Validation of cortisol measurements in canine puppies' hair

- A pilot study -

Sjanne Meinders
Student number: 3796620
January 2017 till March 2017

Supervisors:
Claudia Vinke
Vivian Goerlich-Jansson

Department of Animal in Science and Society
Faculty of Veterinary Medicine, University of Utrecht

Table of contents

Prefatory note

The aim of this pilot study was to collect preparatory data for future studies and to establish a protocol for the collection and treatment of puppy hair for the reliable measurement of cortisol. This report therefore consists of a literature study, the pilot practical investigation and finally two thorough protocols that can serve as standard operating procedure (SOP) for future experimental studies.

The aim of the future study is to investigate if cortisol (and other hormones as testosterone) in hair of dog neonates can reflect information about the bitch gestation time. This additional information can be very important and useful in some veterinary cases, for example, when puppies are confiscated from illegal pup trade or so called the "puppy mills". These pups are often bred under highly stressful circumstances, which could be reflected in hair cortisol concentrations. Therefore, hair could provide extra information to enhance knowledge about the breeding conditions of the dogs. For the puppy itself, information on past exposure to stress can additionally help to estimate a prognosis for the dog more accurately (for example whether this dog is likely to develop behavioural problems) and make an individual specific (behavioural) treatment plan.

Abstract

Hair cortisol measurement has increasingly been used to assess HPA-axis activity retrospectively in different species. The aim of this pilot study was to collect preparatory data and to establish a protocol for the collection and treatment of canine puppy hair for the reliable measurement of cortisol. This study consisted of a literature study, a pilot practical investigation and the establishment of two thorough protocols that can serve as standard operating procedure (SOP) for future studies. The literature study discusses previous findings about hair cortisol measurements from different species, with focussing on dogs. In the pilot practical investigation hair samples from seven canine puppies (< six months of age from different breeds) and one young-adult dog were analysed to test the repeatability of cortisol measurements and the influence of sample mass, sampled body site, gender, age, hair part, hair colour and hair type on hair cortisol concentration. The used method showed highly repeatable results. The minimum amount of hair necessary was 10 mg. No significant differences in hair cortisol concentration were found between 10 mg and 50 mg hair samples ($P = 0.133$). However, a trend was found with 10 mg samples showing higher cortisol concentrations than 50 mg hair samples. Significant differences in hair cortisol concentration between sampled body sites were found within the young-adult dog ($P = 0.021$), but not between the pups ($P = 0.758$). No significant relations were found with hair cortisol concentration and gender ($P = 0.701$) or age ($P = 0.579$). A significant difference in hair cortisol concentration was found between proximal and distal hair segments ($P = 0.043$). This pilot study showed for the first time the possibility to measure cortisol in alive canine puppies' hair, with a reliable method. Both established protocols can be used for future experimental studies when investigating cortisol from canine puppies' hair.

Key words: 'HPA-axis', 'cortisol', 'canine', 'puppy', 'hair', 'protocol'.

Introduction

This introduction is divided into different sections. First it will provide background information about cortisol physiology (with extra attention to cortisol in the perinatal period) and canine's hair physiology. Finally these two aspects will be combined in the section about cortisol deposition in canine hair.

Cortisol physiology

Cortisol is an essential glucocorticoid and is often referred to as the 'stress hormone'. The effects of glucocorticoids are pleiotropic and their effects are the results of activation of their wide distributed intracellular receptors. The hormones act on the glucocorticoid-preferring receptors (GR) and mineralocorticoid-preferring receptors (MR). These receptors interact with DNA sequences on target genes affecting gene expression. Besides genomic effects, cortisol also have direct catabolic, lipogenic, anti-reproductive and immunosuppressive effects. The catabolic and lipogenic effects result in the release of glucose what can be used by many tissues during stress. During an acute stress response, basal blood cortisol increases rapidly within a few minutes and return to baselines after the stressor subsides. So cortisol plays indeed a central role in the stress response, as the referred name suggests (Rijnberk & Kooistra, 2010).

The stress response has both central and peripheral components and is regulated mainly by the hypothalamic-pituitary-adrenocortical axis (HPA axis). However, other factors such as hormones, cytokines and neuronal autonomic information also participate in the regulation of the secretion of cortisol. In a stressful situation, various factors activate the HPA axis which results in the release of corticotrophin-releasing hormone (CRH) and vasopressin (AVP) by the hypothalamus. Following this, adrenocorticotropic hormone (ACTH) is released by the anterior part of the pituitary gland. ACTH has several effects on the main target, the adrenal cortex. ACTH regulates glucocorticoid, androgen and aldosterone secretion by the adrenal gland. The glucocorticoids, including cortisol, are the final effectors of the HPA axis and have also an important role in the regulation of the HPA-axis themselves by the negative feedback on both CRH and ACTH, to limit the duration of previous mentioned effects (Fig.1) (Charmandari, Tsigos, & Chrousos, 2005).

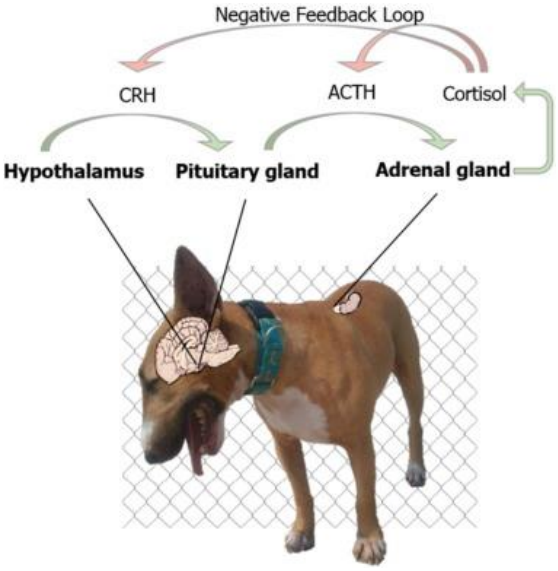

Fig.1. The HPA-axis in a dog (Protopopova, 2016).

The stress system is very important for maintaining the homeostasis of organisms, as it coordinates the adaptive response to stressors. It leads to behavioural and peripheral changes that improve the ability to adapt, and thereby enhances the chance of survival. However, when the stress system is activated inadequately or during a prolonged period of time, negative side-effects can be seen. Direct negative effects of high levels of cortisol are the impeding of wound healing, the higher vulnerability to infections and the triggering of autoimmune diseases among others. These effects can lead to impairment of growth and development early in life and also to a variety of endocrine, metabolic, autoimmune and psychiatric disorders later in life (Charmandari et al., 2005). Moreover, the catabolic and lipogenic effects of elevated cortisol results in high levels of blood glucose, which may be problematic in dogs with diabetes mellitus (Nicholson & Meredith, 2015).

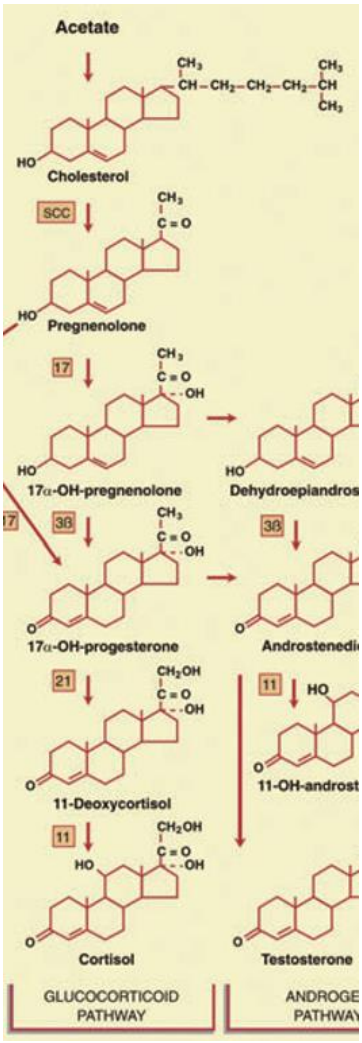

Fig. 2. The conversion of cholesterol into cortisol in the adrenal gland (Rijnberk & Kooistra, 2010).

Glucocorticoids are produced in the fascicular zone of the adrenal gland with cholesterol as the starting compound. Cholesterol is liberated after binding of low density lipoproteins (LDL) on receptors of the adrenal cortex and is also synthetized from acetate within the gland itself. Four rings contribute to the basic structure of the steroids and is also seen in the structure of cortisol. The conversion of cholesterol to steroid hormones is effectuated by cytochrome P-450 enzymes in five steps of enzyme activity (Fig. 2). Steroids are secreted immediately after biosynthesis and are then largely bound to plasma proteins. Approximately 75% and 12% of the total cortisol is respectively bound to corticosteroid-binding globulin (CBG) and to albumin and erythrocytes. These binding proteins serves as buffering and rapid variations in plasma cortisol are so prevented. The free- and bounded fraction are in equilibrium and both fractions are therefore potentially available to tissues. However, only the free fraction of cortisol is biologically active and in the dog this ranges from 6 to 14%. Free cortisol diffuses quickly into the salivary glands. The cortisol concentration in canine saliva is approximately 7-12% of the total blood cortisol, so highly comparable with the free fraction of cortisol. The major sites of the corticosteroid metabolism are the liver and the kidney. These organs inactivate the hormones and increase their water solubility. These metabolites are thereafter excreted by the kidney. Approximately one to two percent of total cortisol is excreted in the urine unaltered (Rijnberk & Kooistra, 2010). Faecal excretion of cortisol also occurs, but the exact percentage is not well known in dogs. One study measured a fraction of 23% of the original added cortisol in the faeces of dogs (Schats & Palme, 2001).

Cortisol in the perinatal period

During gestation, important changes are seen in the HPA axis. For example, in the third trimester of gestation, both maternal and foetal plasma cortisol concentrations rises tremendously (Reynolds, 2013; Sapolsky & Meaney, 1986). The rise of cortisol is due to the release of CRH by the placenta into the maternal blood stream. The placental CRH acts on the systemic HPA axis and also directly on the maternal adrenal gland, providing a positive feedback. Glucocorticoids are lipophilic and can cross the placenta. The rise of cortisol is essential for the growth, the brain development and the organ maturation of the foetus in the last period of gestation (Reynolds, 2013). Normally, 30-40% of the variance of foetal cortisol is originating from maternal cortisol (Egliston, McMahon, & Austin, 2007).

Foetal glucocorticoid levels are always lower than maternal levels because of the placental enzyme HSD2 (11β hydroxy steroid dehydrogenase type 2). This enzyme converts cortisol into the inactive form cortisone, and so protecting the foetus from extensive levels of glucocorticoids. The efficiency of placental HSD2 varies considerably and can be weakened by diet, infection, inflammation, hypoxia and stress, which results in higher foetal levels of

cortisol. Because maternal cortisol levels are much higher than foetal levels, even a little reduction of HSD2 activity results in great alteration of foetal cortisol (Reynolds, 2013)..

When cortisol rises to extended levels during gestation, several negative effects can be seen in foetal tissues, especially on neural structure and function (Fig. 3) (Reynolds, 2013). The hippocampus is a primary target. High levels of cortisol causes neuronal atrophy, inhibition of neurogenesis and even neuronal death in this part of the brain. The foetal brain is developing at a high rate and is therefore highly vulnerable (Egliston et al., 2007). Possible consequences later in life are low birthweight, an activated HPA-axis, cardiometabolic diseases, brain disorders, deficits in learning and memory and sex-atypical behaviours (Reynolds, 2013; Kaiser & Sachser, 2005).

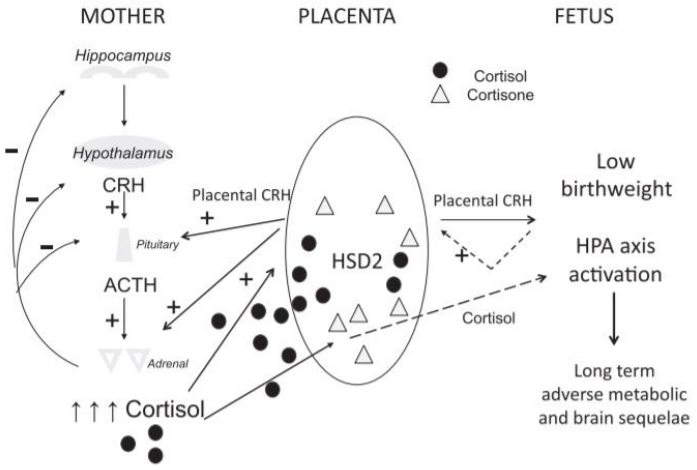

Fig. 3. Cortisol signalling between mother, placenta and foetus. The HSD2 enzyme converts active cortisol into inactive cortisone. When activity of this enzyme is diminished, high cortisol levels results in negative effects in foetal tissue (Reynolds, 2013).

The reprogramming of the foetal HPA-axis by maternal cortisol is investigated by a plethora of studies in humans and animals. A specific study for the dog is unfortunately lacking. Because of the similarity with humans and other animal species, cortisol transfer across the placenta is also expected in the dog and lasting effects of prenatal cortisol excess can be expected in canine offspring's life (Veronesi et al., 2015).

Also after birth, the HPA axis is essential for various physiological processes. The neonatal HPA system develops at a much higher rate than other organ systems and helps the neonate to adapt to the new extrauterine life (Veronesi et al., 2015). The development of the HPA-axis in dogs has not been investigated well. The importance of the HPA-axis early in canines life can, however, surely be expected because of the results of various socialization and isolation studies (Freedman, King, & Elliot, 1961; Scott & Fuller, 1965).

In different species, including rats, cortisol is greatly reduced in the early postnatal period (Fig. 4). In this period, the neonate will enter a stress hyposensitive period (SHRP), which is described in both rats and dogs. Low basal cortisol concentrations can be found during this period because of the relative inactivity of the HPA axis. The negative influence of glucocorticoids on the development of the central nervous system in this stage is the likely reason for the SHRP. High levels of glucocorticoids in this period of life could lead to reduction of brain weight, to altered social behaviours and impaired learning ability. However, a minimal baseline of cortisol is essential for the development of the central nervous system (Sapolsky & Meaney, 1986; Nagasawa et al., 2014).

