrocortisone on innate immune function in adult horses
- *Annual Costs:* $20,000 (July 2013 – June 2014)
- *Overlap:* None. PI.

(4)

- *Source:* University of Georgia Faculty Innovative Instruction Grant

- *Grant Title:* Interactive tools for enhancing instruction of veterinary diagnostic skills
- *Major Goals:* To develop interactive iBook based teaching materials for clinical skills courses in the first-year veterinary curriculum, and evaluate the impact of these materials on student learning.
- *Annual Costs:* $5,000 (July 2013 – June 2015)
- *Overlap:* None. PI.

(5)

- *Source:* University of Georgia Faculty Summer Support Funding
- *Grant Title:* Detecting flagellin and flagellin antibodies in septic foals and horses with gastrointestinal diseases.

  Major Goals: To measure bacterial flagellin and anti-flagellin antibodies in critically ill horses and foals, and determine if circulating bacterial flagellin predicts disease severity and outcome in this population of animals.
- *Annual Costs:* $5,000 (July 2013 – June 2014)
- *Overlap:* None. PI.

Potential Overlap: There is no overlap with regards to scientific rationale, budget, or committed effort between these projects and the proposed study.

XII. Prior MAF Support During the Last Three Years

Dr. Kelsey A. Hart (Co-investigator):

a. **MAF grant ID number: D15-EQ303**

b. Title of the project:

Dendritic cell function in equine neonatal sepsis

c. Amount and period of support (dates):

$86,433; January 2015 to December 2016

d. Brief summary of the objectives:

The objectives of this study are to characterize effects of age, soluble plasma factors, and cortisol on the maturation, activation and function of equine dendritic cells in horses and foals using an *ex vivo* bacterial sepsis model.

e. Summary of the results of the completed work:

Year 1 of this two-year study is currently underway. What follows is a summary from the mid-progress reported filed July 1, 2015:

i. We proposed to assess the effect of age (Specific Aim 1), factors in the blood (Specific Aim 2), and the hormone cortisol (Specific Aim 3) on foal and adult horse

dendritic cells using an ex vivo bacterial sepsis model. To date, we have optimized our *in vitro* culture conditions for adult horse and foal monocyte-derived dendritic cells (MoDCs) to reliably attain >90% cell viability, determined ideal minimum saturating antibody concentrations for all 3 proposed cell surface markers used to characterize equine MoDC maturation via flow cytometry, and optimized bacterial endocytosis/phagocytosis protocols for both adult horse and foal MoDCs to assess MoDC function in live MoDCs *in vitro*. Thus, all of the necessary techniques for assessing equine dendritic cell function are now validated and fully optimized in our lab. We are on schedule to complete these aims as proposed, as we are currently collecting samples to complete Specific Aim 1 during the ongoing foal season, and will complete Specific Aims 2 and 3 during the 2016 foal season next spring as planned.

Preliminary analysis of early data for Specific Aim 1 demonstrates impaired bacteria-induced MoDC maturation and activation in 1 day-old foals as compared to adult horses.

f. List of the publications resulting from this MAF award:

We expect to have sufficient data for the first publication resulting from this study in the next 4-6 months, and anticipate sufficient data for a second publication at the completion of the project in December 2016.

g. List of presentations resulting from this MAF award:

We expect to present the findings from this work at a veterinary immunology or veterinary internal medicine scientific meeting in late 2016/early 2017.

h. List of patents resulting from this MAF award: N/A

XIII. Biographical Data

Name: Dr. Kathern Myrna

Role: Primary Investigator

Current Position: Assistant Professor of Veterinary Ophthalmology

kmyrna@uga.edu

706-206-7584

Department of Small Animal Medicine and Surgery

College of Veterinary Medicine

2200 College Station Drive

Athens, GA 30602

Education/Training:

Institution	Degree	Year Completed	Field of Study
American College of Veterinary Ophthalmologists	Board Certified	2013	Veterinary Ophthalmology
University of	Fellowship	2010	Comparative

Wisconsin, Madison, WI			Veterinary Ophthalmology
University of Wisconsin, Madison, WI	MS	2010	Comparative Biomedical Sciences
Angell Animal Medical Center – Western New England, Springfield, MA	Intern	2006	Ophthalmology
Western College of Veterinary Medicine, Saskatoon, SK	Intern	2005	Medicine and Surgery
VA-MD Regional College of Veterinary Medicine, Blacksburg, VA	DVM	2004	Veterinary Medicine
Vassar College, Poughkeepsie, NY	BA	1998	Biology

Previous Positions: N/A

Selected Honors and Awards:

2014	Zoetis Distinguished Teacher Award, UGA
2014	Class of 2016 Faculty Recognition Award
2013	Lilly Teaching Fellow, UGA
2012	Class of 2014 Faculty Recognition Award
2005	Hills Intern of the Year Award, Western College of Veterinary Medicine, Saskatoon, SK
2004	American College of Veterinary Ophthalmologists Senior award, VMRCVM
2004	Roseanne Robertson Memorial Award for Ophthalmology, VMRCVM
2003	Dr. Robert C. Hammond Award from the Maryland Veterinary Foundation
2003	Florence A. Nocka Award; VMRCVM scholarship
2003	Phi Zeta Inductee (top 10% of class)
2002	Ruth S Fleming Scholarship, VMRCVM

