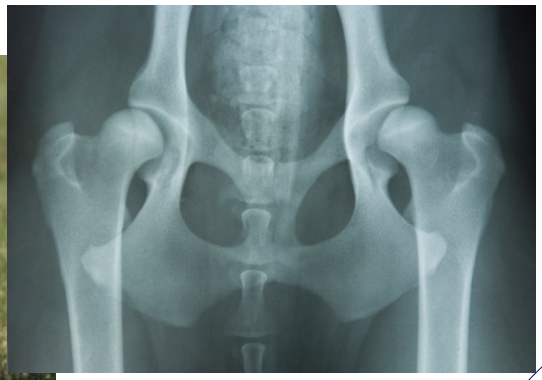
FACULTY OF HEALTH AND MEDICAL SCIENCES
UNIVERSITY OF COPENHAGEN

Master's thesis

Hair and Saliva as Biomarkers for Stress Evaluation in Labrador Retrievers in Relation to HD-scores

Katrine Svendsen (gvq874)

Ann-Sofie Cæcilie Søndergaard (bgp294)



Academic advisors:
Christopher Harold Knight
Department of Veterinary Clinical and Animal Sciences,
University of Copenhagen

Dorte Hald Nielsen
Department of Veterinary Clinical and Animal Sciences,
University of Copenhagen

Submitted: 13th of June 2014

Key words: Cortisol, Canine, Hypothalamic-pituitary-adrenal axis, Saliva, Hair, RIA, Welfare

List of abbreviations

%B/B_0 = The normalized percent bound

ACTH = Adrenocorticotrophic hormone

ANS = Autonomic nervous system

CAR = Cortisol awakening response

CHD = Canine hip dysplasia

CRH = Corticotropin-releasing hormone

DKK = Dansk Kennel Klub

FCI = Fédération Cynylogique Internationale

GS = German Shepherd

HCC = Hair cortisol concentration

HD = Hip dysplasia

HD-score = Hip dysplasia scores

HPA-axis = Hypothalamic-pituitary-adrenal axis

LR = Labrador Retriever

OA = Osteroarthritis

PBS = Phosphatate-buffered saline

RIA = Radioimmunoassay

SCC = Saliva cortisol concentration

Table of content

Abstract

Cortisol measurement is widely used in the monitoring of stress in animals. An increasing attention to animal welfare has led to the development of non-invasive sampling methods suitable for the collection of cortisol. Both hair and saliva are valid non-invasive biomarkers of stress in humans and domestic animals. An acknowledged stressor is the presence of pain. In dogs, hip dysplasia is associated with chronic pain and therefore can be defined as a source of stress resulting in decreased welfare. This study was designed to determine saliva and hair cortisol concentrations of cortisol in Labrador retrievers and to examine possible differences in the hair cortisol concentrations collected from different body regions according to their hip dysplasia scores.

The experiment was performed on forty Labrador retriever dogs divided into 3 groups: Group A: non-clinically disease young dogs (N = 13), Group B: non-clinically diseased older dogs (N = 14), and Group C: older dogs with a unilateral HD score of D or E (N = 13). Hair and saliva concentrations were determined by the RIA method.

None of the factors evaluated had any significant effect on saliva cortisol concentrations and only a slight trend was detected in accordance to place of sampling.
A significant difference in cortisol concentration was found between black and brown hair but the study failed to determine a significant difference in hair cortisol concentrations between the hips of group C, which questions the value of HD-scoring as a valid predictor of the development and severity of hip dysplasia. However, a trend of hair cortisol concentrations rising through group A to C is encouraging and opens up the possibility of further investigation of the value of cortisol measuring in detecting possible subclinical diseases. A combination of cortisol measuring on a repeated basis along with other parameters could be of good value in tracking gradual changes related to slowly progressing diseases within dogs.

Resumé

Måling af cortisol er udbredt i monitoreringen af stress hos dyr. Et øget fokus på dyrevelfærd har ført til udviklingen af non-invasive målingsmetoder i forbindelse med indsamling af cortisol. Både hår og spyt er valide biomarkører for stress hos mennesker og husdyr. En anerkendt stressfaktor er tilstedeværelsen af smerte. Hos hunde er hofteledsdysplasi forbundet med kroniske smerter og kan derfor defineres som en kilde til stress, der kan medføre nedsat velfærd for det pågældende dyr. Dette forskningsstudie blev designet til at undersøge hårkoncentrationer af cortisol i spyt og hår hos Labrador retrievere samt til at undersøge en mulig forskel i cortisolkoncentrationen i hår fra forskellige kropsregioner i forbindelse med dyrenes HD-score.

Eksperimentet blev udført på fyrre Labrador retrievere, der blev inddelt i 3 grupper: Gruppe A: ikke-klinisk syge unge hunde (N = 13), Gruppe B: ikke-klinisk syge ældre hunde (N = 14) og gruppe C: ældre hunde med en unilateral HD-score på D eller E (N = 13). Cortisolkoncentrationen i spyt og hår blev bestemt via en RIA-metode.

Ingen af de undersøgte faktorer havde nogen signifikant indvirkning på cortisolkoncentrationen i spyt, og kun en svag tendens blev fundet i forbindelse med prøvetagningsstedet. En signifikant forskel i cortisolkoncentration blev fundet mellem sort og brunt hår, men studiet fandt ingen signifikant forskel cortisolkoncentrationen i hår mellem hofterne i gruppe C, hvilket stiller spørgsmålstegn ved værdien af HD-scoring som en validforudsigende faktor i forhold til udviklingen samt sværhedsgraden af hofteledsdysplasi. Dog blev der i eksperimentet fundet en tendens i forhold til cortisolkoncentrationen i hår, der steg i værdi fra gruppe A til gruppe C. Denne trend er lovende og åbner op for muligheden for fremtidige studier af værdien af cortisolmåling i forbindelse med fund af mulige subkliniske sygdomme. En kombination af gentagne cortisolmålinger og andre parametre kunne være af god værdi i forbindelse med en løbende evaluering af eventuelle gradvise ændringer i relation til en langsomt udviklende sygdom hos den enkelte hund.

1. Introduction

1.1. Animal welfare

One specific definition of animal welfare does not exist but Sandøe and Christiansen (2008) have reviewed and concluded the existence of three different welfare theories that can form man's assumption on animal welfare; hedonism, preference theory and perfectionism.

According to hedonism, the best life is one in which there are as many positive experiences and as few negative experiences as possible. The more positive experiences compared to negative ones, the better the quality of the life is. The preference theory is based on the necessity of having one's preferences satisfied. Usually a requirement in this welfare theory is that the preferences are pre-considered and well informed. Presumably animals, to a less extent than people, have conscious desires and goals but it is clear that they strive to achieve different objectives and that some desires are more persistent than others (Sandøe *et al*., 2001). From a perfectionist's view a good life equals being able to realize species-specific potentials. According to this theory the key is not to be well but rather to do well, which is based on the assumption that animals have well-defined natures (Sandøe and Christiansen, 2008).

In many contexts the three welfare theories will give the same answer as to what is required for an animal living a good life. Positive experiences will often follow when one achieves what one strives for and moreover manages to live a natural life. Likewise it will lead to a negative experience if one's desires are not met and one doesn't exploit its natural potentials. Choosing one welfare theory as being "the right one" is not possible since all three theories have their own strengths and weaknesses (Sandøe *et al.*, 2001).

Measuring animal welfare

In animal welfare science different parameters for assessing animal welfare exist. In general these parameters can be divided into three main categories; health, behavior and physiology.

The health category is focused on the *absence* of health, rather than health itself. This is based on the assumption that diseases and reduced health in general equals discomfort and suffering of the animal. Behavior is based on observations of the animal's behavior in a natural or semi-natural environment. Knowledge of normal behavior is used to assess welfare by comparing the behavior displayed by animals of the same species in a more restricted environment. The third category,

physiology, indicates whether the animal has a physical or mental burden. Chronic and acute stress falls under this category. Physiological parameters are most often used in experimental research (Sandøe and Christiansen, 2008). One has to bear in mind that obtaining these parameters can be stressful for the animal in itself. A way of minimizing this can be the use of non-invasive collection methods such as monitoring of heart rate (Marques *et al*., 2010) as well as sampling of biological matrixes such as plasma (Beerda *et al*., 1996), feces (Acorsi *et al*., 2008; Bryan *et al*., 2013), urine (Beerda *et al*., 1996), milk (Gygax *et al*., 2006), saliva (Beerda *et al*., 1996; Marques *et al*., 2010) and hair (Bennett and Hayssen, 2010; Bryan *et al*., 2013).

1.2. Stress

No clear definition of stress has yet been established and unlike other diseases no definitive etiology has been defined. Different definitions of stress have been proposed including stress being a biological response elicited when an individual perceives a threat to its normal homeostasis with the threat being defined as the stressor. A stressor can include different events such as illness, infection, and pain (Moberg, 2000; Blackburn-Munro and Blackburn-Munro, 2001). Animal models of chronic pain are associated with an activation of the hypothalamic-pituitary-adrenal (HPA) axis, where chronic pain acts as the stressor. Prolonged periods of stress may affect the HPA axis to such an extent, that the negative feedback mechanism is disrupted making the HPA axis become maladaptive (Blackburn-Munro and Blackburn-Munro, 2001). The International Association for the Study of Pain defines pain as “an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage” (Merskey and Bogduk, 1994). The primary function of pain is to protect the individual from a potentially damaging stimulus to tissues in the organism. Longer-lasting pain, which follows damage to tissue, is usually correlated with hyperalgesia and allodynia. The longer-lasting pain functions to protect the injury from any further trauma. Chronic pain, which exists beyond its biological potential to protect the organism, is believed to compromise the quality of life for the individual (Blackburn-Munro and Blackburn-Munro, 2001). Various sources of stress have now been recognized including: physiological stress, psychological stress, activity or exercise induced stress, environmental stress, stress arising from pathological conditions, and acute vs. chronic stress (Cook, 2012).