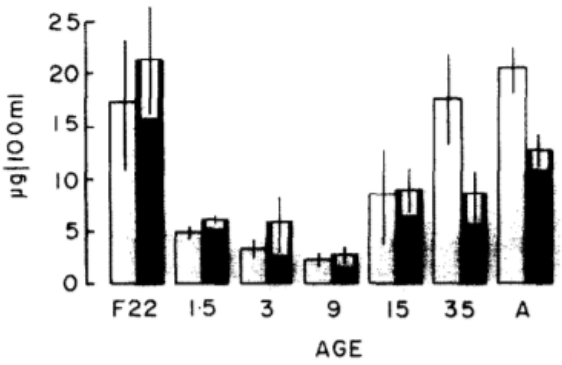

Fig. 4. Glucocorticoids levels in different ages in rats. F: foetal, A: adult. In the late foetal period glucocorticoids levels are high, following by a great reduce in the early postnatal period. Black bars representing male rats, white bars female rats (Sapolsky & Meaney, 1986).

The SHRP in canine puppies last until four weeks of age. Cortisol concentrations show thereafter, during the first six weeks of age, high variations, suggesting immaturity of the HPA axis (Nagasawa et al., 2014). Until six months of puppy age, cortisol levels are still very variable. This period is the most critical learning period for the dog. Because cortisol can hamper memory retrieval, low cortisol concentrations during this period of life are relevant. The amygdala tends to be critical in this phenomenon, with an inhibitory effect on HPA activity during early development. The inhibitory effect of the amygdala results in "developmental switches" and HPA-axis activity is therefore variable in early development. Cortisol levels in puppies under six months of age should therefore not be compared with cortisol levels of adult dogs (Cobb, Iskandarani, Chinchilli, & Dreschel, 2016).

Quantification of cortisol

Cortisol can be measured in blood, saliva, urine, faeces and keratinized tissue such as nails and hairs (Mack & Fokidis, 2017; Mormede et al., 2007).

Measuring cortisol in blood is often used to evaluate animal welfare, but some problems with this method can be seen. One of these problems is the large variation within an individual, and also between individuals. Also, blood collection is associated with an acute stress response, leading to increased cortisol levels. Similarly, salivary cortisol is also influenced by acute stress. Advantages of salivary cortisol are the relatively non-invasiveness of collection and the measurement of only the free fraction of cortisol (Mormede et al., 2007). Current methods for saliva sampling employ, however, yet mild to moderate restraint and current saliva absorption material employs inconsistent cortisol results (Accorsi et al., 2008). Urinary cortisol measurement is a non-invasiveness method, but reflects only a short period of time. This is also the case with faecal samples (Mormede et al., 2007). Both nail- and hair samples contain cortisol with less environmental variability and give information over a longer period of time (Mack & Fokidis, 2017). Measurement of cortisol in foetuses and new-borns was investigated mainly by blood analysis. Because of the invasiveness of this method, new techniques of cortisol measurement in keratin tissues were also performed in new-born dogs (Veronesi et al., 2015).

Because of the large amount of intraindividual- and interindividual variability and external variables on canine cortisol concentrations, reference ranges are difficult to determine and cover a wide range. Therefore, different studies on this topic should be compared with caution at any time and deviating levels from the reference range should be interpreted carefully (Cobb et al., 2016).

The reference range for blood cortisol concentration in adult dogs is established at 10 to 160 nmol/L (Schoeman, Goddard, & Herrtage, 2007). Salivary cortisol concentration ranges from 0 to 33.79 µg/dL (Cobb et al., 2016). Urinary cortisol is always measured in ratio with creatinine, due to variable urinary concentration. Urinary cortisol/creatinine ratio ranges differs per laboratory but the ratio should be under 4-10 x 10^{-6} (Galeandro et al., 2014). In dogs, reference ranges for cortisol in faeces and keratinized tissues are not yet established (Accorsi et al., 2008; Nicholson & Meredith, 2015).

Canines hair physiology

Hair is very important in thermal insulation, for sensory perception and as a barrier against different injuries to the skin (Scott, Miller, & Griffin, 2001).

Hair derives at the epidermis of the skin as a result of stimuli from the hair follicle, which is located in the dermis. The hair follicle forms the hair and two bulges, one for the sebaceous gland and one for the arrector pili muscle. There are two types of hair follicles, the primary and the secondary type. The primary hair follicles are located deeply in the dermis and

a single hair emerges. Secondary hair follicles are smaller and located more at the surface in the dermis. Only hairs of primary hair follicles have sweat glands and arrector pili muscles (Hyttel, Sinowatz, & Vejlsted, 2010). The primary hairs, from primary follicles, form the outercoat, as the secondary hairs, from secondary follicles, form the undercoat. In literature, the outercoat is also referred to as guard hair, where the undercoat is referred to as wool hair. The shape of the hair is controlled by the shape of the hair follicle. Straight follicles give rise to straight hair, curly follicles to curly hairs (Scott et al., 2001).

Hair development will happen in almost the entire body surface of the dog. The body surface will be covered by closely spaced hairs. Different individuals have a variation in hair density, hair type, hair distribution pattern and hair colour (Hyttel et al., 2010). Also within an individual, hair density differs. Dorsolateral aspects of the body have usually a thick haircoat, while the lateral surface of the pinnae, the under surface of the tail and the ventral body have thin haircoat (Scott et al., 2001).

The cycle of hair growth can be separated into different stages (Fig. 5). Hair follicles enter first the anagenic phase, a continuous cycle of growth where the follicle produces hair actively, followed by the catagenic phase (phase of regression) and the telogenic phase (the resting phase in which the hair is retained as a dead hair in the follicle). These phases are then followed by the exogenic phase where shedding of the hair takes place. A new anagenic follicle is formed and the cycle starts again (Scott et al., 2001).

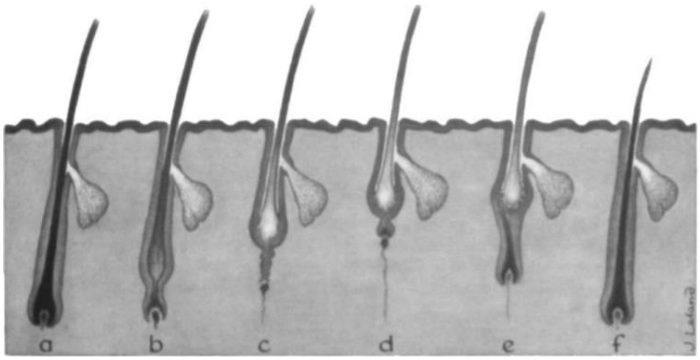

Fig.5. The hair cycle with the anagenic phase (a), the early catagenic phase (b), the catagenic phase (c), the telogenic phase (d), the early anagenic phase (e) and the new anagenic phase (f)(Scott et al., 2001)

In most mammalian species, the telogenic phase dominates the hair cycle (Diaz, Torres, Dunstan, & Lekcharoensuk, 2004). In canine species, however, differences between breeds are found. For example, in Boxers, Labrador Retrievers and Collies the telogenic phase predominates, whereas the anagenic phase predominates in West Highland White Terriers and Cairn Terriers (Arslan, Mackenzie, Brown, & Baxter, 1983).

Both endogenous (hormones and cytokines) and exogenous (photoperiod and temperature) factors influence the hair cycle. The hair cycle has not been studied extensively in canine species but factors that thought to affect the hair cycle are environmental changes, age, sex hormones, body region and breed (Scott et al., 2001).

Hair growth is regulated predominantly by the length of the photoperiod, where seasonal coat changes are seen by the influence of various hormones such as prolactin and melatonin. In winter, melatonin levels increase resulting in the decrease of prolactin levels with consequently the growth of winter pelage. In spring the situation reverses and shedding of the hair takes place (Diaz et al., 2004). Following this, the dog has a moulting period generally in early autumn and another in spring. This differs however between countries (Scott et al., 2001; Favarato & Conceição, 2008). Dogs in countries with a large variation in environmental temperature, show maximal hair follicle activity in summer (50% of the hair follicles in anagenic phase) and minimal activity in winter (10% of the hair follicles in anagenic phase)(Scott et al., 2001). Dogs in countries with less variation in environmental temperature have, on the other hand, less hairs in anagenic phase during summer and more during winter (Favarato & Conceição, 2008).

In dogs, hair grows till it reaches its preordained length and enters then the resting phase, which may last a long time. Each breed and each region of the body has its own finally length of hair and is genetically determined. Following this, the speed of growth also differs between particular sites of the body. Where the ultimate hair length is shorter, the hair will grow slower. In the shoulder region for example, hair length is about 30 mm in mongrel dogs with an average growth rate of 6.7 mm per week. In the forehead region however, hair length is about 16 mm with an average growth rate of 2.8 mm per week. Some studies measured the time of hair growth in canines: in Greyhound dogs for example, hair growth rate was established at 0.04-0.18 mm/day and in Beagle dogs at 0.34-0.40 mm/day (Scott et al., 2001).

Canine coats can be divided into normal (intermediate length), short and long coats (Scott et al., 2001). Each coat differs in which hair type predominates (table 1).

Table 1. Different hair coat types in dogs.

	Intermediate coat	Short coat		Long coat	
		Coarse coat	Fine coat	Coarse coat	Fine coat
Breeds	German Shepherd, Welsh Corgi	Rottweiler, Terriers	Boxers, Dachshunds, Pinschers	Poodle, Bedlington Terrier, Kerry Blue Terrier	Cocker Spaniel, Chow Chow
Predominated hair type	Secondary hairs	Primary hairs	Secondary hairs	Secondary hairs	

Canine coat colour can be divided into two classes, agouti or non-agouti. The classes differ in individual hair pigmentation, which may vary (agouti type) or be uniform throughout the length of the shaft (non-agouti). The agouti-type hair has a white or light tip, a pigmented brown or black body and a light yellow or red-brown base. The non-agouti type hair has black pigment throughout the hair shaft (Scott et al., 2001).

Two types of pigment are known, the eumelanin with the black colour and the pheomelanin with the yellow colour. Non-agouti hairs only have eumelanin, while agouti hairs can have only eumelanin (hair is entirely black), only pheomelanin (hair is entirely yellow) or both (hair is banded) (Scott et al., 2001).

Pigmentation of the hair is controlled by melanocyte-stimulating hormone (MSH). MSH binds on melanocortin receptors peripherally. Dogs having the agouti gene produce a product which is the antagonist of these receptors, thus blocking the receptor. Melanocytes can, in the presence of the agouti gene, switch between the synthesis of pigments eumelanin and pheomelanin. The non-agouti gene is recessive (Bennett & Hayssen, 2010).

Hair in canine puppies

Body hair in canine foetuses begin to grow at 45 days of gestation (Pretzer, 2008). After birth, for the first 12 weeks, the puppies have their puppy coat. This coat exists of mainly simple hair follicles that produce secondary hairs. Puppies do not lose this puppy coat, they gain after 12 weeks an adult coat with both primary and secondary hairs (Scott et al., 2001).

Cortisol deposition in canines hair

As previous mentioned, cortisol can be measured in different samples such as serum, saliva, faeces, urine and also in keratinized tissues (nails and hairs). The benefit of using hair samples is the long-term endocrine profile that is provided (Accorsi et al., 2008; Veronesi et al., 2015). Also, sampling of hair is relatively easy and hair is easily conserved (Accorsi et al., 2008). Hair could be shaved in regular intervals in a research setting to monitor stress (Bryan, Adams, Invik, Wynne-Edwards, & Smits, 2013) and in humans, differences in hormone content along the

length of the hair shaft can even be used to measure differences over a specific period of time (Ouschan, Kuchar, & Möstl, 2013).

Studies showed the possibilities in different species, as well in dogs. Because of the differences between species in cortisol secretions, each species requires their own validation in hair cortisol measurement (Bennett & Hayssen, 2010). In dogs, significant correlations were found between cortisol concentrations in hair and cortisol concentrations in saliva and faeces, suggesting that hair is a representative sample to measure cortisol, also in this species (Veronesi et al., 2015; Accorsi et al., 2008; Bennett & Hayssen, 2010). Recent studies even demonstrated that hair cortisol can function as a tool for the diagnosis of hypercortisolism (Cushing's disease) in the dog (Ouschan, Kuchar, & Möstl, 2013; Corradini et al., 2013).

To date, the incomplete information on hair physiology and the lack of laboratory validation is the main limit of this method (Accorsi et al., 2008). One uncertainty is that cortisol in hair may originate systemically from the HPA axis and/or directly from the hair follicle (Bennett & Hayssen, 2010; Accorsi et al. 2008).

Systemic cortisol from the HPA axis is incorporated, mainly in anagenic phase, into the hair shaft via the capillary blood vessels feeding the hair follicle (Davenport, Tiefenbacher, Lutz, Novak, & Meyer, 2006). The actual function, origin and rate of uptake and deposition of this hormone are yet unknown (Accorsi et al., 2008). Because of the high lipid solubility of steroids, diffusion of the free fraction is possible (Davenport et al., 2006). In telogenic phase, cortisol can enter the hair via sebum and sweat secretions, but in lower rates than in anagenic phase (Gow, Thomson, Rieder, Van Uum, & Koren, 2010).

Besides systemic cortisol, the hair can also contain locally produced cortisol. The hair follicle can produce cortisol itself in coordination with systemic stress, in response to local skin irritation or as functioning as a normal hair follicle (Accorsi et al., 2008). This is also called the peripheral HPA axis (Fig. 6). It is still unclear in which extend peripheral and central HPA axis contribute to the cortisol levels in both human and animal hair (Sharpley, McFarlane, & Slominski, 2011). In guinea pigs, systemically administered radioactive glucocorticoids were used to investigate both origins. This study showed a large percentage of locally produced glucocorticoids in the hairs (Keckeis et al., 2012). A study in Lynxes showed, however, high levels of systemic cortisol in the hairs (Terwissen, Mastromonaco, & Murray, 2013). The uncertainty of cortisol origin in hair suggests caution with interpretation.

