



Master's Thesis

Stress-related Biomarker Measurement in Cavalier King Charles Spaniels with Mitral Valve Disease

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Abstract

With general increasing interest in animal welfare, a reliable method for assessment of animal welfare is needed. Animal welfare can be defined in multiple ways, but one way is the absence of long-term stress. Cortisol has been established as a biomarker for stress, with hair cortisol being a measure of chronic stress, while saliva and plasma are measures of acute stress.

A common chronic disease in Cavalier King Charles Spaniels is mitral valve disease. The purpose of this thesis is to determine whether mitral valve disease have an effect on the biomarkers for long- and short-term stress (hair and plasma/saliva cortisol respectively). Also, to evaluate other factors which might influence hair cortisol concentration and compare cortisol concentrations in different matrices.

In this study, blood, saliva and hair samples were collected from 45 Cavalier King Charles Spaniels and 13 Beagles enrolled in a screening project for Mitral Valve Disease. The dogs were divided into groups according to their disease status.

The thesis concludes that mitral valve disease did not have a significant effect on hair, saliva or plasma cortisol concentration. Plasma and saliva cortisol concentration was significantly correlated, but neither were significantly correlated with hair cortisol concentration. Hair color had a significant effect on hair cortisol concentration. None of the other factors evaluated had any effect on hair cortisol concentration.

Resumé

I takt med stigende interesse for dyrevelfærd er det blevet vigtigt at finde en pålidelig metode til vurdering af denne. Der findes adskillige måder at definere dyrevelfærd på. En af disse er fravær af længerevarende stress. Kortisol er en etableret biomarkør for stress. Kortisol i hår er en biomarkør for kronisk stress, mens kortisol fra plasma og spyt er en biomarkør for akut stress.

Mitralklap insufficiens er en hyppig kronisk lidelse hos Cavalier King Charles Spaniels. Formålet med denne afhandling er at vurdere, om mitralklap insufficiens har en indvirkning på biomarkørerne for kronisk og akut stress (hhv. kortisol i hår og kortisol i plasma-/spyt). Formålet er derudover at evaluere andre faktorer, der kunne have en indvirkning på kortisolkoncentrationen i hår samt sammenligne kortisolkoncentrationen i forskellige matrix. Blod-, spyt- og hårprøver blev indsamlet fra 45 Cavalier King Charles Spaniels og 13 Beagler i forbindelse med et screeningsprojekt for mitralklap insufficiens. Hundene blev inddelt i grupper på baggrund af deres sygdomsstatus.

Afhandlingen konkluderer, at der ikke fandtes nogen signifikant sammenhæng mellem graden af mitralklap insufficiens og koncentrationen af kortisol i hhv. hår-, spyt- og plasma. Kortisolkoncentrationen i plasma og spyt var signifikant korrelerede, men der fandtes ingen sammenhæng mellem koncentrationen af kortisol i hår og koncentrationen af kortisol i hhv. spyt og plasma. Hårfarve havde en signifikant indvirkning på kortisolkoncentrationen i hår. Ingen af de andre faktorer havde en signifikant indvirkning på kortisolkoncentrationen i hår.

Introduction

For many years now, there has been an increasing interest in assessing animal welfare, both in livestock, companion animals and wildlife.

In order to assess whether welfare or “quality of life” is present, one first needs to define what good welfare is. This can be, as in the case of the Five Freedoms (Hewson 2003), that the animal is not hungry or sick. It can also be that the animal can express natural behavior. Most definitions are very general and do not necessarily take the individual animal into account. Some frameworks for welfare assessment even focus more on the environment than the animal itself. It is difficult to develop a perfect method.

Even though the use of frameworks, like the Five Freedoms, makes it easier to compare different welfare assessments, welfare assessments are often very subjective and depend on the person making them.

One way to define welfare is the absence of long-term stress. To evaluate whether an animal is stressed or not, one can either look at the behavioral patterns of the animal or look at the physiology behind it. It would probably be best with a combination. It is important to have in mind, that what might be stressful for one animal might not be stressful for all animals.

In the following pages there will be a review of the theoretical background of this thesis.

HPA axis

When a stressor is present, there is an activation of the hypothalamic-pituitary-adrenal axis (HPA axis).

When activated the anterior hypothalamus releases corticotropin-releasing factor (CRF), which stimulates the pituitary gland to release adrenocorticotropic hormone (ACTH). ACTH stimulates the cortex of the adrenal gland, which releases glucocorticoids including cortisol. (Seaward 2006)

An obvious way to measure HPA-axis activity is to measure the “end-product”, which means the hormones released. In this case the glucocorticoids. In different species there are variations on which glucocorticoid is the predominant in stress physiology. In humans and

dogs it is cortisol (Beerda, Schilder et al. 1999, Davenport, Tiefenbacher et al. 2006). In some other species, e.g. rodents, it is corticosterone (Thanos, Cavigelli et al. 2009).

The golden standard for measuring cortisol is measuring the concentration in plasma. Cortisol has also been measured in a wide variety of other matrices, including saliva, urine, feces and hair (Beerda, Schilder et al. 1996, Accorsi, Carloni et al. 2008). With every matrix there is benefits and drawbacks.

Each matrix shows a different window in time with plasma being the most acute (a measure of cortisol within the last minutes) and hair the most chronic measure of cortisol (a measure of cortisol within the last weeks to years, depending on the length of the hair sample) of the above mentioned (Bennett and Haysen 2010).

Plasma cortisol

Even though measuring blood in plasma is the golden standard, there are some issues with this matrix.

Sampling

Blood sampling is invasive and should be performed by trained personnel. Bringing the dog to the veterinary clinic might be stressful and therefore influence the cortisol concentration.

The blood sampling can by itself also be a stressful event. Since the plasma cortisol reflects the cortisol concentration at the moment of blood sampling, the stress of handling might elevate the cortisol level in the blood sample (Bennett and Haysen 2010).

Ethically, one might also question the fact that we expose the animal to a stressful situation (by taking a blood sample) in order to measure the stress level of the animal.

Diurnal variation

Another aspect of plasma cortisol concentration one has to consider is whether there is a diurnal variation. Diurnal variation of plasma cortisol have been observed in humans, with higher cortisol levels in the mornings (Chrousos and Gold 1998). In dogs, however, several studies have not found any circadian rhythmicity, but there were observed variations in

cortisol levels during a 24-h period (Kemppainen and Sartin 1984, Castillo, Blatter et al. 2009).

Since there is a variation in cortisol concentration during the day, it is important to have this in mind when planning a study. Samples should be taken at the same time of day to eliminate sources of error when comparing acute cortisol concentrations.

If the chronic level of cortisol is needed instead of the acute level, it is important to take multiple blood samples at different times of the day and at different days to even out the variations.

Benefits

One of the benefits of measuring cortisol in plasma is that it is easy to get enough plasma to measure the cortisol level. Another is that blood sampling is often performed anyways during veterinary examination of companion animals when checking for diseases. Plasma is also a matrix that has been used more and is more well known than the other matrices mentioned, making the protocols for handling plasma more reliable.

Saliva cortisol

As an alternative to plasma cortisol, saliva cortisol has been used. It has the same properties as plasma cortisol as an acute measure of cortisol, but it is non-invasive which makes it possible for owners to collect the samples themselves.

The cortisol concentration in saliva is significantly correlated with the concentration of cortisol in plasma, but the values are only about 10% of those in plasma (Vincent and Michell 1992, Beerda, Schilder et al. 1996).

For more information on how cortisol gets into the saliva see the review (Vining and McGinley 1986).

Sampling

Since saliva cortisol is a measure of acute cortisol concentration, it is important to be certain that the sampling does not increase cortisol concentration in the sample.

If sampling is limited to a time interval of less than 2 minutes there is not seen an increase in saliva cortisol concentration. It might even be possible to restrain the dog for up to 4 minutes, but the literature is not consistent on this point (Kobelt, Hemsworth et al. 2003, Oyama, Hyodo et al. 2014).

Food luring can be used to minimize the amount of restraint needed for sample collection (Bennett and Haysen 2010), but dogs should not be allowed to eat before sampling is finished since food contamination of the samples can influence the results (Dreschel and Granger 2009).

One problematic feature of using saliva is how to get enough sample material for analysis. A way of ensuring this is to use citric acid. In small amounts the use of citric acid in the mouth of the dog does not appear to be influencing cortisol concentration, but in vitro tests with larger amounts of citric acid shows a significant increase in measured saliva cortisol (Dreschel and Granger 2009). Therefore it might be a better solution to use food luring.

In humans an effect of the salivary flow rate on the cortisol concentration has been observed with higher cortisol concentration with higher flow rate (Gallagher, Leitch et al. 2006).

It might also be difficult getting all of the saliva out of the sampling material and if the recovery of saliva is very poor, the concentration of salivary cortisol is also affected (Harmon, Hibel et al. 2007).

If multiple saliva samples are wanted in rather short intervals, e.g., every 30 minutes, it is important to know whether the dogs are used to having their teeth brushed. In dogs used to regular teeth brushing the saliva cortisol concentration does not significantly increase in samples taken 30 minutes after the first sample, in contrast to dogs whose teeth were not regularly brushed (Oyama, Hyodo et al. 2014).

