Hair cortisol as a new biological marker for stress in cats

Author: D.W. Zijlstra

Supervisor: H.S. Kooistra

Abstract

Cortisol concentrations have been measured in a variety of sources, including blood, saliva, urine and faeces. A novel method is hair cortisol measurement, which is non-invasive, reflects long-term circulating cortisol concentrations, is insensitive to momentary stressors and is less sensitive to individual circadian patterns. The objectives of the present study were to create reference ranges for cats *(Felis catus)* and to investigate whether breed, coat colour, age and different locations on the body have a significant influence on hair cortisol values. A total of 106 cats from three different breeds (European Shorthair, Burmese and Maine Coon) were used. Cortisol concentration of hair samples from the ventral neck and abdomen was determined using High-performance liquid chromatography (HPLC). There was a significant difference (ANOVA, $p = 0.004$) between the breeds for abdominal hair; between the Burmese and the Maine Coon ($p = 0.003$) and between the European Shorthair and the Burmese ($p = 0.049$). Overall reference ranges were established at 1.38-4.95 pg/mg and 1.38-5.64 pg/mg for the ventral neck and abdomen cortisol value, respectively. Because of the significant difference between breeds, reference ranges were also established for each breed for abdomen hair cortisol. Coat colour did not show a significant difference, while age had a significant ($p = 0.038$) but weak correlation with neck hair cortisol ($R^2 = 0,032$). After making three age groups, this significant difference was no longer seen. Thanks to this study, it is shown that hair cortisol can be determined reliably and with the established reference ranges it will possible to study factors that may result in long-term stress in cats in a non-invasive way.

Introduction

Cortisol, the primary glucocorticoid of most mammals, is a key component of the physiological response to stress (Bennett 2010). In the short term, cortisol may be adaptive and will help the animal to cope with stressful situations. However, chronic exposure to high cortisol levels is maladaptive and may result in impairment of growth and development. It can also elicit endocrine, metabolic, autoimmune or psychiatric disorders (Charmandari 2005).

Over time, several ways to measure the cortisol concentration have been established. It can be measured in blood (Beerda, Schilder et al. 1999, Vincent, Michell 1992), saliva (Davenport, Tiefenbacher et al. 2006, Dreschel, Granger), urine (Stephen, Ledger 2006) and faeces (Accorsi 2008). Blood, saliva and urine reflect systemic cortisol concentrations at the time of sampling and shortly before sampling, but they do not provide an indication of the cortisol concentration with regard to the past (Thomson 2010). Glucocorticoid levels in blood or saliva reflect point samples that are highly variable within the same individual and are strongly affected by, for example, time of day or food intake. Besides that, the sampling method can be stressful, thus raising the cortisol concentration (Bennett 2010). Faeces and urine provide values for some longer periods of hypothalamic-pituitary-adrenocortical (HPA) activity (up to 24 h for urine), but both methods are less sensitive to acute variability in cortisol concentrations than blood or saliva and assess an unknown accumulation of

cortisol (Bennett 2010). In addition, corticosteroids may not be uniformly distributed within faecal samples and are inversely related to faecal output (Hayssen 2002).

Hair sampling is the newest method to quantify chronic cortisol secretion, both in humans and animals. This method is non-invasive, reflects long-term circulating cortisol concentrations, is insensitive to momentary stressors (including handling during sampling) and is less sensitive to individual circadian patterns (Bennett 2010). Hair cortisol analysis presents a complementary means of monitoring stress, capturing systemic cortisol exposure over longer periods of time (Russell, Koren et al. 2012). Cortisol measured in hair may originate either directly from the hair follicle or systemically from the hypothalamic-pituitary-adrenal axis. Hair may also function as a storage area for cortisol, but little is known about the storage or circulation of cortisol in hair (Accorsi 2008). In humans a difference was found between distal and proximal hair segments (Bennett 2010). So far no difference was found in hair segments in rhesus monkeys (Davenport, Tiefenbacher et al. 2006). In dogs, hair cortisol concentrations have been shown to correlate positively to cortisol in saliva and faeces, validating hair for basal cortisol measurements (Accorsi 2008, Bennett 2010). Moreover, a study in 2006 by Davenport et al. reported increased values of hair cortisol in response to environmental stress in 20 rhesus monkeys (Davenport, Tiefenbacher et al. 2006). The mean levels were significantly different before and after relocation, from 81 to 130 picogram per milligram (pg/mg). After one year, the cortisol values had dropped to baseline level. The same pattern was seen in salivary cortisol. Therefore, this study showed that hair cortisol values are valuable for monitoring chronic stress.