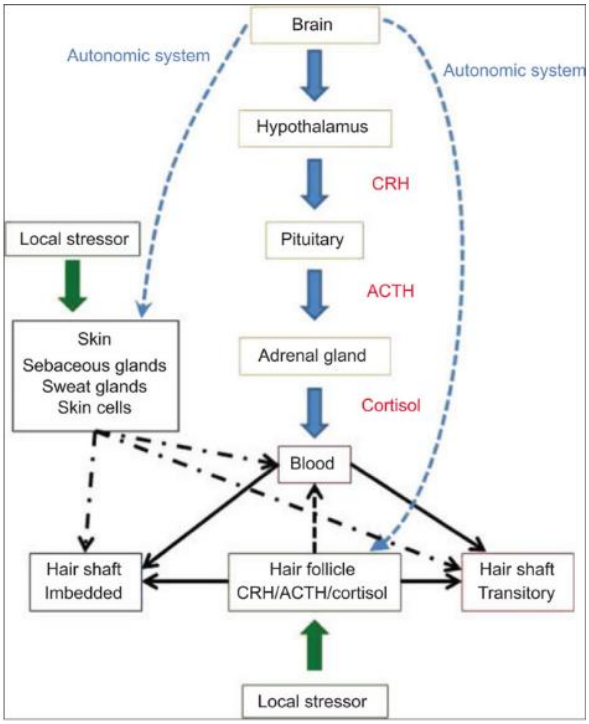

Fig. 6. The possible pathways of cortisol secreted into the hair with both the central- and peripheral HPA axis (Sharpley et al., 2011).

Hair cortisol in neonates

Different studies showed the possibilities of measuring cortisol in hairs of adults. Also, hair cortisol measurement of neonates is proven to be effective in new-born babies, in foals and recently also in canine puppies (Veronesi et al., 2015). This is interesting when combining this with the knowledge of maternal cortisol transfer to the foetus during gestation. Cortisol in neonate hair can consequently reflect information about the gestation time retrospectively. Two

previous studies used this method to obtain information about the period of time during gestation by measuring cortisol in infant hair (Kapoor, Lubach & Ziegler, 2016; Meise et al., 2016). Both studies showed that a moderate prenatal stressor can induce persistent effects on hair hormones in neonates of both monkeys and seals. Additional to this, a relationship between high hair cortisol concentration of infants and impaired learning abilities and anxiety behaviours later in life, was also demonstrated (Dettmer, Novak, Novak, Meyer, & Suomi, 2009; Dettmer, Novak, Suomi, & Meyer, 2012).

Material and Methods

Literature study

More information on canine hair cortisol had to be obtained before starting the practical investigation. A literature study was performed to investigate the most accurate and suitable method for measurement of hair cortisol in canine puppies.

To find additional information about hair cortisol measurement in canines hair, research-questions were formulated. Answers to these questions were collected by critically analysing previously published studies. Different keywords were used for searching articles on PubMed: 'canine', 'dog', 'puppy', 'animal', 'cortisol', 'glucocorticoids', 'welfare', 'stress', 'HPA axis', 'physiology', 'development', 'hair', 'coat', 'blood', 'saliva', 'urine', 'faeces', 'extraction', 'determination', 'measurement', 'protocol'. Keywords were used separately or in combination with another keyword. Different aspects for relevant required information were made to search more specifically. These groups were: (1) influence of age, gender and breed on hair cortisol concentration, (2) sampling site for hair collection, including amount of hair and the collection procedure, (3) influence of hair part, hair colour and hair type on hair cortisol concentration, (4) storage of hairs and hair preparation, (5) cortisol extraction, (6) cortisol determination and (7) cortisol interpretation via previous found concentrations. First, previously published studies on measurement of canine hair cortisol were obtained. When more information was needed, additional studies about hair cortisol in different species were consulted. Also studies concerning cortisol measurement in other biological samples than hair were incorporated for a better understanding. Articles were assessed based on year of publishing, journal of publishing, author names/names of universities, sample size and type of research. Mendeley was used as reference programme and American Psychological Association 6th edition as reference style.

Practical investigation

The aim of the practical investigation was to validate cortisol measurements from canine puppies' hair. Questions that had to be answered by the pilot study were: (1) is the used method reliable for determination of cortisol in canine puppies' hair? (2) are there differences in measured cortisol concentrations when different amounts of hair are tested? (3) what is the amount of hair that should at least be collected from the puppy for cortisol determination? (4) which body site should be used for the collection of puppy's hair to determine cortisol? and (5) are there differences in hair cortisol concentrations between: male and female puppies; proximal and distal hair parts; dark and white hair; and finally between guard and wool hair?

Animals

The pilot study was performed on 7 puppies, 3 females and 4 males from 6 different breeds (Table 2). The puppies ranged from 2 months to 6 months of age. The puppies belonged to owners who were known by the researchers as friends or relatives. The puppy owners gave consent to the collection of hair for research purpose. As hair collection is non-invasive, ethical application was not necessary.

Table 2. Identification, sampling site and description of collected hair from the puppies.

	Pup 1	Pup 2	Pup 3	Pup 4	Pup 5	Pup 6	Pup 7
Date of hair collection	21/01/2017	21/01/2017	24/01/2017	24/01/2017	28/01/2017	04/02/2017	03/02/2017
Name	Storm	Lana	Korrel	Cai	Billie	Cisko	Filo Soof
Date of birth	26/07/2016	31/10/2016	08/09/2016	11/08/2016	01/12/2016	09/08/2016	10/10/2016
Gender	♂	♀	♀	♂	♂	♂	♀
Breed	Husky	Australian labradoodle	Kooiker dog	Labrador Retriever	Jack Russel terrier	Labrador Retriever	Dutch Sheep dog
Sample site	Neck, Lateral thigh	Neck, Lateral thigh, Caudal back	Neck, Lateral thigh, Caudal back, Ventral chest	Lateral thigh, Tail	Neck, Lateral thigh, Caudal back, Ventral chest	Neck, Lateral thigh, Caudal back, Ventral chest	Neck, Lateral thigh, Caudal back, Ventral chest
Hair colour	Dark	Dark	White	Dark	White	Dark	Dark
Hair type	Wool	Guard	Guard	Guard	Guard	Guard	Guard
Hair part	x	At neck site: proximal and distal	x	x	x	x	At neck site: proximal and distal

Hair collection

Hair was collected in the end of January and the beginning of February 2017 on five different days. Hair was collected in the puppy's own home (5 times), after puppy class (1 time) or at the university of Utrecht (1 time). The amount of hair collected from different sites of the puppy's body was 'as much as possible' without leaving a hairless gap in the coat. Different wisps of hair were collected. Sample sites used were the neck, the hip (= ischiatic region/ lateral thigh), the back, the ventral chest and the base of the tail. These sites were chosen because of the use in previous published studies. Hair was cut with scissors as close to the skin as possible (within 5 mm). When the hairs of the neck were long enough, it was directly separated into proximal and distal parts (this was the case in 2 dogs). In addition to the canine puppies, to test reliability of the laboratory procedure, hair was collected from an young-adult dog (1 year and 10 months of age, Dutch Sheep dog, male) from the neck, ischiatic region, ventral chest and flank. This was done to have a large amount of hair available for testing. The hair from the neck from this dog was also separated in a proximal and distal part.

Cortisol analysis

After collection, the hair samples were placed in aluminium foil in paper envelopes which were stored at room temperature in a dark place. The laboratory protocol used for the extraction of cortisol from the hair was based on the protocol described by Davenport et al. (2006). The hairs were washed twice with 20 mL isopropanol and were thereafter dried in a stove at 37 ºC for 96 hours. When the hairs were thoroughly dry, they were clipped into smaller pieces with a scissor and were put into 2 mL Eppendorf tubes. These tubes were first filled with three beads. The amount of hairs in the tubes were accurately weighed and ranged from 6 till 347 mg. To test the repeatability of the used method, hair samples were divided into smaller subsamples when enough hair was available. This was the case for hair samples of the young-adult dog and eleven puppy samples. The number of subsamples ranged from two till five, depending on the amount of hair available. In total, 58 repeats were made from 17 hair samples. All hair samples were then grinded with a Tissue Lyser II at 30 Hz with 15 minutes during grind sessions. After each session, samples were centrifugated for 3 minutes at 13000 rpm. Hair samples were reviewed

after each session for the success of grinding. When no progress of grinding was established, tubes were opened, hair clots were removed and hair was manually pulled apart. When it seemed that too much hair present in the tube prevented adequate grinding, hair samples were split into two. The number of grinding sessions performed on the hair samples ranged from two till five times. Obtained hair powder was weighed to the nearest 0.01 mg. Target powder weight was 50 mg for each sample. Three samples of the young-adult dog were divided into five samples of 10 mg to test the influence of sample mass to the measured cortisol concentration. Extraction of cortisol was established by adding 1.5 mL of 100% methanol to the tubes (after centrifuging the tubes first for 3 minutes at room temperature at 13000 rpm) using a repeater-pipet with a combi-tip 10 mL Eppendorf and a 1 mL pipet-tip. After that, the tubes were incubated at room temperature overnight on an end-over-end roller. The tubes were then centrifugated for 5 minutes at 14000 rpm. The supernatant was transferred (two times 650 µL with a pipet-tip of 1 mL on the pipet) to 1.5 mL tubes which was centrifugated again for 5 minutes at 14000 rpm. 1.0 mL was again transferred to a new 1.5 mL tube by using reverse-pipetting. From some samples less extract was available and 800 µL was then pipetted of by using reverse-pipetting. The extracts were dried in two batches at 42ºC in a Speed Vac Concentrator (CentriVap Concentrator Labconco) for 2.5 hours. Batch 1 was stored at room temperature until batch 2 was finished. The dried extracts were then frozen at -20 ºC.

Two ELISA sets were performed with plates from the same LOT number. In the first set, one sample of each dog, collected from the same body site (the hip), was tested to get an impression on the expected range of variation of cortisol concentrations (11 out of 85 samples). The dried extracts of these 11 samples were dissolved in a phosphate buffer that was provided in the assay kit (High Sensitivity Salivary Cortisol ELISA kit, Salimetrics). 100 µL phosphate buffer was used for 1 mL extracts, 80 µL phosphate buffer for 800 µL extracts. To test the linearity of the samples with the standard curve, serial dilutions of three samples were included as well. These samples were tested undiluted, in a 1:2, 1:4 and 1:8 dilution. The dilution series was measured in duplicate. Cortisol concentration was determined with Salimetrics ELISA for salivary cortisol, the assay protocol was used provided by the manufacturer (Salimetrics, n.d.).

The second ELISA set was performed five days later. In the second set, 200 µL phosphate buffer was used (instead of 100 µL) for 1 ml samples and 160 µL (instead of 80 µL) for 800 µL samples to dissolve the dried extracts. The higher dilution was used because of the cortisol values of the first set being just above the mean of the standard curve. After adding the buffer, samples were stored overnight at 4ºC. Thereafter the previously described protocol was followed. In the second set all samples of the pilot study were measured in duplicate. Two ELISA plates were used. To test inter-assay variations the duplicates of 20 samples were separated and measured on the different plates. Also, the eight samples measured within the first ELISA set were tested again. Intra-assay variation, as the average of the coefficient of variations of the duplicates within the plates, was 2.7% for plate I and 1.2% for plate II. Inter-assay variation, as the average of the coefficient of variations of the duplicates between the plates, was 8.08% (between plate I and plate II of ELISA 2) and 7.81% (between ELISA 1 and ELISA 2). The minimum concentration of cortisol detectable was 0.0076 µg/dL. Cross-reactivity of other steroids was < 0.56%, except for dexamethasone at 19.2%.

Protocols

Different keywords were used for searching for protocols of cortisol determination from canines hair on PubMed: 'hair', 'cortisol', 'measurement', 'analysis', 'determination', 'protocol', 'canine', 'dog'. Keywords were used separately or in combination with another keyword. Obtained protocols and protocols used by previous studies on canine hair cortisol were read in

detail and reviewed. These findings formed the basis for a first test-protocol, which was improved with the findings from the pilot measurements. Based on the literature study and the pilot study, two protocols were established to serve as standard operating procedures (SOP) for the main study. One protocol for the best method for collection of the canine puppies' hair and one protocol for the best method of the laboratory analyses to determine cortisol concentrations.

Statistical analyses of the practical investigation

Data were collected, organized and explored with using Excel. Linearity of the cortisol measurements, intra-assay variation and inter-assay variation were calculated at first. Cortisol concentrations were then calculated in pg/mg hair. The data were subsequently organized and explored by making graphs in the following order: to test 1) repeatability of measurements, 2) the effect of the amount of hair used: 50 vs 10 mg, 3) the effect of the sample site used, 4) the effect of gender, 5) the influence of age, 6) differences between proximal and distal hair part, 7) differences between white and dark hair, and 8) differences between guard and wool hair (Table 2). After the first data exploration, statistics were performed. Statistical analysis were conducted using SPSS, IBM (version 24). The Kolmogorov-Smirnov test was used to test the normality of the dataset. Hair cortisol concentrations were not normal distributed using the raw data. This was mainly due to the samples of one individual (dog 6), which consistently showed higher cortisol concentrations than samples from all other dogs. As we had only a small sample size, dog 6 was excluded from the data to avoid this outlier to influence the results. Without dog 6, the hair cortisol concentrations were normally distributed.

Repeatability of cortisol determinations was evaluated by the coefficient of variation (CV) and the estimated repeatability coefficient (CR) of the repeated samples. The CV (%) was calculated by standard deviation (SD) / average x 100 and 10-15% was set as a reasonable target. The repeatability coefficient was defined by 1.96 x $\sqrt{2}$ x within subject SD. This coefficient is the value under which the difference between any two repeated measurements should fall with 95% probability (Bartlett & Frost, 2008). The distinction between technical- (repeated measurements of the exact same sample after all preparatory techniques) and biological replicates (parallel measurements of distinct samples by dividing the sample before all preparatory techniques) are herein important. The technical repeats of this study were the duplicates from each sample performed in ELISA 2 and were assessed only by the CV. The biological repeats were the repeats made from one hair sample (the 17 hair samples divided into 58 repeats) and were assessed by both the CV and the CR.

The influence of the amount of hair used (50 vs 10 mg) was tested with a general linear model (GLM) with both region and amount of hair as fixed factors. Only one dog (dog 0) was used for this test while, in contrast to the other dogs, multiple hair samples were available from both 50- and 10- mg.