Diurnal variation

It has been shown in humans that there is diurnal variation in saliva cortisol (though not all have the “normal” cycle with higher morning cortisol) (Smyth, Ockenfels et al. 1997), but in dogs the literature is more conflicted. In some studies there does not seem to be systematic circadian differences of saliva cortisol (Bryan, Adams et al. 2013), whereas others showed higher saliva cortisol in the morning (Beerda, Schilder et al. 1999).

Urinary cortisol

Measuring cortisol in urine is also a noninvasive method. It is widely used in humans, where it is also easier to obtain a urine sample, than in animals. It is also easier to collect 24-hour urine in humans. The good thing about collecting 24-hour urine is that you smooth out the diurnal variations there might be in plasma cortisol (Ifedayo and Olufemi 2013).

In dogs (and other animals) it is also quite easy to collect the urine sample at home, thereby not exposing them to the potentially stressful situation of going to the veterinary clinic.

It is important to take into account that excessive intake of fluids will increase the level of cortisol in urine (Ifedayo and Olufemi 2013).

Fecal cortisol

Fecal cortisol represents a timeframe of hours to days depending on the species and is a noninvasive method.

The steroids in feces are not evenly distributed and it is important to homogenize the sample before analysis. The bacterial fauna in feces samples from different animals can vary, therefore the steroid stability might differ if the samples are not frozen directly after collection of fresh feces (Palme 2005).

Another issues with fecal cortisol are the possibility of contamination by the environment. Furthermore it might be difficult to identify which individual the feces samples are from if collected in the wild and whether it might be a mix from more than one animal.

For more information on fecal cortisol see the review by Palme (2005).

Hair cortisol

A rather new approach to determining cortisol concentrations is by measuring the cortisol concentration in hair. Hair cortisol concentrations have been measured in a wide variety of species including humans (Sauvé, Koren et al. 2007), captive rhesus monkeys (Davenport, Tiefenbacher et al. 2006), dogs (Accorsi, Carloni et al. 2008, Bennett and Haysen 2010), cats (Accorsi, Carloni et al. 2008), cattle (Comin, Prandi et al. 2011), horses (Comin, Veronesi et al. 2012), chipmunks (Martin and Réale 2008), rock hyrax (Koren, Mokady et al. 2002), grizzly

bears (Macbeth, Cattet et al. 2010), polar bears(Bechshøft, Sonne et al. 2011), caribou and reindeers(Ashley, Barboza et al. 2011).

In contrast to plasma and salivary cortisol, which both give an acute picture of the cortisol concentration, hair cortisol is a measure of chronic cortisol accumulated over weeks or months (Koren, Mokady et al. 2002).

Several studies have compared hair cortisol concentration with cortisol concentration in other matrices to validate this new approach to measure cortisol levels (Davenport, Tiefenbacher et al. 2006, Sauv , Koren et al. 2007, Accorsi, Carloni et al. 2008, Bennett and Haysen 2010, Ashley, Barboza et al. 2011, Bryan, Adams et al. 2013). Since different matrices shows a different time period of cortisol accumulation, it is not surprising that many of the studies does not find a significant correlation especially between hair and some of the more acute measures of cortisol concentration like saliva (Bryan, Adams et al. 2013), but other studies does find a positive correlation (Bennett and Haysen 2010).

The variability of cortisol measurements in hair is smaller than in other matrices (Stalder, Steudte et al. 2012, Bryan, Adams et al. 2013), making it easier to rely on a single sample for a basic level measurement.

Incorporation of cortisol

It is not known exactly how the cortisol is deposited into the hair shaft, but different theories have been made. It is generally assumed that cortisol enters the hair by passive diffusion from blood capillaries into the growing hair, but diffusion from sweat or sebum secretions is an alternative mechanism. It is very possibly a combination of several mechanisms(Pragst and Balikova 2006).

The cortisol concentration in the hair shaft is probably not only derived from the systemic levels of cortisol, since the hair follicle is also a local source of cortisol (Ito, Ito et al. 2005).

Another important aspect of hair cortisol is how big an elevation in systemic cortisol concentration needs to be to cause an elevation in hair cortisol concentration. Minor ocular surgery did not cause elevation in hair cortisol concentration in a dog even though salivary cortisol was elevated (Bryan, Adams et al. 2013). In another study, administration of ACTH in

caribou and reindeer did not increase hair cortisol concentration, even when the dose was increased (Ashley, Barboza et al. 2011).

According to a study in humans where intra-individual stability of hair cortisol concentrations were measured, hair cortisol were only to a lesser extent influenced by occasion-specific factors (Stalder, Steudte et al. 2012).

Even though one study (Sharpley, Kauter et al. 2009) showed changes in hair cortisol concentration directly after immersing the hand in ice water, this is more likely a result of changes in the cortisol concentration in the sweat or sebum on the outside of the hair shaft. The samples in the study were not washed prior to extraction.

Impact of hair color

When using a new matrix it is important to know what might influence the results. In hair one of these variables is hair color.

In several studies a difference in hair color and hair cortisol concentration has been found. In dogs, black dogs had a hair cortisol concentration that were 24% lower than non-black dogs and black hairs were consistently lower in cortisol than yellow hairs (Bennett and Haysen 2010). In grizzly bears the variation in hair cortisol concentration was not explained by differences in hair color of bears, however there was a difference between hair cortisol concentrations in differently colored hairs from the same bear. Surprisingly, here there was a higher hair cortisol concentration in dark than in light hair (Macbeth, Cattet et al. 2010).

It makes sense that hair color is connected to the amount of cortisol bound in the hair shaft since it is the same family of hormones and receptors which is involved in control of both pigment and cortisol (Bennett and Haysen 2010).

Studies in humans have not shown a difference in hair cortisol concentration in different hair colors (Sauvé, Koren et al. 2007).

Growth rate and body site

In humans the growth rate of hair has been estimated to 1cm/month with a small variability at the different sites of the head (Pragst and Balikova 2006). When the growth rate is known it is possible to use hair cortisol concentration in a retrospective manner and measure cortisol concentration at specific times in the past.

In dogs the growth rate of hair has not been determined and there is reason to believe that the growth rate is dependent on the length of the coat. It has been shown that it takes the same amount of time for complete regrowth despite differences in original hair length (Bennett and Haysen 2010). In dogs it is therefore not possible to determine when a specific part of the hair shaft is from, without more knowledge of the growth pattern in the specific breed.

If it is necessary to obtain a hair sample that corresponds to a specific time period, a shave/reshave method can be used. Here an area is shaved at the beginning of the time period and reshaved in the end, thereby obtaining a hair sample that has grown in that time period. It is important to note that there is a small error in this method since there will be a small part of the hair shaft still in the skin when shaving. Therefore a small part of the hair is from before the time period and there will still be a small part from the time period left, when the reshave is performed.

It would be wise to have a standardized collection method to ensure comparability with other studies. It has been shown that hair cortisol concentration varies greatly between hair samples collected from different body regions, however interbody variability was greater than the intrabody variability (Macbeth, Cattet et al. 2010). There is not, however, a difference between hair samples collected from left and right side of the same area (Bryan, Adams et al. 2013) and in humans there was no difference between samples collected from various parts of the head (Sauvé, Koren et al. 2007).

In bears it has been shown that there is a significant difference in hair cortisol concentration between guard hairs and undercoat (Macbeth, Cattet et al. 2010) making it important that the hairs collected are of the same type.

Hair washing

It has been speculated whether external changes might change the concentration of cortisol already incorporated in the hair. In humans the focus has especially been on hair washing which is associated with a significant loss of hair cortisol (Hamel, Meyer et al. 2011). Most of the effect is due to the water, so it might also be important with wild animals living in wet climates or dogs swimming regularly. A difference in hair cortisol concentration in the proximal and distal part of the hair shaft was not, however, shown in either bears or dogs

(Bennett and Haysen 2010, Macbeth, Cattet et al. 2010). Furthermore, hair left outside for up to 18 days did not show a significant decrease in hair cortisol (Macbeth, Cattet et al. 2010).

Other factors

There has not been found an influence of age, race, weight or neuter status in dogs on hair cortisol concentration (Bennett and Haysen 2010). Most studies did not find an effect of sex on hair cortisol level either (Bennett and Haysen 2010, Macbeth, Cattet et al. 2010, Ashley, Barboza et al. 2011, Gow, Koren et al. 2011) but in polar bears females had significantly higher cortisol concentrations than males (Bechshøft, Sonne et al. 2011).

Storage of samples

In contrast to many other biological matrixes, hair can be stored at room temperature. It has been shown that the cortisol content in hair remains the same when stored intact in dry paper envelopes for at least 17 months (Macbeth, Cattet et al. 2010).

Motivation for this thesis

The literature seems to agree that cortisol is a biomarker for stress. In order to test this I tried to find a situation where the cortisol level might be altered by a stressor.

The animal chosen for this project was the dog. The reasons for this were multiple.

Firstly, I wanted to work with small animals (either dogs or cats) in order to write a project that might be relevant for later work in a small animal clinic.

Secondly, a project with mitral valve disease (MVD) in Cavalier King Charles Spaniels (CKCS) was ongoing at the university and the next round of follow-up echocardiography was already planned, making it easy to get a rather large sample size.

The question was then, whether different stages of MVD might influence the chronic level of cortisol. MVD is a chronic disease, which makes it possible to presume that there might also be a chronic strain on the body and thereby a chronic stressor.