The physiology of stress

The main components of the stress system include the autonomic nervous system (ANS) and the HPA axis (Marques *et al*., 2010). The ANS is part of the nervous system that is not under conscious control. It regulates different subconscious body functions such as blood pressure, heart rate, and intestinal mobility and is essential for homeostasis. The ANS is divided into the sympathetic nervous system and the parasympathetic nervous system that act to maintain a homeostatic balance and these are activated by the hypothalamus (Seaward, 2005; Cunningham and Klein, 2007). The sympathetic nervous system is responsible for the fight-or-flight response through the release of epinephrine (adrenaline) and norepinephrine (noradrenaline). The parasympathetic nervous system is responsible for the rest-and-digest response through the release of acetylcholine. The body has different backup mechanisms to ensure physical survival. These systems are classified as: 1) immediate effects (the release of epinephrine and norepinephrine from the sympathetic nervous system), 2) intermediate effects (release of epinephrine and norepinephrine from the adrenal medulla) and 3) prolonged effects (neuroendocrine pathway involving the HPA axis) (Seaward, 2005).

HPA-axis

The HPA axis begins with the release of corticotropin-releasing hormone (CRH) from the anterior hypothalamus. The CRH then activates the pituitary gland to release adrenocorticotrophic hormone (ACTH), which travels via the bloodstream to the adrenal cortex, which it then activates to release a set of corticosteroids: the major glucocorticoid, cortisol and the major mineralocorticoid, aldosterone (Seaward, 2005; Levine *et al*., 2007). Increased secretion of cortisol in to the blood acts primarily to ensure an adequate supply of blood sugar for energy metabolism. However, when increasingly high levels of cortisol are observed due to chronic stress, this hormone compromises the integrity of several physiological systems such as the cardiovascular, endocrine and gastro-intestinal system (Beerda *et al.*, 1997; Seaward, 2005).

A negative-feedback system exists, whereby glucocorticoids inhibit the release of CRH. This in turn results in a decreased secretion of corticotropin by the pituitary gland. Stress is a factor that can modify the negative-feedback control of glucocorticoids. The glucocorticoid response to stress is immediate, making the concentrations of cortisol increase rapidly to reach values that are several-fold higher than normal values, within minutes. The response is proportional to the severity of the

stress, lower levels of stress result in less cortisol production than do higher levels of stress (Cunningham and Klein, 2007).

The negative-feedback control system does not result in the maintenance of a uniform hormone concentration in blood throughout the day. The HPA axis activity follows a diurnal rhythm with several secretory episodes of short duration and high amplitude. Normally the highest production of cortisol occurs in the second half of the night with peak cortisol levels in the early morning hours. Throughout the day the cortisol levels decline with lowest levels during the first half of the night (Kirschbaum and Hellhammer, 1989; Tsigos and Chrousos, 2002; Fries and Remedios, 2009).

A study by Smyth *et al.* (1997) examining the diurnal cycle of cortisol in humans found that there were individual differences in diurnal cortisol cycles with 17% showing no fluctuations in their diurnal cycles across 2 days, 30% showing variation in their diurnal cycle from day to day, and 51% whose diurnal cycle were considered normal across 2 days. Furthermore, there is an increase in cortisol concentrations within 20-30 minutes after awakening in the morning. This phenomenon is known as the cortisol awakening response (CAR) (Fries and Remedios, 2009).

1.3. Biomarkers for measuring stress

The measurement of cortisol is commonly used as a biomarker of an animal's stress response (Cook, 2012; Bryan *et al.,* 2013). A variety of biological matrixes can be used in measuring cortisol concentrations such as plasma, saliva, feces, milk, urine and hair. Traditionally, plasma cortisol is accepted as the "gold standard" for the assessment of stress responses (Cook 2012) even though the act of obtaining blood samples can be a severe stressor to the animal as it often includes restraint and venipuncture (Davenport *et al.,* 2006; Cook 2012). Concerns about animal welfare have led to the development of non-invasive sampling methods suitable for the collection of cortisol (Vincent and Michell 1992; Beerda *et al.,* 1996; Coppola *et al.,* 2006; Bennett and Hayssen 2010). The main focus in this study is to review saliva and hair as biological biomarkers of canine stress and welfare.

Saliva

Sampling of saliva has the advantage of being easy to collect by using non-invasive, stress free techniques (Riad-famy *et al.,* 1982) and moreover being a valid alternative to plasma for the assessment of HPA axis activity following acute stress (Beerda *et al.,* 1996; Marques *et al.,* 2010).

Saliva is predominantly produced in specialized secretory end pieces also known as acini. These end pieces make up the largest part of the salivary glands. Cortisol concentrations in saliva are essentially the result of passive diffusion of free cortisol across the acinar cells of the lumen of the salivary gland (Vinning and McGinley, 1987). Salivary cortisol concentrations (SCC) have previously shown to be highly correlated with plasma concentrations in dogs (Vincent and Michell, 1992; Beerda *et al.,* 1996). SCC is often reported to be approximately 10% of plasma concentrations (Vincent and Michell, 1992: Cook 2012), which represent the free non-protein bound fraction in plasma (Riad-famy *et al.,* 1982; Vincent and Michell, 1992; Vining and McGinley, 1987; Kirschbaum and Hellhammer, 1989; Kobelt *et al.,* 2003).

Measurement of cortisol in saliva gives an instantaneous view of the adrenocortical activity at the time the sample was collected, reflecting the acutely circulating cortisol levels (Bennett and Hayssen, 2010; Cook, 2012; Stalder and Kirschbaum, 2012). In comparison urine represents a short time frame ranging from < 1 hour to 24 hours (Davenport *et al.,* 2006; Stalder et al., 2012), feces represents a period of multiple hours (Palme, 2005), whereas measurement of hair represents a period of weeks to months (Koren *et al.,* 2002; Ashley *et al.,* 2011; Comin *et al.,* 2012; Cook, 2012; Bryan *et al.,* 2013).

Hair

The hair follicle is located just beneath the skin surface and is in close relation to arterial capillaries and two different types of glands; the sebaceous glands and the apocrine sweat glands (Harkey, 1993; Cone, 1996). These glands secrete sebum and sweat, which they empty into the hair follicle (Cone, 1996). In dogs, hair tends to grow in a cyclical manner. The hair growths until it reaches its predetermined length, which varies according to the different body regions and is genetically determined. This phase is known as the anagen phase and is followed by the catagen phase and ultimately the telogen phase, known as the resting phase (Gunaratnam and Wilkinson, 1983; Wennig, 2000). The mechanism by which cortisol enters the hair is not entirely clear but three different ideas of incorporation routes exist: 1) free cortisol entering the hair follicle through

passive diffusion from the blood capillaries during the anagen phase of hair growth (Harkey 1993; Comin *et al.,* 2011; Cook, 2012; Stalder *et al.,* 2012). 2) Contamination of the hair shaft by endogenous cortisol from sweat and sebaceous gland secretions or by exogenous sources of cortisol such as corticosteroid medication (Cook, 2012; Stalder *et al.,* 2012). 3) An endogenous source of cortisol in the hair follicle via a functional CRH-ACTH-cortisol axis (Ito *et al.,* 2005; Slominski *et al.,* 2007; Cook, 2012; Keckeis *et al.,* 2012).

Local vs. systemic cortisol

It is known that human skin expresses elements of the HPA axis including hormones such as CRH and ACTH, and key enzymes of the synthesis of corticosteroid and synthesizes glucocorticoids (Ito *et al.,* 2005; Slominski *et al.,* 2007). Therefore, skin may have developed a local stress response system resembling the central HPA axis. The most pronounced endocrine and neuroendocrine activity in human skin appears to be located in the skin's major appendages, also known as the pilosebaceous units. These units consist of the hair follicles and the sebaceous glands. The entire pilosebaceous unit has been characterized as a source and target of key elements of the HPA axis (Ito *et al.,* 2005; Arck *et al.,* 2006; Paus *et al.,* 2006). A study by Ito *et al.* (2005) supported the theory of a peripheral equivalent to the HPA axis in human hair follicles and that CRH and ACTH affect the intrafollicular cortisol synthesis and secretion. These findings suggest that human hair follicles do synthesize cortisol even long after having been severed from their neural and vascular connections to systemic endocrine signals. A recent study on hair cortisol synthesis in guinea pigs by Keckeis *et al.* (2012) indicates that measured cortisol levels may not necessarily reflect adrenal cortisol synthesis and secretion, but rather a local synthesis in the hair follicle.

To our knowledge there are no existing or published data concerning local production of cortisol in dogs; therefore it remains unknown whether the cortisol concentration in hair reflects that in the peripheral circulation and/or local production.

In the present study to further investigate the possibility of a local cortisol production and secretion, canine hip dysplasia (CHD) was chosen as the stressor.

1.4. Canine hip dysplasia

CHD is a developmental orthopedic disorder that is characterized by the formation of a loose, ill-fitting coxofemoral joint (Brass, 1989). CHD is a known predisposing cause of osteoarthritis (OA) as a consequence of the malformation of the coxofemoral joint (Wood and Lakhani, 2003; Hou *et al.,* 2010; Lewis, 2010). Primary or idiopathic OA is rare in dogs and will occur without any prior trauma or disease. Secondary OA is much more common and can be caused by trauma of the joint, inflammation or congenital and developmental abnormalities (i.e. hip dysplasia) (McLaughlin, 2000). Secondary OA due to some type of trauma is usually either caused by an abnormal force on a normal joint or a normal force on an abnormal joint. OA occurs when there is disruption of the normal cartilage structure and homeostasis. The cartilage is composed of chrondrocytes and extracellular matrix. The chondrocytes are metabolically active cells, which produce and maintain the extracellular matrix. The extracellular matrix is primarily composed of collagen, proteoglycans, and water and it is the extracellular matrix that distributes force over the underlying subchondral bone and provides a smooth and almost frictionless surface that allows the joint to move When the normal distribution of these components is disrupted, the function of the articular cartilage is altered, leading to the changes associated with OA. During the process of OA, the joint capsule becomes thickened and vascularity increases. The joint capsule undergoes fibrosis and thickening resulting in a decreased range of motion, stiffness and pain (Johnston, 1997; Renberg, 2005).

In Denmark certain HD-requirements exist for Labrador Retrievers: Offspring can only be registered within a breed club if both parent dogs has an official HD status registered in Dansk Kennel Klub. The minimum age for radiographic screening is 12 month for Labrador Retrievers (DKK, n.d.). Denmark is part of the organization Fédération Cynologique Internationale (FCI) and the classification of hip status in dogs is based on the FCI protocol. The FCI protocol includes a five class grading system (FCI, 2006; Kronveit et al., 2010).

Table 1.1 HD-scores (Kronveit *et al.,* 2010)

HD-score	**A**	**B**	**C**	**D**	**E**
Meaning	Excellent	Normal	Mild dysplasia	Moderate dysplasia	Severe dysplasia

The hip score is obtained at an early age and is designed to provide an assessment of the radiographic condition of the hip joints. The score is likely positively correlated with the probability of occurrence and the severity of hip dysplasia (Lust, 1997).

