

**Analysis of the Embedded Cortisol Signature in the Nail of
*Canis lupus familiaris***

Zoe Mack

A Senior Honors Project Submitted for Fulfillment of Requirements
for Honors in Biology

April 2015

Faculty Sponsor: Bobby Fokidis

Rollins College
Winter Park, Florida

Table of Contents

I.	Abstract.....	3
II.	Literature Review	
	a. Introduction.....	4
	b. Hormone Function and The Stress Response.....	5
	c. Cortisol's Influence on Stress-Related Disease.....	9
	d. Established Techniques to Measure Cortisol.....	10
	e. Novel Techniques to Measure Cortisol.....	12
	f. The Potential of the Nail as a Source of Embedded Cortisol.....	13
	g. Proposed Research.....	15
III.	Materials and Methods.....	16
IV.	Results.....	21
V.	Discussion.....	26
VI.	Literature Cited.....	31
VII.	Appendix I.....	35

Abstract

Cortisol is a glucocorticoid steroid hormone that is released from the adrenal gland in response to stress in most mammal species. There is currently no method to measure cortisol levels from the dog nail. Development of such method would allow a more accurate analysis of long-term cortisol secretion in relation to current techniques such as measures of blood and saliva, which measure transient increases in cortisol during acute stress. In this study, a novel technique was developed to extract and quantify cortisol concentrations within the nail of the domestic dog (*Canis familiaris*). Nail samples collected from a local veterinary clinic were homogenized using an attrition mill. Cortisol was then recovered from the homogenized sample using both liquid-liquid and solid phase extraction techniques, and then measured using an enzyme-linked immunoassay. Cortisol was found to be present within the nail at a quantifiable concentration that was variable between both individuals and techniques used. Nail cortisol was also positively correlated with cortisol levels in hair, an already established biomarker. Methodology was refined to maximize cortisol recovery, and this research will lay the groundwork for advancing technology available surrounding cortisol testing in both domestic and wild animals.

Introduction

The endocrine system is a vital physiological system that controls communication throughout the body. Endocrine signaling regulates growth, metabolism, development, and coordinates responses to environmental stimuli. Once a hormone has reached its target cell, the hormone will bind with a specific receptor and elicit one or many physiological response(s) (Norris 2013). Cortisol is a glucocorticoid steroid hormone that plays a role in the stress response in many mammal species, including humans. Cortisol is secreted by the adrenal gland in response to an environmental (e.g., predator) or internal stressor (e.g., disease) and is vital in preparing the body to adapt to the changing conditions associated with the stress. Abnormal variations in cortisol secretion can lead to a variety of detrimental effects on immune and reproductive health, along with growth and metabolism (De Martin 2006). This has spurred the frequent use of cortisol as biomarkers of health and wellness, in both animals and people. Current techniques to analyze cortisol focus on measuring short-term secretion that can vary widely throughout the course of a day and in response to immediate stress in the environment. However, cortisol analysis is important in the diagnosis of a variety of stress-related conditions that chronically raise cortisol levels. The lack of an effective method suggests the need for a technique that accurately reflects patterns of chronic cortisol secretion. This thesis begins by introducing the stress response with respect to regulation by both the endocrine and sympathetic nervous system, and highlighting the specific role of cortisol. Furthermore, the current methods used to measure cortisol will be discussed and the potential of the dog nail as a source of embedded cortisol will then be explained. This thesis then describes a method to analyze cortisol from the nail of the domestic dog and test its validity as a novel method of cortisol testing.

Hormone Function and The Stress Response

The endocrine system involves a system of glands throughout the body that secrete hormones into the bloodstream to ultimately trigger responses in target cells. Thus, hormones are chemical messengers and their release into the blood means they induce changes in cells and act at relatively long distances within the body. Among the major endocrine glands are many familiar structures including the adrenal glands, the thyroid, the pancreas, the ovaries, and the testes (Norris 2013). Once a hormone has reached its target cell, it will bind to a specific receptor, usually a protein, and elicit one or many physiological responses. Among the best-studied hormones, is a class of cholesterol-derived lipids, known as steroids, which includes the stress hormone, cortisol (Charmandari 2005).

When a distressing environmental or internal stimulus is presented to an animal, a “fight-or-flight” response is activated, which induces both behavioral and physiological changes. This response is central to ensuring an animal’s survival to acute, or short-term, stress and is mediated by the coordination of both the endocrine and sympathetic nervous systems (Sapolsky et al. 2000). The adrenal glands, located just above the kidneys, both synthesize and secrete hormones associated with the body’s response to stress. These glands are divided into an inner medulla, which is part of the sympathetic nervous system and releases the neurohormones epinephrine (adrenaline) and norepinephrine (noradrenaline), and an endocrine outer cortex, which secretes steroids, namely cortisol. The response elicited by the release of these medullar neurohormones is very rapid – on the order of seconds. For example, epinephrine levels will increase several-fold, but only for one or two minutes before returning to normal basal levels once again (Norris 2013). Physical adaptations as a response to the

neurohormones include increased blood pressure, heart and respiratory rates, and production of glucose (Chrousos and Gold 1992).

Cortisol is a glucocorticoid steroid hormone, composed of three six-carbon rings and one five-carbon ring, and is hydrophobic as a result of this four-ring structure (Beato and Klug 2000; Engel and Langer 1961). Cortisol is synthesized from cholesterol by the adrenal cortex, the outer layer of the adrenal gland (Figure 1; Figure 2). Cortisol binds primarily to two receptor types: a low-affinity glucocorticoid receptor (GR), found throughout the vertebrate body, and a less widespread, but higher affinity mineralcorticoid receptor (MR) (Charmandari 2005).

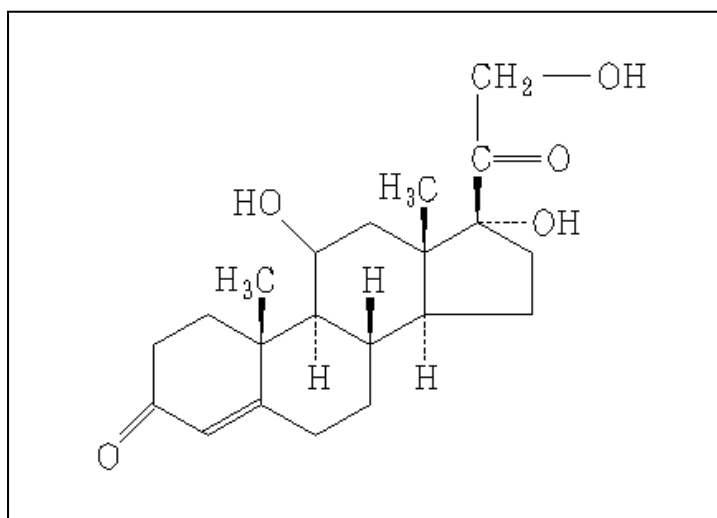


Figure 1 – Molecular structure of cortisol, a glucocorticoid steroid hormone associated with the stress response. Obtained from The Spectral Database for Organic Compounds (SDBS).