Mitral valve disease

MVD is the most common cardiovascular disease in dogs (Detweiler and Patterson 1965, Egenvall, Bonnett et al. 2006) and is known to cause congestive heart failure (Haggstrom, Hoglund et al. 2009). MVD occurs in all dog breeds, but is especially associated with the Cavalier King Charles Spaniel (Thrusfield, Aitken et al. 1985). In the early stages MVD is mostly asymptomatic, but can be diagnosed through auscultation and echocardiography (Borgarelli and Haggstrom 2010). In most breeds MVD develop fairly slowly and it takes years for the disease to evolve to a stage where signs of heart failure appear. Since it has a late onset in most dog breeds, many dogs will not show symptoms during their lifetime (Borgarelli, Savarino et al. 2008).

In Cavalier King Charles Spaniels MVD is associated with early onset and is believed to have a high genetic factor. In a study MVD has been shown to be present in 59% of CKCS 4 years old or older (Darke 1987).

Clinical signs and diagnosis

The most common clinical signs of MVD is coughing, exercise intolerance, restlessness during the night and dyspnea. All of the clinical signs are vague and not specific for MVD, but are rather signs of respiratory problems.

In CKCS in Denmark the presence and severity of MVD in CKCS is determined through auscultation and echocardiography. The regurgitation is rated in the four-chamber-view from the left side in color Doppler as the percentage of regurgitation from the left ventricle into the left atrium (%jet). It is also in the four-chamber-view the presence of prolapse is determined most easily.

The stressor of cardiac disease on cortisol

An elevation in hair cortisol concentration in a 3-month period prior to acute myocardial infarction in humans has been shown (Pereg, Gow et al. 2011). This supports that there is a correlation between cortisol concentration and cardiac disease, but it also seems to indicate that an elevation of cortisol concentration is causing the cardiac event and not the other way around.

High cortisol levels in plasma has also been shown to be a predictor of mortality in humans with chronic heart failure (Güder, Bauersachs et al. 2007)

Psychological acute stress in humans, have been shown to increase the risk for cardiac events like thrombosis, arrhythmia or mechanical cardiovascular events. Chronic stress seems to accelerate the atherosclerotic process (Brotman, Golden et al. 2007).

In humans it would be relevant to presume that the knowledge of having a cardiovascular disease might be a stressor in itself and might therefore elevate the cortisol level.

In dogs, however, it is difficult to know if they are aware of anything being wrong. If the disease is far progressed, the dog might experience several symptoms, but whether this will influence their stress level can only be speculated upon.

In humans there seems to be a definite effect of high cortisol on cardiac disease, but to the my knowledge an effect of heart disease on cortisol have not been shown.

Tissue injury is one of the ways to activate the HPA axis. Since in MVD, the atrial wall can be damaged by regurgitation (Fox 2012), which might cause an activation of the axis and rise in cortisol concentration.

Purpose of the thesis

To summarize, cortisol can be used as a biomarker for stress. Hair cortisol can be used as a measure of chronic stress, showing the cortisol concentration over the last weeks to years. Plasma and saliva cortisol can be used as an acute measure of cortisol, showing the last minutes to an hour. Stressors can elevate the cortisol concentration by activating the HPA axis.

The purpose of this thesis is to determine whether a correlation between the different stages of MVD in CKCS and both acute and chronic levels of cortisol exist. Some of the confounding factors for measurement of cortisol in hair will also be evaluated and cortisol levels in different matrices will be compared.

Materials and Methods

Animals

The animals used in this study were 58 dogs (13 Beagles and 45 CKCS) enrolled in a project about MVD screening. The dogs were chosen for the screening project several years ago and have been examined twice previously. The Beagles were enrolled in the initial study a control group to be sure that there were dogs with no or mild MVD.

The samples were taken during the second follow up examination in the screening project. Information about number of dogs in the home, frequency of baths etc. was obtained from the owners (see Appendix A).

All owners signed a Consent Form.

Definition of groups of MVD

The dogs were divided into 5 groups according to their disease status. The disease status was determined by echocardiography and defined by how large the regurgitation was from the left ventricle into the left atrium. It was measured in the percentage of the atrium that was filled by the regurgitant jet. The groups were defined as control (group 1), jet < 20% (group 2), jet = 20-50% (group 3), jet > 50% without clinical signs (group 4) and jet > 50% with clinical signs (group 5). Group 1 consisted of all the Beagles no matter what their disease status were. In groups 2-5 there were only CKCS. A jet of less than 20% was considered a normal find in healthy dogs.

Saliva sampling

Saliva was collected using Salimetrics Oral Swabs (Salimetrics Europe, Suffolk, UK). The swabs were placed in one side of the buccal cavity and held there for 10-15 seconds (depending on the compliance of the dog). The procedure was repeated in the other side to ensure better absorption of saliva. Plastic gloves were worn throughout the procedure to avoid contamination of the sample. The swabs were placed in storage tubes and stored at -20°C directly after sampling until analysis.

Blood sampling

Blood samples were collected from *V. Jugularis* by the veterinarian in charge of the examination, while one of the veterinary students restrained the dog. The owner was standing next to the veterinarian where the dog could easily see him/her.

There were taken five blood samples from each dog - one for this study, three for other scientific projects and the last for a gene bank. The blood samples for this study were the third of the five blood samples collected from each dog. The blood samples were always taken in the same order.

The blood samples for plasma cortisol analysis were collected in 2 ml Vacutainer tubes containing the anticoagulant K2 EDTA (3.6 mg) (Becton Dickinson A/S, Albertslund, Denmark). Samples were centrifuged at 1700xG for 10 min directly after sampling and plasma was stored at -20°C until analysis.

Hair sampling

Hair samples were collected at the very end of the examination. One hair sample per hair color in each dog was collected. Only one hair sample per dog was collected in the beagles because the hair changed color along the length of the hair in some hairs and it was not possible to collect a hair sample with only one hair color. The hair samples were cut with scissors as close to the skin as possible. Only the 2.5 cm closest to the skin were included in the sample to make the hair length uniform between dogs, since a shave/reshave approach was not used. A shave/reshave approach could not be used here since it was not possible for the owners to bring their dogs more than once. The hair samples were stored in paper envelopes until analysis.

Extraction from hair

Extraction of cortisol from hair samples was performed using the procedure of the laboratory (Comin, Prandi et al. 2011). Briefly, the hair samples were weighed and washed in isopropanol for 2x3 min. They were extracted in methanol for 16 hours at 37°C. Afterwards, the methanol was evaporated and the samples were rehydrated in RIA buffer.

Extraction from saliva

It was not necessary to perform an extraction of cortisol from the saliva samples. The samples were defrosted and centrifuged at 1500 x G for 15 min. The saliva could be loaded directly on the plates for RIA analysis.

Extraction from blood

To extract cortisol from the plasma samples, the samples were defrosted and 50 µl was mixed with diethylether. The samples were centrifuged and 1 ml from the top was transferred to another tube and evaporated. The samples were rehydrated with RIA buffer.

Cortisol assay

The extracted samples were loaded in plates prepared for RIA analysis. See Comin et al. 2011 for more information.

Statistical analysis

All statistical analysis was performed in RStudio (version 0.98.501). The linear mixed models were performed with the package lme4.

Data transformation was performed when data did not meet assumptions for parametric tests.

A linear mixed model was used to analyze data concerning the effect of disease group, plasma cortisol concentration, gender, age, hair color and other dogs in the home on hair cortisol concentration.

A linear model was used to compare saliva cortisol concentration and age with plasma cortisol concentration.

An ANOVA was used to compare the frequency of baths with hair cortisol concentration.

A paired t-test was used to compare cortisol concentration in black/white and red/white hair from the same dogs.

An unpaired t-test was performed to analyze the effect of perceived stress level and age on plasma cortisol.

Results

Hair samples were taken from all of the 58 dogs. 10 of the CKCS were of the Tricolor variety (black and white hair on the main body) and 15 were of the Blenheim variety (red and white), therefore a total of 83 hair samples were collected.

Blood samples were obtained from all dogs except 1 (a Beagle).

Saliva samples were collected from all the dogs but 9 of the samples did not contain any saliva when thawed and centrifuged (5 CKCS and 4 Beagles). Another 16 of the samples did not contain enough saliva for analysis of 2x30 μ l (14 CKCS and 2 Beagles). There they were analyzed with a lower amount on the plates. The last 33 saliva samples had sufficient saliva (26 CKCS and 7 Beagles).

Disease status

Hair cortisol concentration was not affected by disease group (linear mixed model on transformed data, $\text{Chi}^2(3)=2.21$, $p>0.05$). See Fig. 1.

Disease group 1 was not included, to eliminate any influence of breed on the result. Also, some of the dogs in group 1 had a jet > 20%, making group 2 better as a control group.

When looking at fig. 1 there seems to be a tendency for cortisol to rise going from group 2 through group 3 to group 4. The reason that group 5 is not higher than group 4 might be because some of the dogs were medicated.

Analysis was also tried without disease group 5 because most of the dogs in this group were medicated, but this did not make disease group a significant factor of hair cortisol concentration.

Comparison between the animals in group 5 that were medicated and those that were not, did not show a significant difference either.

