Analysis of cortisol in dog hair – a potential biomarker of chronic stress: a review

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ABSTRACT: Cortisol, which is produced in the adrenal glands, is an endogenous glucocorticoid hormone that delivers its hormonal message to cells by acting on glucocorticoid receptors. It is one of the main stress hormones responsible for stress responses in animals and humans, and its overproduction is characteristic of certain diseases. While acute stress disorder can be evaluated by means of measuring the cortisol concentration in blood and urine, chronic stress disorder can be detected by monitoring the cortisol concentration in fur or hair. Hair collection is simple, inexpensive and non-invasive, and can be performed easily and rapidly; thus, it appears to be a suitable method for determining the level of stress in dogs from shelters, abused dogs or dogs involved in different types of animal interactions. Since it is a relatively new method, monitoring cortisol in hair or fur requires further research in order to definitively prove its efficacy, and possibly to determine reference range values for different breeds of dogs.

Keywords: glucocorticoids; lifestyle; stress response; canine; coat colour; welfare

Contents

1. Introduction

The measurement of cortisol concentration in hair is a non-invasive technique used for multiple purposes, both in humans and animals. To date, many studies have reported the determination of the hormonal concentration of cortisol in human hair (Meyer and Novak 2012; Stalder and Kirschbaum 2012), that of domestic animals, e.g., dogs, cats and cows (Accorsi et al. 2008; Galuppi et al. 2013; Cobb

et al. 2016; Peric et al. 2017) and in the hair of wild animals such as squirrels, chimpanzees and bears (Bechshoft et al. 2011; Mastromonaco et al. 2014; Yamanashi et al. 2016). This paper is a summary and overview of the current scientific knowledge concerning cortisol determination in hair obtained from dogs, the factors affecting it, the methods used in the detection and the possible applications of this knowledge in practice.

2. Impact of stressors on organismal homeostasis

One of the characteristics of living organisms that is acquired during development is the ability to adapt to various external influences. This also includes responses to external stimuli in which the body tries to maintain balance, thus ensuring relatively dynamic stability of the internal environment – homeostasis (Kottferova et al. 2008). In current use, the term stress refers to the body's nonspecific adaptive response when trying to adjust. Stressors may be actual or perceived, and can be psychological or physiological in origin (Selye 1950). Furthermore, there are a huge number of external and internal stressors that can induce a stress response. Depending on the length of exposed to the stressors, stress can be either acute or chronic; while the response to short-term stress is adaptive and helps individuals to cope with emerging situations, long-term stress elicits maladaptive responses (Nelson 2005). Overexposure to glucocorticoids, owing to malfunction or long-term (chronic) hyperactivation of the stress system, can damage the body, impair growth and development, and elicit endocrine, metabolic, autoimmune or psychiatric disorders (Charmandari et al. 2005). In humans, different social conflicts may also increase allostatic load reflected in increased concentrations of glucocorticoids (Goymann and Wingfield 2004). Studies in different environments have delineated a range of behaviours indicative of underlying welfare status of the dog (Hiby et al. 2006). Licking, lowered body posture, panting, shaking of the body and paw lifting are generally displayed by dogs in response to short-term stress, whereas repetitive performance of a particular behaviour, a stereotype, is usually observed in response to prolonged stress (Hetts et al. 1992; Beerda et al. 2000).