Bennett et al. described that species differ in cortisol rhythms and secretions, as well as the stress response. They argue that, for that reason, every species requires their own validation (Bennett 2010). Subsequently, it is possible that there could be a difference between different breeds of cats.

Research in deer, mice and dogs, revealed a difference in hair cortisol concentration with differences in coat colour (Bennett 2010, Hayssen 2002). The hormones which control cortisol and pigment are both pro-opiomelanocortin derivatives and the same families of melanocortin receptors are involved (Bennett 2010).

Another potential source of variation in hair cortisol concentration is the anatomical location from which the hair sample is obtained. MacBeth et al. found that the cortisol content of guard hair from the neck area was significantly higher than for samples obtained from the shoulder, rump and abdomen of grizzly bears. Also, the cortisol value of guard hair was significantly higher than for the undercoat (Macbeth 2010).

The main goal of this study is to examine if cortisol can be determined in hair of cats. If so, the goal is to determine reference values. The hypotheses of our study are four-fold: (1) we can determine cortisol in hairs of cats; (2) we expect no significant difference between cat breeds; (3) coat colour has an influence on the value of hair cortisol; and (4) there will be no significant difference in hair cortisol of the different places of the body.

Material and methods

Animals

A total of 106 cats from three different breeds were included in the study. All of the cats were healthy, adult cats (older than one year of age). The cats that were used, were cats homed in their own environment, at the owners' homes, so there were no changes in their daily routine or diet. In addition, four foundlings cats from a shelter were included. Three different breeds were used to investigate the influence of breed on the hair cortisol levels. Most of the cats that are held in households in the Netherlands are of the breed European Shorthair, so that breed was chosen as 'standard'. In addition,

the breeds of Maine Coon and Burmese were chosen. Of the European Shorthair breed 51 cats were sampled, 23 males (2 intact and 21 neutered) and 28 females (5 intact and 23 neutered). Age varied from 1 till 17 years of age (mean = 6.8 years). Of the Burmese breed 26 cats were sampled, 9 males (3 intact and 6 neutered) and 17 females (9 intact and 8 neutered). Age varied from 1 till 11 years (mean = 4.6 years). Of the Maine Coon breed 28 cats were sampled, 10 males (1 intact and 9 neutered) and 18 females (2 intact and 16 neutered). Age varied from 1 till 16 years (mean = 5.6 years). Of one cat all information lacked, so the cortisol values were only included when measuring the mean and to set the reference range (N =1). For other measurements, this cat was excluded.

Via a survey additional information was gathered. With this information, multiple household types were identified and it became clear whether the cat only stays inside the house or also has the opportunity to go outside the house. Also the breed, colour of the hair sample, family composition, behavioural changes, use of medication and presence of other animals were included in the survey (see addendum). The goal of this survey was to gather information to investigate if some of these variables could have a significant correlation with the value for cortisol.

Hair sampling

Due to the fact that cats throughout the country were used, the sampling time took several months, from February 2015 till June 2015. For the sampling of the hairs, two different places were used. The ventral neck area and the area between the nipples, on the ventral abdomen. The goal for measuring cortisol on these two different places, was to see if there was a difference between the outcomes. For the measurement, a minimum amount of one gram was needed. This was obtained by using a (human) shaving device (Babyliss E709E). Depending on the thickness of the fur about 10 cm^2 was shaven, directly from the skin surface. Between each sample the shaver was cleaned, using a toothbrush and a 70% alcohol wipe, to avoid cross-contamination. Hair samples were labelled and stored in plastic bags at room temperature until analysis.

Cortisol hair analyses

Extraction of cortisol and cortisone were performed at the laboratory of clinical chemistry in the University Medical Centre Groningen (UMCG). Hair cortisol concentrations were measured with online-solid phase extraction combined with isotope dilution LC-MS/MS. 50mg of hair was weighted in polypropylene containers and washed with dichloromethane. About 50 steel balls were added, together with 50 µL deuterated cortisol in 1450 µL methanol. The hair was pulverized using the Ball Mill (Retsch, MM400). The suspension was centrifuged and the supernatant was evaporated to dryness at 45°C using a nitrogen flow. The samples were resuspended in 10% methanol. Subsequently, 50µL was injected onto the LC-MS/MS system. For this method the intra-run coefficients were 9.3% at 3.5 pg/mg, 6.2% at 8.8 pg/mg and 4.3% at 30.3 pg/mg. The inter-run coefficients were 6.1% at 3.4 pg/mg, 5.5% at 8.8 pg/mg and 6.0% at 10.6 pg/mg. The lower limit of quantitation for hair cortisol was 0.70 pg/mg hair. For hair, we use the concentration of cortisol in picogram per milligram (pg/mg) of hair.