1.5. Hair color, hair wash, growth rate and body site

Multiple factors can influence the measurement of HCC. In two animal studies the relationship between HCC and hair color has been investigated. Bennett and Hayssen (2010) found differences in cortisol concentration; across all subjects in their study, black dogs had a lower concentration of cortisol than the non-black dogs. Furthermore they found a difference in the individual dog, black (eumelanin) hair had less cortisol than yellow (phenomelanin) hair. In contrast, Macbeth *et al.* (2010) found no significant difference in HCC in grizzly bears among the different color categories (ranging from nearly white to black) across all subjects. However, a tendency of a higher HCC in dark hair than in light hair was found within several of the individual grizzly bears.

Another factor worth mentioning is washing of the hair, which has been shown to reduce the HCC in rhesus monkey hair. The washing effect was primarily due to the exposure of water rather than shampoo (Hamel *et al.,* 2011). In the present study, the frequency of showers both with and without shampoo was registered for each dog.

Knowledge of the rate of hair growth is another important factor in retrospective studies in order to identify the specific time frame represented by the hair sample. To our knowledge no study on growth rate has been conducted on hair from dogs but a mean of approximately 1 cm/month is generally accepted in humans (Wennig, 2000). Because of this it is not possible to predict which specific time the dog hair represents. One way to overcome this is to use a shave/re-shave method, such as the ones used in Accorsi *et al. (*2008), Comin *et al. (*2011) and Bryan *et al. (*2013) measuring the HCC in the new grown hair.

The localization of the hair is also something to consider, since rate of growth may vary among different body sites. In a study by Bryan *et al.* (2012) measuring HCC in healthy dogs, no difference was found between hair collected from the right and left side of each dog. This is consistent with Comin *et al.* (2012) who found no significant difference in cortisol concentration in hair samples collected from different body sites within individual New Zealand white rabbits. In contrast Macbeth *et al.* (2010) found that neck hair had higher cortisol levels than hair from the shoulder, rump and abdomen in free-ranging grizzly bears.

2. Materials and methods

The research protocol was approved by The Danish Ethical Administrative Committee.

2.1. Dogs

This paper is based on data from Labrador Retrievers, whose owners volunteered them for the research. Forty dogs, 15 males and 25 females, aged 1-14 years were selected and divided into 3 groups (see table 2.1.)

Table 2.1 Grouping of dogs.

Group	Number of dogs	Criteria
A	13	Screened for Hip Dysplasia at the age of 12 months.
B	14	> 5 years of age with an HD score of A or B (score obtained at the age of 12 months).
C	13	> 5 years of age with a unilateral HD score of D or E (score obtained at the age of 12 months).

Group A

Group A is a control group based on non-clinically diseased young dogs. The dogs in this group were sampled at a veterinary clinic prior to radiographic screening for HD.

Group B

The B group is a control group based on non-clinically diseased older dogs. All dogs but one were sampled in their homes, and no changes to their normal routine were required. All dogs were recruited through breeders of Labrador Retrievers.

Group C

The C group is the research group based on older dogs with a unilateral HD score of D or E and A, B, or C on the contralateral hip. All dogs in this group were sampled in their homes, and no changes to their normal routine were required. All dogs were recruited through Dansk Kennel Klub's database.

2.2. Hair and saliva sampling

All sampling occurred between February and April 2014. A questionnaire was used to gather information about each dog, see appendix A.

A patch of hair (2 x 2 cm) was collected from the ischiatic region on both the left and right side, and from the front side of the neck of each dog. Electrical clippers were used shaving close to the skin. Shaving was chosen over plucking as suggested by Gow *et al.* (2010) since the plucking method will include the hair follicle possibly distorting the cortisol concentration in the hair shaft.

Saliva samples were collected on the same day as the hair samples. The owners were informed not to feed their dog at least 2 hours prior to the sampling since Dreschel and Granger (2009) found that food particles could contain products, which interfere with cortisol measurement. Two saliva samples were collected from each dog. While gently restraining the dog, a sterile sponge (Salimetrics, State College, PA, USA) was placed in the dog's mouth. After swabbing the sponge inside the dog's cheeks and mouth it was removed and the entire procedure was completed within 4 minutes. According to Kobelt *et al.* (2003) this brief restraint likely would not have affected the salivary cortisol measurement. None of the dogs had any previous experience with saliva sampling.

2.3. Saliva cortisol assay

All samples were kept cool using an insulated container with icepacks after sampling and then frozen at -18°C. The samples were brought to room temperature and then centrifuged for 15 min. at approximately 3.000 RPM (1500 x g). Saliva cortisol was determined using a radioimmunoassay (RIA) method. A 96-well microtitre plate (Optiplate, Perkin-Elmer Life Science, Boston, MA, USA) was coated with anti-rabbit γ-globulin serum raised in a goat, diluted 1:1000 in 0.15 mM sodium acetate buffer, pH 9, and incubated overnight at 4°C. The plate was washed 2 times with RIA-buffer, pH 7.4 and incubated overnight at 4°C with 200 µL of the anti-cortisol serum diluted 1:12000. The rabbit anti-cortisol antibody used was obtained from Biogenesis (Poole, UK). After washing the plate with RIA buffer, standards, a quality control extract, the test extracts and tracer were added and the plates were incubated overnight at 4°C, see appendix B.

Bound hormone was separated from free hormone by decanting and washing the wells in RIA-buffer. After the addition of 200 µL scintillation cocktail, the plates were counted on a beta-

counter (Top-count, Perkin-Elmer Life Sciences, Boston, MA, USA). Some samples could not be successfully assayed, due to limited sample volumes (< 2 x 30 µL) (Comin *et al.,* 2011).

2.4. Hair cortisol assay

The hair samples were stored in paper envelopes at room temperature until analysis. From each hair sample approximately 70 mg of hair strands were put in a dry glass vial and washed 2 times for 3 minutes in 2mL of isopropanol and dried until cortisol extraction of the hair. 2 mL of methanol were added, and vials were incubated at 37°C with gentle shaking for 16 h. The vial content was then centrifuged (15 minutes / 1000 x g) and evaporated to dryness under an air-stream suction hood at 37°C. Dry residue was dissolved into 0.6 mL of phosphatate-buffered saline (PBS) 0.05 M, pH 7.5 (0,1% bovine serum albumin). Hair cortisol concentrations were determined using the same RIA-method described for the saliva cortisol assay. Some samples could not be successfully assayed, due to limited sample volumes.
Intra-assay and inter-assay coefficients of variations were 5.54% and 9.71% respectively.

After counting of the microtitre plates, the concentration of analyte in each of the hair and saliva samples was determined by interpolation from a standard curve, created by the following steps.
The count for each sample was averaged as the mean and the average NET count for all standards and samples were calculated by subtracting from each the average non-specific binding counts. Next the normalized percent bound (%B/B_0) for each standard and samples was determined from the following equation:

$$\%B/B_0 = (\text{NET cpm of standard or samples/NET cpm of "0" standard}) \times 100$$

The %B/B_0 for each standard was plotted versus the corresponding of unlabeled ligand calibrator added in picograms. The amount of analyte in each sample could then be determined by interpolation from the standard curve (Perkinelmer, n.d.). All samples with concentrations above the range of the standard curve were diluted with assay buffer and re-assayed.

2.5. Statistical analysis

All statistical tests were performed using the program SPSS for MAC, version 19, Inc. 1989-2010. The animals were used as the experimental unit and the level of significance was set at $P < 0.05$.

The data came from two sources: owner-derived data (e.g. age, sex, neuter status) and experimenter-measured data (cortisol concentrations in hair and saliva).

The normality of data distribution was tested using the Shapiro-Wilk test. When data was not normally distributed it was logarithmically transformed to safety assumption of normal distribution and homogeneity of variance. Unless otherwise indicated, results are presented as means ± standard error (SE).

A one-way ANOVA was performed to compare the mean values of HCC for the three groups (A, B, and C) and to investigate the relationship between HCC and coat color (black, yellow, brown). A one-way ANOVA was also used to compare the SCC for group A, B, and C. When a statistically significant difference was found, a post-hoc test (Scheffe) was run to see between which of the three groups the difference existed.

The relationship between HCC and bad versus good hip scores within the individual dog was tested using a paired t-test and a difference score was calculated for each dog.

Student's t-test was used for pairwise comparisons of categorical variables (sex, neuter status, pain symptoms, place of sampling, effect of hair wash) with hair or salivary cortisol.

A Pearson's correlation test was used to test the association of cortisol concentrations and age for both saliva and hair, and saliva and hair cortisol in order to test for correlation between the two.

3. Results

Overall, cortisol concentrations were variable among dogs and more variable in hair than in saliva. Mean cortisol content in 115 hair samples was 3.225 ± 0.243 pg/mg where as mean cortisol content determined in 36 saliva samples was 0.738 ± 0.045 ng/ml. All relevant statistical SPSS results are shown in appendix C.

3.1. Hair cortisol

No statistically significant difference was found between group A, B, and C's means for concentration of cortisol (p = 0.132). The mean concentrations of cortisol for the three groups were as follows: Group A (n=13): 0.385 ± 0.024 pg/mg, Group B (n=14): 0.438 ± 0.031 pg/mg and Group C (n=13): 0.488 ± 0.048 pg/mg. There seems, however, to be a tendency of cortisol rising from group A to group C (Fig. 1).