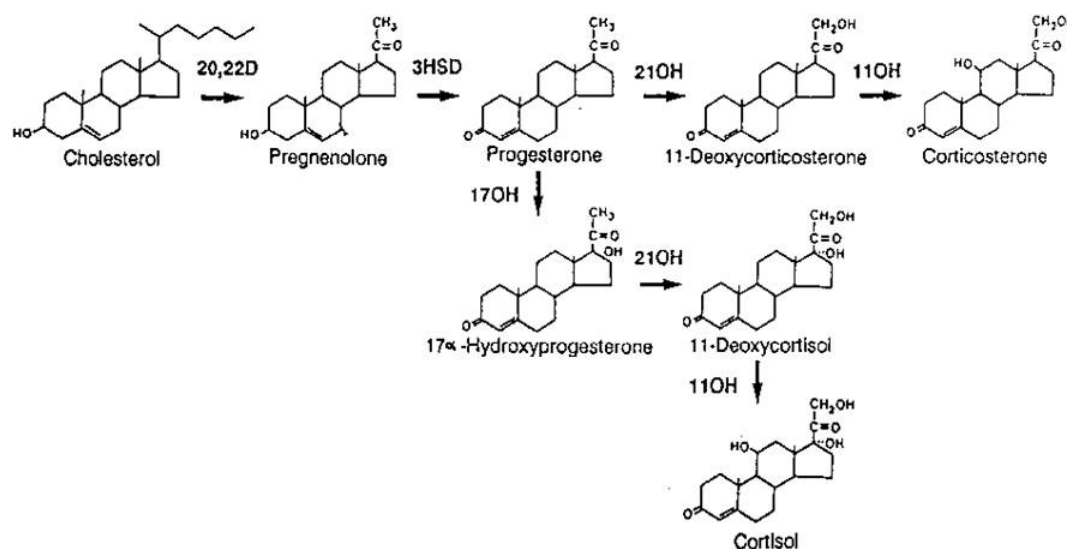


Figure 2 – Steroid synthesis from cholesterol to cortisol (steroid of interest) and the primary avian and reptile glucocorticoid, corticosterone. Obtained from King 2014.

The release of cortisol during stress is regulated by the hypothalamic-pituitary-adrenal, or HPA axis (Figure 3). This hormone cascade pathway begins with a stressor promoting increased secretion of both corticotropin-releasing hormone (CRH), and vasopressin (VP), by the hypothalamus of the brain (Salata 1988). The hypothalamus is a “master” neuroendocrine regulator that receives information directly from various parts of the brain to initiate an appropriate response to a stressor (Sapolsky et al. 2000). These hormones bind to CRH and VP receptors on the anterior pituitary gland, which in turn promote the release of adrenocorticotrophic hormone (ACTH) into the blood stream. ACTH then binds to specific ACTH receptors located on the plasma membranes of adrenocortical cells, causing them to secrete cortisol (Sapolsky et al. 2000). Cortisol in

circulation has a variety of functions that include promotion of the synthesis and release of glucose from energy stores, and the modification of behavior to encourage vigilance, and foraging behavior (Boonstra 2004).

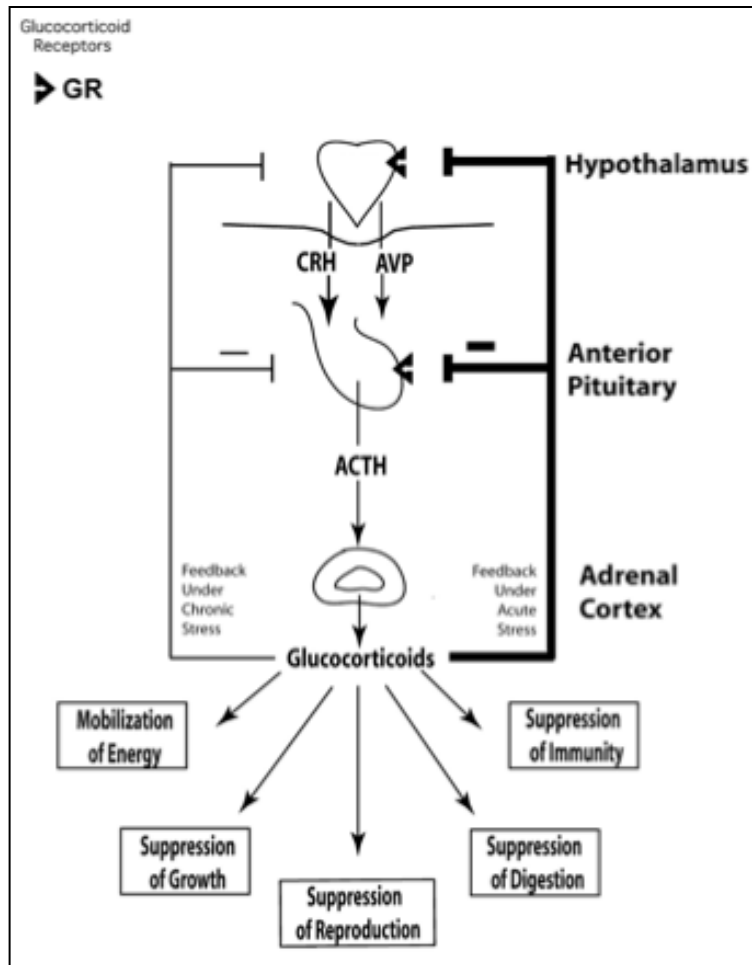


Figure 3 – The hypothalamic-pituitary-adrenal axis. A stressor promotes secretion of both corticotropin-releasing hormone (CRH) and vasopressin (AVP) by the hypothalamus of the brain. This leads to the production of adrenocorticotrophic hormone (ACTH) from the anterior pituitary gland. ACTH cortisol. Modified from Boonstra 2004.

After activation by a stressor, the acute stress response is followed by a recovery period, where cortisol returns to a basal level (Charmandari et al. 2005). The temporary nature of acute stress is ultimately beneficial to the animal and results in no long-term

negative effects (Chrousos and Gold 1992). Chronic stress occurs when there is a lack of this recovery and the detrimental effects of long-term secretion are observed, due to continued exposure to elevated cortisol concentrations above the normal baseline level. This is what people often refer to when talking about being “stressed.” This lack of recovery may be explained by a debilitated negative feedback loop, as cortisol mediates hormone secretion by both the hypothalamus and pituitary gland in a concentration dependent manner (Kozlov and Kozlova 2014). Chronic exposure to cortisol can suppress the immune system, growth, development, reproduction, and metabolism (Charmandari et al. 2005; Sapolsky et. al. 2000).

Cortisol’s Influence on Stress Related Disease

Abnormal cortisol secretion patterns are linked to a variety of stress related conditions. Hyperadrenocorticism, a condition also known as Cushing’s disease, is seen often in dogs and involves the overproduction of cortisol. This disease is seen relatively frequently, with 1-2 cases per every 1000 dogs diagnosed each year (Willeberg 1982). Two forms of hyperadrenocorticism are recognized in veterinary medicine and must be distinguished in order to provide proper treatment. The majority of cases involve pituitary-dependent hyperadrenocorticism. This occurs as the result of a disruption in the negative feedback loop exhibited by the pituitary gland on ACTH secretion, resulting in more ACTH release (Castinetti et al. 2012). A second less common form of hyperadrenocorticism involves the presence of an adrenocortical tumor, resulting in excessive cortisol secretion (Kool et al. 2013). This form of hyperadrenocorticism occurs in approximately 15% of all cases (Galac 2010). Hyperadrenocorticism leads to a variety of complications in dogs, including a weakened immune system, and hyperglycemia that can eventually lead to a diabetic state (Peterson 2007).

An opposite effect of reduced cortisol production (i.e., hypoandrenocorticism) can lead to Addison's disease, or chronic adrenal insufficiency. This condition tends to be more common in younger animals, and is characterized by hypotrophy (i.e., shrinking) of the adrenal gland and insufficient cortisol secretion (Feldman and Nelson 2013). Generally, Addison's disease is more rare than its counterpart, Cushing's disease, in both dogs and people (Brooke and Monson 2013). Any growths, tumors, or damage on the adrenal glands are also very likely to alter long-term cortisol secretion patterns, yet currently employed methods primarily focus on shorter-term assessments of cortisol secretion (Castinetti et al. 2012).