3. Stress hormones

Glucocorticoids, also known as stress hormones, are a key physiological tool in ecological studies and aid in the assessment of the health and condition of populations as they cope with and respond to change (Busch and Hayward 2009). Their primary role lies in basic energy regulation (acquisition, deposition, mobilisation), and only at high levels do they orchestrate the changes associated with stress (Busch and Hayward 2009). They are commonly used as biomarkers of stress (Russell 2012). Cortisol and corticosterone are the endogenous glucocorticoids in humans and rodents, respectively (Terao and Katayama 2016), and the major glucocorticoid hormone in birds is corticosterone (Holmes and Phillips 1976). Cortisol has long been considered to be a reliable physiological measure of the stress response in domestic mammals such as guinea pigs (Kunzl et al. 2003), pigs (Turner et al. 2005), sheep (Smith and Dobson 2002), goats (Aoyama et al. 2008), cows (Christison and Johnson 1972) and horses (Visser et al. 2008) and in wild mammals, e.g., *Antechinus* (Bradley et al. 1980), *Macaca* (Davenport et al. 2008), *Odocoileius* (Millspaugh et al. 2002), *Peromyscus* (Hayssen et al. 2002), *Procavia* (Koren et al. 2008), *Puma* (Bonier et al. 2004), *Saimiri* (Lyons et al. 1995) and *Spermophilus* (Mateo 2008; Delehanty and Boonstra 2009). The stress response in vertebrates, mediated by the HPA axis, involves the release of adrenocorticotrophic hormone (ACTH) into the blood stream, which in turn acts on the adrenal cortex to activate the secretion of glucocorticoids (Tsigos and Chrousos 2002). In humans, the skin is, among other organs, a producer of hormones and the main mediator of the systemic stress response, in which the hormones are components of the HPA axis (Slominski and Wortsman 2000). There is only scant information about the incorporation of hormones into the hair shaft or possible factors influencing local cortisol production (Russell 2012). Many studies have been performed regarding the mechanism of cortisol incorporation into the hair shaft, and several hypotheses have been advanced. One potential mechanism is the diffusion of cortisol from blood during the hair shaft's growth period but hair cortisol may also originate from excretion of sebum or sweat (Cone 1996; Russell et al. 2014). An *in vitro* study (Ito et al. 2005) using organ-cultured follicles of human scalp has already demonstrated the

existence of an equivalent to the central HPA-axis in the hair follicle. Further, autonomous production of cortisol in human keratinocytes has been confirmed in the study of Cirillo and Prime (2011). Later studies (Keckeis et al. 2012; Stubsjoen et al. 2015) also suggest a possibility of local production of cortisol and intrafollicular autonomous cortisol synthesis. In addition, canine hair follicle cells *in vitro* are able to metabolise progesterone to cortisol (Bamberg et al. 2005).

The *in vivo* study of Salaberger et al. (2016) demonstrated local production of cortisol in sheep resulting in an elevation of cortisol concentration in wool. Elevated local production in this study (Salaberger et al. 2016) was reached through applying extensive brushing (stimulation of mechanical irritation) or glucocorticoid treatment (solution with dexamethasone) on the skin of sheep.

Thus, local synthesis of cortisol could interfere with the hair cortisol concentration (HCC) as a parameter for evaluating long-term stress and caution is advised with respect to the interpretation of HCC.

4. Matrices for cortisol measurement

Generally, cortisol concentrations are assessed by sampling blood plasma or serum (Haverbeke et al. 2008; Giannetto et al. 2014), saliva (Kobelt et al. 2003; Giannetto et al. 2014), urine (Zhang et al. 2017), faeces (Accorsi et al. 2008; Salaberger et al. 2016) and milk (Grey et al. 2013).

Although blood sampling itself can promote increased glucocorticoids concentrations, blood samples are most commonly used for the analysis of glucocorticoids concentrations (Boiti and Yalow 1978). Saliva and serum samples provide a measurement of the cortisol concentration at a single point in time and they can therefore be used to test the acute changes. Data on salivary cortisol concentrations correlate well with the levels in serum (Vining et al. 1983). In healthy individuals, plasma cortisol concentration peaks early in the morning, and gradually decreases thereafter (Russell 2012). The concentration of faecal glucocorticoid metabolites exhibits a medium-term (between short-term and long-term) elevation of glucocorticoid production. The advantage offered by faecal samples is that they can be easily collected without stressing the animals. In a previous

study, faecal concentrations of cortisol metabolites reflected the total amount excreted and therefore cortisol secretory patterns better than did blood concentrations, which changed quickly (Palme et al. 2005). In terms of cortisol in urine, its concentration reflects unbound and biologically active cortisol in plasma. Measurement of urinary free cortisol is clinically important in the diagnosis of Cushing's syndrome. A 24-h urine sample is usually collected for assessing cortisol in this biological matrix because of the diurnal rhythm of cortisol excretion (Turpeinen and Hamalainen 2013). Cortisol can also be detected in excreted milk (Tucker and Schwalm 1977). A clear disadvantage is that milk can be sampled only in lactating female animals or humans (Peric 2014).