Statistical Analysis

All statistical analyses were conducted using SPSS Statistics version 20 (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp.). Performing multiple two-way ANOVA with a Bonferroni correction (for multiple comparisons).

Regression analysis was used for comparison of the continuous variables age with hair cortisol. General Linear Models (GLM) were used to compare individual attributes (hair colour, sex, neuter status, age groups, household status and the presence of other animals). To investigate if colour has a correlation with hair cortisol, two different groups were made; ‘white coat colour’ and ‘non-white coat’ colour.

The data came from two sources: owner-derived data via the survey (age, household status, neuter status, presence of other animals) and experimenter-measured data (cortisol concentrations in hair).

To get a good overview of the average value of cortisol in hair, outliers with an unreasonable high or low outcome, were removed from the dataset. A high pregnant cat scored very high (mean = 3.11 including all cats, pregnant cat = 42.11 pg/mg) in the sample hair abdomen cortisol. So this cat got excluded, for that particular sample. For the sample hair neck, this cat was included. In the samples hair neck, no cats were removed from the population. After this selection, further detection of outliers was performed by using both the Z-scores and the outlier labelling rule by using a factor 2.2 (Hoaglin, Iglewicz 1987). These methods are comparible. Both showed the same outliers and can therefore be seen as a valid method for detecting outliers. Values of Z-scores above 3.29 (99.9% of z-scores lie between -3.29 and +3.29) were seen as an outlier. By using this methods, several cats were excluded. In the sample abdomen hair two cats were excluded and two were missing (N = 102). In the sample neck hair two cats were excluded (N = 104).

All the results are expressed as: mean (±) standard deviation (and the range) with the concentration of cortisol in picogram per milligram (pg/mg) of hair. $P < 0.05$ was considered significant.

Results

Breed and cortisol

Mean cortisol from neck area hair were 2.66 ± 0.90 pg/mg (range = 1.00-5.10 pg/mg); 2.70 ± 0.87 pg/mg (range = 1.39-5.17 pg/mg); 2.31 ± 0.64 pg/mg (range = 1.37-4.18 pg/mg) for the European shorthair (N = 50), Burmese (N = 26) and Maine Coon (N = 27), respectively.

Mean cortisol from hair of the abdomen area were 2.51 ± 1.02 pg/mg (range = 0.91-6.33 pg/ml); 3.40 ± 2.45 pg/mg (range = 1.45-9.64 pg/mg); 2.01 ± 0.89 pg/mg (range = 0.94-5.20 pg/mg) for the European shorthair (N = 48), Burmese (N = 25) and Maine Coon (N = 28), respectively.

There was no significant difference between the breeds according to the ANOVA with regard to the outcomes from the neck area hair ($p = 0.152$). There was, however, a significant breed-related difference in the abdomen hair ($p = 0.004$). ANOVA with Bonferroni correction revealed no significant difference in abdominal hair cortisol between European Shorthair and Maine Coon cats ($p = 0.483$), but there was a significant difference between Burmese and Maine Coon ($p = 0.003$) and between the European Shorthair and the Burmese cats ($p = 0.049$).

Age and cortisol

To determine if age had a significant correlation with the values of cortisol, a regression analysis was performed. Age and neck hair cortisol had a significant correlation ($p = 0.038$) with a R^2 of 0,032 (N = 100, missing N = 4). Abdomen hair cortisol did not have a significant correlation with age.

Three different age groups were defined, to make this more clinical applicable. Junior: age 1-2 years (N = 26), mature: age 3-9 years (N = 54), senior: 10 years and higher (N = 20) (table 1). ANOVA, with a Bonferroni correction, showed no significant differences between the groups ($p = 0.112$). ‘Young’ and ‘mature’ ($p = 1$), ‘young’ and ‘senior’ ($p = 0.257$), ‘mature’ and ‘senior’ ($p = 0.132$).

Age groups	Mean in pg/mg	Std. Deviation	N
Junior age: 1-2 years	2,5050	0,85128	26
Mature age: 3-9 years	2,4900	0,82462	54
Senior age: 10 years and higher	2,9335	0,81497	20
Total	2,5826	0,84003	100

Table 1: Age groups

Hair colour and cortisol

After making groups of 'white coat colour' (N = 53 and N = 38 in neck and abdomen, respectively) and 'non-white coat colour' (N = 47 and N = 62 in neck and abdomen, respectively), no significant difference was seen in all outcomes.