Established Techniques to Measure Cortisol

There are a variety of samples and methods that have been used to analyze levels of cortisol. The use of blood, urine, and saliva analysis is most commonly employed by the veterinary industry to record and monitor cortisol levels in patients. Current methods that screen for the presence of hyperadrenocorticism by measuring blood cortisol secretion in dogs include the exogenous ACTH stimulation test to determine the adrenal cortisol response to injection, and the low-dose dexamethasone suppression test to test the efficacy of the negative feedback at the level of the pituitary. The low-dose dexamethasone suppression test is currently the test of choice that utilizes blood due to its accuracy in recording cortisol concentration (approximately 85 to 100%) in comparison to similar testing methods (Gilor et al. 2011). Other methods include the high-dose dexamethasone suppression test and measuring the amount of ACTH directly with an assay. The high-dose test (8 mg per day for 2 days) is successful in diagnosing Cushing's disease with a high level of specificity, whereas the low-dose test (1 mg per day for 2 days) is able to rule out the disease with a high sensitivity

(Castinetti et al. 2012). All of these techniques rely on processing blood samples to differentiate the cause of hyperadrenocorticism (De Martin et al. 2006). Such blood sampling is accurate in measuring current levels of cortisol, but they require restraint of the animal in order to obtain, which can be a stressor on its own. This could lead to secretion of cortisol and thus inaccuracy in determining the baseline level in testing results (Blackshaw and Blackshaw 1989).

Cortisol is also excreted in urine and has also been used as a direct measurement of cortisol levels. The measurement of the urinary cortisol to creatinine ratio (UCCR) was proposed in a study by Rijnberk et al. (2008), and is currently the most common metric for measuring urine cortisol. This method takes advantage of the ratio between creatinine and cortisol in the urine. Creatinine is a product derived from the organic acid creatine that is excreted in the urine at a constant level in vertebrate species. By comparing this value to cortisol, fluctuations can be detected (Smiley and Peterson 1993). Although this method is widely used, the test is only accurate within 24 hours, as it is indicative of previously circulating cortisol that has been metabolized and secreted. In addition, the results of UCCR may be influenced by current physical and internal stress that alter both stress and urine output (van Vonderen et al. 1998). Thus, test results are more accurate when samples are collected in a home environment, which may be inconvenient. Furthermore, UCCR may be unreliable for diagnosing hyperadrenocorticism in dogs, since both polyuria (i.e., excessive urine production) and polydipsia (i.e., excessive thirst due to dehydration) can lead to similar results thus confounding diagnosis (Feldman and Mack 1992; Petrie G. 2014).

The measurement of saliva cortisol is a less invasive method of analyzing a dog's baseline cortisol levels. The concentration of cortisol in saliva is significantly lower than that in plasma (~8-10%). In circulation, cortisol is largely bound to transport proteins

(namely the cortisol binding globulin or CBG) due to their hydrophobic nature. The lower levels in saliva are due to only the free fraction of cortisol that is unbound to such proteins being transferred into the saliva from the blood (Beerda et al. 1996). As with blood and urine, saliva cortisol is a measure at a specific time point that makes the interpretation of acute stress possible, but may limit inference into chronic cortisol secretion (Kobelt et al. 2003). Measurement of cortisol in blood, saliva, and urine can fluctuate throughout the day and as they are indicative of short-term secretion are highly susceptible to the current stress state of the animal. Often simply arriving at the veterinary clinic can induce a robust stress response for some dogs and this can be coupled with the stress induced from physically obtaining the sample.

Novel Techniques to Measure Cortisol

Recently, studies have begun to explore alternative tissues as sources to measure cortisol secretion, independent of the impacts of the external environment. Corticosterone, the primary avian glucocorticoid, has been found in feathers and used to investigate the effects of chronic stress in both captive and wild birds (Fairhurst et. al. 2011). The measurement of feather corticosterone has been achieved using a methanol-based liquid-liquid extraction technique and subsequent determination with radioactive immunoassay (Bortolotti et. al. 2008). Corticosterone has also recently been successfully recovered from the claws of painted turtles (*Chrysemys picta*) using a methanol-based extraction method (Baxter-Gilbert et al. 2014). In another keratinized tissue, the baleen plates of bowhead whales (*Balaena mysticetus*), cortisol has been recovered and levels are consistent with established patterns of glucocorticoid production in other whale species (Hunt et al. 2014).

Long-term assessments of cortisol concentrations have also been made through the sampling of hair, another keratinized structure. Hair cortisol testing in humans has been the subject of many correlational studies with major life stress events, and is thought to give insight into the “holistic” stress response (Karlén et. al. 2011). Hair cortisol testing is also established in canine species; research has shown dogs with hyperadrenocorticism have a significantly higher concentration of cortisol in the hair follicle compared to healthy dogs (Corradini et. al. 2013). Although hair testing is successful, a variety of external factors may affect the validity of this method. Hair can be sampled from various regions of the body, however it is unknown whether levels fluctuate based on the collecting area. As the technique has only been recently developed, the extent to which washing with various hair care products impacts hormone results remains unknown (Corradini et. al. 2013). Furthermore, cortisol levels may vary with hair color even from the same dog and this could lead to inefficient testing and bias based on hair color (Bennett and Hayssen 2010). Finally, local glucocorticoid production may occur within the hair follicle independent of adrenal cortisol synthesis, thus decoupling circulating levels with that found in hair. (Accorsi et al. 2008; Keckeis et. al. 2012). Collectively, these issues may complicate accurate analysis of cortisol secretion patterns resulting from the HPA axis.

The Potential of the Nail as a Source of Embedded Cortisol

The canine nail is composed of a keratinized epithelium with a vascularized inner corium, or “quick” that provides nutrients to the constantly growing tissue (Evans and de Lahunta 2013). The presence of these blood vessels suggests the potential for cortisol accumulation within the nail from the systemic bloodstream. As new cells are produced from the corium, cortisol may be deposited from the blood supply, creating

an embedded cortisol signature in the keratinized nail that can vary in proportion with circulating levels over the growing time of the nail (Figure 4). Cortisol is highly stable due to its four-ring structure, and thus may remain embedded for an extended period of time as it diffuses into the nail (de Berker et al. 2007).

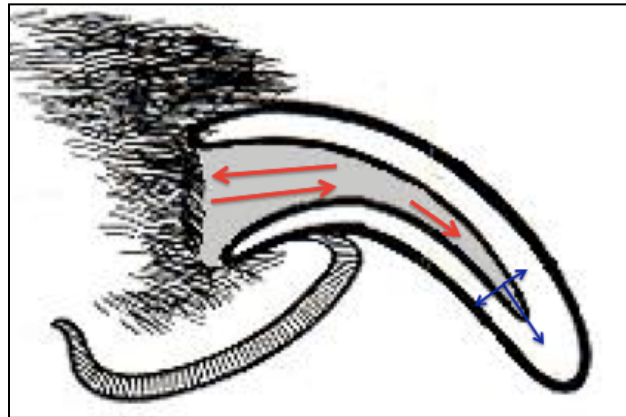


Figure 4 – Proposed mechanism for cortisol secretion within the nail. Cortisol is present as blood circulates through the corium (red). As new cells are produced, cortisol is deposited from the blood supply into the keratinized nail (blue).