All these matrices are subject to major daily physiological fluctuations and for that reason the assessment of overall long-term systemic cortisol exposure is difficult. Assessment of long-term glucocorticoid concentrations in various studies has included different keratinised tissues such as hair (Van Uum et al. 2008; Veronesi et al. 2015; Yamanashi et al. 2016), hoof (Comin et al. 2014), claws (Veronesi et al. 2015), balleen plates (Hunt et al. 2014), turtle claws (Baxter-Gilbert et al. 2014) and feathers (Romero and Fairhurst 2016). In feathers, corticosterone is measured; the concentration of corticosterone deposited is correlated with the stresses experienced by the bird during feather growth (Romero and Fairhurst 2016). Mammalian nail contains a keratinised epithelium with a vascularised inner corium, where cortisol passively accumulates from the bloodstream (Mack and Fokidis 2017). In dogs, cortisol concentrations increased with the amount of claw sample extracted, as was previously documented in a study of corticosterone in turtle claws (Baxter-Gilbert et al. 2014).

Each sampling procedure has some constraints. Ideally, in order to minimise the stress associated with the capture and handling of animals, the sample collection method should be non-invasive (Delehanty and Boonstra 2009). Blood and saliva provide instantaneous data on cortisol concentrations. However, the restraint required for blood collection, in particular, causes stress which raises the concentration of cortisol in patients. In dogs, saliva sampling is generally regarded as a "non-invasive" method of collection that employs mild to moderate restraint. Saliva absorption materials can be flavoured with beef stock to make them

more palatable, but this method yields inconsistent cortisol results, and citric acid is sometimes added to increase salivation but is not well tolerated (Dreschel and Granger 2009). A number of basic principles are followed prior to the collection of saliva samples, among them, that dogs are not fed before sampling and that the dog is not allowed to drink prior to the testing (Matos 2015). While blood collection for cortisol in plasma should be completed within three minutes so as to not affect the cortisol concentration in plasma, saliva sampling has to be completed in less than four minutes to prevent the stress of restraint from elevating salivary cortisol concentrations (Kobelt et al. 2003). Principles for milk sample collection in humans have also been described (Grey et al. 2013), and faecal sample collection in dogs and cats should be done with an emphasis on cross-contamination prevention and immediate identification, labelling and storing (Accorsi et al. 2008). Compared with the application of assay techniques for other types of biological sampling, hair, and in the most up-to-date research, also claw (Veronesi et al. 2015) sampling is a non-invasive and painless method (Ouschan et al. 2013). Therefore, these analyses of cortisol represent robust, non-invasive methods that hold promise for the study of prolonged changes in HPA axis activity.

5. Cortisol in hair

Measuring cortisol in hair is an innovative method to determine long-term cortisol elevation, and is free of many of the methodological difficulties associated with other matrices (Dowlati et al. 2010). The interest in hair as a record of endocrine activity derives from the various unique features of hair compared to more traditional substrates (e.g., blood, stool, urine, saliva), that offer advantages to researchers interested in using cortisol levels as a proxy for chronic stress (Fourie et al. 2016). Cortisol is incorporated into the hair shaft through two principal mechanisms. The free, unbound fraction of cortisol is incorporated during growth of the follicle (Cone 1996). In addition, sweat and sebum excreted by glands of the skin contain cortisol which can become incorporated into the hair shaft (Anielski 2008). Hair provides an attractive alternative for evaluating physiological stress for numerous reasons, as follows: (1) it is thought to

incorporate blood-borne hormones during the growth phase of hair; (2) it is relatively stable and (3) it can be stored and transported at room temperature. Moreover, even if hair sampling may require capture or handling in certain cases, the stress potentially experienced during this event will not affect glucocorticoid concentration in the collected hair (Mastromonaco et al. 2014). Whereas in humans the average rate of hair growth is approximately 1 cm per month (Wennig 2000), in dogs, the normal hair growth rate depends on a number of internal and external factors, e.g., seasonal variations or body region (Muntener et al. 2011). The rate of hair growth varies not only between individual dogs but also between different regions in the same dog. In the study of Gunaratnam and Wilkinson (1983), the rate of hair growth was most rapid in the shoulder region, followed by the flank and then the forehead regions, and was slightly more rapid in the shoulder and flank regions of three of the dogs during summer than in winter.