Reference ranges

To create a reference range, the 95% interval was determined. Boundaries were set at 2.5% and 97.5% of the dataset. Normal 95% interval could not be used, due to the fact the data was not normal distributed. The overall reference ranges of the neck and abdomen cortisol value are 1.38-4.95 pg/mg and 1.38-5.64 pg/mg, respectively. Due to the fact there was a significant difference between the breeds in the abdomen cortisol sample, for the different breeds all reference ranges were calculated. The reference ranges were 1.19-4.71 pg/mg; 1.45-9.60 pg/mg and 0.97-4.33 pg/mg for the European shorthair (N = 48), Burmese (N = 25) and Maine Coon (N = 28), respectively.

Cortisol and individual subject attributes

A few stray cats, that were found and homed in a shelter, were included. One of these cats was excluded in the abdomen hair samples, with a value 6.5 times (17.18 pg/mg) the average. The other stray cats had values within the calculated reference range. Also a pregnant cat (59 days at that moment) was excluded from the dataset because of the high abdomen hair value. The value of cortisol in the pregnant cat was 42.11 pg/mg, a factor 16 above the average (2.60 pg/mg).

Individual attributes (sex, neuter status, household status, type cat) were compared with the cortisol levels. No significant differences were found in these variances. But pairwise comparisons showed several trends, that has to be investigated in further research.

Abdomen hair cortisol sample

Males (mean = 2.42 pg/mg, N = 38) had a lower hair cortisol value than females (mean = 2.59 pg/mg, N = 61), but this difference was not significant (p = 0.50). This does not take into account the neuter status. Looking at neuter status (sex not taken into account), non-neutered cats (mean = 2.84 pg/mg, N = 20) had a higher cortisol value than neutered cats (mean = 2.45 pg/mg, N = 79), also not a significant difference (p = 0.57). There is a difference between single-cat household (mean = 2.65 pg/mg, N = 19) or a multi-cat household (mean = 2.46 pg/mg, N = 77). Indoor/outdoor cats (mean = 2.87, N = 49) have a higher value compared with indoor cats (mean = 2.14, N = 19) and cats that have the availability to a demarcated garden (mean = 2.16, N = 28) (p = 0.057). The presence of a dog in the household (mean = 2.16, N = 9) shows a lower value than the absence of one or more (mean = 2.53, N = 87), with a non-significant p-value of 0.1.

Neck hair cortisol sample

Males (mean = 2.47 pg/mg, N = 38) had a higher hair cortisol value than females (mean = 2.56 pg/mg, N = 61), but this difference was not significant (p = 0.54). This does not take into account the neuter status. Looking at neuter status (sex not taken into account), non-neutered cats (mean = 2.59 pg/mg, N = 20) had a higher cortisol value than neutered cats (mean = 2.50 pg/mg, N = 79), also not a significant difference (p = 0.48). There is a difference between single cat household (mean = 2.77 pg/mg, N = 23) or a multi cat household (mean = 2.46 pg/mg, N = 77*),* but this difference was not significant (p = 0.45). Indoor/outdoor cats have a higher value (mean = 2.61, N = 53) compared with indoor cats (mean = 2.48, N = 19) and cats that have the availability to a demarcated garden (mean = 2.39, N = 28), but these differences were nog significant (p = 0.46). The presence of a dog in the household (mean = 2.66, N = 9) shows a higher score than the absence of one or more (mean = 2.47, N = 87) with a non-significant p-value of 0.17.

Discussion

Evaluating the method used in this research, cortisol extraction from hair using HLPC can be seen as a useful method to measure cortisol in hairs of cats. HLPC was already used in other studies for measuring cortisol in hairs in both animals and humans. The results of this study indicate that cortisol concentrations can be measured in cat hair. The next challenge was to create reference ranges for this species. This could possibly be used in further studies to see whether cats are stressed or not, for example in shelters or in multicat-households. In order to measure a 'basal' cortisol value, almost all cats were sampled at home, where no changes were made in their daily routine or diet. So daily stressors are included. Despite the fact that hair cortisol reflects a long-term concentration of cortisol, these measures were made.

In order to determine whether cortisol reflects stress, the results in some of the cats are very informative. Bonanni et al. described that stress levels can rise during pregnancy and lactation, due to the increased energy demands during these periods (Bonanni, Cafazzo et al. 2007). This is supported by several studies that show that glucocorticoid levels are higher in humans, intact female mammals and primates (Setchell, Smith et al. 2008, Gesquiere, Khan et al. 2008, Cavigelli, Dubovick et al. 2003, Schradin 2008, Kirschbaum, Tietze et al. 2009). As one of the sampled cats was pregnant and was 16 times higher than average, this suggests that pregnancy in cats is associated with higher cortisol secretion, which may mean stress. The fact that the level of cortisol in one of the stray cats was increased, suggests that this cat had a higher stress level than other cats. More research is needed to support this conclusion.