Measurement of cortisol secretion in nails has begun to develop within a human context. Warnock et al. (2010) found that the nails of subjects under long-term stress conditions had higher levels of cortisol compared to when the subjects were at a resting state. Indeed, methods are currently being developed that can analyze small quantities of cortisol in human nail at a high level of recovery (Khelil et al. 2011). Adapting the nail as a source for long-term cortisol analysis in a veterinary context would allow for a variety of practical applications. Dog nails are easily clipped without negatively affecting outward appearance, which has often been a concern of similar diagnostics using hair (Corradini et. al. 2013). The collection of nails is much less invasive than the collection of either blood or saliva, and is routinely done without requiring additional

timing or restraint. Indeed, owners could potentially clip nails at home and send the samples in for further analysis. Potential applications of the measurement of nail cortisol beyond use in the veterinary industry exist as well. The embedded cortisol signature could be analyzed in wild mammals, including possibly extinct species, to gather information surrounding the long-term secretory patterns in response to environmental stressors. Such information may also be relevant in determining an animal's physiological state in legal cases surrounding animal neglect and abuse.

Proposed Research

Lack of an effective measure of long-term cortisol secretion in the veterinary industry calls for development of a method to accurately analyze hormone levels. This research aims to explore the presence of cortisol in the nail of the domestic dog and validate its use as a variable and biologically relevant measure of long-term stress. A combined solid phase extraction and enzyme-linked immunoassay protocol has been established to isolate and analyze corticosterone from bird feathers and cortisol from human nails (Khelil et al. 2011; Warnock et al. 2010). This general methodology will be the basis towards the development of the proposed nail cortisol biomarker in this project.

To prepare the nail samples for cortisol measurement, a homogenization protocol will be employed to enable access to liberate steroids from their matrix by subsequent extraction. Homogenization is a process that unifies the composition of a sample and is commonly employed prior to biochemical extraction. Both liquid-liquid and solid phase extraction protocols will be used to recover cortisol from the nail. Organic solvents such as methanol can be used to elute impurities, such as other lipids that could interfere with the hormone assay, from the sample and retain the steroid product in the

stationary sorbent due to cortisol's non-polar and hydrophobic nature. As this methodology revolves around the development of a technique, the specifics of the protocol will be manipulated to maximize cortisol recovery from sample. Enzyme-linked immunoassays (EIA) will be used to determine the recovery of cortisol from the tissue sample. The EIA will be validated by running duplicate samples to determine both interassay and intrassay variation, and by testing parallelism between a serially diluted nail sample and the standard curve. Steroid recovery will be determined by spiking nail samples with a cortisol standard of known concentration. To assess the biological relevance of this measure, samples obtained from dogs of varying breeds, ages, sexes, and weights will be compared to identify cortisol fluctuations both within and between individuals. Finally, comparison of nail cortisol concentrations with the already well-established hair cortisol concentrations will be made to assess the comparability of the method.

Materials and Methods

Collection of Samples

Nail samples were obtained from 27 dogs at Loch Haven Veterinary Hospital with written permission from both the animal and facility owners (Appendix I). Samples were collected solely by facility employees; the sex, age, weight, breed, and whether the dog was spayed or neutered was recorded (Table 1). Nails were clipped below the quick and stored at room temperature. In addition, hair was also collected from the surface coat of each dog to facilitate comparison with nail concentrations.

Table 1 - Subject information obtained from Loch Haven Veterinary Hospital. All individuals listed were utilized in nail and hair cortisol analysis. All animals were spayed or neutered.

Subject Number	Age (yrs.)	Sex	Weight (lbs.)	Breed
102	9	M	165	Lab Mix
103	1	M	18	Boston Terrier/ French Bulldog
104	5	F	48.5	Harrier Hound
105	13	M	69	Collie Mix
106	5.5	M	80	Lab Mix
107	4	M	35.5	King Charles Cavalier
108	2	F	14.5	Mini Dachshund
109	5	M	12	Pomeranian
110	7	M	Unknown	Rat-Terrier Mix
111	3	F	28.5	Beagle
112	5	M	37	Beagle
113	8	F	72	Husky
114	5	F	45	Mixed Breed
115	5	F	45	Mixed Breed
116	8	M	33	Cocker Spaniel
117	8	F	101.5	Tibetan Mastiff
119	3	M	69	Lab Mix
120	9	F	14.5	Dachshund
121	10	F	28.5	Fox Terrier
122	9	F	13	Shih-Tzu
126	13	F	76	Shepard Mix
127	6	F	16	Jack Russell Terrier Mix
128	16	M	17	Pug

Nail Sample Preparation

To extract steroids, samples first had to be ground down to a powder form, and a variety of methods were tested. A pooled sample of nails from multiple individuals was used to optimize the grinding procedure. These methods included: the use of a mortar

and pestle with samples exposed to liquid nitrogen; a Precellys 24-bead ruptor (Bertin Technologies Corp., Rockville, MD, USA) with tubes and beads of varying material; and a hand-held attrition mill (Glas-Col LLC, Atlanta, GA, USA). These methods varied in their ability to grind down the nail samples (Table 2). Another method involved washing the nails with 100% ethanol in an attempt to dry them before further breakdown with the bead ruptor, as described in Khelil et al. (2011). The most success in generating a powdered nail sample was found to be with the hand-held attrition mill, and this method was adopted for the duration of the study. Each sample was then ground down and 50 mg from each individual sample was collected and stored for future analysis.

Hair Sample Preparation

Hair Samples were cut into 1-4mm fragments to effectively break down the hair for cortisol extraction. 30-50 mg of fractioned hair from each individual sample was collected and stored for later analysis.

Hormone Extraction

Powdered samples were suspended in a 19:1 ratio of 84% methanol in water (μL) to sample mass (mg) and left overnight. This liquid-liquid extraction between methanol (an organic solvent) and water (an aqueous solvent) partitions compounds on the basis of polarity, with steroids accumulating in the methanol fraction. Samples were then centrifuged at 3000 g for 10 minutes at 4 °C. The methanol supernatant was collected and suspended in 10 mL of distilled water.

Solid phase extraction was performed using C18 filter column cartridges (Agilent Technologies, Santa Clara, CA, USA) on a vacuum manifold at a constant flow rate of 2 drops per second. Columns were first primed with 3 mL of 100% ethanol, and then equilibrated with 10 mL of distilled water. The columns were then loaded with the 10 mL of samples. Impurities, such as bioinactive sulphated and gluconurated steroids, were then eluted out with 10 mL of 40% methanol and the columns were allowed to run dry at maximum vacuum pressure for 5 minutes. The final purified samples were collected with 5 mL of 90% methanol and samples were dried in a speed vacuum concentrator (Thermo Fisher Scientific Inc., Pittsburgh, Pennsylvania, USA) at 60 °C for 4 hours. Dried extracts were stored at -20 °C until assayed.

Enzyme-linked Immunoassay

Cortisol concentration was determined through a cortisol enzyme immunoassay kit (EIA) (Arbor Assays Inc., Ann Arbor, Michigan, USA). Dried extracts were each re-suspended with 2 µL of 100% ethanol and 60 µL of the assay buffer provided by the kit. The assay was carried out as outlined by the manufacturer. The 96-well plate was read using a spectrophotometer at 495 nm wavelength to provide optical density measurements. Final concentrations were calculated using raw absorbance data interpolated from the standard curve generated with GraphPad Prism version 6 (GraphPad Software Inc., La Jolla, California, USA). All subsequent statistical analyses were conducted using SigmaPlot 13 (Sysat SYSTAT Software Inc., San Jose, CA, USA).

Testing Parallelism

To determine whether any substances within the nail matrix interferes with the ability of cortisol to be measured using an EIA, the relationship between the assay

standard curve and a serial dilution of a nail sample pool was compared. If no interference occurs then both the standard curve and the serial dilution should be parallel. The standard curve was generated from serial dilution of a cortisol stock (3200 pg/mL) provided by the EIA kit. The serial dilution was generated from a pooled nail sample that ranged from 1.5 to 95 mg of total nail mass. Concentration as a function of percent mass was presented, with percent mass acting as a percentage of the total pooled sample's mass. These serial samples were separately processed using SPE and assayed together. Parallelism was statistically compared using a multiple curvilinear regression comparison in GraphPad Prism 6.