6. Analyses of hair cortisol concentration (HCC) in animals

The first study examining cortisol concentration in hair was performed by Koren et al. (2002) using hair from wild hyraxes. In this study, the hyraxes were briefly captured so that 7–20 mg of hair could be plucked. Subsequently, the cortisol was extracted from the hair with methanol, and the concentration was determined using the modified salivary ELISA assay. Cortisol was found in detectable amounts in the hair with a significant positive correlation found between hair cortisol concentration and social ranking of the hyraxes. Therefore, this study based on hair cortisol analysis supports the "stress domination" hypothesis. Basal cortisol concentrations were higher in dominant animals than in the subordinate ones. Thus, hair cortisol concentrations were predictive of social dominance-related variables (Koren et al. 2008). A number of studies have reported the determination of hair cortisol in dogs. In 2008, Accorsi et al. studied cortisol in hair and faeces from domestic cats and dogs. Their data show that in both species, there was a significant positive correlation between the concentration of cortisol determined in the hair and in the faeces. This correlation seems to support the hypothesis that both faecal and hair

cortisol measurements reflect at least in part the same adrenal activity. The mean cortisol content in 29 hair samples was 2.10 ± 0.22 pg/mg (Accorsi et al. 2008). The HCC results obtained from the study of Bennet and Hayssen (2010) were higher than the results of Accorsi et al. (2008). The mean cortisol concentration of old hair samples (obtained from each dog on day 1 by shaving from the ischiatic region) was 12.6 pg/mg and in new hair samples (obtained within the same region after approximately 6–12 weeks) HCC was 10.9 pg/mg. The different hair cortisol concentrations recorded in the two studies may be due to the powdering of hair, which was shown to result in a 3.5-fold increase in cortisol recovery compared to the chopping method used by Accorsi et al. (2008). Furthermore, the study of Bennet and Hayssen (2010) showed that the distal hair segments in dogs are much more likely to harbour higher concentrations of cortisol metabolites than the proximal segments (seven of nine dogs), which is the opposite of what would be expected if cortisol were leached from the hair as it aged. In dogs, little is known about the possible leaching effects that washing and sunlight exposure may have on hair over time and throughout the length of the hair shaft. However, in other species the differences in cortisol concentrations between the proximal and distal ends of the hair of macaques were not significant (Davenport et al. 2006). Kirschbaum et al. (2009) reported a decline in cortisol concentration throughout the length of the hair from scalp to tip in a study of female subjects.

It is not only the long-term effects of various kinds of non-specific stressors that act to increase the concentration of cortisol in dog hair; the concentration of cortisol may also become higher in various disease states. Chronic stress, often stemming from disease, elevates cortisol above the normal baseline concentrations (Mack and Fokidis 2017). Hair cortisol concentrations were significantly higher in dogs with different diseases, e.g., in Cushing's syndrome (Corradini et al. 2013). Hair cortisol concentration was 4.53 pg/mg (0.32–74.62 pg/mg), 1.49 pg/mg (0.13–14.19 pg/mg) and 1.28 pg/mg (0.34–5.38 pg/mg) in dogs with hypercortisolism, sick dogs and healthy dogs, respectively (Corradini et al. 2013). Atopic dermatitis is another disease leading to elevated cortisol concentrations in dog hair (Park et al. 2016). Increased HCC reflects excessive endogenous cortisol secretion and corresponds with studies performed

in humans; Cushing's syndrome patients could be distinguished from healthy controls on the basis of HCC diagnosis (Corradini et al. 2013). These data are comparable to the results of Ouschan et al. (2013). In dogs with suspected pituitary-dependent HAC (hyperadrenocorticism), the measurement of cortisol in hair offers numerous benefits. It is a non-invasive and painless method in which the hair can be easily collected at any time of the day or year and then stored at room temperature. Measurement of cortisol in hair thus represents a practical method for assessing glucocorticoid overproduction in dogs and should be explored further as a non-invasive method for diagnosing HAC in dogs (Ouschan et al. 2013).