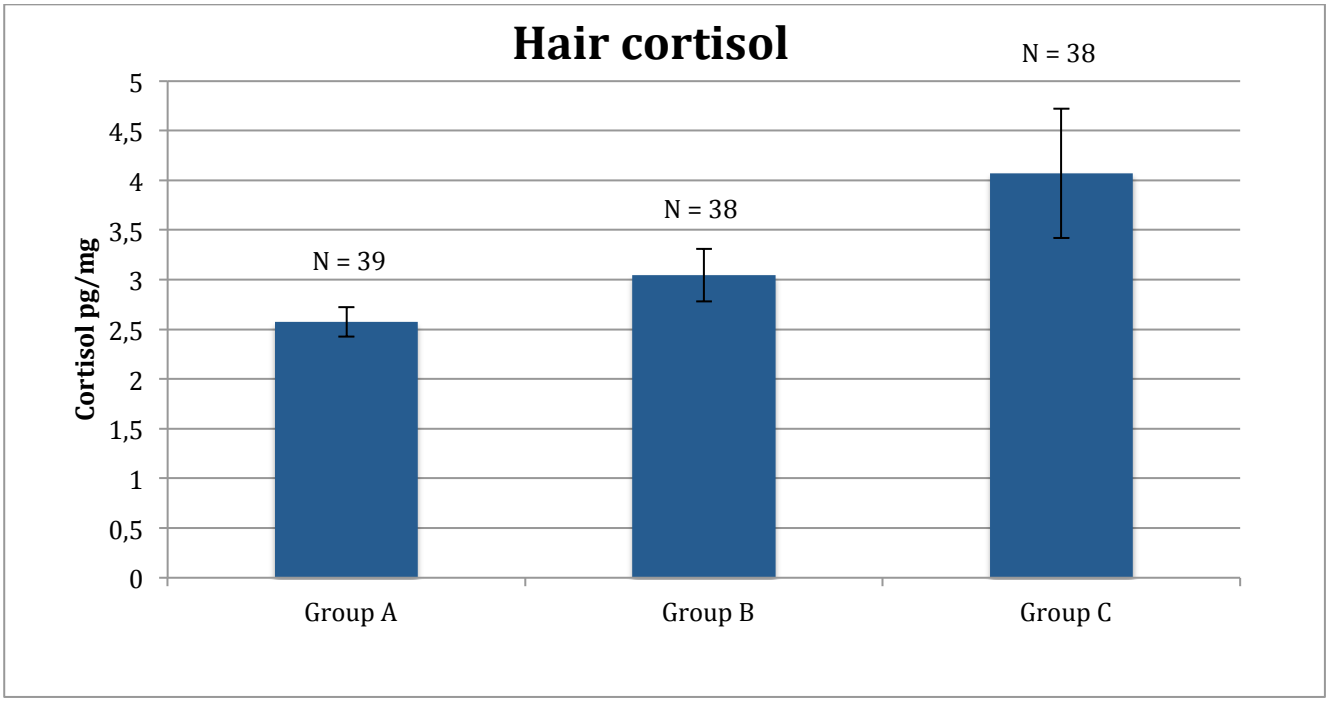


Figure 1 Mean ± SE for each group. Shown with original data. Analysis was performed on log-transformed data.

A statistically significant difference between coat color and cortisol was found (p = 0.010). The following post-hoc test revealed that the difference existed between brown hair and black hair (p = 0.022) (Fig. 2).

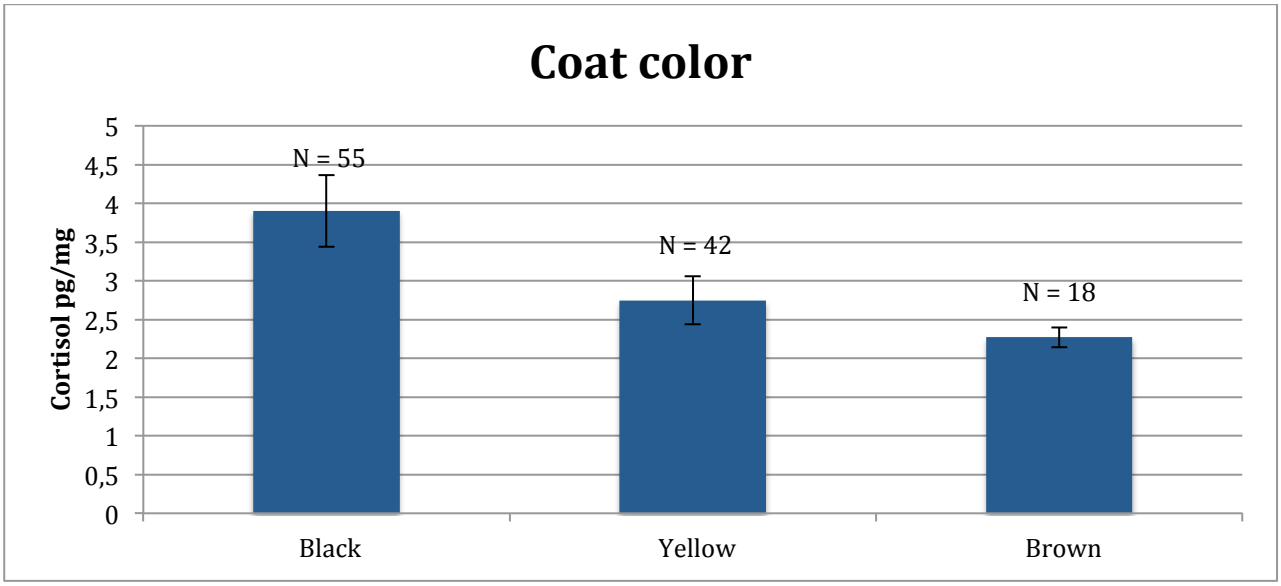


Figure 2 Mean ± SE for each group. Shown with original data. Analysis was performed on log-transformed data.

There was no effect of hair wash on HCC: Group A (p = 0.127), Group B (p = 0.426) and Group C (p = 0.872) (Fig. 3).

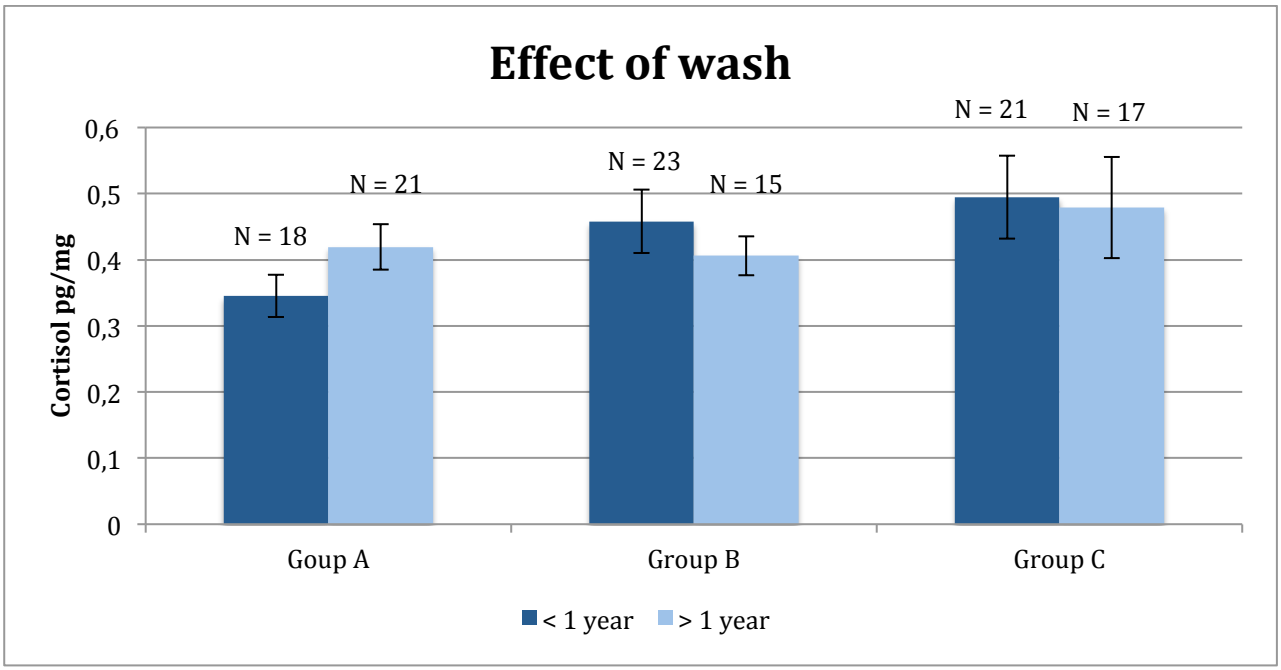


Figure 3. Mean ± SE for each group. Shown and analyzed with log-transformed data.

The dogs in group C showed a slight difference in mean values for cortisol concentration: Good hip scores (N = 13): 0.5008 ± 0.0902 and bad hip scores (N = 13): 0.5127 ± 0.0952. This difference, however, was not statistically significant (p = 0.751). Difference scores for HCC in mean values for the bad hip versus the good hip were non consistent. If the hypothesis was correct, then the average difference score for all the individuals should be bigger than zero (Fig. 4 and 5).

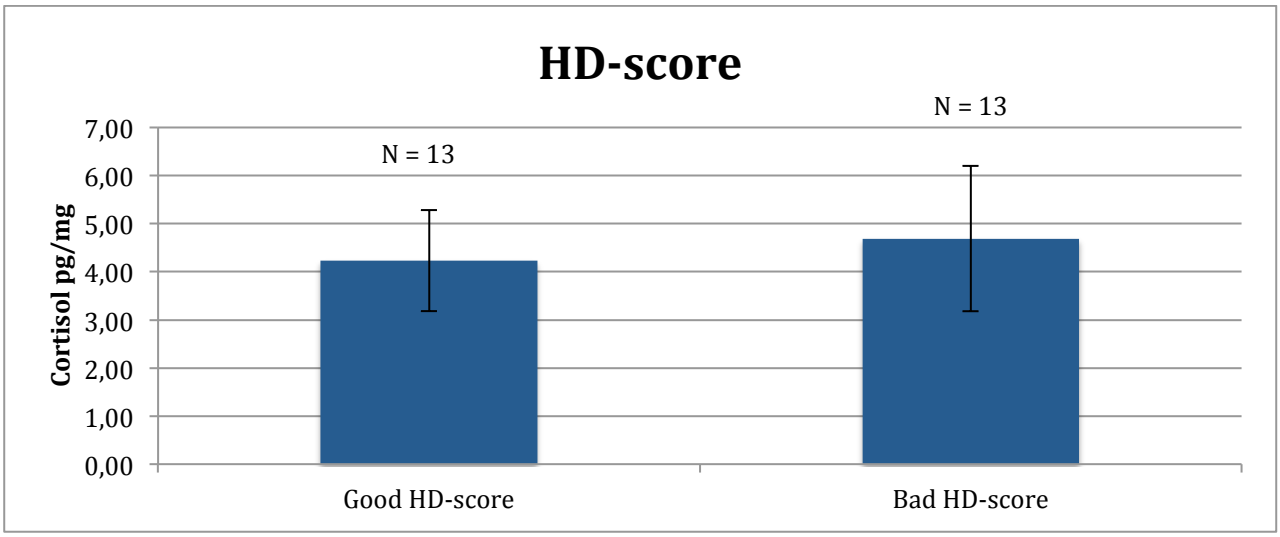


Figure 4 Mean ± SE for each group. Shown with original data. Analysis was performed on log-transformed data.