Cortisol Recovery

To estimate the recovery of steroid using the above extraction methods, pooled nail samples were spiked with a known concentration of cortisol. Samples were spiked with cortisol standard at the following concentrations: 113, 22, 16, and 2 ng/g. These values were selected as they represented a wide range of physiologically relevant concentrations consistent with preliminary observations. Furthermore, pooled samples were pretreated with a 2 to 1 ratio of dextran-coated charcoal (DCC) to sample and left to incubate overnight prior to they assay, in order to absorb cortisol in the sample to serve as a negative control.

Assay Precision

Inter-assay and intra-assay variation were determined by calculation of coefficients of variation across duplicates of the standard curve, either between or within plates respectively. These measures indicate the degree to which sample

placement within and between plates at different well locations provides consistency in results.

Comparison of Nail and Hair Cortisol Concentrations

Pearson's correlations were done to determine whether cortisol concentrations obtained from nail tissue is comparable to that sampled from hair. Hair cortisol levels were determined through an identical hormone extraction protocol as outlined above.

Relationship between Nail Cortisol and Canine Characteristics

One-way analysis of variance (ANOVA) was completed to determine variation between nail cortisol concentration and sex, size, and age. Size classes were divided into small, medium, and large, with a categorization of 10-24 lbs, 25-49 lbs, and 50+ lbs, respectively.

Results

Several homogenization methods were conducted using plasma pools generated from multiple individual samples to maximize steroid recovery (Table 2). The use of an attrition mill was the most successful at grinding the nail samples to a powder, and this facilitated increased extraction efficiency resulting in the highest mean cortisol recovery (Table 2). Intra-assay variation (expressed as % CV) was 14.4%, with an inter-assay variation of 15.0% of the standard curve duplicated in each plate (n=6 microplates).

Steroid recovery was highly variable and proportional to the concentration of the "spike" (Table 3). The highest recovery of 92.99% was obtained using a spike concentration of 113 ng/g, which is 10x the mean concentration found in nail tissue

(10.94 ng/g from Table 2). The dextran-coated charcoal pooled sample was found to have a specific binding of 120.4%, consistent with the blank at 132.4% binding.

A serial dilution of pooled samples was successfully modeled using curvilinear regression approach ($R^2 = 0.999$, $F_{1,3} = 2979.602$, $P < 0.001$). These pooled samples were within linear range of and significantly parallel to the assay kit's standard curve ($R^2 = 0.994$, $F_{1,5} = 691.073$, $P < 0.001$, $n=10$) (Figure 5A). Furthermore, cortisol concentrations increased as a function of the percent of the total nail mass for a pooled sample that was extracted (Figure 5B).

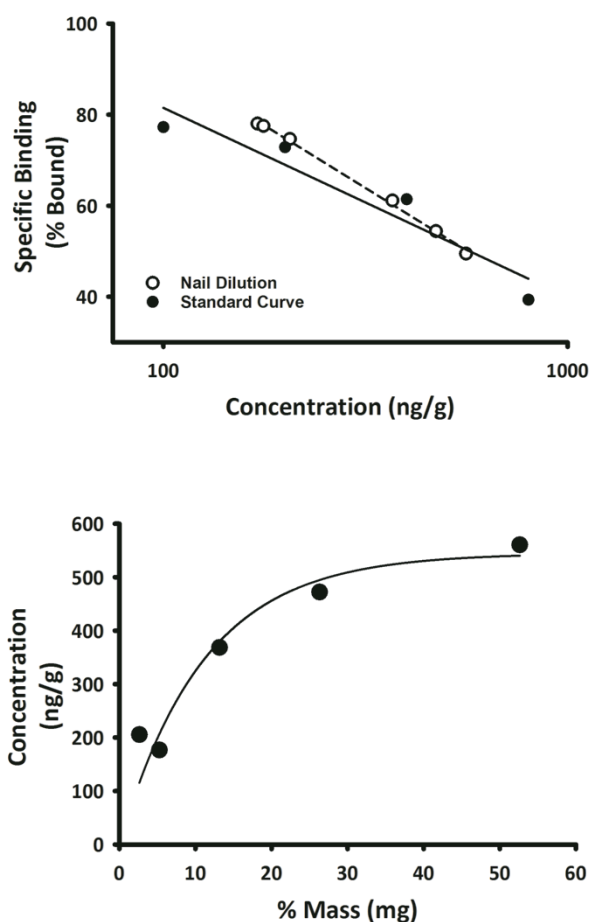


Figure 5. (A) The percent of cortisol specifically bound to the assay antibody as a function of cortisol concentrations of a nail sample pool. Samples within the linear range were parallel to the standard curve provided by the assay kit ($R^2 = 0.994$, $F_{1,5} = 691.073$, $P < 0.001$, $n=10$). This suggests no substances were present in the nail matrix that could interfere with the assay. (B) Cortisol concentrations of the sample pool in relation to percent mass of a pooled sample ($R^2 = 0.909$, $F_{1,4} = 30.003$, $P < 0.01$, $n=10$).

Table 2. Mean cortisol concentrations from a common sample pool extracted using different methods of nail homogenization. Numbers in parentheses indicate sample size and values are given as means + standard error.

Homogenization Treatment	Selected Beads	Mean Cortisol Concentration (ng/g)
Stainless Steel with Liquid Nitrogen	6 Zirconia Beads (3)	1.27 ± 0.76
Stainless Steel	6 Zirconia Beads (6)	1.36 ± 0.52
Stainless Steel	4 Chrome Steel Beads (3)	1.82 ± 0.94
Reinforced Plastic with Liquid Nitrogen	6 Zirconia Beads (3)	0.42 ± 0.19
Reinforced Plastic	6 Zirconia Beads (2)	4.37 ± 3.58
Hard Tissue Lysing Kit	Zirconia Oxide Beads (3)	1.87 ± 0.90
Hard Tissue Lysing Kit Washed With Ethanol	Zirconia Oxide Beads (1)	1.63
Ethanol Wash	No beads (2)	0.20 ± 0.01
Attrition Mill	No Beads (38)	10.94 ± 1.79

Table 3. Determination of percent steroid recoveries in nail cortisol spiked samples from a sample pool (n = 10).

Concentration Spike (ng/g)	% Recovery
113	93
22	70
13	1.7
6	0.1

Nail concentrations were not significantly correlated to hair cortisol concentrations ($r = 0.366$, $P < 0.15$, $n=19$), however five data points stood out as outliers based on a Chavenaut's test (> 2 standard deviations from the mean) (Figure 6).

When these were removed there was a significant positive correlation between cortisol concentrations measured from nail and from hair ($r = 0.776$, $P < 0.001$, $n=14$; Figure 6).