7. Previous studies of cortisol concentrations in dogs

A number of studies have been carried out to determine hair cortisol concentration in dogs. Most of the analyses performed in order to assess HCC in the hair of dogs are summarised in Table 1. The table shows the number of dogs on which the determinations were performed, the breed differences, the number of dogs and the place of sampling as well as the method that was used to determine whether the hair was washed or not, and, finally, the authors of the different studies.

8. Collection and storage of dog hair samples

Hair should always be cut or shaved close to the skin rather than plucked so as to avoid including follicles in the sample and to prevent possible blood contamination. Shaving is well tolerated by most domestic species. Although shaving may require some restraint or sedation of wild animals such as wolves, hair should be easily collected during routine capture or release procedures (Bennet and Hayssen 2010). In animals, each hair shaft grows to a certain length after which the growth stops and the final cycle of shedding begins. Consequently, hair samples obtained without previous shaving will contain a mixture of hairs that have incorporated cortisol over different time periods. This problem can be solved by using a shave-reshave approach that establishes a known timeline of cortisol incorporation (Meyer and Novak 2012).

Table 1. Previous studies reporting cortisol concentration in dog hair

Sample (n)	Breed	Amount of hair used for analysis	Sampling area	Analysis	Extraction metodology/ extractant	Washing of hair sample	Authors
29	mixed-breed	60 mg	the ischiatic region	RIA	Koren et al. (2002)/ methanol	not washed	Accorsi et al. (2008)
48	German shepherd, Labrador retrievers	250 mg	the ischiatic region	EIA	Davenport et al. (2006)/ methanol	isopropanol	Bennet and Hayssen (2010)
7	mixed-breed	25 mg	the shoulder region	RIA	Davenport et al. (2006)/ methanol	2 × with distilled water, 2 × with isopropanol	Bryan et al. (2013)
90	various breeds + mixed-breed	60 mg	the xiphoid region	RIA	Koren et al. (2002)/ methanol	not washed	Corradini et al. (2013)
22	various breeds	50 mg	the region of the vena cephalica	EIA	modified extraction technique/methanol	hexane	Ouschan et al. (2013)
14	various breeds	300 mg	the ischiatic region	RIA	modified extraction technique/methanol	not washed	Siniscalchi et al. (2013)
40	Labrador retrievers	70 mg	the ischiatic region, front side of the neck	RIA	not mentioned/ methanol	2 × with isopropanol	Svendsen and Sondergaard (2014)
33	various breeds	approximately 300 mg	not mentioned	EIA	Davenport et al. 2006 + Bennet and Hayssen 2010/methanol	not washed	Nicolson and Meredith (2015)
165	32 different canine breeds/purebred puppies	approximately 20 mg	the back and the dorsal portion of the neck	RIA	not mentioned/methanol	isopropanol	Veronesi et al. (2015)
20	Border collie	7–8 mg of guard hair (3–6 mg)	the neck	EIA	not mentioned/ methanol	not washed	Rosen 2016
59	German shepherd	5–10 mg	the chest and the neck	RIA	modified extraction technique/methanol	not washed	Roth et al. 2016
26	various breeds + mixed-breed	25–150 mg	the ischiatic region	EIA	modified extraction technique/methanol	isopropanol	Park et al. 2016
121	various breeds + mixed-breed	approximately 250 mg	the underside of the upper right chest region	RIA	Davenport et al., 2006/ethanol	2 × with isopropanol	Willen et al. 2017

EIA = enzyme immunoassay, RIA = radioimmunoassay

The body areas used for hair sampling prior to HCC analysis have varied slightly in different studies. Hair was taken from the cephalic vein area (Ouschan et al. 2013) at two sites – the chest and neck (Veronesi et al. 2015; Rosen 2016; Roth et al. 2016; Willen et al. 2017), the ischiadic region (Accorsi et al. 2008; Bennet and Hayssen 2010; Siniscalchi et al. 2013; Svendsen and Sondergaard 2014; Park et al. 2016), right and left shoulders (Bryan et al. 2013) or from the xiphoid region (Corradini et al. 2013). Hair was collected either by an electric shaving razor or surgical scissors, and is gathered by cutting as close to the skin as possible without injuring the dog. The shaving area was approximately 10 × 10 cm in almost all studies and resulted in the gathering of 3–500 mg of dog hair. Collected hair samples from dogs are then identified, labelled and stored at room temperature or in the freezer (–80 °C).