The effect of sample site, gender and age were tested with mixed model analysis. The individual dog was set as subject to control for dependence of multiple samples within the dog. Sample site and gender were set as fixed factor and age as covariate. Hair from the flank and tail was only collected from one dog each, these sample sites could therefore not be included in the mixed model analysis. Hair from one dog (dog 5) was only available in a minimum amount, because of the earlier found possible trend between amount of hair and cortisol concentration, this dog was therefore also not included in this statistical test. Because some hair samples were measured several times to test repeatability, the average cortisol concentration of the repeats was calculated for this model.

The effect of hair part (proximal versus distal) was tested with a paired sample T test. The effect of hair colour (dark versus white) and hair type (guard versus wool) were only tested and presented graphically, because the sample sizes were too small for statistical tests (only two dogs with white hair and only one dog with wool hair).

Table 3 summarizes the research questions and samples used for statistical analyses. Results were considered significant with $\alpha < 0.05$.

Table 3. Overview of research questions and samples used for statistical analyses

Research issue	**Selected cases**	**Number of cases (N)**	**Cortisol concentration (individual vs average)**	**(Statistical) test used**
Repeatability	Dogs with repeated hair samples	58 hair samples from 17 repeated samples (6 dogs)	Individual	Coefficient of variation and estimated repeatability coefficient
Amount of hair (50 vs 10 mg)	Hair part = entire hair Sample site: Hip, Flank and Ventral	50 mg = 15 10 mg =15 (1 dog)	Individual	General linear model, univariate
Effect of sample site	Hair part = entire hair Sample site = Neck, Hip, Back and Ventral Dog ~ = Dog number 5	14 samples Neck = 2 Hip = 6 Back = 3 Ventral = 3 (6 dogs)	Average	Mixed model analysis
Effect of gender	Hair part = entire hair Dog ~ = Dog number 5	14 samples Males = 3 Females = 3 (6 dogs)	Average	Mixed model analysis
The relation with age	Hair part = entire hair Dog ~ = Dog number 5	14 samples (6 dogs)	Average	Mixed model analysis
Effect of hair part (distal/proximal)		Distal = 3 Proximal = 3 (3 dogs)	Individual	Paired sample T-test
Effect of hair colour (dark/white)	Hair part = entire hair	Dark = 12 (5 dogs) White = 5 (2 dogs)	Average	-
Effect of hair type (guard/wool)	Hair part = entire hair	Guard = 15 (5 dogs) Wool = 2 (1 dog)	Average	-

Additional study material

After the pilot study, also hair of another canine litter was collected. This was done (1) to see whether it was possible to collect the minimum amount of hair (10 mg), established in the pilot study, from very young puppies, (2) to collect hair repeatedly to see the change of hair cortisol concentrations in time and (3) to collect hair that can be used in the main study to represent a control litter. The litter was from a Dutch Sheep dog and consisted of five puppies, three males and two females (one male puppy died however within 12 hours after birth). The bitch was four years old and it was her first litter. At four weeks of puppy age, hair from the neck was collected from the four puppies and the mother. This was repeated at seven- and nine weeks of puppy age close to the sampling site used before. The procedure of collecting and storing the hairs was the same as mentioned for the pilot study.

Results

Literature study

A recent study demonstrated for the first time the possibility of measuring cortisol in canine puppies' hair (Veronesi et al., 2015). Veronesi et al. (2015) collected hair from 165 spontaneously dead puppies from three age classes; 25 premature puppies, 97 fresh term born-dead puppies or died within 24 hours and 43 puppies who died after 1 till 30 days of age. At least 20 mg of hair coat was collected by shaving hair from the puppies' back and neck. In each hair sample cortisol was detectable and the mean hair cortisol concentration was 65.2 ± 52.23 pg/mg. Significant higher cortisol concentrations were demonstrated in hairs of premature born puppies (123.22 ± 103.41 pg/mg) than in puppies born dead (66.97 ± 36.56 pg/mg) or puppies died between 1 and 30 days of age (42.97 ± 18.84 pg/mg).

Other available studies on measuring cortisol in canine's hair are only performed with adult dogs. A lot of differences between these studies are seen. The following will reflect each study per different aspect of relevant information (dog identification; hair collection; hair identification; storage of hair and preparation; cortisol extraction; cortisol determination; and cortisol concentrations). A conclusion is made in the end of each paragraph. Table S1 (see appendix) summarises the methods of previous studies on canine hair cortisol.

Dog identification factors (age, gender, breed, neutering status, lifestyle)

Age Cortisol concentrations in hair are variable during life. Especially early in life when the HPA-axis is still immature. The development of the HPA-axis and the hormonal changes throughout canine life are still not completely clear.

Veronesi et al. (2015) demonstrated in canine puppies a decline of cortisol concentration when the puppies got older. Puppies delivered prematurely had significant higher cortisol values than puppies dead at birth or within the first 30 days of age. The hair collected at birth reflects cortisol accumulated from first coat appearance (45 days of gestation) till the time of delivery. This cortisol could be produced by the mother, by the foetus or by both. Because of the incomplete information about the physiological mechanisms of cortisol deposition in hair, the proportions of these sources remain unknown. The higher cortisol levels in the premature puppies could possibly be the result of increased maternal cortisol, what already is demonstrated in women in the third trimester of pregnancy (Veronesi et al., 2015; Kirschbaum, Tietze, Skoluda, & Dettenborn, 2009). Comin et al. (2012) also found a decrease of hair cortisol with age (Fig. 7). In this study, hair cortisol of horse foals was measured from birth to 60 days of age. In humans, high hair cortisol variability in neonates and infants is demonstrated. Hair cortisol concentration decreased with early age and increased subsequently up to adulthood (Wester & van Rossum, 2015).

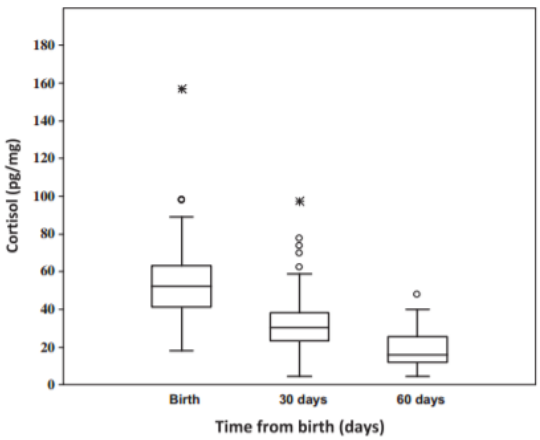

Fig. 7. Significant decrease of hair cortisol concentration in foals from birth to 60 days of age (Comin et al., 2011).

Hair cortisol levels in canine puppies' seems to be much higher than in adult dogs (Veronesi et al., 2015). When only looking at adult dogs, several studies did not find an effect of age on hair cortisol concentrations (Bennet & Hayssen, 2010; Nicholson & Meredith, 2015).

In dogs, studies with different biological samples than hairs have also been performed to test the changes of cortisol levels with age. Cortisol concentrations in serum and saliva seems to be lower in puppies than in adult dogs (Palazzolo & Quadri, 1987; Cobb et al., 2016). Another study on salivary cortisol, however, showed that cortisol levels were decreasing with age (Morrow et al., 2015).

High variability in cortisol levels in young puppies has been demonstrated by different studies using different biological samples (Palazzolo & Quadri, 1987; Cobb et al., 2016; Nagasawa et al., 2014). This variability of cortisol in young puppies can be explained by the immaturity of the HPA axis. Additionally, until four weeks of age, puppies do not yet produce cortisol due to the timing of the SHRP (Fig. 8) (Nagasawa et al., 2014).

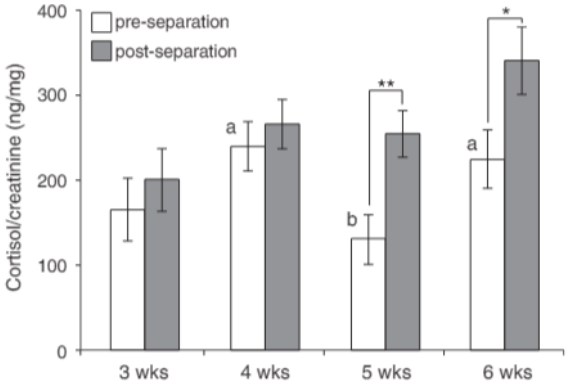

Fig. 8. Significant increase in urinary cortisol levels after separation in 5- and 6- weeks old puppies, but not yet in 3- and 4- weeks old puppies suggesting less production of cortisol <4 weeks of age by the puppy (Nagasawa et al., 2014).

Because of the differences in hair cortisol concentrations between different ages, age specific reference ranges are needed (Noppe et al., 2014).

In conclusion, different correlations between age and cortisol concentrations are found. Age specific reference ranges of hair cortisol are therefore needed. It is important to keep in mind that hair cortisol from canine puppies under 4 weeks of age probably reflects mainly cortisol levels of the bitch during gestation.

Gender Sex hormones and the HPA axis are possibly related to each other. Sex hormones can namely influence the development of the HPA axis and stress can also influence the sex hormones (Carpenter, Grecian, & Reynolds, 2017; Renard, Rivarola, & Suárez, 2007; Kapoor et al., 2016).

Some studies showed (in different species, testing different biological samples), significant differences in cortisol levels between males and females. Results are however inconsistent. Several studies showed higher cortisol levels in males (Mongillo, Prana, Gabai, Bertotto, & Marinelli, 2014; Rijnberk & Kooistra, 2010), while other studies showed higher cortisol levels in females (Cobb et al., 2016).

Studies with hair cortisol specifically are also inconsistent (Macbeth, Cattet, Stenhouse, Gibeau, & Janz, 2010; Wester & van Rossum, 2015). Looking at canine hair cortisol, no differences between males and females have yet been found (Veronesi et al., 2015; Nicholson & Meredith, 2015).

In conclusion, different correlations between gender and cortisol levels are found in different species. Up to date, no correlation between gender and canine hair cortisol has yet been found. Nevertheless, because of the lack of consensus between studies, the effect of gender should be taken into account for analyses.

Breed The mechanism of hair growth suggests consideration of breed differences on hair cortisol, such as specific hair growth rates and number of hairs at particular stages. Breeds with more hairs in anagenic phase and/or with a fast hair growth rate will have more incorporation of cortisol in the hairs (Terwissen et al., 2013; Arslan et al., 1983; Scott et al., 2001).

Different studies investigated the possible influence of breed/body size to cortisol concentrations. With canine hair cortisol, no correlation with breed has yet been found (Veronesi et al., 2015; Ouschan et al., 2013; Bennet & Hayssen, 2010). Testing salivary cortisol on the other hand, significant differences between breeds were found (Morrow et al., 2015; Sandri, Colussi, Perrotta, & Stefanon, 2015). In humans, a positive correlation has been found

between body mass and urinary cortisol and can be explained by the higher sensitivity to ACTH in obesity (Fraser et al., 1999).

In conclusion, different correlations between breed/body size and cortisol levels have been found. No correlation has been found up to date between canine hair cortisol and breed, but it can be presumed because of the mechanisms of hair growth. Breed differences should therefore be taken into account when interpreting hair cortisol concentrations.

Neutering status Because of the influence of sex hormones on the HPA axis (see above), the neutering status can also be important for cortisol levels. Studies that investigated the possible influence of neutering status on hair cortisol concentration are, however, missing.

Two studies showed the effect of neutering status on salivary cortisol levels in dogs. Intact dogs seems to have higher cortisol levels than neutered dogs (Cobb et al., 2016; Sandri et al., 2015).

In conclusion, neutering status can possibly influence cortisol levels and should be taken into account in future studies. However, to date is has not yet been demonstrated with hair samples. Moreover, as young puppies are regularly still intact, this possible influencing parameter is likely irrelevant.

Lifestyle Several parameters related to lifestyle can potentially influence cortisol concentrations.

Potential influential parameters on canine hair cortisol are housing conditions (lower cortisol levels can be found in single housed dogs compared to multidog households) (Bennet & Hayssen, 2010), the occupation of the dog (lower cortisol levels can be found in companion- and working dogs compared to competition dogs) (Roth et al., 2016), season (lower cortisol levels can be found in May and September compared to January) (Roth et al., 2016), time of the day (lower cortisol levels can be found in the morning compared to the evening) (Sinischalchi et al., 2013), and diseases (higher cortisol levels can be found in diseased dogs compared to healthy dogs) (Corradini et al., 2013; Ouschan et al., 2013; Park et al., 2016; Rijnberk & Kooistra, 2010). However, inconsistent results also have been found. For example, Nicholson & Meredith (2015) showed no significant differences in hair cortisol concentration between dogs with different housing conditions and also no significant difference between chronical ill- dogs and healthy dogs. Also studies with different species reveal different results. For example, in foals, no correlation between hair cortisol concentration and season has been found (Montillo et al., 2014), where in bears the same correlation has been found as in dogs (Bechshøft et al., 2013) .

In humans, hair treatment seems to be associated with lower hair cortisol concentration. Possible factors are special shampoos and lotions, frequent washing or frequent swimming (Ouschan et al., 2013). In dogs, no studies have specifically tested the effects of hair treatment, though Bennet & Hayssen (2010) did not find a correlation between bathing frequency and hair cortisol.

Moreover, it is very important to keep in mind whether the dog is treated with medications, especially (local or systemic) corticosteroid treatment while this will logically increase hair cortisol concentration (Wester & van Rossum, 2015).

In conclusion, a lot of various parameters related to lifestyle and their influence on hair cortisol are described in literature. These parameters include housing conditions, season, time of the day, diseases, hair treatment and medications among others. When performing future studies on canine hair cortisol, these influential parameters should be taken into account.

Hair collection (sampling site, the amount of hair and collection method)

Previous studies used different hair sampling sites to determine cortisol. Studies on different species than the dog suggest that there are differences in hair cortisol concentration depending on body location (Yamanashi et al., 2016; Terwissen et al., 2013; Macbeth et al.,

2010; Ashley et al., 2011). In canine species significant differences in hair cortisol and sampling site has, however, not yet been found (Roth et al., 2016). Also in canine species it is however expected. Hair will grow quicker when the site's specific ultimate hair length is longer and cortisol will then incorporate more extensively, resulting in different hair cortisol concentrations between different regions. Body sites with high hair growth rates are the neck, the lateral thigh and the flank (Scott et al., 2001; Diaz, 2004).