The elevated samples in the pregnant and stray cat, were both samples taken from the abdomen. Looking at the differences between the levels at the abdomen and the neck hair sample in these cats, something striking can be seen. The values of the pregnant cat were 42.11 and 4.84 pg/mg for the abdomen and neck hair, respectively. The values of the stray cat were 17.18 and 4.57 pg/mg for the abdomen and neck hair respectively. A possible conclusion can be, that the level of cortisol from the abdomen shows values of cortisol of (sub)acute period, while the level of cortisol neck shows a more constant value and can thus be seen as a value of stress over a longer period back in time. If so, the neck hair cortisol is a more clinical relevant value. Less fluctuations will be seen and it will be in that way more likely to use as an indicator for, possibly, chronic stress. More research is needed to evaluate this suggestion and to see what correlation between the values of the different locations is.

Another possible explanation could be that growth speed of hair in different areas of the body differ from each other. Gunaratnam et al. investigated that there is a different growth speed of hair in dogs (Gunaratnum, Wilkinson 1983). In this study, it has been shown that between individuals and between different locations, growth speeds varied. It was most rapid in the shoulder region, followed by the

flank and then the forehead region. For cats, a research like this has never been done. This could influence the storage function of cortisol in hair. While the mechanism of incorporation of cortisol in hair is not clearly defined, it has been said that it can be incorporated via the blood capillaries during formation as well as through sebum and sweat secretions into the hair follicle after formation (Salaberger, Millard et al. 2016, Henderson , Cone 1996). Passive (or active) diffusion from blood to the hair follicle is thought to be the primary mechanism (Gow, Thomson et al. 2010). Further research is needed to investigate this supposition.

Looking at the references ranges between the different breeds, the clinical relevance of these values must be evaluated. As the cortisol level during pregnancy in one cat shows a high value of 42.11 pg/mg and the baseline level of cortisol in abdomen hair of the different breeds differ significant from 2.01 pg/mg in Maine Coon till 3.40 pg/mg in Burmese cats, the clinical relevance of this significant difference has to be taken in doubt. To validate the calculated reference values of the neck (1.38-4.95 pg/mg) and abdomen (1.38-5.64 pg/mg), further research is needed. In cats with proven stress (as measured in saliva or faeces and with behavioural tests), the hair cortisol concentration must be measured. These cats must have a higher value of hair cortisol than the upper boundary set in this research. Then these reference values (without taking breed into account) can be used.

Another possibility to make the reference ranges clinical applicable, is to make only use of the neck hair cortisol. Then the reference range can be used for all breeds, instead of using different frame of references for different breeds. And as an additional benefit, it could perhaps be that neck hair cortisol will be less sensitive for fluctuations, as suggested earlier. Further research is needed to look at the stability of the value of neck hair cortisol between the different breeds.

Looking at the age and the value of cortisol, a significant correlation was seen when age is set out against neck hair cortisol (and no significant correlation is seen when looking at abdomen hair cortisol). The low correlation coefficient of 0.032 means that it is a very weak correlation, despite the fact that it is significant. To date, no research has been done on whether older cats have higher cortisol levels in comparison with younger cats. A possible explanation could be that storage of cortisol in older animals is different than in young animals. To make this more clinical applicable, age groups were made. Distinct age groups in cats are not well defined. The American Animal Hospital Association has developed ‘Life Stages Guidelines (Amy Hoyumpa Vogt, Ilona Rodan 2010). Partly based on these guidelines and the number of cats available, own age groups were made. Making different age groups, the significant correlation between age and cortisol disappears. This makes the significant correlation in the first place less strong. As mentioned before, the clinical relevance must be evaluated. If the difference is so small, no effects to the usage of the frame of reference must be carried out, then age is not of clinical relevance.

Due to the fact that the sampling period was several months and the extraction of cortisol from the hair lasted longer, storage has been done in plastic bags. Steroids are stable at room temperature for over a year (Davenport, Tiefenbacher et al. 2006, Macbeth 2010, Raul, Cirimele et al. 2004, Sauvé 2007) so specific storage was not needed.

Due to the fact a lot of different cats were used, specific selection on colours was not performed. This resulted in a very diverse range of colours. From black to white, red, brown, crème, tricolour and brown. To investigate whether colour has a correlation with hair cortisol, groups were made. As the group of ‘white-coat cats’ had more or less the same size as ‘non-white-coat cats’, this selection was made. No significant difference is shown. Perhaps, with different sample sizes, other outcomes will be seen.