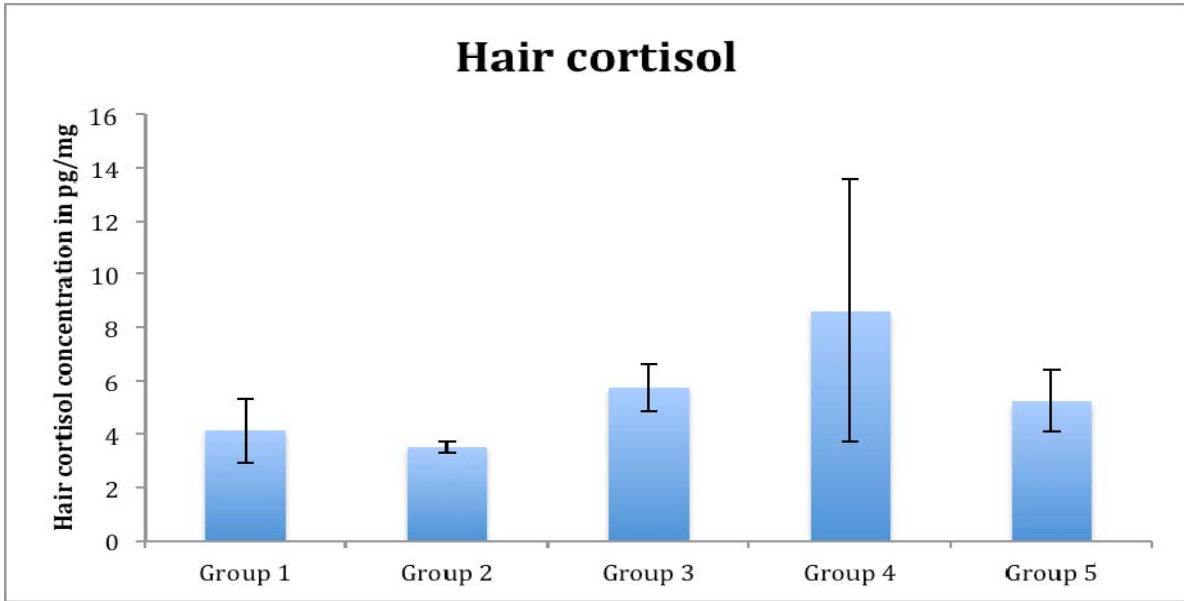


Figure 1. Mean \pm SE for each disease group. Here shown with original data. Analysis was performed on transformed data and only on group 2-5. One Beagle was excluded since its hair cortisol concentration was more than 3 standard deviations away from the mean.

Neither saliva or plasma cortisol concentration were significantly influenced by disease group (ANOVA with transformed data, $F(3,36)=0.55$ $p>0.05$ and ANOVA, $F(3,41)=0.91$ $p>0.05$, respectively). See Fig. 2 and 3.

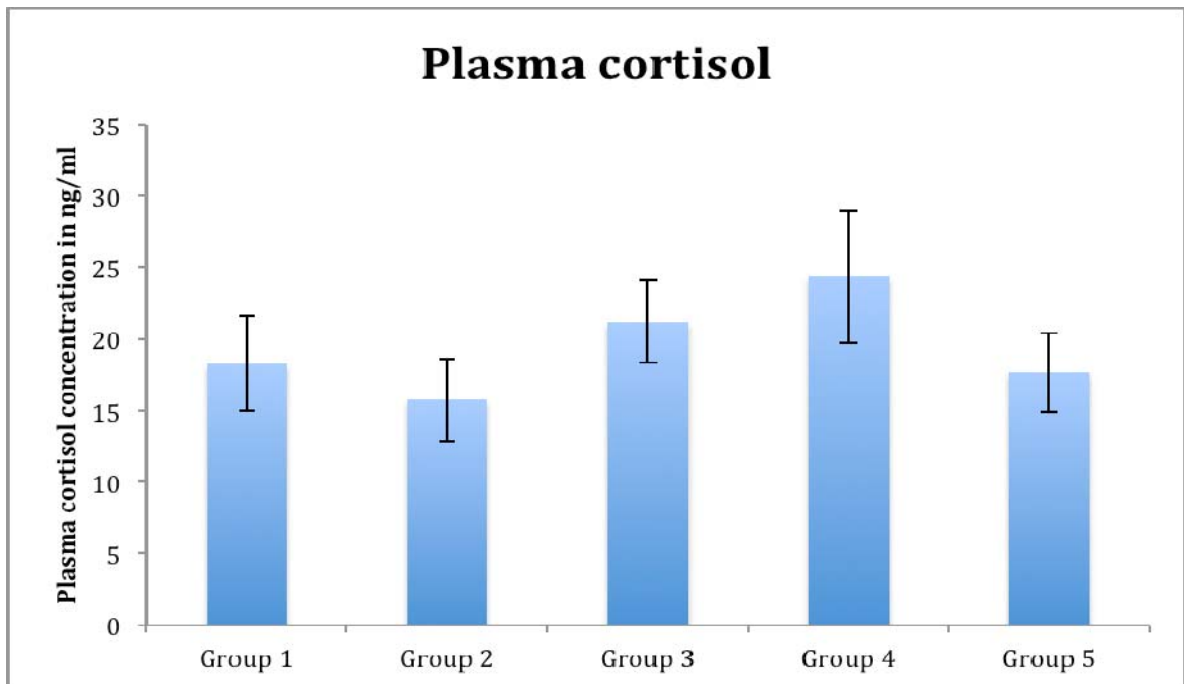


Figure 2. Mean \pm SE for each disease group. Here shown with original data. Analysis was performed on transformed data and only on group 2-5.

In plasma cortisol, a similar tendency to the one observed in hair cortisol is noted. There is an increase in plasma cortisol from group 2 to group 3 to group 4 and then a fall from group 4 to group 5.

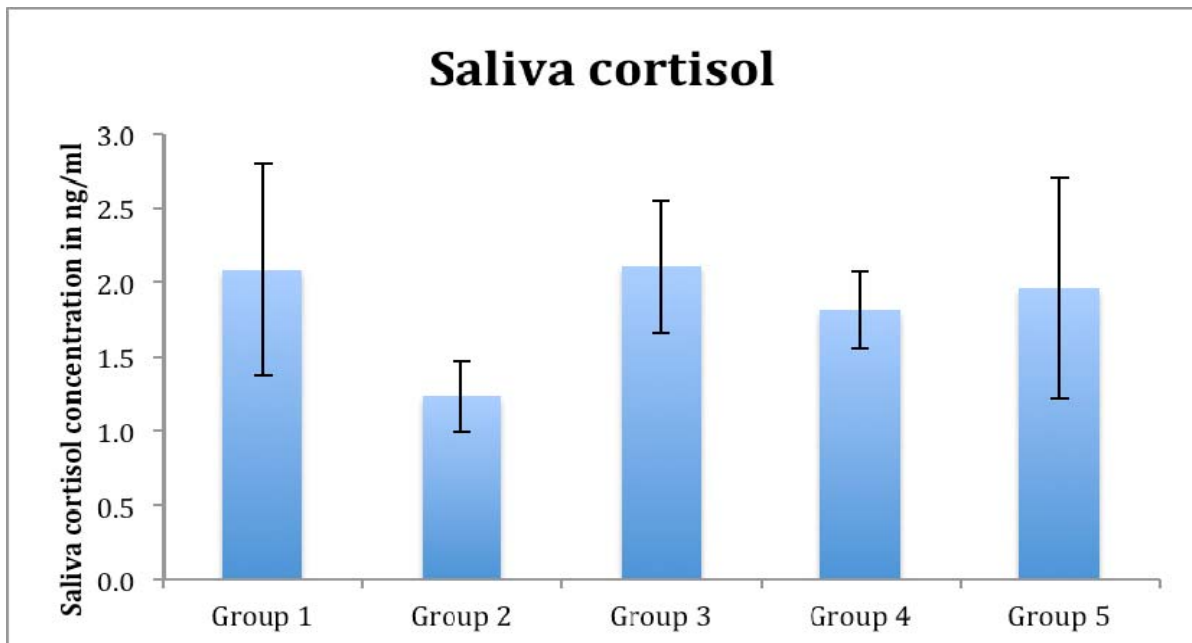


Figure 3. Mean \pm SE for each disease group. Here shown with original data. Analysis was performed on transformed data and only on group 2-5.

The same tendency as in hair and plasma cortisol is not observed in saliva cortisol. Though there is an increase in saliva cortisol from group 2 to group 3, the mean saliva cortisol in group 4 is lower than group 3.

The reason a similar tendency is not observed in saliva, might be because the dataset is incomplete (saliva samples from several of the dogs were missing as mentioned previously). Fig. 4 is made to illustrate whether the dogs without a saliva sample are the ones with high or low plasma cortisol concentration.

As seen in fig. 4 the dogs without saliva samples in group 4 have a much higher mean plasma cortisol concentration than the dogs in the same group with a saliva sample. When depicting the means of each group using only the dogs with a corresponding saliva sample, the same pattern as in fig. 3 is seen (see fig. 5). As with the saliva cortisol, a rise is seen from group 2 to group 3, with a fall in group 4 and roughly the same mean in group 4 and 5.

There might therefore have been the same pattern with the saliva cortisol concentration, if analysis could have been made on all samples.