Previous studies used also different amounts of hair fort the extraction of cortisol. This is important while sample mass can possibly affect measured cortisol concentration (Bortolotti, Marchant, Blas, & German, 2008).

Finally, different hair collection methods are used such as clipping, shaving or pulling. Hair follicles contain large amounts of cortisol and can produce this hormone locally. Therefore, clipping and shaving is recommended (Gow et al., 2010).

Sampling site and sample mass Canine studies on hair cortisol used different sampling sites, different amounts of hair and different collection methods. Veronesi et al. (2015) shaved > 20 mg of puppy hair from the back and neck. Bennet & Hayssen (2010), Accorsi et al. (2008), Sinischalchi et al. (2013) and Park et al. (2016) collected hair from the ischiatic region (hip) by clipping or shaving. Other sampling sites used in the canine species were the shoulders (Bryan et al., 2013), the chest (Roth et al., 2016), the foreleg (Ouschan et al., 2013) and the region of the sternum (Corradini et al., 2013). These hairs were also clipped or shaved. In contrast to all other canine studies, Nicholson & Meredith (2015) collected the hairs by brushing. Different amounts of hair were used, ranging from 25 till 500 mg.

In other species than dogs, also different hair sampling sites were used. In chimpanzees, lynxes, bears, caribous and reindeers significant differences in hair cortisol concentrations between sampling sites were found (Yamanashi et al., 2016; Terwissen et al., 2013; Macbeth et al., 2010; Ashley et al., 2011). In chimpanzees, hair samples from the animals flank contained more cortisol than hair samples from the arms or back (Yamanashi et al., 2016). In lynxes, hair cortisol concentrations differed even within the foot/leg region (Fig. 9) (Terwissen et al., 2013). In bears, hair samples from the neck contained more cortisol than hair samples from the shoulders, rump and abdomen (Macbeth et al., 2010). This has also been found in caribous, but the opposite was found in reindeers (Ashley et al., 2011). In these species, also different amounts of hair were used. In chimpanzees and grizzly bears only 5 mg was required (Yamanashi et al., 2016; Macbeth et al., 2010; Meyer et al., 2014). This corresponded with only 5-7 hairs in chimpanzees (Yamanashi et al., 2016).

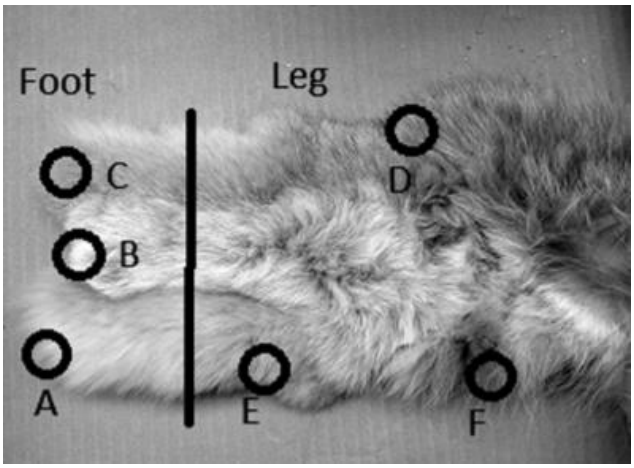

Fig. 9. Different hair cortisol levels found in different body regions in Lynxes. Distal sites (sites ABC) showed significantly lower hair cortisol concentrations than proximal sites (sites DEF) (Terwissen et al., 2013).

Because of the different hair growth rates between body sites, each body site might reflect a different period of time. This can be important when using canine puppies' hair to retrospectively reflect the time of gestation. Body sites with high hair growth rates might contain both puppy and maternal cortisol, where body sites with slow hair growth rates might only contain maternal cortisol.

In conclusion, different sampling sites can be used to collect hair for the determination of cortisol in dogs. It should always be taken into account that cortisol levels can differ between sampled body sites. Preferable sample sites are the neck, the hip or the flank. Lowest minimum

amount of hair required for cortisol analysis is 5 mg, studies with this little amount has however not yet been performed in dogs. Hair should always be cut or clipped and not be pulled out as the incorporation of hair follicles will increase hair cortisol concentration.

Hair identification factors (hair part, hair colour and hair type)

Hair part Some studies showed differences in cortisol concentration between distal and proximal hair parts (Kirschbaum et al., 2009; Yamanashi et al., 2016). Kirschbaum et al. (2009) measured lower cortisol concentration in distal hair than in proximal hair suggesting cortisol to degrade over time in humans. Yamanashi et al. (2016) measured on the other hand the opposite in chimpanzees, discouraging the fact of cortisol leaching from hair as it aged. Human hair is typically longer than animals and degradation is therefore more likely. Other studies also did not find significant differences between proximal and distal hair parts, for example in dog hair (Bennet & Hayssen, 2010) and in monkey hair (Davenport et al., 2006). Two explanations can be given for constant cortisol concentration along the hair shaft: (1) because of the stable past environmental circumstances of the animal, or (2) because of diffusion of the steroid in the shaft. However, because the hair shaft is filled with spaces of air, diffusion along the hair shaft is not possible and this latter hypotheses can therefore be discouraged (Davenport et al., 2006). Interesting was that Bennet & Hayssen (2010) found significantly more dogs with more cortisol in the distal hair segment.

In humans, specific periods of time can be represented in differences in hormone content along the hair shaft when calculating with an average hair growth rate of 1 cm per month (Ouschan et al., 2013). This has never been investigated in animals.

In conclusion, different correlations between hair part and cortisol concentration are found in different species. Because of the inconsistency between studies, hair part should be taken into account when measuring hair cortisol concentration.

Hair colour Both hair pigment and cortisol are controlled by the same families of hormones and the same family of receptors (melanocortin receptors). Because the agouti gene produces an antagonist of these receptors, it controls both coat colour and cortisol production. Because of this implication studies were set to investigate. In mice, the first demonstration was made with cortisol differences associated with the agouti gene. Mice with an agouti-gene released more cortisol in faeces than non-agouti mice (Hayssen, Harper, & Defina, 2002).

Bennet & Hayssen (2010) found for the first time in dogs a significant correlation between hair colour and hair cortisol concentration. Dogs with black hair had less hair cortisol than non-black dogs. Within the same dog, black hairs (eumelanin) had also less cortisol than yellow hairs (pheomelanin), with agouti (banded) hairs intermediate. Two explanations of this phenomenon can be given: (1) different similar control mechanisms underlie the interaction between hair colour and hair cortisol, and (2) more space is present in yellow hair than in black hair because of the lack of pigment. In chimpanzees it has also been found that white hairs contain higher levels of cortisol than black hairs (Yamanashi et al., 2016). Veronesi et al. (2015) and Nicholson & Meredith (2015) did, on the other hand, not find a significant correlation between hair colour and hair cortisol in dogs.

In conclusion, different correlations are found in previous studies between coat colour and cortisol concentration. Black hairs contain probably less cortisol than non-black hairs. Hair colour should therefore be taken into account in future studies.

Hair type Only a few studies investigated the role of hair type on hair cortisol concentration. In grizzly bears differences between types of hair were found, with guard hair (outercoat) containing significantly more cortisol than wool hair (undercoat). According to the authors, guard hair can better be used than wool hair because of three reasons: (1) guard hair reflects a longer period of time because of the longer hairs, (2) guard hair is less variable between different body sites and (3) guard hair can better be cleaned and ground to powder (Macbeth,

Cattet, Stenhouse, Gibeau, & Janz, 2010). In dogs, no differences in cortisol concentrations were found between guard and wool hair (Roth et al., 2016).

In conclusion, to make results as comparable as possible, focussing on only guard hair is wisely for future studies on hair cortisol. However, no studies on canine hair are yet been published that demonstrated significant differences in hair cortisol between different hair types.

Summarizing Different parameters are (probably) influencing cortisol in canine hair. This should be taken into account when performing more studies on canine hair cortisol in the future. Different correlations are found and are summarized in table 4 (with * representing studies with dogs, but with a different biological sample than hair and with ** representing studies with different species than dogs).

Table 4. Summary of correlations found with influential parameters on hair cortisol.

	Significant **positive** correlation	Significant **negative** correlation	**No** significant correlation found
Age: increasing	Palazzolo et al. (1987)*, Cobb et al. (2016)*, Wester & van Rossum (2015)**, Noppe et al. (2014)**	Veronesi et al. (2015), Morrow et al. (2015)*, Comin et al. (2012)**, Noppe et al. (2014)**, Montillo et al. (2014)**	Bennet & Hayssen (2010), Nicholson & Meredith (2015), Macbeth et al. (2010)**, Bechshoft et al. (2013)**
Gender: male	Mongillo et al. (2014)*, Rijnberk & Kooistra (2010)*, Wester & van Rossum (2015)**	Cobb et al. (2016)*, Macbeth et al. (2010)**	Veronesi et al. (2015), Nicholson & Meredith (2015), Comin et al. (2012)**, Macbeth et al. (2010)**, Bechshoft et al. (2013)**, Montillo et al. (2014)**
Breed/body size: big	Morrow et al. (2015)*, Fraser et al. (1999)**	Sandri et al. (2015)*	Veronesi et al. (2015), Ouschan et al. (2013), Bennet & Hayssen (2010)
Lifestyle: hair treatment, single dog, companion dog, winter, evening, disease	Roth et al. (2016), Corradini et al. (2013), Ouschan et al. (2013), Park et al. (2016), Rijnberk & Kooistra (2010)*, Bechshoft et al. (2013)**	Bennet & Hayssen (2010), Roth et al. (2016)	Ouschan et al. (2013), Bennet & Hayssen (2010), Nicholson & Meredith (2015), Roth et al. (2016), Siniscalchi et al. (2013), Nicholson & Meredith (2015), Montillo et al. (2014)**
Sample site positive correlation = a correlation	Scott et al. (2001), Diaz et al. (2004), Macbeth et al. (2010)**, Yamanashi et al. (2016)**, Terwissen et al. (2013)**, Ashley et al. (2011)**		Roth et al. (2016)
Hair colour: black	Macbeth et al. (2010)**	Bennet & Hayssen (2010), Hayssen et al. (2002)**, Yamanashi et al. (2016)**	Veronesi et al. (2015), Nicholson & Meredith (2015), Macbeth et al. (2010)**
Hair part: distal	Yamanashi et al. (2016)**	Kirschbaum et al. (2009)**	Bennet & Hayssen (2010), Davenport et al. (2006)**, Macbeth et al. (2010)**
Hair type: guard	Macbeth et al. (2010)**		Roth et al. (2016)

The following will describe the different laboratory procedures for cortisol extraction from canine hair described by previous published studies. A schematic view of the procedure is seen in Fig. 10.

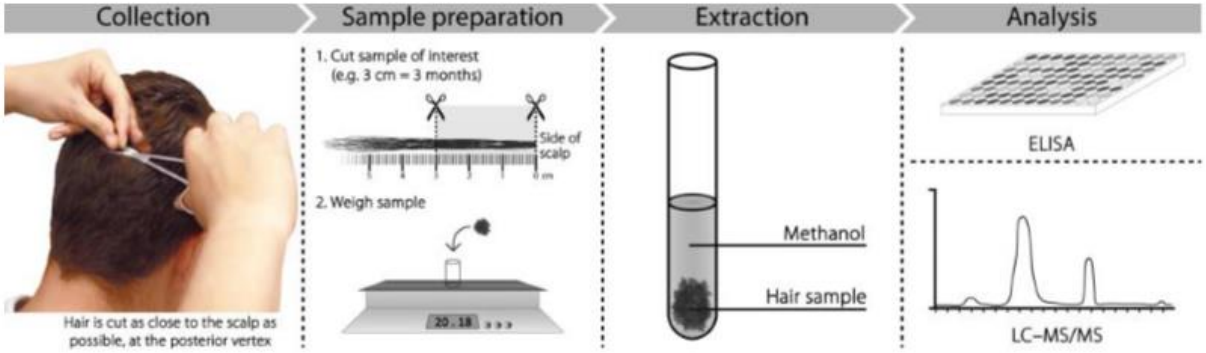

Fig. 10. An overview of cortisol analysis from human hair (Wester & van Rossum, 2015).

Hair storage and sample preparation

Storage Hair samples from previous studies on canine hair cortisol were all stored at room temperature. Most studies stored the hair samples in a simple envelope or paper bag. Some studies suggested storage in aluminium foil also.

Because steroids in hair are stable at room temperature, hair samples can be stored without conservation (Bryan et al., 2013). The possibility to store intact hair samples for a long period of time (couple of years) was showed by several studies (Park et al., 2016; Yamanashi et al., 2016). It is important to keep in mind that cortisol levels in hair powder will decrease quicker (Macbeth et al., 2010).

In conclusion, the hairs can be stored at room temperature without conservation for a long time.

Preparation The hairs in previous published studies were mostly washed (for removing contaminated steroids on the outer shaft of the hair) with isopropanol as a preparation before extraction. Davenport et al. (2006) determined the appropriate wash procedure for animal hairs and isopropanol was the final choice used as washing medium. Methanol and water should both not be used as washing medium because it also removes the internal cortisol. Washing with methanol does, however, not remove inner shaft cortisol from bear hairs, so differences between species are convincing (Macbeth et al. 2010). Two washes of isopropanol are sufficient (Davenport et al., 2006; Meyer, Novak, Hamel, & Rosenberg, 2014). Accorsi et al. (2008), Nicholson & Meredith (2015) and Siniscalchi et al. (2013) did not wash or prepare the hairs at all. The majority of studies appointed however contamination of the exterior of the hair shaft with steroids and these should be washed away for a more accurate measurement of internal hair cortisol (Gow et al., 2010). After washing the hairs, the hairs should be dried in a stove for 2-3 days before starting the extraction procedure.

In conclusion, the hairs should be washed with isopropanol before extracting cortisol.

Cortisol extraction

The cortisol extraction method is somewhat similar between previous published studies with canine hair. Differences are seen when looking specifically, for example in centrifuging rate, -time and/or temperatures.