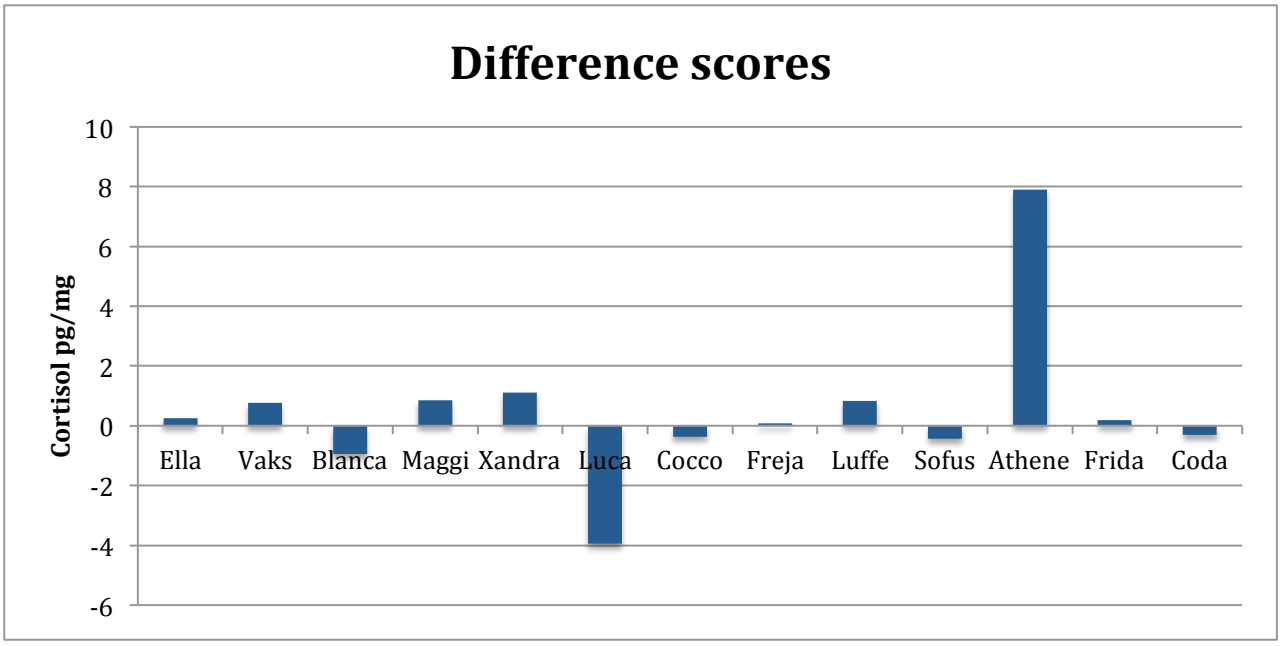


Figure 5 Calculated difference scores for dogs in group C. Shown with original data.

There was no statistically significant correlation between age and HCC (p = 0.179), between sex and HCC (p = 0.769), between neuter status and HCC (p = 0.664) and between dogs with observed pain (N = 10) and dogs with no observed pain (N = 68) (p = 0.667).

3.2. Saliva cortisol

Comparison of the SCC between the three groups showed no statistical significance (p = 0.689). The mean concentrations were as follows: Group A (N = 13): -0.129 ± 0.044 ng/mL, group B (N = 12): -0.170 ± 0.045 ng/mL, and group C (N = 11): -0.176 ± 0.041 ng/mL (Fig.6).

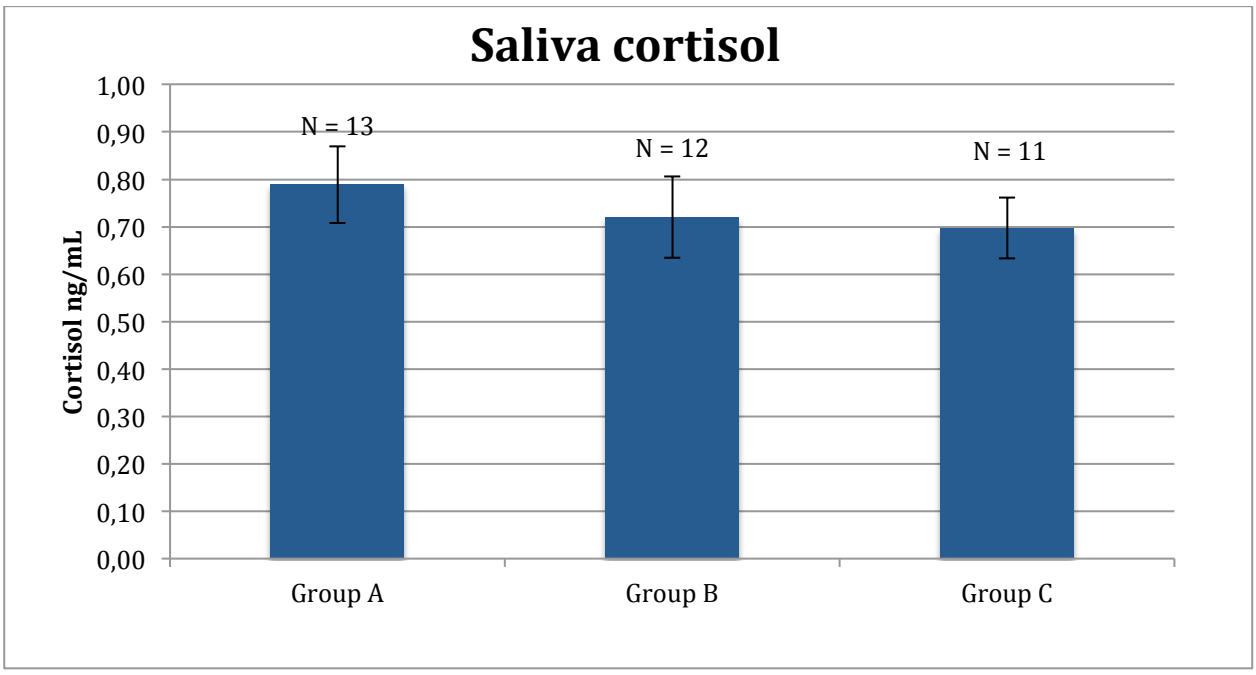


Figure 6 Mean ± SE for each group. Shown with original data. Analysis was performed on log-transformed data.

No statistically significant correlation was found between SCC and age (p = 0.632), sex (p = 0.610), time of sampling (p = 0.085) or place of sampling (p = 0.409) (Fig. 7).

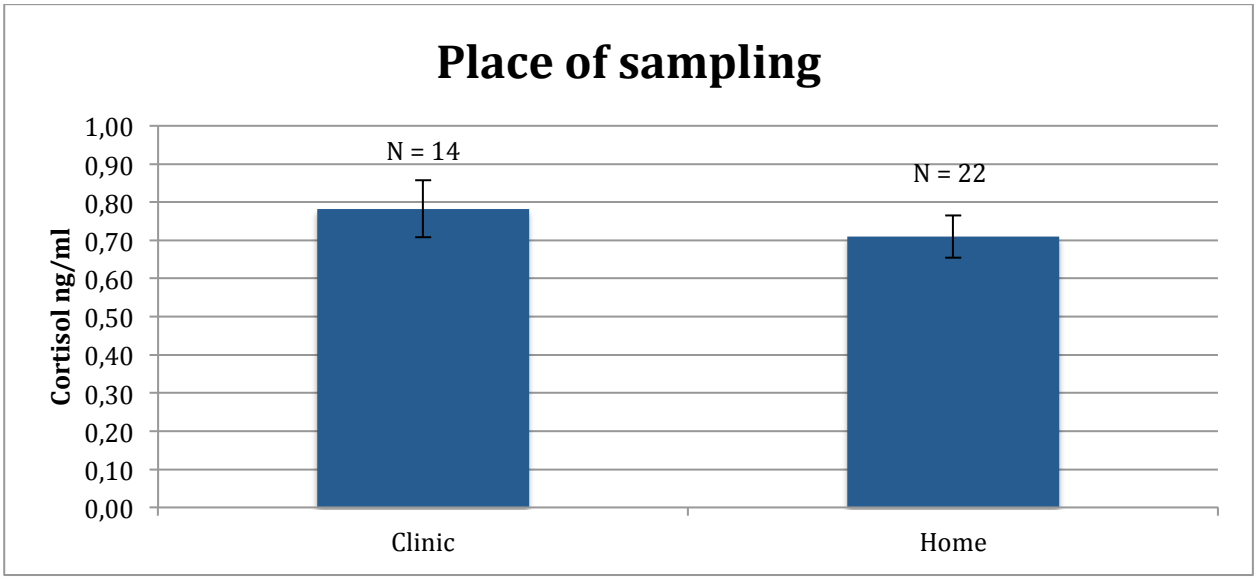


Figure 7 Mean ± SE for each group. Shown with original data. Analysis was performed on log-transformed data.

3.3. Hair and saliva cortisol

Pearson's correlation test was performed to test the correlation between cortisol concentration in saliva and neck hair (N = 40, r = 0.054, p > 0.01), saliva and mean hip hair (N = 40, r = -0.306, p > 0.01) and mean hip hair and neck hair (N = 40, r = 0,129, p > 0.01). No correlation was found in any of the above-mentioned combinations but a comparison of hair samples from the left and right hip revealed a significant correlation (N = 39, r = 0.930, p < 0.01) (Fig. 8 and 9, see appendix B).