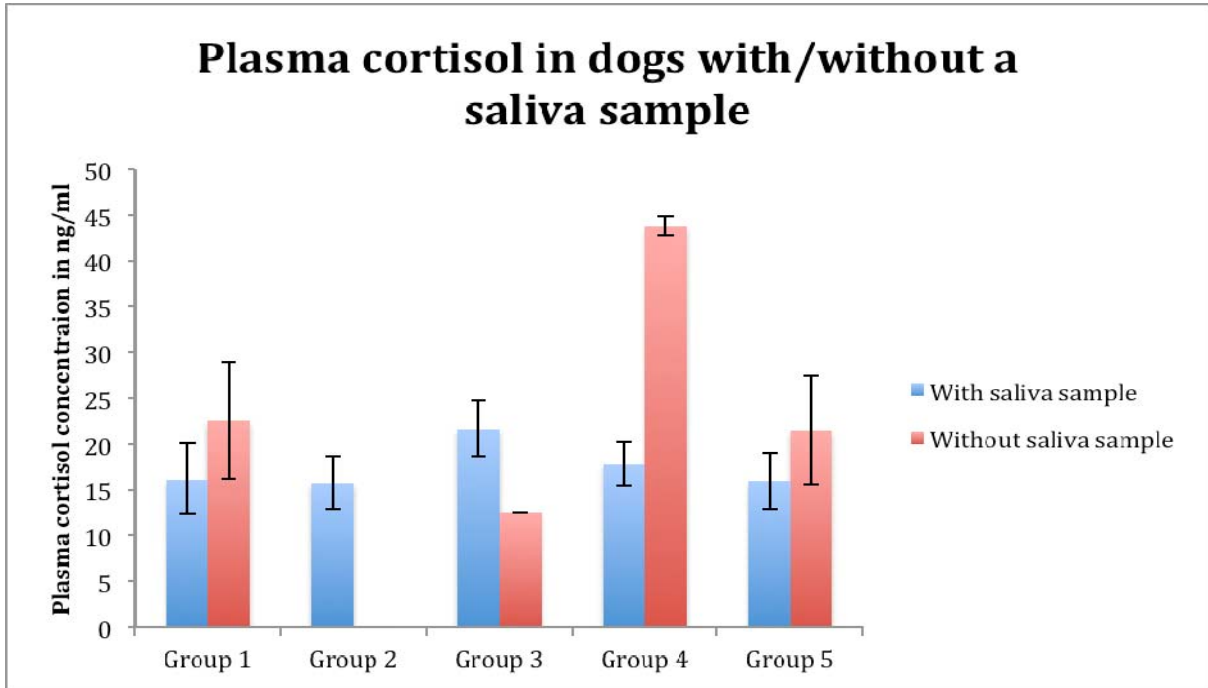


Figure 4. Mean \pm SE for the dogs in each group with and without a corresponding saliva sample. All the dogs in group 2 had a saliva sample.

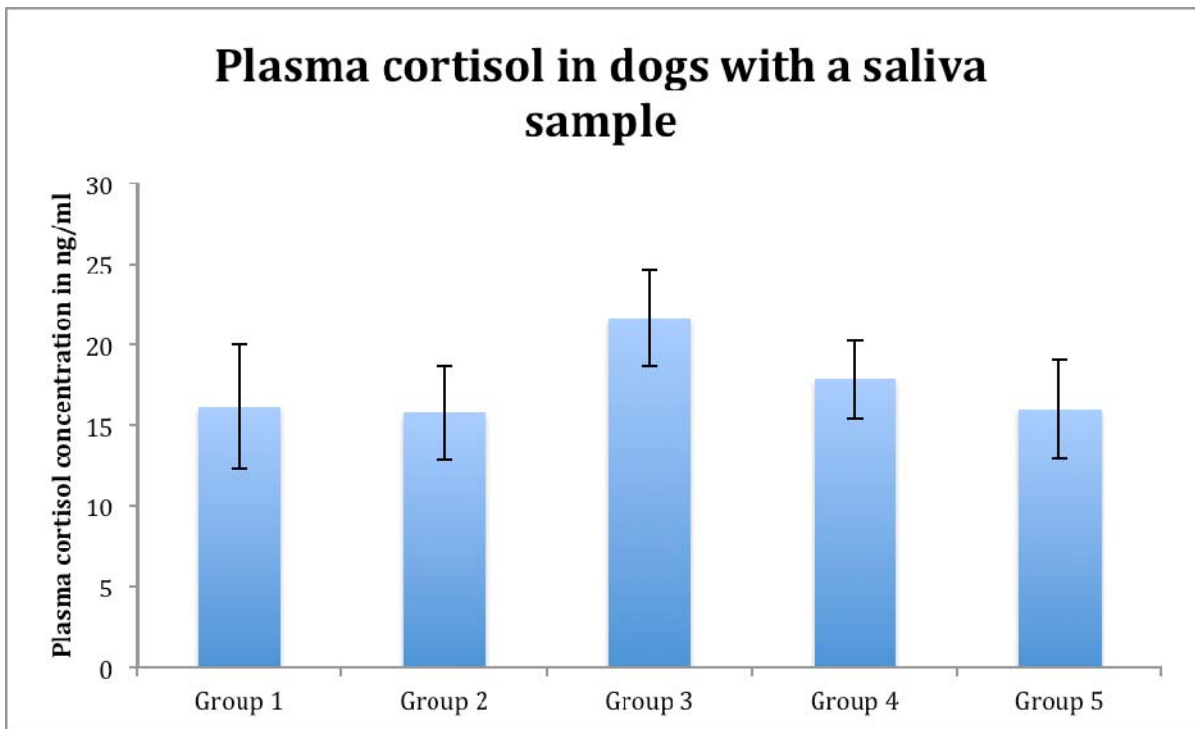


Figure 5. Mean \pm SE for each disease group, but only the dogs with a corresponding saliva sample has been included. Here shown with original data.

Hair color

In Tricolor CKCS there was a significant difference between the cortisol concentration in the black and white hair (paired t-test on transformed data, $t(9)=4.33$ $p<0.01$) (See Fig. 6).

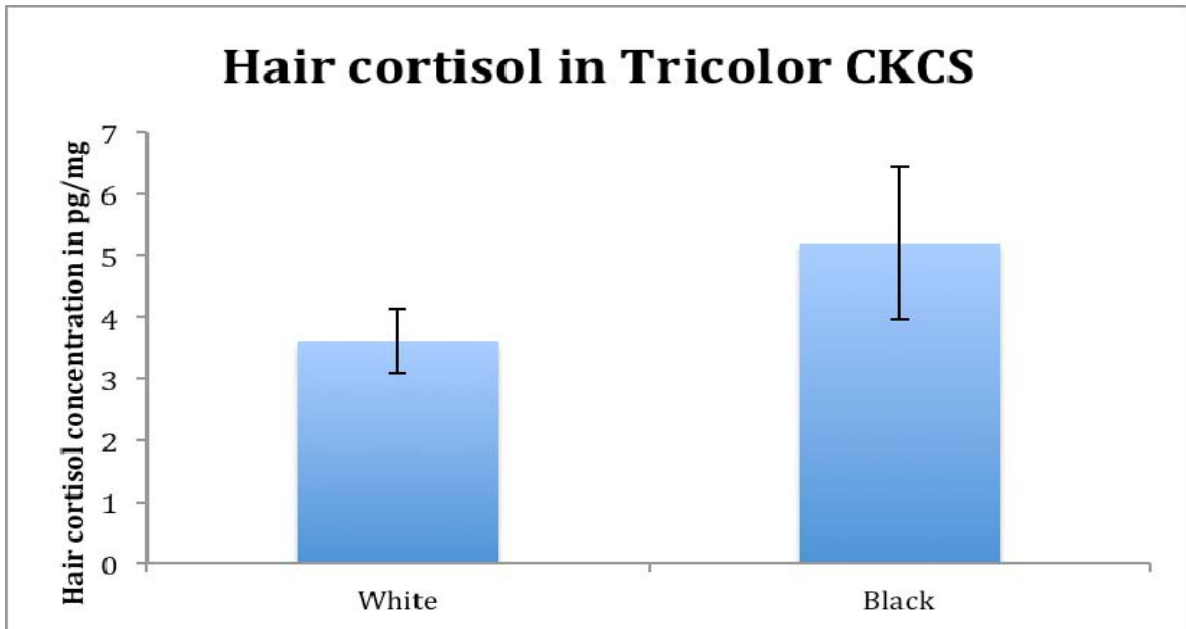


Figure 6. Mean \pm SE for each color. Here shown with original data. Analysis was performed on transformed data.

There was no significant difference between white and red hair in Blenheim CKCS (paired t-test, $t(14)=-0.43$ $p>0.05$).

When compared across all CKCS, black hair color affected hair cortisol concentration (linear mixed model on transformed data, $\text{Chi}^2(2)=15.61$, $p<0.001$), elevating it by about 1.35 pg/mg hair ± 1.06 (SE) (see Fig. 7).