The first step after the preparation of the hairs is to cut the hairs into 1-3 mm fragments, or to grind the samples into powder. Slominiski et al. (2015) found that grinding the hair does not extract more cortisol than finely cutting. Meyer et al. (2014) and Davenport et al. (2006), however, noted a significant increase of cortisol extraction from the sample after grinding (3.5

times), due to the breakdown of the hair matrix. Two different methods of hair grinding are described (Meyer et al., 2014). The hair powder or hair fragments are then put in a glass vial and methanol is added for extraction. This is stored at room temperature overnight or incubated for >10 hours. Gow et al. (2010) demonstrated an optimal incubation time of 16 hours. Slominiski et al. (2015) suggested repeated extractions (3 times) to maximize cortisol extraction. These samples are then centrifugated and the supernatant is pipetted of/decanted. This supernatant is evaporated to dryness under a stream of air or nitrogen. The dried residue is finally dissolved with a buffer. Meyer et al. (2014) described a step-by-step validated protocol for hair to extract and analyse cortisol from hairs. This protocol is also been performed in canine hairs (Meyer et al., 2014).

Extraction Specifically speaking, studies on canine hair cortisol used methods described by other authors. Veronesi et al. (2015) followed the procedures by Comin et al. (2014). Bennet & Hayssen (2010), Bryan et al. (2013) and Nicholson & Meredith (2015) followed the procedures described by Davenport et al. (2006). Accorsi et al. (2008) and Corradini et al. (2013) followed the procedures described by Koren et al. (2002). Roth et al. (2016) followed the procedures described by Karlen et al. (2011). Siniscalchi et al. (2013) followed the procedures described by Sharpley et al. (2009). Ouschan et al. (2013) and Park et al. (2016) did not refer to a specific used protocol.

In conclusion, previous published studies on canine hair cortisol used broadly the same method to extract cortisol from hairs.

Cortisol determination

Determination of cortisol after extraction can be achieved by enzyme-linked immune-absorbent assay (ELISA), radioimmunoassay (RIA) or chromatography. Hair samples have to be prepared and stored in a similar way prior to these different techniques (Gow et al., 2010).

The majority of published studies used the ELISA technique. In the Netherlands, Salimetrics ELISA from the United Kingdom is mostly used. The ELISA is an often used method because of the low costs, quickly results and good sensitivity. Unfortunately, variation in results is seen among similar ELISA methods and the validity and accuracy of this method should therefore be questioned. Besides ELISA, the RIA method has also frequently been used. It is a highly specific and sensitive method to analyse cortisol in hair, but is however expensive and requires special precautions. The last method, mass spectrometry is also highly specific and sensitive. This technique is seen as the "golden standard" for hair analysis. In hair analysis gas chromatography/mass spectrometry (GC/MS) is widely used, but to measure cortisol in hair only high performance-liquid chromatography/mass spectrometry (HPLC/MS or LC/MS) has been used previously. This technique is very accurate but is relatively expensive. Between these methods, different minimum amounts of hair are required: at least 10 mg for the ELISA, 25 mg for the RIA and 30 mg for the mass spectrometry (Gow et al., 2010).

It is not sure yet if results obtained from different methods can be compared. In humans, one study investigated the correlation between the different methods on the same hair. The immunoassays measured substantially higher hair cortisol concentrations than LC-MS/MS methods, but the levels were significant correlated. The higher cortisol concentrations from immunoassays ranged between 2.5-fold and 20-fold that of chromatography methods. This allows different laboratories to use a correction factor to compare results with other laboratories (Russell et al., 2015; Slominski, Rovnaghi, & Anand, 2015).

Determination The three methods for hair cortisol determination were al used in previous studies in canine hair cortisol. Bennet & Hayssen (2010), Ouschan et al. (2013), Park et al. (2016) and Nicholson & Meredith (2015) used the ELISA method. Veronesi et al. (2015), Accorsi et al. (2008), Roth et al. (2016), Corradini et al. (2013) and Siniscalchi et al. (2013)

used the RIA method. Bryan et al. (2013) used, as first in dogs, analysis of cortisol in hair by LC-MS/MS.

In conclusion, ELISA, RIA and mass spectrometry can all three be used to analyse cortisol from canine hairs. Results from the different methods can best be interpreted separately because the comparability of these methods is not yet fully clear. The ELISA technique is preferable in canine puppies because of the minimal amount of hair (at least 10 mg) needed, the quick results and the high sensitivity.

One study on chimpanzees hair tested the variability of several methods for cortisol measurement. This revealed that variations in hair cortisol concentration can be caused by degree of grinding, extraction time and various ELISA systems. In contrast, different methods of storage and drying of the hairs did not affect absolute hair cortisol concentrations (Yamanashi et al., 2016).

Cortisol concentrations

Cortisol concentration Big differences were found in canine hair cortisol concentration measured by previous studies. These differences are due to the high individual variability in dogs and the high variability between used methods.

Hair cortisol concentration from canine puppies (65.2 ± 52.23 pg/mg) seems to be higher than in adult dogs (Veronesi et al., 2015). In adult dogs, discrepancy of different hair cortisol concentrations can be due to hair powdering, while this results in a 3.5-fold increase in cortisol conservation (Bennet & Hayssen, 2010; Davenport et al., 2006). For example, some studies grinded the hairs and measured higher cortisol concentrations (Bennet & Hayssen, 2010; Bryan et al., 2013; Nicholson & Meredith), while other studies did not and measured therefore lower cortisol concentrations (Accorsi et al., 2008; Ouschan et al., 2013; Corradini et al., 2013). However, this is not always consistent while also higher hair cortisol concentrations can be measured without grinding the hairs (Roth et al., 2015; Park et al., 2016). One study measured a lot higher hair cortisol concentrations than previous studies, possibly due to experimental settings with stressful acoustic stimuli (Siniscalchi et al., 2013).

Reference ranges of hair cortisol concentrations in dogs are not yet available. In human hair, cortisol levels in non-pathological situations are ranging from 5 to 91 pg/mg (Binz, Braun, Baumgartner, & Kraemer, 2016).

In conclusion, canine hair cortisol concentrations measured by previous studies differ tremendously. These differences are probably due to inter-individual variability and variability between different analyse methods. Because of these variabilities, comparing of different studies is very difficult. Comparing dogs within a study is therefore probably more meaningful than comparing dogs between studies.

From this, it is clear that hair cortisol concentrations are highly variable in dogs. Comparing results of different studies is difficult, due to inter-individual variability and the different determination methods of cortisol. The great amount of potential influential parameters on hair cortisol (as age, gender, breed, sample site, hair part, hair colour, hair type), makes it even more difficult. All these parameters should be taken into account when assessing and evaluating hair cortisol concentration in dogs.

Finally, it is important to keep in mind that cortisol in canines hair possibly find its origin both systemically and locally. The exact amount of local produced cortisol is unknown but with our knowledge so far, hair cortisol should not be interpreted as a 100% reflection of systemic cortisol.

Additional literature for future studies

The aim of the future study is to reflect retrospectively information about the gestation time by the measurement of cortisol in canine puppies' hair. Two previous studies, with seals and monkeys, used this method to obtain information about the period of time during gestation (Kapoor, Lubach, Ziegler, 2016; Meise et al., 2016). Both studies showed that a moderate prenatal stressor can induce persistent effects on hair cortisol in neonates.

Kapoor et al. (2016) demonstrated significant lower cortisol levels in the hair of infant monkeys born from stressed mothers, compared to infant monkeys born from control mothers (Fig. 11). The lower cortisol levels in prenatal stressed infants differs than previously suspected. An explanation of this can be found in the suppression of foetal HPA-axis because of the elevated maternal cortisol. Because of the summative view the hair is given, this prolonged suppression can overview the previous elevated levels in the foetus. This is an important finding while it suggests that when the prenatal stressor is before the actual start of foetal hair growth, lower hair cortisol levels can be found. In this study also a significant difference in hair testosterone level was found. Neonates born from stressed mothers showed significant lower hair testosterone levels.

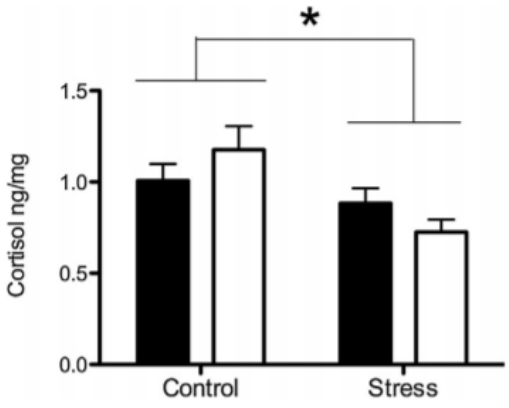

Fig. 11. Significant lower hair cortisol concentration in neonates born to mothers from stressed pregnancies. No differences between sexes were found (black bar: male, white bar: female) (Kapoor, Lubach, Ziegler, 2016).

Meise et al. (2016) found significantly higher levels of cortisol in the hair of marine mammals when exposing to stress in high density colonies during gestation. Significant higher hair cortisol levels in neonate marine mammals of these mothers, however, were not demonstrated. Representing of early pregnancy in the seal mothers hair and of late pregnancy in the offspring's hair, and/or an high concentration of HSD2 enzymes in seals can be explanations for the uncorrelation. The marine pups did show a significant difference in hair testosterone levels. Lower testosterone levels were found in the hairs of pups from low density breeding colony. As also found by Kapoor et al. (2016), offspring's hair cortisol levels were higher as maternal cortisol levels (Fig. 12).

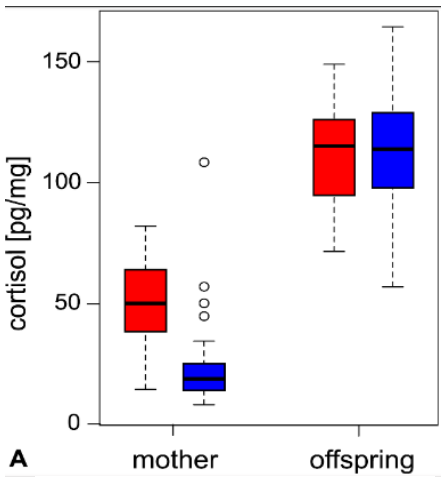

Fig. 12. Significant higher cortisol levels in offspring's hair than in maternal hair. High density colonies (red bars) only results in significant increase in cortisol levels in maternal hair (Meise et al., 2016).

Further investigation on neonate hair reflecting maternal stress during gestation should be performed in future studies to reconfirm or disconfirm results of previous studies.

When performing a similar study on canines, hair of puppies under four weeks of age could be used. These hair samples will mainly reflect cortisol levels of the bitch during gestation (last two till three weeks) because of the absence of own cortisol production by the pup. When older puppies are tested, distal hair can possibly be measured to reflect this period of time, but this should be done very carefully. Also, because different body sites have different hair growth rates it is probably possible to test both maternal and neonatal cortisol separately from one pup. Hypothesized is that puppies delivered by bitches suffered from stress or diseases during gestation, will have higher hair cortisol concentrations due to the inhibition of the HSD2

enzyme. However, when the prenatal stressor was present before 45 days of gestation, lower hair cortisol concentration can be found because of the suppression of the foetal HPA axis.

Additionally, also other hormones such as testosterone could be measured in the hairs of the puppies to investigate all possible effects of prenatal stress. The control group should be standardized (e.g. age, gender, breed, sampling site, hair part, hair colour, hair type) as much as possible to reduce inter-individual variation.

Practical investigation

Protocol adaptations

Adaptations to the current protocol available for hair cortisol extraction at the Utrecht University (Davenport et al., 2006) were established for hair cortisol extraction, specific for dog hair.

At first, collecting 500 mg of hair as described by Davenport et al. (2006) is unnecessary for cortisol extraction from canine puppy hair, at least 10 mg of initial hair can be used in this species. A maximum of 90 mg of hairs should be placed in the Eppendorf tubes before grinding sessions. Maximal 55 mg of hair should be used when testing wool hair, because of the light weight of these hairs. Two grinding sessions will then be sufficient. To optimize grinding, the hairs should be clipped into small pieces before putting into the tubes. These hairs should be hold with a forceps to minimize hair loss. When very less hair is available (< 20 mg), this step should be skipped, while hair loss should be prevented at best. After grinding, no more than 55 mg of hair powder should be put into the tubes for extraction. When more hair powder is used, less extract will be available. To reduce variability, using the same amount of hair powder for each sample is recommended. Collecting the extract should be performed in two steps of pipetting with centrifuging in between to lose most of the residue. For the first step, as much as possible of the extract should be collected, 2 times pipetting of 650 µL is sufficient. For the second step, reverse pipetting of exact 1 mL should be used. To dry the extracts, 2.5 hours are sufficient at 42 ºC on a Speed Vac Concentrator. To dissolve the extracts, 200 µL of phosphate buffer should be used. The cortisol measurements will then be on the middle of the standard curve of the ELISA.

A protocol that can be used as standard operating procedures for future studies for canine hair cortisol extraction is now available, with the incorporation of these adaptations.

ELISA 1

In the first ELISA, concentrations were detectable in all but one sample. It is likely that too little hair was present in this sample (3 mg powder) to determine cortisol. Cortisol values were just above the mean of the standard curve. Cortisol concentrations of each puppy were respectively 7.2, 13.7, 14.5, 18.6, 20.8 and 52.8 pg/mg. The serial dilutions ran parallel to the standard curve (see Fig. 13, Fig. 14 and Fig. 15). The variation of the diluted samples ranged from 100% to 137% (Fig. 15).

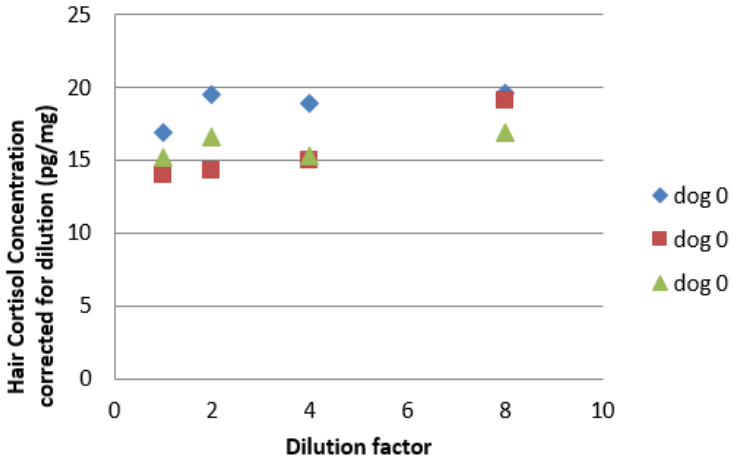

Fig. 13. Influence of sample dilution on final dog hair cortisol concentrations (pg/mg). Three different hair samples from the same sampling site of dog 0 were tested.