9. Preparation of dog hair samples and methods used to determine HCC

There are several methods for preparing samples for hair analysis. The most commonly used methods are those reported by Davenport (2006), Koren at al. (2002) and Sauve et al. (2007), as well as several modifications thereof. The first step for preparing the hair sample is washing. To wash the hair sample, either distilled water, to remove water-soluble substances such as faeces or urine from the exterior of the hair, or isopropanol, to remove steroids that might contaminate the exterior of the hair, are used (Bryan et al. 2013).

Following steroid extraction according to Davenport et al. (2006), approximately 250 mg of hair are washed twice in 5 ml of isopropanol by gentle rotation for 3 min. Then, the hair is dried at room temperature for approximately five days, before being ground to a fine powder with a Retsch ball mill for 5 min at 30 Hz. Finally, the cortisol is then extracted from the powdered hair. To this end, 1 ml of methanol is added to 50 mg of powdered hair and incubated at room temperature with slow rotation for 24 h. After spinning in a microcentrifuge for 30 s, a 0.6-ml aliquot is taken from the top, and then dried using a vacuum centrifuge. The dried extract is reconstituted with phosphate buffer.

The most widely used method is the preparation technique according to Sauve et al. (2007). In this method, a minimum of 10 mg of hair sample required for the analysis of hair are weighed. The hair is cut into small pieces using small surgical scissors and then inserted into separate glass vials. Subsequently, 1 ml of methanol is added to the vial which is then sealed and incubated overnight at 52 °C for 16 h with gentle shaking. After incubation, the methanol is removed, transferred into a disposable glass tube and evaporated under a constant stream of nitrogen. The dried extracts are dissolved in 250 μl of phosphate buffered saline (pH 8.0) and vortexed for 1 min. The samples are vortexed and centrifuged again for 30 s prior to analysis.

Hair cortisol in dogs was analysed using the enzyme immunoassay (EIA) and radioimmunoassay (RIA) methods, whereas in other animal species and in humans the chemiluminescent immunoassay (CLIA) and high-pressure liquid chromatography-mass spectrometry assay with high-pressure liquid chromatography fluorescence detection (Meyer and Novak 2012) were also used.

10. Factors affecting hair cortisol concentration in dogs

A great deal of research in this area has addressed various factors that could have a potential impact on hair cortisol concentration in dogs, among them, coat colour, age, spay/neuter status, period of the year, lifestyle and well-being and health; finally, effects that are not directly related to the amount of cortisol like washing the hair sample prior to analysis and the method used to determine the cortisol concentration have also been studied.

10.1 Variations in coat colour

The relationship between variations in coat colour and HCC in dogs was confirmed for the first time by Bennet and Hayssen (2010) who detected statistically significant differences among German shepherd dogs, with black hair containing lower amounts of cortisol than non-black hair. Statistically significant differences between hair colour and HCC in dogs were also confirmed by Svendsen and Sondergaard (2014). These differences were determined in samples of brown and black hair from 40 Labrador retrievers, but in the groups that were compared the dogs were not of

the same size, which could also affect the obtained results. On the other hand, the research of hair cortisol concentration by Rosen (2016) did not show differences between black and white coat colour either within a population of Border collies or within individuals as was also found by other authors (Nicolson and Meredith 2015). Nicolson and Meredith (2015) analysed cortisol concentrations in dogs with black hair, in different breeds of dogs with different hair colours (Jack Russell terrier, Labrador retriever, Cavalier King Charles spaniel, Shih Tzu and Springer spaniel) and crossbreeds. They did not observe any differences between the two groups when comparing dogs with and without black hair. However, that some breeds, such as the German shepherd, may have more cortisol concentration in their non-black hair coat compared to the black hair coat should still be taken into account (Bennet and Hayssen 2010). In humans, no differences between the hair colour and hair cortisol concentration were detected in the studies of Raul et al. (2004) and Sauve et al. (2007). On the other hand, the research of Wosu et al. (2015) showed that blacks and Hispanics have higher hair cortisol concentrations compared to whites and other ethnicities.