Selected Peer-Reviewed Publications:

1. Dorbandt DM, Moore PA, **Myrna KE**. Outcome of conjunctival flap repair for corneal defects with and without an acellular submucosa implant in 73 canine eyes. *Veterinary Ophthalmology*. Epub 22 Jul 2014.
2. Utter ME, Gemensky-Metzler AJ, Scherrer NM, Stoppini R, Latimer CA, MacLaren NE, **Myrna KE**. Corneal dystrophy in Fresian horses may represent a variant of pellucid marginal degeneration. *Veterinary Ophthalmology*. 2014; 17(s1) 186-94.

3. Bergstrom BE, Labelle AL, Pyde ME, Hamor RE, **Myrna KE**. Prevalence of Ophthalmic Disease in Blue-Eyed Horses. *Equine Veterinary Education*. 2014; 26(8) 438-40.
4. Monk CS, Hart KA, Berghaus RD, Norton NA, Moore PA, **Myrna KE**. Detection of endogenous cortisol in equine tears and blood at rest and after simulated stress. *Veterinary Ophthalmolgy*. 2014; 17(s1) 53-60.
5. Delk KW, Mejia-Fava J, Jimenez DA, Kent M, **Myrna KE**, Mayer J, Divers S. Diagnostic Imaging of Peripheral Vestibular Disease in a Chinese Goose (*Anser cygnoides*). *Journal of Avian Medicine and Surgery*. 2014; 28(1) 31-7.
6. **Myrna** KE, Mendonsa R, Russell P, Pot SA, Liliensiek SJ, Jester JV, Nealey PF, Brown D, Murphy CJ. Substratum Topography Modulates Corneal Fibroblast to Myofibroblast Transformation. *IOVS*. 2012; 53(2):811-6.
7. Mathes RL, Burdette EL, Moore PA, **Myrna** KE. Concurrent clinical intraocular findings in horses with depigmented punctate chorioretinal foci. *Veterinary Ophthalmology*. 2011 15(2):81-5
8. **Myrna KE**; Bentley, E; Smith, LJ. Effectiveness of injection of local anesthetic into the retrobulbar space for postoperative analgesia following eye enucleation in dogs. *JAVMA*. 2010; 237(2): 174-7.
9. **Myrna, KE**; Pot, SA; Murphy, CJ. Meet the corneal myofibroblast: the role of myofibroblast

   transformation in corneal wound healing and pathology. *Veterinary Ophthalmology*, 2009; 12(s1): 25.

10. Pot, SA; Liliensiek, SJ; **Myrna, KE**; Bentley, E; Jester, JV; Nealey, PF; Murphy, CJ. Nanoscale topography modulates fundamental cell behaviors of rabbit corneal keratocytes, fibroblasts and myofibroblasts. *IOVS*. 2010; 51(3): 1373-81.

11. Myrna KE, Pot S, Bentley E, Adkins E, Miller P, Murphy C. Toxic anterior segment syndrome and are we missing it? *Veterinary Ophthalmology*. 2009; 12(2):138.

12. **Myrna, KE**; Herring, I. Constant rate infusion for topical ocular delivery in horses: a pilot study; *Veterinary Ophthalmology*.2006; 9(1):1-5.

2. Name: Kelsey A. Hart

   Role**:** Co-Investigator

   Current Position: Assistant Professor of Large Animal Internal Medicine

   Department of Large Animal Medicine and Surgery

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Education/Training:

Institution	**Degree**	**Year Completed**	**Field of Study**
University of Georgia, Athens, GA	PhD	2010	Physiology

American College of Veterinary Internal Medicine	Board Certification	2008	Large Animal Internal Medicine
University of Georgia, Athens, GA	Residency	2008	Large Animal Internal Medicine
University of Georgia, Athens, GA	Internship	2005	Large Animal Medicine & Surgery
Cornell University, Ithaca, NY	DVM	2004	Veterinary Medicine
Cornell University, Ithaca, NY	AB	1998	Biology

Previous Positions:

2008 – 2011 (Jan): Clinician and Graduate Fellow (PhD Candidate), Large Animal Internal

Medicine Department of Large Animal Medicine, College of Veterinary Medicine, University of Georgia, Athens, GA.