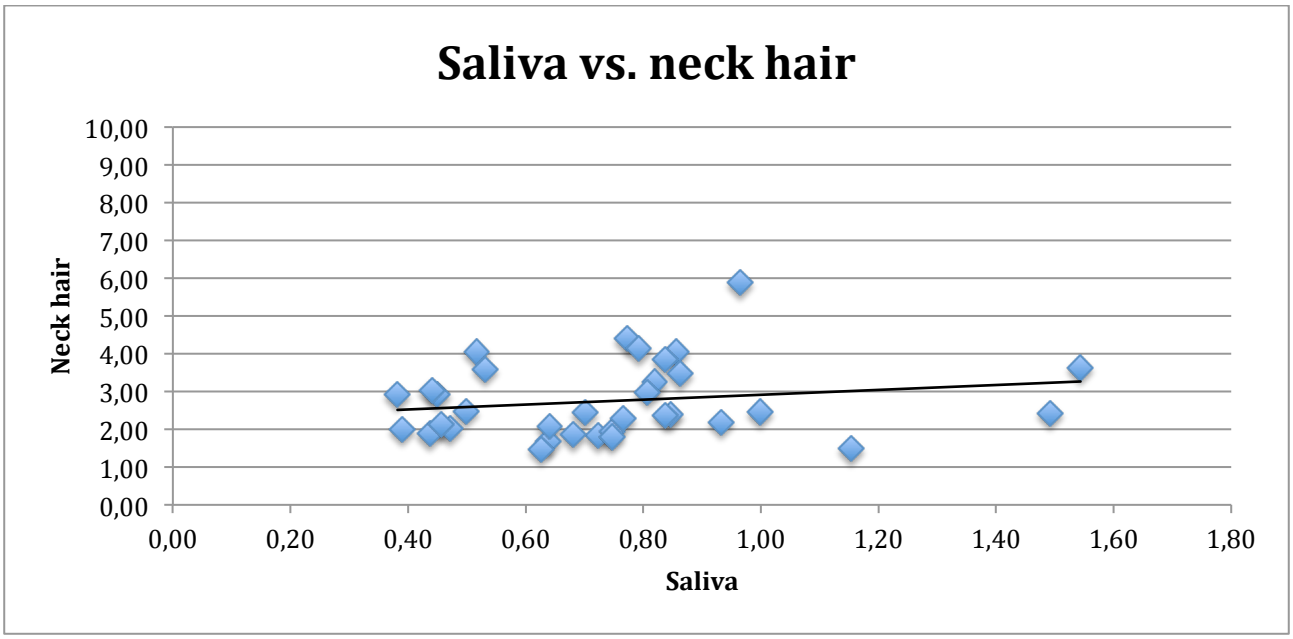


Figure 8 Linear model of correlation between saliva and neck hair cortisol concentration. Shown with original data. Analysis was performed on log-transformed data.

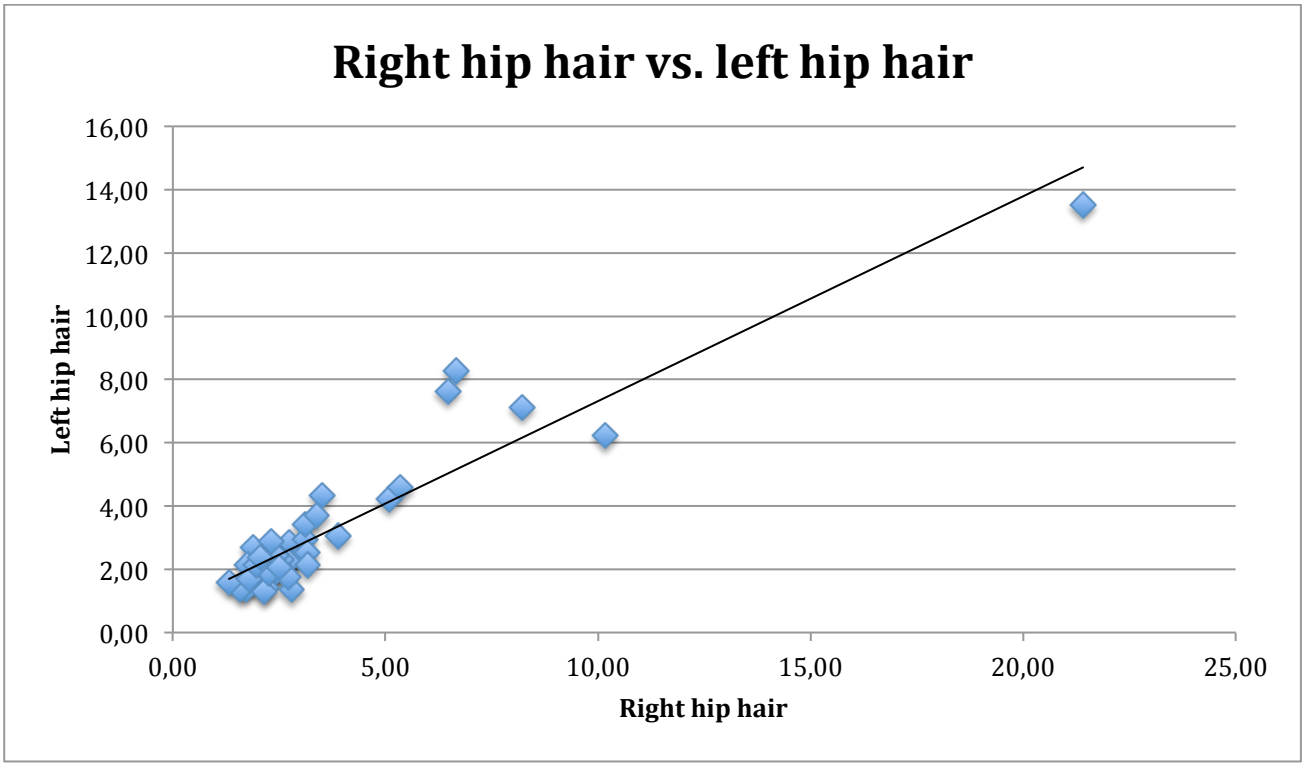


Figure 9 Linear model of correlation between right hip hair and left hip hair cortisol concentration. Shown with original data. Analysis was performed on log-transformed data.

4. Discussion

4.1. HD-scores between and within groups

No difference in the means for cortisol concentrations between group A, B, and C was found. However, when looking at fig. 1 there seems to be a trend of cortisol rising from group A to group C. The mean of HCC in the young, healthy dogs (group A) was lower than the mean HCC for the older, healthy dogs (group B), which again was lower than the mean HCC for the older dogs with a bad HD-score (group C). This trend between the three groups did not exist with SCC; in this case it was rather a slightly opposite trend declining from group A to group C (Fig. 6).

A possible explanation for the trend observed in HCC could be that cortisol levels increase with age but a correlation between HCC and age was not found in the present study, which is consistent with the finding in studies on grizzly bears (Macbeth *et al.,* 2010), polar bears (Bechshøft *et al.,* 2011) and humans (Kirschbaum *et al.,* 2009). This, however, would not explain the rise from group B to C, which instead could be indicative of a subclinical problem in the dogs

from group C. Based on this finding the next logical step, would be to test HCC in healthy dogs versus diseased dogs of same age with clinical symptoms to see, whether a similar trend would appear.

To further assess the effect of a bad hip-score, analysis within the individual dog was performed to see if hair from a hip with a bad HD-score would have a higher cortisol concentration than hair from a hip with a good HD-score. No statistically significant difference was found between the left and right hip. This raises speculations as to whether bad HD-scores have an influence on the local HCC and questions the value of HD-scoring as a valid predictor of the development and severity of hip dysplasia.

4.2. Age, sex, and neuter status

No correlation was found between age and cortisol concentration in neither hair nor saliva. This is consistent with the findings of Bennett and Hayssen (2010) who found no significant relationship between age and cortisol concentrations in dogs in both saliva and hair. Coppola *et al.* (2006) also reported no effect of age on cortisol concentrations in canine saliva. Studies in other animals (Macbeth *et al.,* 2010; Bechshøft *et al.,* 2011) and humans (Kirschbaum *et al.,* 2009) support this.

No statistically significant difference between sex, neuter status, and cortisol concentrations in neither hair nor saliva was found in the present study. This is consistent with the findings of Bennett and Hayssen (2010). Macbeth *et al.* (2010), Webb *et al.* (2010) and Ashley *et al.* (2011) who also found no significant difference between sex and HCC in grizzly bears, humans, caribou and reindeer respectively. A study on shelter dogs by Coppola *et al.* (2006) also reported no effect of sex on cortisol concentrations in canine saliva. In polar bears Beschøft *et al.* (2011) reported that females had significantly higher cortisol concentration than males, which is in contrast to a study by Tryland *et al.* (2002) who found no difference in HCC in relation to sex in polar bears.

4.3. Place of sampling and diurnal variation

Another objective in the present study was to see, whether place of sampling had an effect on SCC. The dogs were either sampled at a veterinary clinic or at home. Being at the veterinary clinic, which is an unfamiliar environment with multiple dogs present, could have an effect on the dog's

stress level. This effect could possibly be detected in the SCC. The effect of a veterinary waiting-room environment on canine serum cortisol was studied by Perego *et al.* (2013). The study concluded that the indoor waiting room appeared to be stressful for the dogs. This conclusion is supported by van Vonderen *et al.* (1998), showing that a visit to a veterinary practice could be considered a stressful condition for dogs but that a large variation in stress responses was observed. In the present study, place of sampling showed no significant effect on SCC. However, a slight increase in SCC was detected as shown in fig. 7. An explanation to why the effect wasn't higher could be that saliva sampling was done within 4 minutes of arriving to the clinic for most of the dogs. As described by Kobelt *et al.* (2003), up to 4 minutes can be taken to collect a saliva sample without the sampling itself having any influence on the SCC.

In studies investigating the possible effect of the diurnal rhythm on cortisol concentrations, samples are often taken at intervals throughout the day for each subject being studied (Kirschbaum and Hellhammer, 1989; Sharpley *et al.,* 2010; Esposito *et al.,* 2012). In the present study only point-samples were obtained. No statistically significant difference was found between time of sampling and cortisol concentration. This could be explained by the findings of Kirschbaum and Hellhammer (1989) recommending the use of certain time intervals. Within these periods relatively small changes in cortisol values were observed. Most of the sampling in the present study occurred within these time intervals. As previously mentioned there exists a cortisol awakening response (CAR) leading to a sudden rise in cortisol concentrations within 20-30 min after awakening in the morning. In the present study none of the subjects were sampled in the morning just after awakening. Over all, time of sampling didn't seem to affect the SCC.