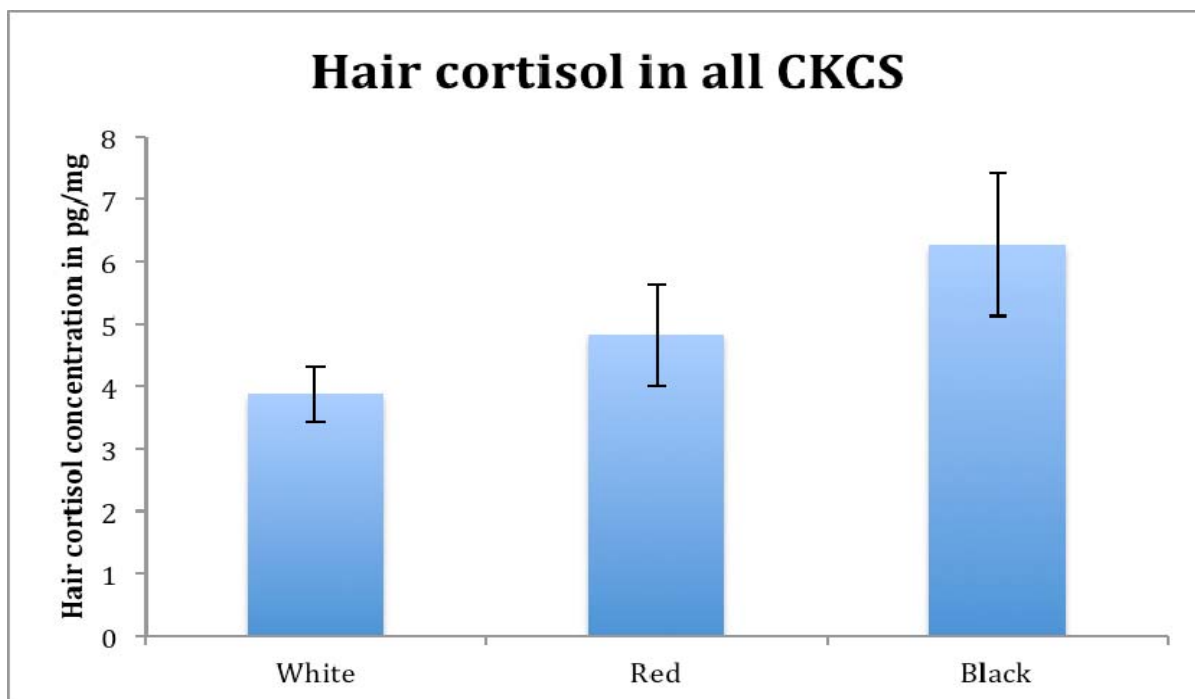


Figure 7. Here shown with original data. Analysis performed on transformed data. One of the red dogs was excluded since its hair cortisol concentration was more than 3 standard deviations away from the mean.

Other factors on hair cortisol

There was no significant effect of plasma cortisol, age, gender, frequency of wash or other dogs in the home on hair cortisol concentration.

Analysis for significance of wash on hair cortisol concentration was performed on transformed data with samples from dogs with white hair only, to remove the effect of color (ANOVA, $F(2,20)=0.31$, $p>0.05$).

Saliva/Plasma

Saliva cortisol is significantly correlated with plasma cortisol (linear model on transformed data, $F(1,46)=25.24$, $r^2=0.34$, $p<0.01$). Analysis was performed on data both with and without the values from the saliva samples where there were not enough to charge 2x30 microliter on the plates. Excluding those values did not change the result, so they were included. Both CKCS and Beagles were included, but dogs with missing saliva samples were excluded.

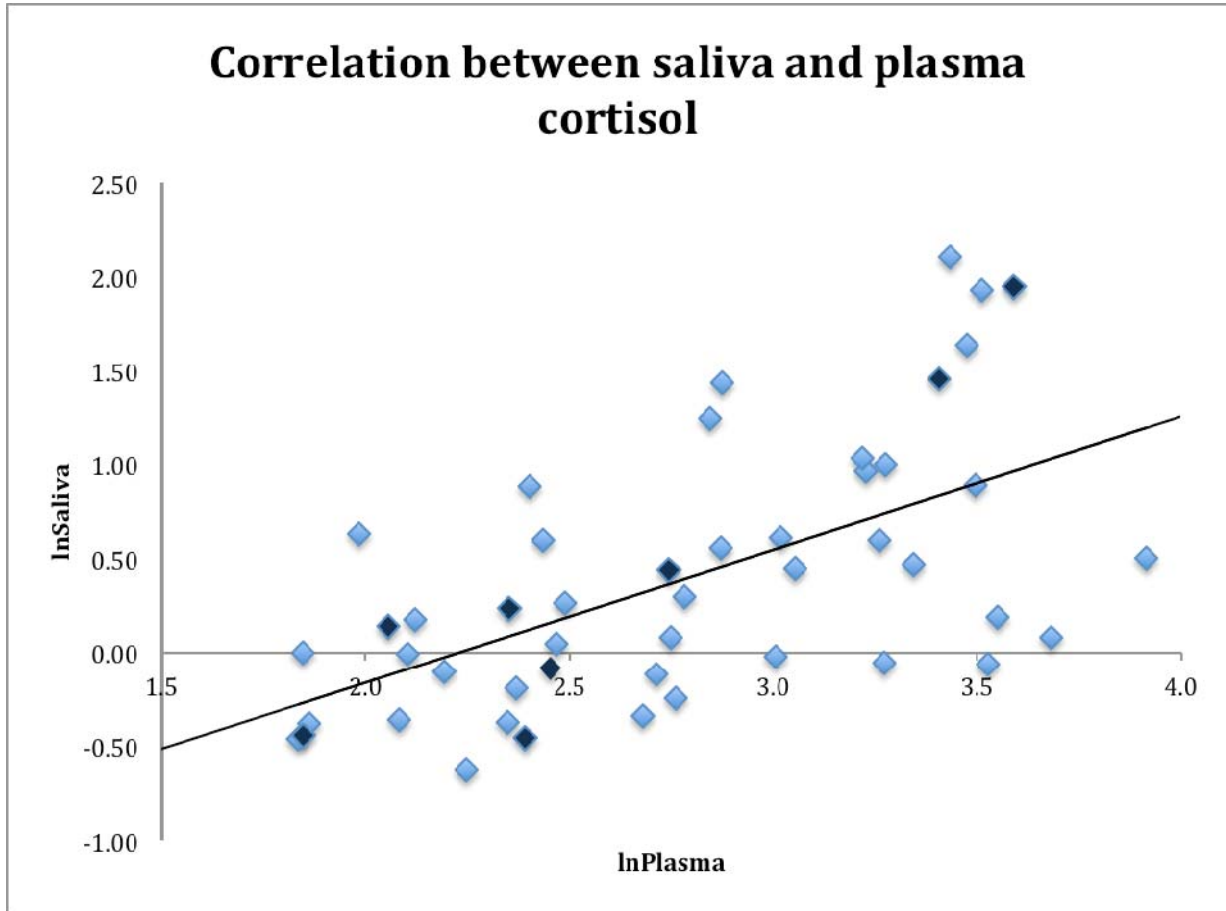


Figure 8. Linear model of the correlation between saliva and plasma cortisol levels. Shown with transformed data. The dark blue data points are Beagles. The light blue data points are CKCS.

Perceived stress level

The dogs were scored for their stress level when the blood sample was taken. All CKCS except 1 was either in the category “0% stressed” or “25% stressed”.

An unpaired t-test was performed to investigate if stress level had an influence on plasma cortisol concentration. No significant difference between the two groups was observed (unpaired t-test, $t(42)=0.99$ $p>0.05$).

Discussion

The purpose of this thesis was to determine whether MVD in CKCS has an influence on cortisol concentration, either acute or chronic. Also, to compare acute and chronic measures of cortisol concentration and to evaluate some of the other factors that might influence hair cortisol concentration.

Furthermore a comparison with reference values from other studies and a discussion of the animals used this study and the experimental design will be made.

Mitral valve disease

There is no significant difference of cortisol concentration, either in plasma, saliva or hair, in the different MVD groups.

There seems to be a tendency toward higher cortisol concentration, both in plasma and hair, with a more severe MVD, at least within group 2-4.

The lower mean cortisol concentration in group 5 (both in plasma and hair) might be caused by the relatively small sample sizes. Potentially the cortisol concentration in group 5 could be lower because some of the dogs in the group was medicated to cope with their heart disease. When comparing the mean of the medicated and unmedicated dogs in group 5, this did not seem to be the case.

With a larger sample size there might be a significant positive correlation between disease status and cortisol concentration, both acute and chronic.

If MVD would influence the HPA axis there would most likely be seen an effect on hair cortisol concentration, since MVD is a chronic disease. Plasma and saliva cortisol are both acute measures of cortisol and more natural fluctuations in cortisol concentration would be expected, thereby masking the effect of MVD. On the other hand, a chronic disease like MVD might amplify the HPA axis response of the dog to other stressors.

Acute versus chronic cortisol concentration

There was a significant correlation between plasma and saliva cortisol concentration, but no significant correlation was found between hair and plasma cortisol.

A significant correlation between plasma cortisol and hair cortisol was not to be expected, since one is a measure of acute stress and the other of chronic stress. On the other hand a significant positive correlation between saliva and plasma cortisol was to be expected, as they are both measures of acute stress. Since the saliva sample were taken before the blood sample, the stressful situation of sampling and being at the examination could potentially influence the blood sample more than the saliva sample.

Hair color and other factors

There was a significant difference between the cortisol concentration in black hairs and in hairs of other colors (white or red) in CKCS.