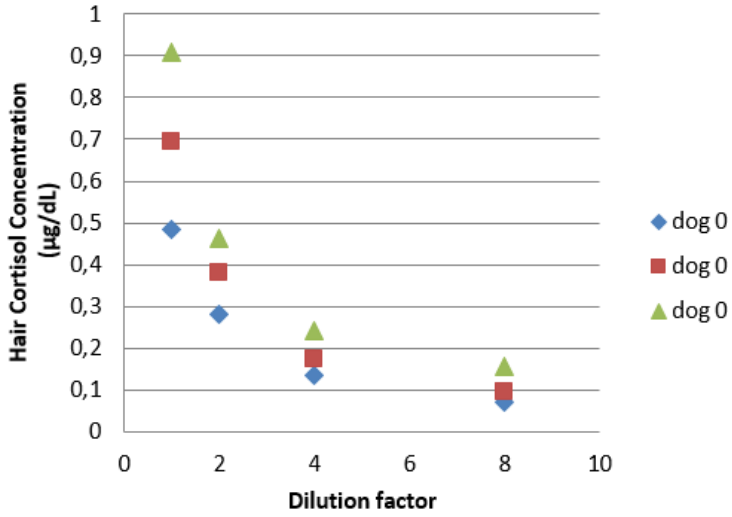

Fig. 14. Influence of sample dilution on assay concentrations of cortisol (µg/dL). Three different hair samples from the same sampling site of dog 0 were tested.

Hair sample	Dilution Factor	Observed cortisol (µg/dL)	Percentage (%)
1	1:1	0.49	100
	1:2	0.56	116
	1:4	0.54	112
	1:8	0.56	116
2	1:1	0.91	100
	1:2	0.93	102
	1:4	0.98	107
	1:8	1.24	137
3	1:1	0.70	100
	1:2	0.76	110
	1:4	0.70	100
	1:8	0.78	112

Fig. 15. Serial sample dilutions of three hair samples from one dog (dog 0).

ELISA 2

In the second ELISA, all the samples were in the middle of the standard curve. Cortisol concentrations could be measured from all the tested samples. Hair cortisol concentrations ranged from 7.30 pg/mg to 119.44 pg/mg. Mean cortisol content from the puppies' hair was 26.15 ± 26.67 pg/mg. One dog (dog 6) had consistently higher hair cortisol concentrations than the others (four outliers of 119.44, 109.40, 50.43 and 34.38 pg/mg). Excluding this dog, the hair cortisol concentrations ranged from 7.30 pg/mg to 26.30 pg/mg. Mean hair cortisol content without this pup was 17.06 ± 3.69 pg/mg.

The minimum amount of hair necessary to measure cortisol in canine hair was 5 mg of hair powder, corresponding with initial 10 mg of hair. This was available at all the tested sampling sites from the canine puppies. In general, less hair was available at the ventral site compared to the neck, hip and back (Fig. 16). When testing the canine litter, the necessary minimum amount of hair was available repeatedly at the puppies' neck at four-, seven- and nine- weeks of age.

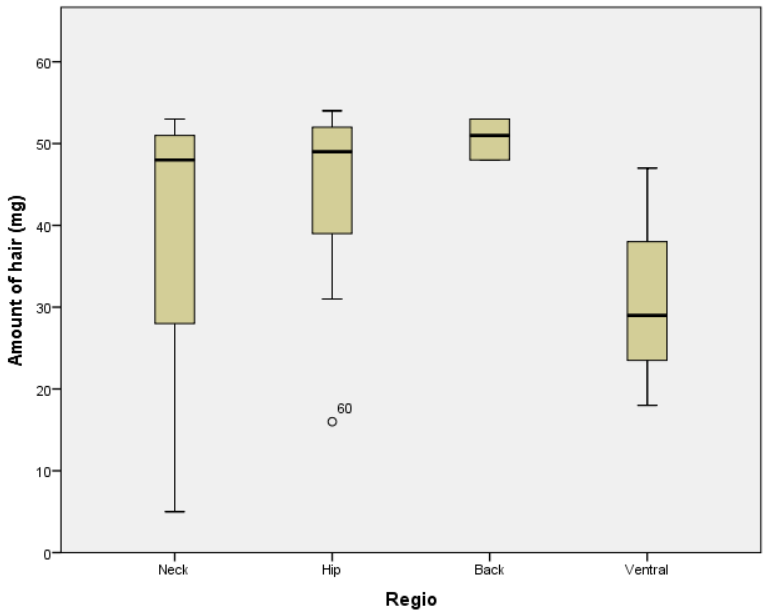

Fig. 16. Amount of hair (mg) collected from different body sites. On the ventral body site less hair is available from a canine puppy compared to the neck, hip and back.

The repeatability of the cortisol measurements, using the technical repeats, was assessed by the CV. The CV of the duplicates within one plate were 2.7% for plate I and 1.2% for plate II. The CV of the duplicates between two plates were 8.08% (between plate I and plate II of ELISA 2) and 7.81% (between ELISA I and ELISA 2). The repeatability of the cortisol measurements, using the biological repeats, was assessed by the CV and the CR (Fig. 17 and Fig. 18). Four samples (samples 4, 10, 13 and 16) exceeded the 15% CV maximum, with two samples only minimal. Two samples (samples 4 and 6) contained repeats with more deviation between the repeats than the coefficient of repeatability. This was however less than 95% of the repeats.

The GLM revealed no significant effect of the interaction amount of hair*region to the hair cortisol concentration (F (2) = 1.826, P = 0.183). After removing this interaction, also the amount of hair had no significant effect on hair cortisol concentration itself (F (1) = 2.405, P = 0.133) (Fig. 19). However, when this was plotted, it seemed that lower amounts of hair display higher cortisol concentrations (Fig. 20). When more dogs were included (dogs that were first excluded because of too less samples on both ends of the scale), the same trend was seen (Fig. 21). The final predictor of the GLM, region, was significant (F (2) = 4.453, P = 0.021). Post

Hoc test (LSD) revealed significant differences between the regions hip and flank (P = 0.041) and hip and ventral (P = 0.008), no significant difference was present between the regions flank and ventral (P = 0.474).

The mixed model analysis of region, gender and age, did not show any significant results. The interaction gender*region was not significant (F (2,6) = 0.255, P = 0.783) at first. This was followed by region (F (3,4) = 0.405, P = 0.758) and by age (F (1,3) = 0.402, P = 0.579). Also the final predictor, gender, was not significant (F (1,2) = 0.195, P = 0.701).

A significant difference was found between proximal and distal tested hair parts (t (2) = -4.67, P = 0.043). Distal hair parts showed consistently higher hair cortisol concentrations (18.97 ± 0.31 pg/mg) than proximal hair parts (15.98 ± 0.90 pg/mg) (Fig. 22 and Fig. 23).

Only a small sample size was available to test the differences between hair colours (dark versus white) and hair types (guard versus wool) and therefore only graphs were made (Fig. 24 and Fig. 25).

Repeated sample	Number of repeats	Coefficient of variation (CV)	Coefficient of repeatability (CR)	Maximum deviation between repeats
1	5	6.91	3.91	3.29
2	5	13.30	7.62	5.49
3	5	9.81	4.24	3.57
4	5	**24.57**	13.56	**13.66**
5	5	11.85	5.48	5.24
6	5	14.38	6.74	**6.81**
7	2	7.34	4.72	2.41
8	2	2.15	0.96	0.49
9	2	2.84	1.47	0.75
10	4	**15.10**	7.52	6.6
11	2	6.13	2.35	1.2
12	4	5.51	1.27	1.01
13	4	**15.56**	3.76	2.7
14	2	2.97	1.23	0.63
15	2	3.05	1.63	0.83
16	2	**26.45**	13.15	6.71
17	2	4.26	2.49	1.27

Fig. 17. Repeatability of cortisol measurements. Bold numbers represents deviating results.

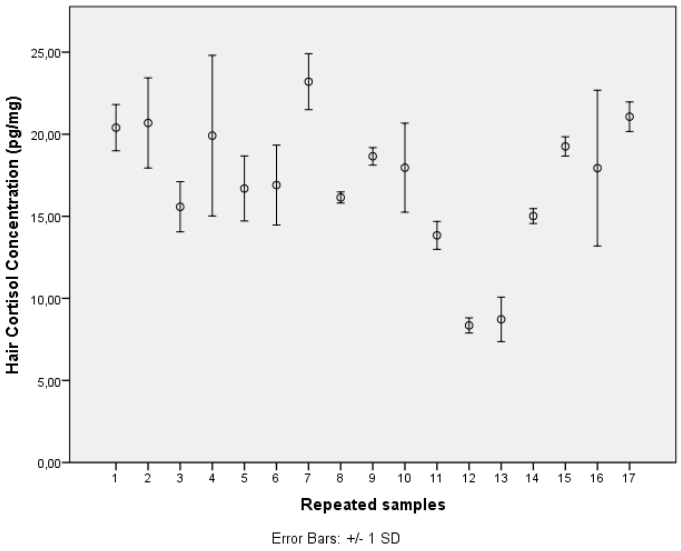

Fig. 18 Simple error bars for repeatability of cortisol measurements.

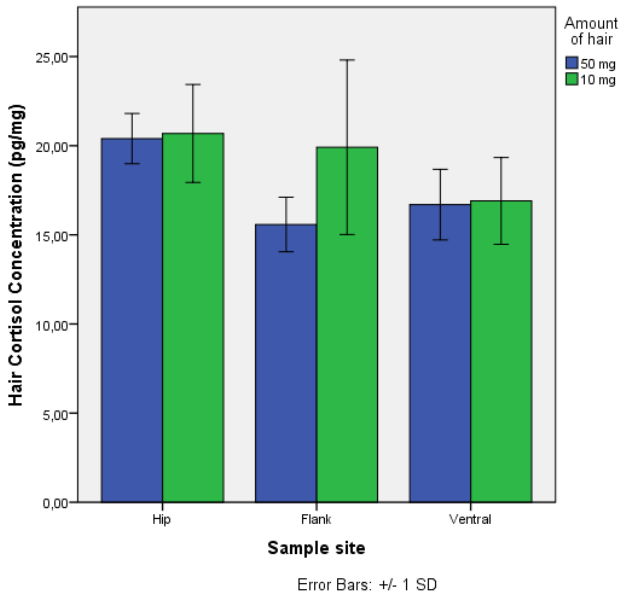

Fig. 19. Hair cortisol concentration (pg/mg) from different regions of 1 dog with different amounts of hair tested. No significant differences were found between 10 mg and 50 mg hair samples.

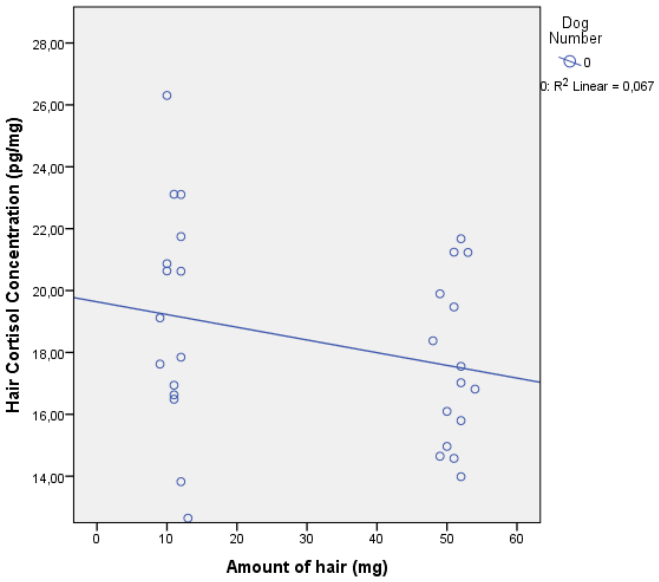

Fig. 20. Scatter plot and regression line for cortisol concentration (pg/mg) versus amount of hair (mg) tested. It seems that lower amounts of hair display higher cortisol concentrations. Samples of one dog are represented in this graph.

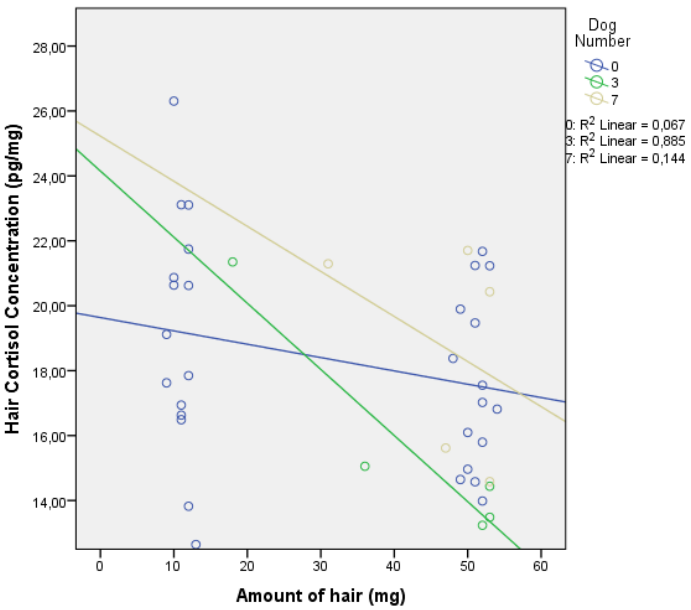

Fig. 21. Scatter plot and regression line for cortisol concentration (pg/mg) versus amount of hair (mg) tested. It seems that lower amounts of hair display higher cortisol concentrations. This trend can also be seen when dog 3 and dog 7 are included in the graph.