4.4. Coat color and hair wash

A statistically significant difference between coat color and cortisol was found. The following post-hoc test revealed that the difference existed between brown hair (N = 18) and black hair (N = 55). One has to bear in mind, that the two groups being compared are of unequal size, which could have affected the result. A study by Bennett and Hayssen (2010) who examined 48 dogs (23 Labrador Retrievers (LR) and 25 German Shepherds (GS)) also revealed a difference in cortisol in relation to coat color. Across all subjects, black dogs had less cortisol than nonblack dogs. In addition, within an individual dog, black hair had less cortisol than yellow hair. This however does

not translate to differences between breeds or coat color within a breed, which might be explained by differences in the genetic control that produces yellow, black, or agouti (banded) hair in LR versus GS. Further studies exploring whether cortisol concentrations may be influenced by the agouti locus is needed (Hayssen *et al.,* 2002). In contrast, Macbeth *et al.* (2010) found no significant difference in cortisol concentration among five different color categories ranging from nearly white to black. These findings agree with studies in humans reporting no difference in cortisol concentrations among blond, brown, or black hair (Raul *et al.,* 2004; Sauvé *et al.,* 2007).
The differences in HCC in different hair colors could limit the value of intra-individual HCC measurements in animals with mixed hair color and is worth considering when designing a study.

The effect of shampoo and water washing on HCC was studied with the grouping variables: 1: showered < 1 year ago, 2 showered > 1 year ago. No significant relationship was found between washing of the hair and HCC. This is inconsistent with a study by Hamel *et al.* (2011) who found a washout effect on HCC in rhesus monkeys when repeatedly exposing the hair to hair wash. The majority of dogs in the present study had never been showered with or without shampoo, which might explain the findings.

4.5. Correlation between hair and saliva

There was no statistically significant correlation between saliva and hair cortisol concentrations. A significant correlation between saliva and hair cortisol concentrations was not to be expected. Saliva represents an instantaneous view of the adrenocortical activity at the time the sample was collected, whereas hair represents a period of weeks to months. This makes saliva useful for assessing acute stress and hair for chronic stress. In Bennett and Hayssen (2010) a positive correlation was found between saliva and hair cortisol concentration. However, in this study a shave/re-shave method was used, ensuring that only new hair (6-12 weeks old) was included.

A correlation between hair samples and different body regions was expected but a comparison of hair from the neck with the mean value of hair from hips, showed no correlation. A possible explanation for this inter-body region variability could be a difference in growth rate between hair from the neck and ischiatic region including differences in moulting patterns. In grizzly bears,

Macbeth *et al.* (2010) also found variations in HCC between different body regions with the highest level of cortisol in neck hair and the lowest in rump, abdomen and shoulder.

There was a significant correlation when comparing hair from the left and right hip. In a canine study by Bryan *et al.* (2013) the hair from the left and right side of each dog was well correlated, which was consistent with the findings by Comin *et al.* (2012) who found no difference in HCC between different body sites in the New Zealand white rabbit. This leads to a suspicion that the anatomically location plays a role when analyzing HCC in dogs and in order to fully investigate the HCC in dogs, sampling should be standardized to a specific body region.

4.6. Reference values

In the present study the mean cortisol content in 115 hair samples was 3.225 ± 0.243 pg/mg.

In two canine studies the mean cortisol content in hair was 12.63 ± 5.45 pg/mg (Bennett and Hayssen, 2010) and 2.10 ± 0.22 pg/mg (Acorsi *et al.,* 2008) respectively.

The mean cortisol content determined in 36 saliva samples was 0.738 ± 0.045 ng/ml in the present study. In two canine studies studying saliva cortisol the mean values of cortisol was 1.56 ± 0.61 ng/mL (Bennett and Hayssen, 2010) and 1.7 ± 0.6 ng/mL (Dreschel and Granger, 2009) respectively. Both means found in the present study was in close relation to the means obtained by the above-mentioned canine studies.

5. Conclusion and perspective

Since no *statistically* significant difference in HCC was found between the good and bad hip, the influence of HD-scores on the local HCC is questionable and speculations of the value of HD-scoring as a valid predictor of the development and severity of hip dysplasia arise. However, the trend of HCC rising through the three different groups as shown in the present study is encouraging. Even though it wasn't statistically significantly supported, it still opens up the possibility of further investigation of the value of HCC as a biomarker in dogs. Hair cortisol on its own, may not have value as a definitive test in determining whether a dog has health problems or not but could be used in combination with other parameters. Measuring cortisol on a repeated basis (i.e. yearly) may be particularly useful in tracking gradual changes related to slowly progressing diseases within the individual dog. Certain limitations exist as found in the present study. A significant difference in HCC was evident between black and brown hair color. This would limit the intra-individual measurement of cortisol concentration in dogs with mixed hair color (i.e. German Shepherds) but not in dogs with a uni-colored coat such as the Labrador retriever.

No significant difference was found between SCC and the three groups in the present study. Despite this one has to bear in mind that when measuring saliva cortisol, external conditions in the study design could have an effect on the SCC. It is important to consider time of sampling and place of sampling to avoid external sources of stress and the possible CAR

Acknowledgements

The authors would like to thank Christopher Harold Knight and Dorte Hald Nielsen for supervision and support and are grateful to the staff at the Department of Food Science, University of Udine, Udine, Italy for their help in the analysis of biological samples. The authors would also like to acknowledge their funding from Dansk Kennel Klub.

References

Acorsi, P.A., Carloni, E., Valsecchi, P., Viggiani, R., Gamberoni, M., Tamanini, C. & Seren, E. (2008): Cortisol determination in hair and feces from domestic cats and dogs. *General and Comparative Endocrinology*. Vol. 155, pp. 398-402.

Arck, P.C., Slominski, A., Theoharides, T.C., Peters, E.M.J. & Paus, R. (2006): Neuroimmunology of Stress: Skin takes center stage. *Journal of Investigative Dermatology*. Vol. 126, pp. 1697-1704.

Ashley, N.T., Barboza, P.S., Macbeth, B.J., Janz, D.M., Cattet, M.R.L., Booth, R.K. & Wasser, S.K. (2011): Glucocorticosteroid concentrations in feces and hair of captive caribou and reindeer following adrenocorticotropic hormone challenge. *General and Comparative Endocrinology*. Vol. 172, pp. 382-391.

Bechshøft, T.Ø., Sonne, C., Dietz, R., Born, E.W., Novak, M.A., Henchey, E. & Meyer, J.S. (2011): Cortisol levels in hair of East Greenland polar bears. *Science of the Total Environment.* Vol. 409, pp. 831-834.

Beerda, B., Schilder, M.B.H., Janssen, N.S.C.R.M. & 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.* Vol. 30, no 0033, pp. 272-279.

Beerda, B., Schilder, M.B.H., van Hooff, J.A.R.A.M & de Vries, H.W. (1997): Manifestations of chronic and acute stress in dogs. *Applied Animal Behaviour Science.* Vol. 52, pp. 307-319.

Bennett, A. & Hayssen, V. (2010): Measuring cortisol in hair and saliva from dogs: coat color and pigment differences. *Domestic Animal Endocrinology.* Vol. 39, pp. 171-180.

Blackburn-Munro, G. & Blackburn-Munro, R.E. (2001): Chronic Pain, Chronic Stress and Depression: Coincidence or Consequence? *Journal of Neuroendocrinology.* Vol. 13, pp. 1009-1023.

Brass, W. (1989): Hip dysplasia in dogs. *Journal of Small Animal Practice*. Vol. 30, pp. 166-170.

Bryan, H.M., Adams, A.G., Invik, R.M., Wynne-Edwards, K.E. & Smits, J.EG. (2013): Hair as a Meaningful Measure of Baseline Cortisol Levels over Time in Dogs. *Journal of the American Association for Laboratory Animal Science.* Vol. 52, no 2, pp. 189-196.

Comin, A., Prandi, A., Peric, T., Corazzin, M., Dovier, S. & Bovolenta, S. (2011): Hair cortisol levels in dairy cows from winter housing to summer highland grazing. *Livestock Science*. Vol. 138, pp. 69-73.

Comin, A., Zufferli, V., Peric, T., Canavese, F., Barbetta, D. & Prandi, A. (2012): Hair cortisol levels determined at different body sites in the new zealand white rabbit. *World Rabbit Science*. Vol. 20, pp. 149-154.

Cone, E.J. (1996): Mechanisms of Drug Incorporation into Hair. *Therapeutic Drug Monitoring*. Vol. 18, no 4, pp. 438-443.

Cook, N.J. (2012): Review: Minimally invasive sampling media and the measurement of corticosteroids as biomarkers of stress in animals. *Canadian Journal of Animal Science.* Vol. 92, pp. 227-259.

Coppola, C.L., Grandin, T. & Enns, R.M. (2006): Human interaction and cortisol: Can human contact reduce stress for shelter dogs? *Physiology & Behavior*. Vol. 87, pp. 537-541.

Cunningham, J.G. & Klein, B.G. (2007): *Veterinary Physiology*. Fourth edition. Saunders Elsevier. St. Louis, Missouri, USA.

Davenport, M.D., Tiefenbacher, S., Lutz, C.K., Novak, M.A. & Meyer, J.S. (2006): Analysis of endogenous cortisol concentrations in the hair of rhesus macaques. *General and Comparative Endocrinology.* Vol. 147, pp. 255-261.

DKK (n.d.): *Avl/Sundheds restriktioner,* [online]. Hundeweb, Solrød Strand. [June 9th 2014]. Available online: http://www.hundeweb.dk/dkk/public/openIndex?ARTICLE_ID=115.

Dreschel, N.A. & Granger, D.A. (2009): Methods of collection for salivary cortisol measurement in dogs. *Hormones and Behavior.* Vol. 55, pp. 163-168.

Esposito, P.L., Comin, A., Peric, T., Montillo, M., Mascolo, M., Tubaro, G. & Prandi, A. (2012): Experimental indicators of ergonomic wellness and quality of life: salivary and hair cortisol. *Work*. Vol. 41, pp. 5442-5445.

FCI (2006): *FCI Requirements for Official Hip Dysplasia Screening*, [online]. DKK, Copenhagen. [June 9th 2014]. Available online: http://www.dkk.dk/xdoc/120/46-2009-annex1.pdf.

Fries, C.L. & Remedios, A.M. (1995): The pathogenesis and diagnosis of canine hip dysplasia: A review. The *Canadian Veterinary Journal*. Vol. 36.

Gow, R., Thomson, S., Rieder, N., Van Uum, S. & Koren, G. (2010): An assessment of cortisol analysis in hair and its clinical applications. *Forensic Science International.* Vol. 196, pp. 32-37.

Gunaratnam, P. & Wilkinson, G.T. (1983): A study of normal hair growth in the dog. *Journal of Small Animal Practice.* Vol. 24, pp. 445-453.