Selected Honors and Awards:

2013	Faculty Clinical Research Award, UGA College of Veterinary Medicine
2013	David Tyler Award for Advances in Teaching, UGA College of Veterinary Medicine
2013	Lilly Teaching Fellow, UGA
2012	Fellow, UGA Teaching Academy
2008	Clinical Research Committee Graduate Enhancement Award (Individual Fellowship), University of Georgia, College of Veterinary Medicine
2008	American Association of Equine Practitioners Foundation, Past Presidents' Research Fellowship
2008	Outstanding House Officer Award, University of Georgia, College of Veterinary Medicine
2008	ACVIM Resident Research Award, Equine Category. ACVIM Annual Forum
2008	Resident Research Award, 2nd Place, Equine Category. IVECC Symposium.
2008	American Association of Veterinary Clinicians' Clinical Resident Award
2004	Physiology Award, Cornell University College of Veterinary Medicine

2004 Pfizer Equine Clinical Proficiency Award, Cornell University College of Veterinary Medicine

2004 The Daphne Award, for recognition of clinical proficiency and excellence in the practice of veterinary medicine, Cornell University College of Veterinary Medicine

2004 Merck Manual Award (top 10 in class), Cornell University College of Veterinary Medicine

2004 Degree Marshall (top 2 in class), DVM Class of 2004 Cornell University

Selected Peer-Reviewed Publications:

1) Mullen KR, Furness C, Johnson AL, Norman TE, **Hart KA,** Burton AJ, Bicahlo RC, Ainsworth DM, Thompson M, Scrivani PV. Adverse reactions in horses that underwent general anesthesia and cervical myelography. *Journal of Veterinary Internal Medicine*. 29 (3): 954-960. 2015. Collicutt NB, Garner B, Berghaus R, Camus M, **Hart K.** Effects of delayed serum separation and storage temperature on serum glucose concentration in four species. *Veterinary Clinical Pathology.* In press. 2014.
2) **Hart KA**, Sherlock CE, Davern AJ, Lewis TH, Robertson TP. Effect of N-butylscopolammonium bromide (Buscopan^{TM}) on equine ileal smooth muscle activity in an *ex vivo* model. *Equine Veterinary Journal*. Epub ahead of print: doi: 10.1111/evj.12293. 2014.

3) McConachie EL, ***Hart KA,*** Whelchel DM, Schroeder EL, Schott HC II, Sanchez S. Pulmonary disease potentially associated with *Nicoletella semolina* in 3 young horses. *Journal of Veterinary Internal Medicine.* 28(3): 939-943. 2014.

4) Monk CS, **Hart KA**, Moore PA, Myrna KE. The concentration of endogenous cortisol in equine tears and blood at rest and after simulated stress. *Veterinary Ophthalmology*. DOI: 10.1111/vop.12128. 2013.

5) **Hart KA,** Linnenkohl W, Mayer JR, House AM, Gold JR, Giguère S. Medical management of sand enteropathy in 62 horses. *Equine Veterinary Journal.* 45 (4): 465-469. 2013.

6) **Hart KA,** Dirikolu L, Ferguson DC, Norton NA, Barton MH. Daily endogenous cortisol production and hydrocortisone pharmacokinetics in adult horses and neonatal foals. *American Journal of Veterinary Research*. 73(1): 68-75. 2012.

7) **Hart KA,** Barton MH, Vandenplas ML, Hurley DJ. Effects of low-dose hydrocortisone therapy on immune function in neonatal horses. *Pediatric Research*. 70(1): 72-77. 2011

8) Ellis AE, **Hart KA**, Elfenbien J. Pathology in Practice: Proliferative enteropathy in a miniature horse foal. *Journal of the American Veterinary Medical Association*. 238(11): 1417-1419. 2011

9) **Hart KA,** Barton MH, Ferguson DC, Berghaus R, Slovis NM, Heusner GL, Hurley DJ. Serum free Cortisol fraction in healthy and septic neonatal foals. *Journal of Veterinary Internal Medicine*. 25(2): 345-355, 2011.

10) **Hart KA,** Slovis NM, Barton MH. Hypothalamic-pituitary-adrenal axis dysfunction in hospitalized neonatal foals. *Journal of Veterinary Internal Medicine*, 23(4): 901-912, 2009.

11) **Hart KA,** Heusner GL, Norton NA, Barton MH. Hypothalamic-pituitary-adrenal axis assessment in healthy term neonatal foals utilizing a paired low dose / high dose ACTH stimulation test. *Journal of Veterinary Internal Medicine*. 23(2): 344-351, 2009.

12) **Hart KA,** Felippe (Flaminio) MJB, Leroy BE, Williams CO, Dietrich UM, Barton MH. Successful resolution of cryptococcal meningitis and optic neuritis in an adult horse with oral fluconazole therapy. *Journal of Veterinary Internal Medicine*, 22(6): 1436-1440, 2009.

13) **Hart KA,** Barton MH, Williams KJ, Felippe (Flaminio) MJB, Howerth EW. Multinodular pulmonary fibrosis, pancytopenia and Equine Herpesvirus-5 infection in a Thoroughbred gelding. *Equine Veterinary Education*, 20(9): 470-476, 2009.

14) **Hart KA,** Ferguson DC, Heusner GL, Barton MH. Synthetic adrenocorticotropic hormone stimulation tests in healthy neonatal foals. *Journal of Veterinary Internal Medicine*, 21(2): 314-321, 2007.

15) Gardner R, **Hart K**, Stokol T, Divers T, Felippe (Flaminio) MJB. Fell Pony Syndrome in North America. *Journal of Veterinary Internal Medicine*, 20(1): 198-203, 2005.

3. Name: Brittany M. Wynne

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Cornell University, Ithaca, NY	BS	2011	Animal Science