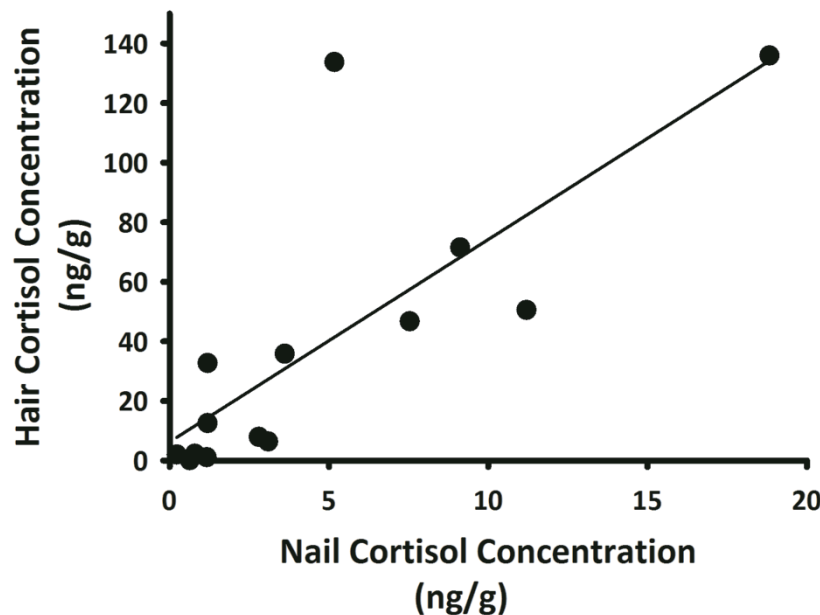


Figure 6. Correlation between cortisol concentrations in nail and hair analysis collected from the same individual dogs.

There was no statistically significant difference in nail cortisol concentrations between male and female subjects ($F_{1,16} = 0.235$, $P = 0.635$; Figure 7). There was a definite trend of increasing nail cortisol concentrations with decreasing size in dogs, however this was also not statistically significant ($F_{2,14} = 1.913$, $P = 0.190$; Figure 8). There was a trend towards a positive correlation between age and cortisol concentration ($r = 0.4453$, $F_{1,16} = 3.711$, $P = 0.073$; Figure 9).

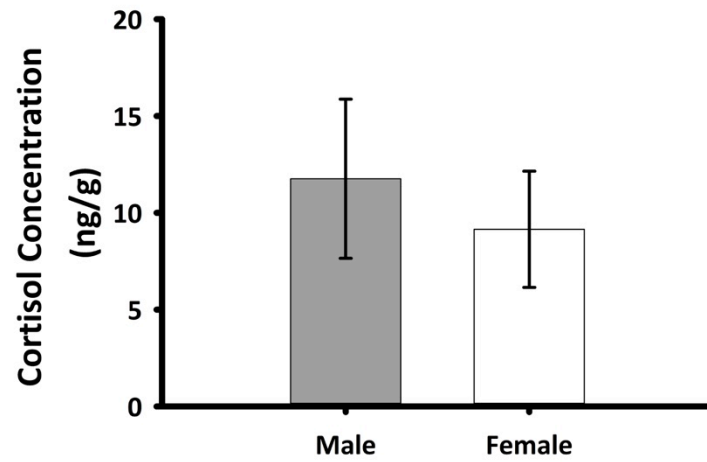


Figure 7 – Variation of nail cortisol between male and female individuals.

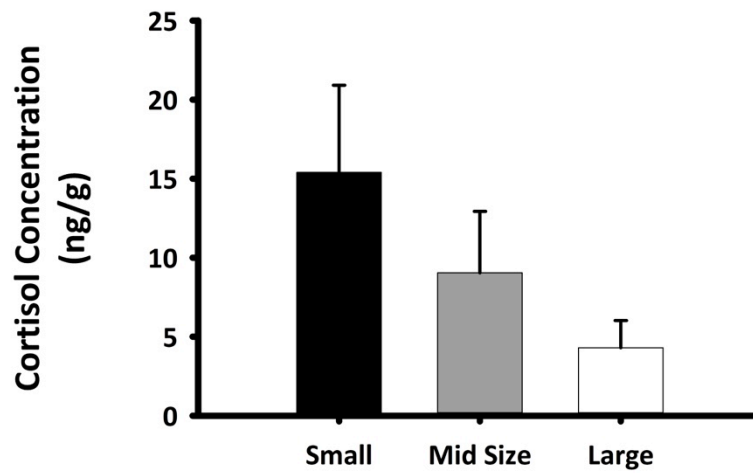


Figure 8 – Variation in nail cortisol between small, mid-size, and large individuals. Small dogs were classified as 1-24 lbs, mid-size dogs as 25-49 lbs and large dogs as 50+ lbs.

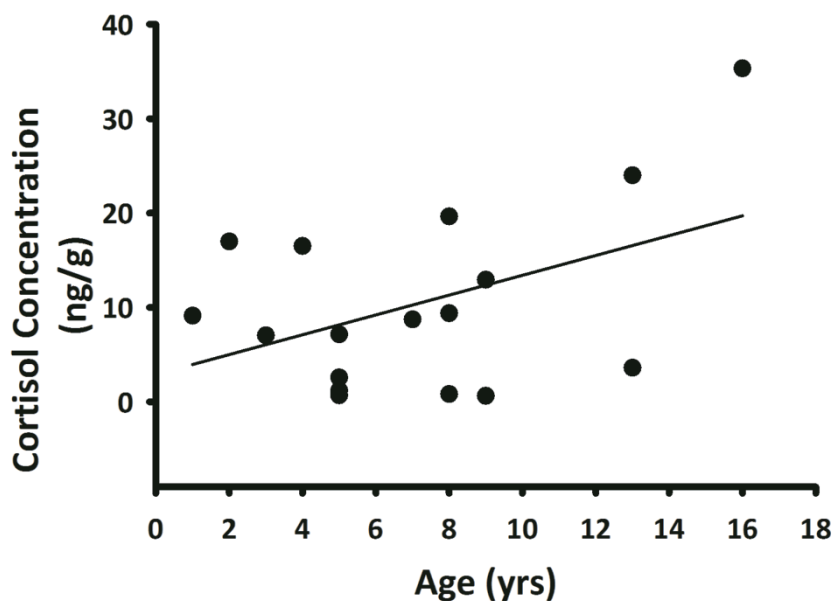


Figure 9 – Correlation between cortisol concentration in nail and age of individual subjects. Samples exhibited a slight trend but were not of statistic significance.

Discussion

Cortisol plays an integral role in the stress response of many mammal species, yet few noninvasive methods exist for measuring chronic levels of this steroid. An accurate method to analyze long-term cortisol secretion patterns would allow for the correct diagnosis of a variety of cortisol-linked diseases and patterns of chronic stress in dogs. The nail is a promising avenue for cortisol testing, and this thesis aimed to develop a technique to measure cortisol levels in the nail of the domestic dog. The results obtained from this study indicate that the dog nail contains a measurable amount of cortisol that can be extracted and measured with a good degree of reliability and precision using a combination of a liquid-liquid and solid phase extraction.

Cortisol concentrations based on a serial dilution pool were found to be well within range of and parallel to the standard curve generated by the immunoassay. Cortisol concentrations increased with the percent mass of the nail sample that was extracted. Collectively, these data suggest no substances in the nail matrix interfere with the EIA. This same pattern of mass and cortisol concentration was found in Baxter-Gilbert et al. (2014) with corticosterone in turtle claws. As corticosterone concentration increased in relation with the standard curve, so did the mass of claw associated with that sample. This suggests that the keratinized nail exhibits patterns of cortisol secretion in line with that seen in blood, saliva, and urine analysis.

Steroid recovery was variable, with other efforts of steroid spike recovery proving to be unsuccessful, and may be due to the concentrations of cortisol chosen for recovery determination. Higher recovery estimates were obtained using a higher concentration spike with the 113 ng/mg spike providing the highest recovery. According to Linsinger (2008), compound recovery is most accurate using a concentration that is 10x to 50x that of normally observed concentrations, which supports our pattern of cortisol recovery. Dextran-coated charcoal is typically used to adsorb steroids and other lipids from a solution while preserving the proteins in the matrix (Linsinger 2008). In this study, pretreatment with DCC removed steroids from the nail samples to an undetectable concentration equivalent to the assay blank. Thus, the majority of the cortisol appears to have been successfully eliminated from the sample. These collective results suggest that cortisol values obtained using the proposed methodology reflected actual cortisol, and not spurious results or non-specific binding of the anti-CORT antibody found in the assay kit.