Gygax L., Neuffer, I., Kaufmann, C., Hauser, R. & Wechsler, B. (2006): Milk cortisol concentrations in automatic milking systems compared with auto-tandem milking parlors. *Journal of Dairy Science*. Vol. 89, pp. 3447-3454.

Hamel, A.F., Meyer, J.S., Henchey, E., Dettmer, A.M., Suomi, S.J., & Novac, M.A. (2011): Effects of shampoo and water washing on hair cortisol concentrations. *Clinica Chimica Acta*. Vol. 412, pp. 382-385.

Harkey, M.R. (1993): Anatomy and physiology of hair. *Forensic Science International. Vol. 63, pp. 9-18.*

Hayssen, V., Harper, J.M. & DeFina, R. (2002): Fecal corticosteroids in agouti and non-agouti deer mice. *Comparative Biochemistry and Physiology*. Vol. 132, pp. 439-446.

Hou, Y., Wang, Y., Lust, G., Zhu, L., Zhang, Z. & Todhunter, R.J. (2010): Retrospective Analysis for Genetic Improvement of Hip Joints of Cohort Labrador Retrievers in the United States: 1970-2007. *Plos One.* Vol. 5, issue 2.

Ito, N., Ito, T., Kromminga, A., Bettermann, A., Takigawa, M., Kees, F., Straub, R.H. & Paus, R. (2005): *Human hair follicles display a functional equivalent of the hypothalamic-pituitary-adrenal (HPA) axis and synthesize cortisol.* [online]. The Journal of the Federation of American Societies for Experimental Biology, USA. [June 10th 2014]. Available online: http://www.fasebj.org/content/19/10/1332.full.pdf+html

Johnston, S.A. (1997): Osteoarthritis, Joint Anatomy, Physiology, and Pathobiology. *Veterinary Clinics of North America: Small Animal Practice*. Vol. 28, no 4.

Keckeis, K., Lepschy, M., Schöpper, H., Moser, L., Troxler, J. & Palme, R. (2012): Hair cortisol: a parameter of chronic stress? Insight from a radiometabolism study in guinea pigs. *Journal of Comparative Physiology B*. Vol. 182, pp. 985-996.

Kirschbarum, C. & Hellhammer, D.H. (1989): Salivary Cortisol in Psychobiological Research: An Overview. *Neuropsychobiology*. Vol. 22, pp. 150-169.

Kirschbaum, C., Tietze, A., Skoluda, N. & Dettenborn, L. (2009): Hair as a retrospective calendar of cortisol production – increased cortisol incorporation into hair in the third trimester of pregnancy. *Psychoneuroendocrinology*. Vol. 34, pp. 32-37.

Kobelt, A.J., Hemsworth, P.H., Barnett, J.L. & Butler, K.L. (2003): Sources of sampling variation in saliva cortisol in dogs. *Research in Veterinary Science.* Vol. 75, pp. 157-161.

Koren, L., Mokady, O., Karaskov, T., Klein, J. Koren, G. & Geffen, E. (2002): A novel method using hair for determining hormonal levels in wildlife. The Association for the Study of *Animal Behaviour*. Vol. 63, pp. 403-406.

Kronveit, R.I., Nødtvedt, A., Sævik, B.K., Ropstad, E., Skogmo, H.K. & Trangerud, C. (2010): A prospective study on Canine Hip Dysplasia and growth in a cohort of four large breeds in Norway (1998-2001). *Preventive Veterinary Medicine*. Vol. 97, pp. 262-263.

Levine, A., Zagoory-Sharon, O., Feldman, R., Lewis, J.G. and Weller, A. (2007): Measuring cortisol in human psychobiological studies. *Physiology & Behavior.* Vol. 90, pp. 43-53.

Lewis, T.W., Blott, S.C. & Woolliams, J.A. (2010): Genetic Evaluation of Hip Score in UK Labrador Retrievers. *Plos one*. Vol. 5, issue 10.

Lust, G. (1997): An overview of the pathogenesis of canine hip dysplasia. *Journal of the American Veterinary Medical Association*. Vol. 210, pp. 1443-1445.

Marques, A.H., Silverman, M.N. & Sternberg, E.M. (2010): Evaluation of Stress Systems by Applying Noninvasive Methodology: Measurements of Neuroimmune Biomarkers in the Sweat, Heart Rate Variability and Salivary Cortisol. *Neuroimmunomodulation.* Vol. 17, pp. 205-208.

McBeth, B.J., Cattet, M.R.L., Steenhaus, G.B., Gibeau, M.L. & Janz, D.M. (2010): Hair cortisol concentrations as a non-invasive measure of long-term stress in free-ranging grizzly bears (*Ursus arctos*): considerations with implications for other wildlife. *Canadian Journal of Zoology*. Vol. 88, pp. 935-949.

McLaughlin, R. (2000): Management of chronic osteoarthritic pain. *Veterinary Clinics of North America: Small Animal Practice*. Vol. 30, no 4.

Merskey, H. & Bogduk, N. (1994): Classification of chronic pain – descriptions of chronic pain syndromes and definitions of pain terms. *International Association for the Study of Pain.* Seattle, USA. 2.ed. Part III, pp. 210.

Moberg, G.P. (2000): Biological Response to Stress: Implications for Animal Welfare. *The Biology of Animal* Stress, 1-3 ed. G.P. Moberg and J.A. Mench, UK, CABI Publishing.

Palme, R. (2005): Measuring Fecal Steroids – Guidelines for Practical Application. *Annals New York Academy of Science*. Vol. 1046, pp. 75-80.

Paus, R. Theoharides, T.C. & Arck, P. (2006): Neuroimmunoendocrine circuitry of the "brain-skin connection". *Trends in Immunology*. Vol. 27, pp. 32-39.

Perego, R., Proverbio, D. & Spada, E. (2013): Increases in heart rate and serum cortisol concentrations in healthy dogs are positively correlated with an indoor waiting-room environment. *Veterinary Clinical Pathology.* Vol. 43/1, pp. 67-71.

Perkinelmer (n.d): *Radioimmunoassays*, [online]. Perkinelmer, USA. [June 9th 2014]. Available online: http://www.perkinelmer.com/resources/technicalresources/applicationsupportknowledgebase/radiometric/radioimmunoassay.xhtml.

Raul, J.S., Cirimele, V., Ludes, B. & Kints, P. (2004): Detection of physiological concentrations of cortisol and cortisone in human hair. *Clinical Biochemistry*. Vol. 37, pp. 1105-1111.

Renberg, W. (2005): Pathology and Management of Arthritis. *Veterinary Clinics Small Animal Practice*. Vol. 35, pp. 1073-1091.

Riad-Fahmy, D., Read, G.F., Walker, R.F. & Griffiths, K. (1982): Steroids in Saliva for Assessing Endocrine Function. *The Endocrine Society.* Vol. 3, no 4.

Sandøe, P., Johnsen, P.F. & Christiansen, S.B. (2001): Dyrevelfærd – Definitioner, målemetoder og etisk relevans. *Dansk Veterinærtidsskrift. No. 0122.*

Sandøe, P. & Christiansen, S.B. (2008): *Ethics of animal use*. Blackwell Publishing, Oxford, UK.

Seaward, B.L. (2005): *Managing Stress – Principles and Strategies for Health and Well-Being*. Fifth edition. Jones & Bartlett Learning, LLC, Burlington MA, USA.

Sharpley, C.F., Kauter, K.G. & McFarlane, J.R. (2010): Diurnal Variation in Peripheral (Hair) vs Central (Saliva) HPA Axis Cortisol Concentrations. *Clinical Medicine Insights: Endocrinology and Diabetes.* Vol. 3, pp. 9-16.

Slominski, A., Wortsman, J., Tuckey, R. & Paus, R. (2007): Differential expression of HPA axis homolog in the skin. *Molecular and Cellular Endocrinology*. Vol. 265-266, pp. 143-149.

Smyth, J.M., Ockenfels, M.C., Gorin, A.A., Catley, D., Porter, L.S., Kirschbaum, C., Hellhammer, D.H. & Stone, A.A. (1997): Individual differences in the diurnal cycle of cortisol. *Psychoneuroendocrinology*. Vol. 22, no 2, pp. 89-105.

Stalder, T. & Kirschbaum, C. (2012): Analysis of cortisol in hair – State of the art and future directions. *Brain, Behaviour, and Immunity*. Vol. 26, pp. 1019-1029.

Stalder, T., Steudte, S., Miller, R., Skoluda, N., Dettenborn, L. & Kirschbaum, C. (2012): Intraindividual stability of hair cortisol concentrations. *Psychoneuroendocrinology*. Vol. 37, pp. 602-610.

Suavé, B., Koren, G., Walsh, G., Tokmakejian, S. & Van Uum, S.H.M. (2007): Measurement of cortisol in human hair as a biomarker of systemic exposure. *Clinical & Investigative Medicine.* Vol. 30, no 5.

Tryland, M., Brun, E., Derocher, A.E., Arnemo, J.M., Kierulf, P., Ølberg, R.A. & Wiig, Ø. (2002): Plasma biochemical values from apparently healthy free-rangin polar bearsfrom Svalbard. *Journal of Wildlife Diseases*. Vol. 38, no 3, pp. 566-575.

Van Vonderen, I.K., Kooistra, H.S. & Rijnberk, A. (1998): Influence of Veterinary Care on the Urinary Corticoid:Creatinine Ratio in Dogs. *Journal od Veterinary Internal Medicine*. Vol. 12, issue 6, pp. 431-435.

Vincent, I.C. & Michell, A.R. (1992): Comparison of cortisol concentrations in saliva and plasma of dogs. *Research in Veterinary Science.* Vol. 53, pp. 342-345.

Vinning, R.F. & McGinley, R.A. (1987): The measurement of hormones in saliva: possibilities and pitfalls. *Journal of Steroid Biochemistry*. Vol. 27, no 1-3, pp. 81-94.

Webb, E., Thomson, S., Nelson, A., White, C., Koren, G., Rieder, M. & Vanuum, S. (2010): Assessing individual systemic stress through cortisol analysis of archaeological hair. *Journal of archaeological science*. Vol. 37, pp. 807-812.

Wennig, R. (2000): Potential problems with the interpretation of hair analysis results. *Forensic Science International.* Vol. 107, pp. 5-12.

Wood, J.L.N. & Lakhani, K.H. (2003): Effect of month of birth on hip dysplasia in Labrador retrievers and Gordon setters. *The Veterinary Record*. Vol. 152, pp. 69-72.