In conclusion, thanks to this study an applicable reference range for hair cortisol in cats is set. The clinical usage must be evaluated after more research is done. Furthermore, a significant difference is seen between two different anatomical places on the body. To interpret this difference, the value of a 'stressed cat' must be measured. If this value is much higher than the set baseline value, this significant difference is less strong. The same applies to the significant correlation between age and the value of cortisol. Further research therefore is needed.

Acknowledgement

We want to thank the 'Stichting Overleg Platform Nederlandse Cat Fancy' for the generous financial support of the study. Without them this study would not have been possible. Furthermore, we want to thank all the generous cat owners who volunteered their pets for our study.

References

ACCORSI, P.A., 2008. Cortisol determination in hair and faeces from domestic cats and dogs. *General and comparative endocrinology,* **155**(2), pp. 398-402.

AMY HOYUMPA VOGT, ILONA RODAN, 2010. AAFP–AAHA Feline Life Stage Guidelines. *Journal of the American Animal Hospital Association,* **46**, pp. 70-85.

BEERDA, B., SCHILDER, M.B., BERNADINA, W., VAN HOOFF, J.A., DE VRIES, H.W. and MOL, J.A., 1999. Chronic stress in dogs subjected to social and spatial restriction. II. Hormonal and immunological responses. *Physiology & Behavior,* **66**(2), pp. 243-254.

BENNETT, A., 2010. Measuring cortisol in hair and saliva from dogs: Coat color and pigment differences. *Domestic animal endocrinology,* **39**(3), pp. 171-180.

BONANNI, R., CAFAZZO, S., FANTINI, C., PONTIER, D. and NATOLI, E., 2007. Feeding-order in an urban feral domestic cat colony: relationship to dominance rank, sex and age. *Animal Behaviour,* **74**(5), pp. 1369-1379.

CAVIGELLI, S.A., DUBOVICK, T., LEVASH, W., JOLLY, A. and PITTS, A., 2003. Female dominance status and fecal corticoids in a cooperative breeder with low reproductive skew: ring-tailed lemurs (Lemur catta). *Hormones and behavior,* **43**(1), pp. 166-179.

CHARMANDARI, E., 2005. Endocrinology of the stress response. *Annual Review of Physiology,* **67**, pp. 259-284.

CONE, E.J., 1996. Mechanisms of drug incorporation into hair. *Therapeutic drug monitoring,* **18**(4), pp. 438-443.

DAVENPORT, M.D., TIEFENBACHER, S., LUTZ, C.K., NOVAK, M.A. and MEYER, J.S., 2006. Analysis of endogenous cortisol concentrations in the hair of rhesus macaques. *General and comparative endocrinology,* **147**(3), pp. 255-261.

DRESCHEL, N.A. and GRANGER, D.A., Physiological and behavioral reactivity to stress in thunderstorm-phobic dogs and their caregivers. *Applied Animal Behaviour Science,* **95**(3), pp. 153-168.

GESQUIERE, L.R., KHAN, M., SHEK, L., WANGO, T.L., WANGO, E.O., ALBERTS, S.C. and ALTMANN, J., 2008. Coping with a challenging environment: Effects of seasonal variability and reproductive status on glucocorticoid concentrations of female baboons (Papio cynocephalus). *Hormones and behavior,* **54**(3), pp. 410-416.

GOW, R., THOMSON, S., RIEDER, M., VAN UUM, S. and KOREN, G., 2010. An assessment of cortisol analysis in hair and its clinical applications. *Forensic science international,* **196**(1–3), pp. 32-37.

GUNARATNAM, P. and WILKINSON, G., 1983. A STUDY OF NORMAL HAIR-GROWTH IN THE DOG. *Journal of small animal practice,* **24**(7), pp. 445-453.

HAYSSEN, V., 2002. Fecal corticosteroids in agouti and non-agouti deer mice (Peromyscus maniculatus). *Comparative biochemistry and physiology.Part A, Molecular & integrative physiology,* **132**(2), pp. 439-446.

HENDERSON, G.L., Mechanisms of drug incorporation into hair. *Forensic science international,* **63**(1), pp. 19-29.

HOAGLIN, D.C. and IGLEWICZ, B., 1987. Fine-Tuning Some Resistant Rules for Outlier Labeling. *Journal of the American Statistical Association,* **82**(400), pp. 1147-1149.

KIRSCHBAUM, C., TIETZE, A., SKOLUDA, N. and DETTENBORN, L., 2009. Hair as a retrospective calendar of cortisol production—Increased cortisol incorporation into hair in the third trimester of pregnancy. *Psychoneuroendocrinology,* **34**(1), pp. 32-37.