10.2 Dog age

It had been assumed that the amount of cortisol in a dog's coat increases with increasing age, but the research has not confirmed this hypothesis (Bennet and Hayssen 2010; Svendsen and Sondergaard 2014; Roth et al. 2016). No correlation between HCC and age was found in grizzly bears (Macbeth et al. 2010), polar bears (Bechshoft et al. 2011) or humans (Kirschbaum et al. 2009). In a recent study (Gonzales-de-la-Vara et al. 2011), a correlation between age and HCC in cattle (HCC was higher in heifers than in 2-year-old cows) was described. These results were explained by high serum cortisol concentrations in late pregnancy that were stimulated by the fetal pituitary-adrenal axis (Gonzales-de-la-Vara et al. 2011). In the study of Montillo (2014) performed in foals, a correlation between age and HCC was also described, namely, a decrease in hair cortisol concentration in the period from birth up to three months of age. Such a decrease in HCC is probably due to the interruption of the foetal-placental connection (Montillo 2014).

10.3 Sex and neuter status

No statistically significant difference between the sexes, neuter status and HCC in dogs was found in the studies of Svendsen and Sondergaard (2014) and Bennet and Hayssen (2010). Additional studies in grizzly bears (Macbeth et al. 2010), humans (Webb et al. 2010), caribou and reindeer (Ashley et al. 2011) support these findings. On the other hand, Lafferty et al. (2015) determined variations in HCC between sexes in black bears, with female HCC ranging from 0.6 to 10.7 pg/ml compared to male HCC which ranged from 0.5 to 35.1 pg/ml. These differences are likely to be a consequence of multiple interacting factors, e.g., differences in the use of food resources, differences in nutritional needs and sex-based social environment differences (Lafferty et al. 2015).

10.4 Different regions of the body

The location of the hair on the dog's body is also one of the indicators that could influence cortisol concentration. HCC in healthy dogs was measured by Bryan et al. (2013), but no difference was found between the hair samples taken from the right side compared to the left side of a particular dog. In the study of Svendsen and Sondergaard (2014), no correlation was observed between the hair taken from the neck and the mean value of the hair taken from the hips. A possible explanation for hair region variability may be differences in the rate of growth between the hair from the neck and the ischiatic region, including differences in moulting patterns (Svendsen and Sondergaard 2014). No differences in HCC from different body regions in individual New Zealand rabbits were found in the work of Comin et al. (2012). This is in contrast to the data of Macbeth et al. (2010), who found that the neck hair had higher HCC than the hair from the shoulder, rump and abdomen in free-living grizzly bears, which may be due to the fact that different regions of the bear's body are covered by different hair types and guard hair contains more cortisol than the undercoat in this species.

10.5 Lifestyle of the dog

The lifestyle of the dog and its levels of physical exertion have a significant impact on the cortisol

concentration in its hair since lifestyles can affect HPA-axis activity. Competition dogs had higher hair cortisol concentrations than both companion and working dogs and this was especially obvious in January (Roth et al. 2016). Cortisol increases could possibly be related to variations in the number of training days, as competing dogs usually train less during the winter and more during the spring, in accordance with the competition season. Alternatively, the dogs might simply be less exercised after the competition season, which might induce restlessness (Roth et al. 2016). Due to the gradual growth of dog hair after trimming or shaving, hair regains its initial length at varying rates (for example, in Labradors kept indoors, the hair grows back in approximately 14.5 weeks according to Diaz et al. 2004). In a recent study with foals (Montillo 2014), no effect of either temperature or day length on the HCC was found over the course of three foaling seasons. However, Bechshoft et al. (2013) reported that hair cortisol in polar bears was dependent on fluctuations in the climate and in the ice extent.

10.6 Health status of the dog

In the human population, individuals with chronic pain were found to have higher cortisol concentrations in hair than those who did not suffer any pain (Van Uum et al. 2008). Moreover, in diseases associated with adrenal hypo- or hyperfunction, such as Addison's or Cushing's disease, altered concentrations (lower or higher) of hair cortisol were confirmed by the results of more recent studies conducted by Thomson et al. (2010) and Corradini et al. (2013). Cortisol concentrations in hair were significantly increased in canine atopic dermatitis (CAD) patients, especially in groups with moderate and severe CAD disease state. These increased concentrations might be a response to chronic physical discomfort from CAD – such as itchy, dry and inflamed skin conditions (Park et al. 2016). Nicolson and Meredith (2015) found that the hair cortisol concentrations of healthy and chronically ill dogs did not differ significantly.