Nail cortisol concentrations were also correlated to the well-established hair cortisol markers, after outliers were removed from the data. Interestingly, the absolute

concentrations of cortisol in nail are substantially lower than that recorded for hair. One possible explanation is the window of opportunity for cortisol to diffuse from circulation into the nail matrix, may be significantly shorter than for diffusion into the hair follicle. However, this correlation is important in that it suggests cortisol extracted from the nail was likely in circulation, as opposed to locally synthesized by steroidogenic cells located near the nail. To date, no steroidogenic cells have been identified in the canine paw. However future studies should test for the presence of the necessary steroidogenic enzymes to rule out this hypothesis. The reliability of hair cortisol testing is also in need of further review. It has been suggested that cortisol concentration fluctuates in hair between the coat colors of individual dogs, which suggests that pigment within the hair may store cortisol variably (Bennett and Hayssen 2010). Chemicals from products such as shampoo may also have affected hair cortisol concentration (Corradini et. al. 2013).

When looking at variation within and between individual dogs, a few patterns were observed that were in line with previously recognized trends in circulation. There was not a statistically significant difference between the nail cortisol concentrations found in male or female dogs. It is interesting to note that older females who had not been spayed were found to have a much higher level of circulating cortisol in previous studies (Mongillo et al. 2014). An expansion of sample size to include individuals who have not been spayed or neutered would allow for a more accurate analysis of nail cortisol concentrations between the sexes in comparison to literature findings. The results of this thesis indicated that there is a trend where nail cortisol concentration appears to increase with age. This is also consistent with the findings of Frank et al. (2015), who found that circulating plasma cortisol concentration is elevated in older dogs exhibiting hyperadrenocorticism.

Using the nail as a biomarker for estimating patterns of long-term cortisol secretion may allow for a variety of future applications. Nails can be clipped without affecting appearance and is less invasive than the collection alternative samples such as blood. This reduces the overall stress the animal experiences in addition to obtaining a sample indicative of patterns of long-term stress. Beyond use in the veterinary industry, cortisol could be analyzed in wild mammals and even potentially extinct species to gather information surrounding stress prior to the animal's extinction. This information may also be relevant in determining an animal's physiological state in legal cases surrounding animal neglect and abuse through the analysis of nail growth at specific time points. Dogs that have been moved from a high to low stress environment will show differences in cortisol secretion that may be used in court when claiming proper ownership of the animal.

This initial study must be followed up with further research aimed at assessing the efficiency of this method and determine whether or not the detected cortisol is a result of local glucocorticoid production. Furthermore, determining whether this biomarker is effective in measuring differences both between and within individuals is also essential. Studies comparing nail cortisol signatures in dogs that experience chronic stress with those exposed to little stress are necessary to determine the functional validity of this measurement and to establish this method as valuable to veterinary medicine.

This thesis has confirmed that cortisol is present in the nail of the domestic dog and can be measured using the technique developed here. It has been determined that cortisol is present in the nail of the domestic dog and can be detected at a variable level. In addition, there is a potential relationship between nail cortisol, dog size, and age that suggests nail cortisol testing may be a biologically useful biomarker of chronic stress in

dogs. The foundation has been laid for the further refinement of a technique that may become vital to the veterinary industry, along with providing other significant applications connected to the analysis of chronic stress in vertebrate mammal species.

Acknowledgements

I would like to thank my thesis advisor Dr. Bobby Fokidis for his continued guidance with carrying out this project. I would also like to thank my thesis committee, consisting of Richard Gregor, Kasandra Riley, and Claire Strom for their constructive feedback throughout the process of this thesis. I would like recognize Dr. Jim Martin and his staff for their continued provision of nail samples from Loch Haven Veterinary Hospital. This research was funded through the Edward W. and Stella C. Van Houten Memorial Fund, the Office of the Dean of Arts and Sciences, and funds provided by the Department of Biology.

Literature Cited

- Accorsi P.A., Carloni E., Valsecchi P., Viggiani R., Gamberoni M., Tamanini C., and Seren E. 2008. Cortisol determination in hair and faeces from domestic cats and dogs. *General and Comparative Endocrinology*. 155: 398-402.
- Baxter-Gilbert J.H., Riley J.R., Mastromonaco G.F., Litzgus J.D., Lesbarrères D. 2014. A novel technique to measure chronic levels of corticosterone in turtles living around a major roadway. *Conservation Physiology*. 2(1): 1-9.
- Beato M., and J. Klug. 2000. Steroid hormone receptors: an update. *Human Reproduction Update*. 6(3): 225-236.
- Beerda B., Schilder M., Janssen N., and Mol J.A. 1996. The use of saliva cortisol, urinary cortisol, and catecholamine measurements for a noninvasive assessment of stress responses in dogs. *Hormones and Behavior*. 30: 272-279.
- Bennett A. and Hayssen V. Measuring cortisol in hair and saliva from dogs: coat color and pigment differences. 2010. *Domestic Animal Endocrinology*. 39(3): 171-180.
- Blackshaw J.K. and Blackshaw A.W. 1989. Limitations of salivary and blood cortisol determination in pigs. *Veterinary Research Communications*. 13: 265-271.
- Boonstra R. 2004. Coping with changing northern environments: the role of the stress axis in birds and mammals. *Integrative and Comparative Biology*. 44(2): 95-108.
- Bortolotti G.R., Marchant T.A., Blas J., and German T. 2008. Corticosterone in feathers is a long-term integrated measure of avian stress physiology. *Functional Ecology*. 22: 494-500.
- Brooke A.M. and Monson J.P. 2013. Addison's disease. *Medicine*. 41(9): 522-525.
- Castinetti F., Morange I., Conte-Devoix B., and Brue T. 2012. Cushing's disease. *Orphanet Journal of Rare Diseases*. 7(41): 1-9.
- Charmandari E., Tsigos C., and Chrousos G. 2005. Endocrinology of the stress response. *Annual Reviews Physiology*. 67: 259-284.
- Chrousos G.P., and P.W. Gold. 1992. The concepts of stress and stress system disorders: overview of physical and behavioral homeostasis. *Journal of the American Medical Association*. 267(9): 1244-1252.
- Corradini S., P.A. Boari A., Beghelli V., Mattioli M., Famigli-Bergamini P., and Fracassi F. 2013. Evaluation of hair cortisol in the diagnosis of hypercortisolism in dogs. *Journal of Veterinary Internal Medicine*. 27: 1268-1272.
- de Berker D.A., Andre J., and Baran R. 2007. Nail biology and nail science. *International Journal of Cosmetic Science*. 29(4): 241-275.