MACBETH, B.J., 2010. Hair cortisol concentration as a noninvasive measure of long-term stress in free-ranging grizzly bears (Ursus arctos): Considerations with implications for other wildlife. *Canadian journal of zoology,* **88**(10), pp. 935-949.

RAUL, J., CIRIMELE, V., LUDES, B. and KINTZ, P., 2004. Detection of physiological concentrations of cortisol and cortisone in human hair. *Clinical biochemistry,* **37**(12), pp. 1105-1111.

RUSSELL, E., KOREN, G., RIEDER, M. and VAN UUM, S., 2012. Hair cortisol as a biological marker of chronic stress: Current status, future directions and unanswered questions. *Psychoneuroendocrinology,* **37**(5), pp. 589-601.

SALABERGER, T., MILLARD, M., MAKAREM, S.E., MÖSTL, E., GRÜNBERGER, V., KRAMETTER-FRÖTSCHER, R., WITTEK, T. and PALME, R., 2016. Influence of external factors on hair cortisol concentrations. *General and comparative endocrinology,* **233**, pp. 73-78.

SAUVÉ, B., 2007. Measurement of cortisol in human hair as a biomarker of systemic exposure. *Clinical and Investigative Medicine,* **30**(5), pp. E183-E191.

SCHRADIN, C., 2008. Seasonal changes in testosterone and corticosterone levels in four social classes of a desert dwelling sociable rodent. *Hormones and behavior,* **53**(4), pp. 573-579.

SETCHELL, J., SMITH, T., WICKINGS, E.J. and KNAPP, L., 2008. Factors affecting fecal glucocorticoid levels in semi-free-ranging female mandrills (Mandrillus sphinx). *American Journal of Primatology,* **70**(11), pp. 1023-1032.

STEPHEN, J.M. and LEDGER, R.A., 2006. A longitudinal evaluation of urinary cortisol in kennelled dogs, Canis familiaris. *Physiology & Behavior,* **87**(5), pp. 911-916.

THOMSON, S., 2010. Hair analysis provides a historical record of cortisol levels in cushing's syndrome. *Experimental and clinical endocrinology & diabetes,* **118**(2), pp. 133-138.

VINCENT, I.C. and MICHELL, A.R., 1992. Comparison of cortisol concentrations in saliva and plasma of dogs. *Research in veterinary science,* **53**(3), pp. 342-345.

Addendum 1: data

Missing values = 999

Number	Neck Cortisol in pg/mg	Abdomen Cortisol in pg/mg	Neck Cortisone in pg/mg	Abdomen Cortisone in pg/mg
1	1.83	1.22	2.28	1.44
2	4.86	999	4.01	999
3	2.51	2.8	7.14	5.56
4	2.17	1.87	3.16	2.43
5	6.88	6.33	6.23	5.65
6	3.54	2.22	2.13	1.61
7	2.56	2.13	3.55	3.57
8	2.24	2.2	2.76	3.12
9	1.84	1.49	2.72	1.64
10	2.59	2.83	2.66	2.77
11	1.95	1.69	3.99	2.58
12	2.13	1.76	4.65	4.35
13	4.02	3.22	7.04	5.54
14	2.09	1.89	4.9	4.03
15	1.6	999	1.34	999
16	2.27	1.5	2.48	1.64
17	1.57	3.56	2.82	3.85
18	2.23	2.26	3.41	3.93
19	1.99	1.52	3.58	3.31
20	2.57	1.83	3.63	2.56
21	1.75	2.71	1.61	1.55
22	1.62	1.16	1.68	0.81
23	2.61	2.14	2.26	2.27
24	2.13	2.33	1.87	2.95
25	2.04	2.31	1.63	2.2
26	2.06	1.72	1.32	0.74
27	2.4	1.68	1.5	1.1
28	2.97	2.11	5.36	4.03
29	2.49	2.26	2.07	3.03
30	3.38	2.32	2.54	1.65
31	4.01	3.64	8.65	8.26
32	2.13	4.42	1.49	1.59
33	2.98	0.91	1.2	999
34	3.51	3.49	2.86	2.91
35	4.1	3.51	1.56	1.59
36	2.8	3.69	2.13	1.28
37	4.57	17.18	4.01	3.84
38	3.41	2.53	10.18	6.71
39	2.72	2.07	3.3	2.12
40	2.15	1.92	1.75	1.92