The fact that there is an influence of hair color on cortisol concentration in hair is supported by previous studies (Bennett and Haysen 2010, Macbeth, Cattet et al. 2010). Like this study, Macbeth et al. found that darker hair had a higher cortisol content than lighter hair within the same animal (bears were used in their study). Bennett et al., however, found that black dogs had less hair cortisol than nonblack dogs. This was between different dogs however, but still a very different result. More studies should be made to test the effect of hair color on hair cortisol concentration.

The lacking influence of gender or age on hair cortisol compares to results from other studies (Bennett and Haysen 2010). That there was not found any effect of washing on the hair cortisol concentration might simply be because the dogs were not washed often enough to observe an effect.

Regarding the factor of “other dogs in the home” this did not seem to have any influence on hair cortisol concentration either.

Reference values

The mean cortisol concentrations measured in this study seem to correlate well with other studies of cortisol in dogs.

In two different dog studies the mean value of cortisol was 2.10 pg/mg hair (Accorsi, Carloni et al. 2008) and 10.88-12.63 pg/mg hair (Bennett and Haysen 2010), respectively. In the study by Accorsi et al. 2008 the hair was minced into 1-3mm fragments whereas Bennett et al. 2010 powdered the hair. It makes sense that the highest values are found in the study where the hair is powdered, since the other process might not get all the cortisol from the core of the hair shaft. Another difference in extraction method is the use of isopropanol wash before the extraction to remove steroid contamination on the hair shaft. This was used by Bennett et al. 2010, but not Accorsi et al. 2008. If an isopropanol wash is not used, the cortisol concentration could be falsely elevated. The difference in extraction methods makes it difficult to compare the cortisol values found in different studies. It would be beneficial for future studies if a consensus was made concerning extraction method.

The mean value of hair cortisol (in CKCS) in this study was 5.71 ± 0.95 (SE) pg/mg hair. The extraction method used in this study is more similar to the method used by Accorsi et al. 2008, excepting the fact that they did not use an isopropanol wash. Since they only used healthy dogs, it is not surprising that the mean hair cortisol concentration in our study is higher.

Mean plasma cortisol has been measured at 58.5 ± 16 (SE) nmol/l (Kemppainen and Sartin 1984) and 86.2 ± 10.03 (SE) nmol/l (Vincent and Michell 1992). Using a converting factor (Schenk, Nachreiner et al. 2011) it is 21.20 ± 5.79 (SE) ng/ml and 31.24 ± 3.64 (SE) ng/ml. In our study the mean plasma cortisol in CKCS was 20.13 ± 1.70 (SE) ng/ml. Both of the studies mentioned used RIA. The mean plasma cortisol concentration in this study seems to correlate well with the values in the other two studies.

Mean saliva cortisol in this study (in CKCS) was 1.88 ng/ml ± 0.26 (SE) ng/ml. In other studies with dogs the control group had a mean salivary cortisol concentration of 1.7 ± 0.6 (SE) ng/ml (Dreschel and Granger 2009) and 1.92 ± 0.31 ng/ml (Vincent and Michell 1992) (using the

same converting from Schenk et al. 2011). There seem to be a consensus in the saliva cortisol concentration.

Animals

Most of the dogs used in the study are of an advanced age, the mean age being 8.93 ± 0.28 (SE) years. Because of this, it is quite possible that many of the dogs suffer from minor health issues that have not been diagnosed, but might influence their cortisol level, e.g. mild arthritis. This could be a possible confounding factor.

The reason for using Beagles as controls in the screening project was to be sure to have some dogs with a low jet percentage, even when the dogs were screened at follow up examinations years after the initial examination. Beagles are not as likely as CKCS to develop MVD, but are dogs of roughly the same size. Since there were actually several dogs in group 2 (CKCS with jet<20%), there was no reason to use group 1 thereby eliminating breed as a factor. Also not all of the Beagles had a jet<20% better qualifying group 2 as control group.

Experimental design

Overall, I believe the methods used for this study were successful. However, there are some issues that should be kept in mind and could be improved upon.

Ideally, the time of sampling should be the same for all the dogs, e.g., they should all be sampled in the morning. Since it was not possible to influence the time of sampling in this study, it is important to have in mind that some variation can be caused by different sampling times. Even if it was possible to take the samples at the same time of the day there might still be some day-to-day variation (Matsuda, Yamaguchi et al. 2012). In the study by Matsuda et al. there was a day-to-day variation of between 43.1 and 100.3% in human saliva samples.

The saliva and plasma samples in this study were plotted against the time of day they were sampled, but no obvious pattern was observed (see Appendix B).

This is only in the case of blood and saliva. There should not be any influence of sampling time on hair cortisol, since this is a retrospective measure of cortisol and the cortisol produced at

the time around sampling will be incorporated in the part of hair shaft, which is still within the hair follicle.

Some of the samples that seemed to be saturated at sampling did not contain any saliva at all when defrosted and centrifuged. Other samples contained a very small amount. Something in the process must have gone wrong even though the procedure recommended from the manufacturer was followed (Salimetrics 2008). To ensure that there was enough saliva, the saliva samples should have been centrifuged directly after sampling. Alternatively, two samples should be collected from each dog to ensure adequate sampling material.

Some of the saliva samples had enough saliva to be analyzed, but not enough to load 2x30 μl . To test if this had any influence on the result, statistical analysis were performed both with and without the samples with less than 60 μl . No significant difference was observed and the samples were therefore included in results stated in this thesis.

The hair samples were not clipped in smaller pieces or powdered, because it was not the procedure at the laboratory where analysis was performed. Difference in extraction method will influence the amount of cortisol measured. This should not cause any problems when comparing dogs in the same study since the same procedure have been used, but it should be kept in mind when comparing with cortisol concentrations in other studies.

Conclusion

In conclusion, MVD did not have a significant influence on neither acute nor chronic cortisol concentrations. A non-significant tendency for higher hair and plasma cortisol in more severe MVD was noted. There was a significant correlation between plasma and saliva cortisol concentration, but no significant correlation was found between hair cortisol concentration and either saliva or plasma cortisol concentration.

Black hair color had a significant effect on hair cortisol concentration, elevating it compared to red or white hair color. No significant effect of gender, age, other dogs in the home or frequency of washing on hair cortisol concentration was found.

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Appendix A

Dog No	Name	Breed	Gender	Intact	Age	Other dogs	Frequency of baths	Medicine for heart condition	Date	Time (Blood sample)	Time (Saliva sample)	MVD group	Plasma cortisol (ng/ml)	Saliva cortisol (ng/ml)	Hair cortisol White hair (pg/mg)	Hair cortisol - red hair (pg/mg)	Hair cortisol - black hair (pg/mg)	Hair cortisol - Beagles (pg/mg)
1	Trygve	CKCS	M	Yes*	10.7	Yes	Never	Yes^	30.10.2013	10:23	10:10	Group 5	10.46	0.69	NA	NA	4.66	NA
2	Sverren	CKCS	M	Yes	6.25	Yes	Never	No	30.10.2013	11:17	11:14	Group 2	6.33	1.00	2.94	NA	3.16	NA
3	Herbert	CKCS	M	Yes*	5.9	Yes	Never	No	30.10.2013	12:03	11:58	Group 3	7.27	1.88	NA	NA	6.53	NA
4	Albert	CKCS	M	Yes	7.33	Yes	1-2/month	No	30.10.2013	13:26	13:20	Group 3	6.27	0.63	NA	1.80	NA	NA
5	Thorkild	CKCS	M	Yes	6.75	Yes	Never	No	30.10.2013	14:10	14:06	Group 2	8.97	0.90	NA	3.07	NA	NA
6	Fridtjof	CKCS	M	Yes	6.33	Yes	Never	No	30.10.2013	14:20	14:15	Group 3	17.18	3.47	NA	4.27	NA	NA
7	Donna	CKCS	F	No	12.5	Yes	1-2/month	Yes^	31.10.2013	11:20	11:10	Group 3	26.08	1.82	3.98	NA	4.66	NA
8	Emily	CKCS	F	Yes	6	Yes	1-2/month	No	31.10.2013	12:24	12:20	Group 3	39.72	1.08	3.23	5.03	NA	NA
9	Oscar	CKCS	M	Yes	8.5	Yes	1-2/month	No	31.10.2013	14:06	14:00	Group 3	20.22	0.98	3.75	3.28	NA	NA
10	Selina	CKCS	F	Yes	10.9	Yes	1-2/year	No	01.11.2013	12:25	12:00	Group 2	25.17	2.62	3.14	NA	4.37	NA
11	Athos	CKCS	M	Yes	5.83	No	1-2/year	No	01.11.2013	13:28	13:20	Group 3	24.98	2.81	12.95	9.66	NA	NA
12	Nana	CKCS	F	Yes	6.25	No	1-2/year	No	01.11.2013	14:35	14:30	Group 2	26.34	0.94	3.50	3.91	NA	NA
13	Athene	CKCS	F	Yes	8.17	Yes	1-2/year	No	01.11.2013	15:55	15:50	Group 3	8.35	1.19	NA	NA	18.95	NA
14	Freja	CKCS	F	Yes	10.9	Yes	1-2/year	Yes^	01.11.2013	15:45	15:40	Group 5	15.09	0.89	NA	NA	16.74	NA
15	Cassi	CKCS	M	Yes	11.67	Yes	1-2/year	Yes^	04.11.2013	9:24	9:20	Group 4	44.91	NA	NA	NA	6.13	NA
16	Dagmar	Beagle	F	Yes	7.4	Yes	1-2/year	No	04.11.2013	10:36	10:34	Group 1	11.63	0.92	NA	NA	NA	17.70