- De Martin M., Giraldi F. P., Cavagnini F. 2006. Cushing's disease. *Pituitary*. 9(4): 279-287.
- Engel LL. and L.J. Langer. 1961. Biochemistry of Steroid Hormones. *Annual Review of Biochemistry*. 30: 499-524.
- Evans H.E. and de Lahunta A. *Miller's anatomy of the dog*. St. Louis (MO): Saunders; 2013.
- Fairhurst G.D., Frey M.D., Reichert J.F., Szelest I., Kelly D.M., and Bortolotti G.R. 2011. Does environmental enrichment reduce stress? An integrated measure of corticosterone from feathers provides a novel perspective. *PLoS ONE*. 6(3): 1-10.
- Feldman E.C. and Mack R.E. 1992. Urine cortisol:creatinine ratio as a screening test for hyperadrenocorticism in dogs. *Journal of the American Veterinary Medical Association*. 11: 1637-1641.
- Feldman E.C. and Nelson R.W. 2003. *Canine and Feline Endocrinology and Reproduction*. St. Louis (MO): Saunder; 2003.
- Frank L.A., Henry G.A., Whittemore J.C., Enders B.D., Mawby D.I., and Rohrbach B.W. 2015. Serum cortisol concentrations in dogs with pituitary-dependent hyperadrenocorticism and atypical hyperadrenocorticism. *Journal of Veterinary Internal Medicine*. 29: 193-199.
- Galac S., Reusch C.E., Kooistra H.S., and Rijnberk A. Adrenals. 2010. *Clinical Endocrinology of Dogs and Cats: An Illustrated Text*.
- Gilor C. and Gaves T. K. 2011. Interpretation of laboratory tests for canine cushing's syndrome. *Topics in Companion Animal Science*. 26(2): 98-108.
- Hunt K.E., Stimmelmayer R., George C., Suydam R., Brower Jr. H., Rolland R. M. 2014. Baleen hormones: a novel tool for retrospective assessment of stress and reproduction in bowhead whales (*Balaena mysticetus*). *Conservation Physiology*. 2(1): 1-12.
- Karlén J., Ludvigsson J., Frostell A., Theodorsson E., and Faresjö T. 2011. Cortisol in hair measured in young adults – a biomarker of major life stressors? *BMC Clinical Pathology*. 11(12): 1-6.
- Keckeis K., Lepschy M., Schöpfer H., Moser L., Troxler J., and Palme R. 2012. Hair cortisol: a parameter of chronic stress? Insights from a radiometabolism study in guinea pigs. *Journal of Comparative Physiology*. 182: 985-996.
- Khelil M.B., Tegethoff M., Meinlschmidt G., Jamey C., Ludes B., and Raul J.S. 2011. Simultaneous measurement of endogenous cortisol, cortisone, dehydroepiandrosterone, and dehydroepiandrosterone sulfate in nails by use of UPLC-MS-MS. *Analytical and Bioanalytical Chemistry*. 401: 1153-1162.

- King M.W. 2014. *Integrative Medical Endocrinology: Examination and Board Review*. McGraw-Hill Professional, Bloomington, IL.
- Kobelt A.J., Hemsworth P.H., Barnett J.L., and Butler K.L. 2003. Sources of sampling variation in saliva cortisol in dogs. *Research in Veterinary Science*. 75: 157-161.
- Kool M. M.J., Galac S., Spandauw C.G., Kooistra H.S., and Mol J.A. 2013. Activating mutations of GNAS in canine cortisol-secreting adrenocortical tumors. *Journal of Veterinary Internal Medicine*. 3(27): 1486-1492.
- Kozlov A.I. and Kozlova M.A. 2013. Cortisol as a marker of stress. *Human Physiology*. 40(2): 224-236.
- Linsinger T.P.J. 2008. Use of recovery and bias information in analytical chemistry and estimation of its uncertainty contribution. *Trends in Analytical Chemistry*. 27(10): 916-923.
- Mongillo P., Prana E., Gabai G., Bertotto D., and Mrinelli L. 2013. Effect of age on sex on plasma cortisol and dehydroepiandrosterone concentrations in the dog (canis familiaris). *Research in Veterinary Science*. 96: 33-38.
- Norris D.O. and Carr J. 2013. *Vertebrate Endocrinology*. Academic Press, London, UK.
- Peterson M.E. 2007. Diagnosis of hyperadrenocorticism in dogs. *Clinical Techniques in Small Animal Practice*. 22(1): 2-11.
- Petrie G. 2014. Canine cushing's disease: diagnosis and management. *Veterinary Nursing Journal*. 25(2): 32-34.
- Rijnberk A. and Wees A. 1988. Assessment of two tests for the diagnosis of canine hyperadrenocorticism. *Veterinary Record*. 122(8): 178-180.
- Salata R.A., Jarrett D.B., Verbalis J.G., and Robinson A.G. 1988. Vasopressin stimulation of adrenocorticotropin hormone (ACTH) in humans. In vivo bioassay of corticotropin-releasing factor (CRF) which provides evidence for CRF mediation of the diurnal rhythm of ACTH. *Journal of Clinical Investigation*. 81(3): 66-774.
- Sapolsky R.M., Romero M., and Munck A.U. 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. *Endocrine Reviews*. 21(1): 55-89.
- Smiley L.E. and Peterson M.E. 1993. Evaluation of a urine cortisol:creatinine ratio as a screening test for hyperadrenocorticism in dogs. *Journal of Veterinary Internal Medicine*. 7(3): 163-168
- van Vonderen I.K., Kooistra H.S., and Rijnberk A. 1998. Influence of veterinary care on the urinary corticoid:creatinine Ratio in Dogs. *Journal of Veterinary Internal Medicine*. 12: 431-435.

- Warnock F., Kevin M., Seo R. J., McIsaac S., Seim D., Ramirez-Aponte T., Macritchie K. A., and Young A. H. 2010. Measuring cortisol and DHEA in fingernails: A pilot study. *Neuropsychiatric Disease and Treatment*. 6: 1-7.
- Willeberg P. and Priester W.A. 1982. Epidemiological aspects of clinical hyperadrenocorticism in dogs (canine Cushing's syndrome). *Journal of the American Animal Hospital Association*. 18: 717-724.

Appendix I

VETERINARY INFORMATION RELEASE WAIVER

Client Name: _____

Patient Name: _____

Age: _____ Sex: _____ Spayed/Neutered? _____ Weight: _____ Breed: _____

Dear Participant,

You will be aiding in a study conducted by a senior research student, Zoe Mack, at Rollins College in Winter Park, FL. This study will explore the potential of the canine nail in recording levels of long-term cortisol secretion. Cortisol is a steroid hormone released from the adrenal gland as a result of stress. Current veterinary techniques do not utilize the nail as a measure of cortisol, but rather measure short-term stress through sampling of blood, urine, and saliva. There can, however, be limits in the accuracy to these methods of sampling due to current stress an animal might be experiencing during sample collection. Methods of evaluating cortisol secretion in hair have been developed, which provide a measure of long-term cortisol release, but issues surround the reliability of this method. A measure of cortisol in the nail would introduce a novel technique that would aid the veterinary industry in the diagnosis of a variety of veterinary conditions.

In order to proceed with this project, nail and hair samples are required to be provided by an outside party. The collection of hair provides a basis for comparison through the use of an effective measure of long-term cortisol secretion. Loch Haven Veterinary Hospital will be collecting hair and nail samples from patients that are in grooming for use in the study. Nail clippings will be collected through routine nail trimming, and a very small sample of hair will be collected either from a routine shave scheduled in grooming or from hair removed from brushing the coat (hair length permitting). The option to provide nail clippings and withhold the collection of hair is also an option if needed. No risk is posed towards the animal beyond that of a normal grooming session. Your participation would greatly assist in the development of this research.

I, _____ (please print) permit the use of the above information in a research study conducted by Zoe Mack and Dr. Bobby Fokidis in conjunction with the Department of Biology at Rollins College. I certify that I am the rightful owner of the patient and that information is accurate to the best of my knowledge. I authorize the use of a small sample of nail, collected by Loch Haven Veterinary Hospital, to be used for purposes of the study.

Client Signature_____
Date

In addition to a small sample of nail, I ____DO / ____ DO NOT authorize the use of a small sample of hair, collected by Loch Haven Veterinary Hospital, to be used for purposes of the study.

Client Signature_____
Date

A detailed outline of the research project can be obtained upon request.