41	3.16	3.01	1.85	3.38
42	1.83	3.01	1.42	3.01
43	1	1.45	0.38	0.77
44	3.49	2.92	3.54	2.74
45	1.68	2.26	1.81	1.89
46	2.79	2.39	4.72	3.42
47	2.64	2.36	2.99	2.87
48	5.1	4.94	4.85	3.63
49	2.16	2.04	4.19	3.37
50	2.77	2.87	5.25	2.84
51	3.75	3.97	2.74	2.51
52	3.82	2.77	7.81	1.08
53	2.45	1.91	3.67	2.92
55	2.42	8.2	2.33	2.33
56	2.7	9.51	3.77	2.88
57	4.84	42.11	7.02	999
58	5.17	4.53	3.45	4.07
59	3.22	9.64	1.41	1.92
60	2.99	3.14	3.46	2.48
61	2.36	2.57	4.55	5.06
62	2.1	5.46	2.83	2.27
63	3.23	4.69	2.87	3.02
64	1.93	1.53	2.18	1.8
65	1.82	1.7	3.53	4.03
66	1.83	1.45	2.33	2.05
67	1.39	1.47	2.63	2.58
68	2.76	2.08	2.54	2.61
69	2.54	2.05	2.57	1.89
70	3.52	4.2	4.7	8.57
71	2	1.46	2.47	1.81
72	2.38	1.88	2.7	1.68
73	2.83	4.21	2.52	1.87
74	2.73	2.75	3.69	2.9
75	2.47	2.14	1.94	1.6
76	2.26	2.19	1.91	1.69
77	2.45	1.77	2.35	1.46
78	2.09	1.72	1.67	1.06
79	2.2	1.92	3.61	2.72
80	1.69	1.61	2.77	2.34
81	6.27	5.2	5.76	4.38
82	2	1.9	2.1	1.58
83	2.56	2.1	5.55	3.52
84	2.22	3.26	3.31	2.19
85	2.9	2.38	6.5	4.84
86	1.96	2.26	4.92	5.37
87	1.37	1.08	3.15	2.31

88	2.07	1.34	5.95	3.58
89	1.52	0.94	3.36	1.56
90	3.38	2.88	6.3	4.91
91	1.68	1.01	3.7	1.7
92	2.17	1.13	3.63	2.09
93	2.48	2.35	3.67	3.59
94	1.79	1.09	1.7	1.08
95	1.71	1.19	1.51	0.67
96	1.81	1.34	2.8	1.01
97	2.69	2.93	3.53	3.22
98	1.64	1.91	3.34	2.82
99	2.11	1.64	2.82	1.73
100	3.1	2.22	3.64	2.52
101	2.94	1.65	6.22	2.94
102	2.87	1.97	5.17	2.89
103	2.57	1.96	4.39	3.82
104	4.18	3.08	3.91	2.21
105	2.36	2.08	4.21	3.17
106	2.4	1.94	4.22	3.14
107	2.83	2.74	3.58	2.77

Addendum 2: survey

Enquête Onderzoek: cortisol meten in kattenharen			
Naam van de kat:	Klik hier als u tekst wilt invoeren.	Geslacht:	Kies een item.
Geboortedatum van de kat:	Klik hier als u een datum wilt invoeren.	Ras:	Kies een item.

	Vragen	Antwoord
1	Gezinssamenstelling (in aantallen) Aantal mensen? Andere huisdieren	Volwassenen Kinderen Dieren
	Opmerkingen: Klik hier als u tekst wilt invoeren.	
2	Is uw kat een binnen-of een buitenkat?	Kies een item.
	Opmerkingen: Klik hier als u tekst wilt invoeren.	
3	Is uw kat de afgelopen twee maanden alleen in zijn eigen, vertrouwde omgeving geweest?	Kies een item.
	Zo niet, waarom niet? Klik hier als u tekst wilt invoeren.	
4	Is uw kat de afgelopen twee maanden ziek geweest?	Kies een item.
	Zo ja, wat had uw kat? Klik hier als u tekst wilt invoeren.	
5	Heeft uw kat de afgelopen twee maanden medicijnen toegediend gekregen?	Kies een item.
	Zo ja, wat voor medicijnen waren dit? Klik hier als u tekst wilt invoeren.	
6	Heeft u de afgelopen twee maanden stress gerelateerde veranderingen in het gedrag van uw kat waargenomen (bijvoorbeeld in huis plassen of overmatig likken?	Kies een item.
	Zo ja, welk gedrag heeft u opgemerkt? Klik hier als u tekst wilt invoeren.	
7	Hebben er in de afgelopen twee maanden grote veranderingen plaatsgevonden waardoor uw kat meer stress ervaren zou kunnen hebben (bijvoorbeeld verhuizen, nieuw gezinslid, ander huisdier)?	Kies een item.
	Zo ja, wat is er veranderd? Klik hier als u tekst wilt invoeren.	