Appendix A

Dog No	Name	Breed	Gender	Intact	Age	Other dogs	Frequency of baths	Medicine for heart condition	Date	Time (Blood sample)	Time (Saliva sample)	MVD group	Plasma cortisol (ng/ml)	Saliva cortisol (ng/ml)	Hair cortisol White hair (pg/mg)	Hair cortisol - red hair (pg/mg)	Hair cortisol - black hair (pg/mg)	Hair cortisol - Beagles (pg/mg)
17	Jette	Beagle	F	No	9.4	Yes	1-2/year	No	04.11.2013	10:29	10:26	Group 1	30.15	4.28	NA	NA	NA	402.00
18	Charlie	CKCS	M	Yes	8.5	No	1-2/year	No	04.11.2013	12:43	12:36	Group 4	26.41	2.71	4.36	NA	5.26	NA
19	Rommy	CKCS	M	No	9.83	Yes	1-2/year	No	08.11.2013	9:23	9:18	Group 4	20.42	1.84	NA	4.08	NA	NA
20	Thor	CKCS	M	No	7.4	Yes	1-2/year	No	08.11.2013	11:20	11:15	Group 3	30.97	8.22	NA	5.14	NA	NA
21	Lady	CKCS	F	Yes	9.33	Yes	1-2/year	No	08.11.2013	13:22	13:18	Group 3	50.13	1.64	4.14	4.86	NA	NA
22	Kikkie	CKCS	F	Yes	6.08	Yes	1-2/month	No	11.11.2013	10:05	9:55	Group 3	10.70	0.82	NA	7.79	NA	NA
23	Olivia	Beagle	F	No	11	Yes	1-2/year	No	11.11.2013	No	13:25	Group 1	NA	1.35	NA	NA	NA	3.38
24	Biker	Beagle	M	Yes	6.75	Yes	1-2/year	No	11.11.2013	13:42	13:35	Group 1	6.35	0.64	NA	NA	NA	3.54
25	Tjalfe	Beagle	M	Yes	10.5	Yes	1-2/year	No	13.11.2013	9:18	9:14	Group 1	10.94	0.63	NA	NA	NA	3.01
26	Galaxy	Beagle	F	No	6.25	Yes	1-2/year	No	13.11.2013	9:22	9:10	Group 1	10.52	1.26	NA	NA	NA	2.51
27	Dina	CKCS	F	Yes	5.9	Yes	1-2/month	No	13.11.2013	11:40	11:36	Group 3	6.44	0.68	NA	3.39	NA	NA
28	Panik	CKCS	M	Yes	7.08	Yes	1/week	No	13.11.2013	11:47	11:42	Group 5	17.65	1.74	3.59	3.66	NA	NA
29	Max	CKCS	M	Yes	9.33	Yes	1-2/month	Yes^	13.11.2013	11:53	11:50	Group 5	33.00	2.43	NA	NA	5.05	NA
30	Kikki	CKCS	F	Yes	9.9	Yes	1-2/month	No	13.11.2013	12:52	12:48	Group 2	15.61	1.08	5.12	4.06	NA	NA
31	See Me	CKCS	F	Yes	10.5	Yes	1/week	Yes^	13.11.2013	12:55	12:53	Group 5	33.44	6.87	4.10	3.98	NA	NA
32	Chikita	CKCS	F	No	8.25	Yes	1-2/year	No	18.11.2013	11:23	11:21	Group 5	13.32	NA	7.53	NA	16.17	NA

Appendix A

Dog No	Name	Breed	Gender	Intact	Age	Other dogs	Frequency of baths	Medicine for heart condition	Date	Time (Blood sample)	Time (Saliva sample)	MVD group	Plasma cortisol (ng/ml)	Saliva cortisol (ng/ml)	Hair cortisol White hair (pg/mg)	Hair cortisol - red hair (pg/mg)	Hair cortisol - black hair (pg/mg)	Hair cortisol - Beagles (pg/mg)
33	Diana	CKCS	F	Yes	7.67	No	1-2/year	No	18.11.2013	11:28	11:26	Group 4	11.03	2.41	NA	62.61	NA	NA
34	Fenja	CKCS	F	Yes	5.9	Yes	1-2/year	No	18.11.2013	11:33	11:31	Group 2	12.06	1.30	3.03	NA	4.36	NA
35	Candy	CKCS	F	Yes	6.08	Yes	1-2/month	No	21.11.2013	9:24	9:10	Group 3	12.54	NA	3.47	3.60	NA	NA
36	Dino	CKCS	M	Yes	9.75	No	1-2/year	No	21.11.2013	11:19	11:16	Group 3	8.19	0.99	2.94	NA	4.06	NA
37	Trunte	CKCS	F	Yes	11.9	No	1-2/year	No	21.11.2013	11:15	11:10	Group 4	11.79	1.04	3.13	2.44	NA	NA
38	Tindra	CKCS	F	No	8.83	No	1-2/month	No	22.11.2013	10:57	10:54	Group 3	17.73	4.18	NA	22.54	NA	NA
39	Mjølner	CKCS	M	Yes	9.5	No	1-2/year	No	22.11.2013	11:54	11:50	Group 3	8.31	1.59	NA	6.81	NA	NA
40	Siggi	CKCS	M	Yes	9.33	Yes	1-2/month	No	22.11.2013	12:54	12:52	Group 3	32.31	5.14	NA	2.72	NA	NA
41	Olivia	CKCS	F	Yes	8.33	No	1-2/year	No	22.11.2013	14:05	14:03	Group 4	21.21	1.56	2.15	2.46	NA	NA
42	Gyda	Beagle	F	No	10.08	Yes	1-2/year	No	22.11.2013	15:00	14:57	Group 1	20.47	NA	NA	NA	NA	1.73
43	Oliver	CKCS	M	Yes	12.08	No	1-2/year	Yes^	23.11.2013	11:19	11:15	Group 5	14.60	0.71	1.68	NA	2.77	NA
44	Mitzi	CKCS	F	No	11.83	No	1-2/month	Yes^	23.11.2013	12:22	12:13	Group 3	34.82	1.21	NA	NA	3.32	NA
45	Helge	Beagle	M	Yes	15	Yes	No	No	24.11.2013	12:57	12:53	Group 1	7.81	1.15	NA	NA	NA	1.88
46	Sine	Beagle	F	Yes	8.25	Yes	No	No	24.11.2013	11:34	11:32	Group 1	36.28	6.99	NA	NA	NA	2.71
47	Leah	Beagle	F	Yes	8.25	Yes	No	No	24.11.2013	11:25	11:25	Group 1	38.60	NA	NA	NA	NA	2.54
48	Marianne	Beagle	F	No	9.33	Yes	No	No	24.11.2013	11:17	11:10	Group 1	23.66	NA	NA	NA	NA	3.34

Appendix A

Dog No	Name	Breed	Gender	Intact	Age	Other dogs	Frequency of baths	Medicine for heart condition	Date	Time (Blood sample)	Time (Saliva sample)	MVD group	Plasma cortisol (ng/ml)	Saliva cortisol (ng/ml)	Hair cortisol White hair (pg/mg)	Hair cortisol - red hair (pg/mg)	Hair cortisol - black hair (pg/mg)	Hair cortisol - Beagles (pg/mg)
49	Smarty	CKCS	F	No	13.9	Yes	1-2/month	No	25.11.2013	9:53	9:50	Group 2	15.81	0.78	2.66	2.50	NA	NA
50	Teddy	CKCS	M	No	8.08	Yes	1-2/month	No	25.11.2013	9:45	9:42	Group 5	9.46	0.54	1.88	3.14	NA	NA
51	Toffée	CKCS	F	No	9	No	1-2/month	Yes^	25.11.2013	11:28	11:25	Group 5	18.31	NA	3.15	2.71	NA	NA
52	Holly	CKCS	F	Yes	7	Yes	1-2/month	No	25.11.2013	13:38	13:09	Group 3	8.03	0.70	2.04	NA	2.66	NA
53	Timmy	CKCS	M	Yes	11.58	No	1-2/year	Yes^	27.11.2013	12:45	12:35	Group 5	11.41	1.81	NA	NA	2.98	NA
54	Hera	CKCS	F	No	12.4	Yes	1-2/year	No	27.11.2013	9:19	9:16	Group 4	16.14	1.34	4.26	3.87	NA	NA
55	Lady	CKCS	F	Yes	10.17	Yes	1-2/month	No	27.11.2013	14:50	14:44	Group 3	33.98	0.94	4.38	NA	4.46	NA
56	Victor	Beagle	M	Yes	9.58	Yes	1-2/year	No	27.11.2013	16:31	16:28	Group 1	7.46	NA	NA	NA	NA	3.66
57	Oscar	Beagle	M	Yes	8.75	Yes	1-2/year	No	27.11.2013	16:24	16:21	Group 1	15.54	1.55	NA	NA	NA	3.41
58	Sveske	CKCS	F	Yes	8.25	Yes	1-2/month	No	28.11.2013	11:17	11:07	Group 4	42.78	NA	NA	NA	2.97	NA

*=Cryptorchid

^=Furex, Vetmedin, Furosemid, Prilactone, Gabapentin, Fortekor. One or a combination.

Appendix B