10.7 Washing-out effect

No studies concerning the effects of washing in dogs are currently available. In humans, the washing-out effect was observed in several studies (Kirschbaum et al. 2009; Dettenborn et al. 2010; Gao et al. 2010). It was suggested that structural damage of distal hair segments might have been caused by liquids like water and cosmetics (Kirschbaum et al. 2009), or alcohol used to wash the samples before analysis (Manenschijn et al. 2011). In monkey hair, the amount of cortisol remaining after washing was inversely related to the number of shampoo exposures (Meyer et al. 2014). Assuming that hair in humans grows approximately 1 cm per month and that humans typically wash their hair at least several times per week, hair located several centimetres from the scalp might be exposed to over-shampooing, which appears to be a significant contributor to variation in cortisol levels (Meyer et al. 2014). Stalder and Kirschbaum (2012) suggested that frequent washing procedures may partially influence HCC in humans but it is unknown whether dogs are similarly affected. However, the substantially lower frequency of hair washing in dogs compared with humans makes it improbable that this aspect could significantly influence the HCC in dogs (Corradini et al. 2013).

10.8 External factors

In humans, not only an extreme dose of UV light, but also repeated sunlight exposure decreases the cortisol concentration of scalp hair (Wester et al. 2016). External factors, such as extensive brushing or treatment with various dermatological preparations may also have a significant influence on cortisol and its concentration in hair, as was shown in sheep, where hair exposed to either extensive brushing or dexamethasone fluid had significantly higher concentrations of immunoreactive cortisol after three weeks of treatment (Salaberger 2016). It can be assumed that external factors could have some influence on HCC in dogs, but currently, there is a lack of studies in this area.

10.9 Different methods of processing hair samples and the various methods used to evaluate hair cortisol concentrations

The processing and preparation of the samples for analysis, as well as the subsequent assay method used, could have some influence on the hair cortisol

concentration measured. In preparing hair samples, the hair is either powdered to maximize cortisol recovery (Davenport 2006), or is cut into the smallest possible pieces (Accorsi et al. 2008; Nicholson and Meredith 2015; Rosen 2016). So far, the RIA and EIA techniques have been the methods of choice for determining HCC in dogs. For the future, it is appropriate to consider further research that would undertake a comparison of the different methods of hair sample processing or the used methods. Difficulties in interpretation of HCC data are due to an absence of a physiological range of canine hair cortisol and the lack of standardisation in hair cortisol extraction and assay methods.

11. Conclusions

Hair cortisol analysis may become an effective assessment tool to measure chronic stress in dogs, and thus also a suitable complementary measure for the detection of particular hormonal diseases and for the assessment of canine welfare. The main advantages of this method are that hair sampling is an easy and simple non-invasive procedure that does not require long-term restraint of the animal and that hair samples may be stored for a prolonged period of time. HCC determination is thus an appropriate method for assessing the long-term overproduction of glucocorticoids in dogs. Hair cortisol analysis could become a particularly useful tool for welfare assessment in working dogs, whose excellent physical and mental health is crucial for their ability to carry out their assigned tasks, or in shelter dogs, kennel dogs or laboratory dogs in order to better understand their histories and treat their behavioural problems.

However, assessing and interpreting the results of cortisol concentration determination in the hair should be approached with caution as HCC can potentially be influenced by a number of factors, such as coat colour variations, dog age, period of the year, lifestyle and welfare of dog, health status and external factors (e.g. washing-out effect, seasonal changes).

Sampling for the HCC assay should be performed from several different regions of the body, but areas with a higher probability of increased mechanical irritation (e.g., in the neck which suffers from mechanical irritation due to the wearing of a collar) and corticosteroid-treated sites should be avoided.

Sampling should not be carried out immediately after washing hair with shampoo or in the period of moulting.

When interpreting the results, it is necessary to take into account not only the factors mentioned above but also the health status of the dog and what the animal is used for (e.g., racing dog, pet dog, competition dog).

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