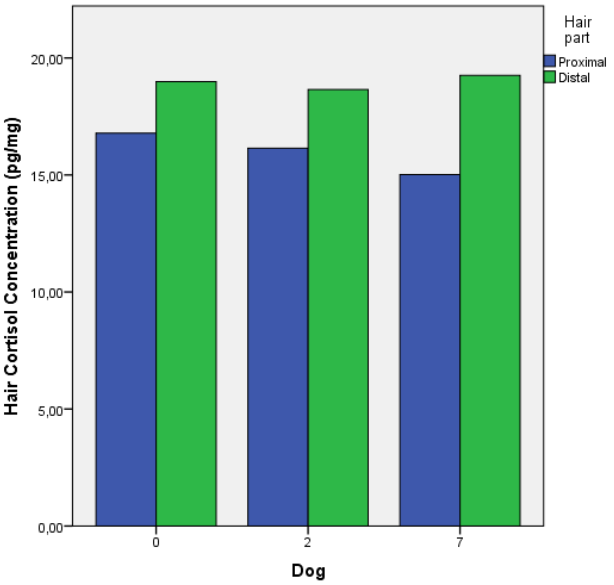

Fig. 22. Hair cortisol concentrations (pg/mg) in proximal (blue bars) and distal (green bars) parts of hair from 3 dogs did differ significantly (P = 0.043).

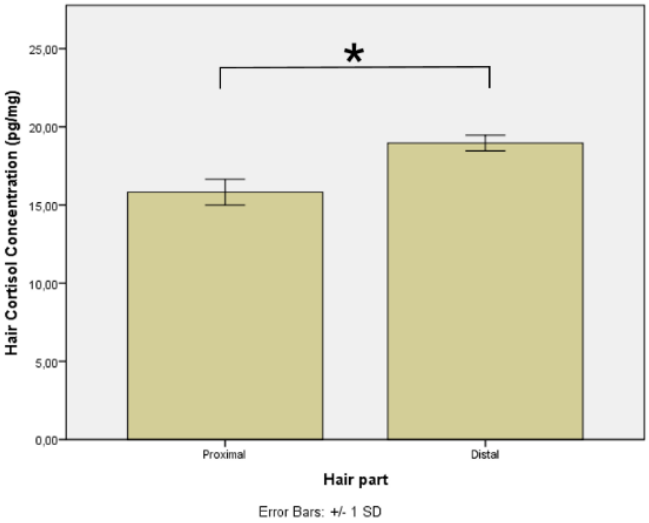

Fig. 23. Hair cortisol concentrations (pg/mg) in proximal and distal parts of hair from 3 dogs did differ significantly (P = 0.043).

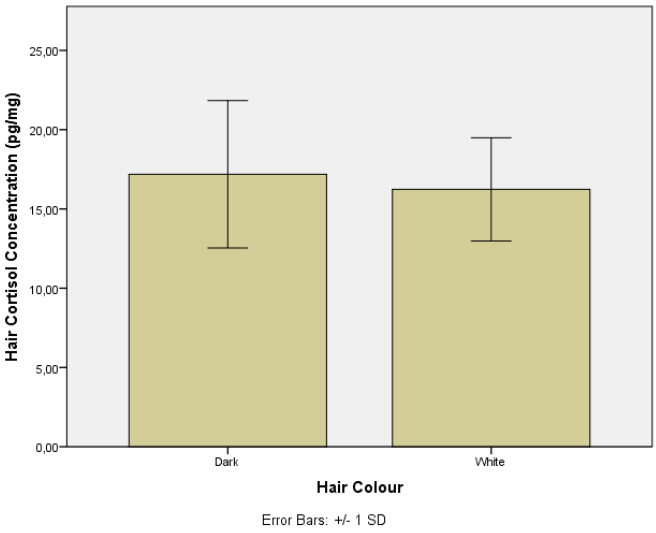

Fig. 24. Hair cortisol concentrations (pg/mg) in dark and white hair. Dark hair was represented in 12 samples from 5 dogs, white hair was represented in 5 samples from 2 dogs.

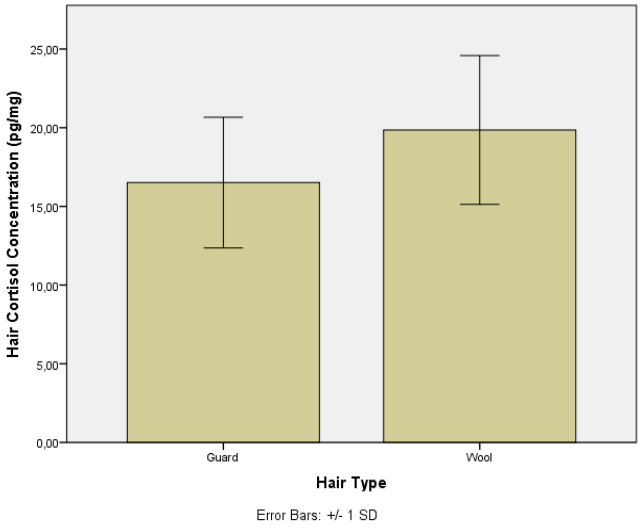

Fig. 25. Hair cortisol concentrations (pg/mg) in guard and wool hair. Note that two hair samples of wool hair are used from one dog (responsible for the SD). Guard hair was represented in 15 samples from 5 dogs, wool hair was represented in 2 samples from 1 dog.

Protocols

See appendices for both protocols.

Discussion

Literature study

From the literature study it is clear that hair cortisol concentrations are highly variable in dogs. A great amount of potential influential parameters on dog hair cortisol concentrations are described such as age, gender, breed, sampling site, hair part, hair colour and hair type. In addition, different laboratory analyses techniques contribute to even higher variability between studies. Despite the contradiction between studies, all influential parameters should be taken into account when assessing and evaluating hair cortisol concentrations in dogs. Interpreting hair cortisol concentrations of dogs is still difficult due to the lack of reference ranges. Larger experimental studies should therefore be set up to establish such a reference range. For young puppies, the establishment of a reference range will be more difficult due to the immaturity of the HPA axis. However, when a large sample size of canine puppies can be tested, a better understanding of hair cortisol concentration in dog pups and the existence of the immature HPA axis, can be achieved. Testing young puppies within a small age range is recommended.

When interpreting hair cortisol concentration it is also important to keep in mind that cortisol in canines hair possibly find its origin both systemically and locally. The exact amount of local produced cortisol is unknown, but with our knowledge so far, hair cortisol should not be interpreted as a 100% reflection of systemic cortisol. Further investigations on this aspect are therefore necessary for a better understanding.

Pilot practical investigation

Specific objectives of the pilot practical investigation were to determine the repeatability and reliability of cortisol measurements from canine puppies' hair, to determine the minimum amount of hair necessary for cortisol quantification and the influence of sample mass, to determine the best sampling site for hair collection and to asses hair cortisol with respect to gender, age, hair part, hair colour and hair type.

The first objective of this study was to investigate the repeatability of cortisol measurements from canine puppies' hair. This was tested with both the technical- and biological repeats. Intra-assay and inter-assay CV's from the technical replicates (2.7%, 1.2% respectively 8.08%, 7.81%) were strongly beneath the maximum of 10 – 15%. This was the same for the majority of the biological repeats, with the exception of four samples that exceeded the 15% maximum. The mean CV of the biological repeats was, however, 10.13%, so beneath the 15% maximum. When looking at the repeatability coefficient, all the biological repeats met the requirements. From this, it can be concluded that the cortisol measurements were reliable. Also, biological variability was greater than technical variability, what is in line as previously described in literature. In addition, the reliability of the 1:8 diluted samples were probably less than the other samples, because of the very low cortisol concentrations. These concentrations were placed at the end of the standard curve of the assay, resulting in less precision. For future studies, it is therefore important to dilute samples in a way that they will end up in the middle of the standard curve of the assay, to optimize precision.

Intra-assay and inter-assay CV's from the technical replicates were very similar to previous published studies for cortisol determination from canine's hair. Biological replicates have also been tested by Bryan et al. (2013) as an additional quality control. Hair from seven dogs was divided in three parts and measured separately. The CV's of these hair samples were strongly corresponding with this pilot study (between 24.57% and 2.15%), as they ranged between 25.1% and 3.6%.

The second objective of this study was to determine the minimum amount of hair necessary for cortisol determination and the possible influence of sample mass. This pilot study describes for the first time the possibility of cortisol extraction with the smallest sample mass in dogs. Five mg of hair powder, resulting from 10 mg intact hair, was the minimum amount necessary to determine cortisol. It was not possible to determine cortisol from a three mg hair powder sample (initial seven mg of intact hair). A four mg hair powder sample was, however, not tested.

During extraction of the hair samples, efforts were made to prevent the loss of sample material. If this process could be optimized, even smaller initial hair samples may be sufficient. While collecting hair from the canine litter it was possible to collect > 10 mg of hair several times (at 4-, 7- and 9 weeks of age on the same sampling site), thus the need to determine the minimum amount of hair might not be urgent. Notable, however, is that it was a Dutch sheep dog litter, a long coated breed. Maybe it is not possible to collect this amount of hair from other (short coat) breeds. The minimal amount of hair necessary, mentioned in the literature, is five mg of intact hair, however, this is only described in monkeys. Previous studies on dog hair cortisol used larger hair samples, ranging from 20 mg till 500 mg. Veronesi et al. (2015) mentioned the difficulty to collect 20 mg of hairs from canine puppies < 30 days of age, regarding to the small size of the new-borns. In the current study only puppies from ≥ 30 days were tested, future studies should therefore investigate if sample mass will actual be of concern.

Results from this pilot show that lower amounts of hair possibly result in higher cortisol concentrations measured in the ELISA assay. Although the amount of hair did not influence hair cortisol concentrations significantly, a trend was seen. The same trend was seen with corticosterone levels in feathers of chickens by Bortolotti et al. (2008). Therefore, sample mass should be taken into account in future studies. When comparing hair cortisol concentrations within a study, approximately the same amount of hair from each dog should be used to compensate for the sample mass effect until additional studies clarified this question.

The third objective of this study was to investigate the most suitable body site to collect hair from a canine puppy to determine cortisol. Surprisingly, significant differences between sampling sites were found within one dog, but no significant effect of sampling site was evident when all puppies were tested. The one dog was a juvenile dog, while the puppies were all younger than 6 months of age. Possibly, differences in hair cortisol concentrations between body regions become evident only after a certain age in dogs. According to Scott et al. (2001) different regions contain different cortisol concentrations due to different hair growth rates between body parts. This is also the case in puppies, maybe even in an higher extent, and significant differences are therefore also expected in this class of age. Only Roth et al. (2016) tested the effect of region on cortisol concentrations in adult dogs but did not find significant differences. These authors used however different regions (neck and chest) than the current study. Also, different regions were tested within this pilot study. The regions tested from the young-adult dog were the hip, flank and ventral site, whereas the regions tested from the puppies were the neck, hip, back and ventral site. The different tested regions might be the reason for the deviating results. Studies on different species than dogs also reported contradictory results about this subject. Because of the inconsistency between previous studies, it would be wise to use the same sampling site between dogs when comparing hair cortisol concentrations. The neck and the hip were often used in previous studies and can therefore serve as standard sampling sites.

Finally, the fourth objective of this study was to asses hair cortisol concentrations with respect to gender, age, hair part, hair colour and hair type. No significant effect of gender was detected in this pilot study, which corresponds to Veronesi et al. (2015) and Nicholson & Meredith (2015), who also did not found an effect of gender on dog hair cortisol concentrations.

Similarly, no significant effect of age was present in this pilot study, in line with Bennet & Hayssen (2010) and Nicholson & Meredith (2015). However, Veronesi et al. (2015) found that hair cortisol concentrations decreased when the puppies got older (between day 0 and day 30 of age). Veronesi et al. (2015) tested younger dogs than other dog studies, including the current study, and this could be the reason for the different results. The young puppies tested by Veronesi et al. (2015) had also a lot higher hair cortisol concentrations than the adult dogs tested by other studies. As mentioned above, a high variability is found in cortisol concentrations and the interpretation of individual cortisol concentrations is therefore difficult. In this pilot study, one dog consistently showed much higher hair cortisol concentrations compared to the other puppies but was not much younger than the other puppies. The reason for this outlier is not known due to the lack of background information, nevertheless it is possible that this dog was exposed to more stressful circumstances than the other puppies. More research should be performed to determine whether this could be the case.

A significant difference was found between proximal and distal hair parts in the current study. Distal hair parts contained consistently higher cortisol concentrations than proximal hair parts. Bennet & Hayssen (2010) also studied this with canine hair, but did not found a significant difference. They noticed, however, that the majority of the dogs had more cortisol in the distal portions of the hair than in the proximal parts. Distal hair segments might represent the last trimester of gestation and high cortisol levels can be found in this period, according to Kirschbaum et al. (2009) and Sapolsky et al. (1986). This might be the reason for higher cortisol levels in the distal hair segments. However, further investigation is necessary, as only three dogs were included in this pilot study for this particular test.

Because of the small sample size, no statistical test could be performed on both hair colour and hair type and therefore no statements about these parameters can be made. Bennet & Hayssen (2010) found significant differences in cortisol concentrations between different hair colours, with black hair containing less cortisol than white hair. Also Veronesi et al. (2015) and Nicholson & Meredith (2015) tested the influence of canine hair colour on cortisol concentration but did not find significant differences. An relation between hair colour and cortisol seems reasonable, as cortisol is involved in the melanocortin system. Regarding hair type, only Roth et al. (2016) tested this with dog hair and did not find any significant differences. Differences have however been reported in other species and therefore only guard hair should be analysed to make the results as comparable as possible.

In conclusion, (1) both technical and biological repeats from the dog hair samples showed similar cortisol concentrations, therefore the method can be assessed as reliable, (2) the amount of dog hair tested had no significant effect on hair cortisol concentrations, however a trend was seen with lower amounts of hair displaying higher cortisol concentrations, (3) a minimum of 10 mg of hair was required for cortisol determination, (4) hair should be collected from the same region from dogs to compare as accurate as possible, (5) distal hair segments contained higher cortisol concentrations than proximal hair parts and (6) hair should be collected from the same length, the same colour and the same type (guard) to make the results as comparable as possible.

The protocol established in this pilot study can be used for future studies on hair cortisol extraction in canine puppies. This protocol was described for the laboratory of the department of Animal in Science and Society at the University of Utrecht specifically, and probably some adaptations are still necessary, when using this protocol in another laboratory. Also, it can function as a basis for protocols used in other species. Because of hair differences between species, specific protocols for each species should be made to optimize accuracy and efficiency.

Hair cortisol measurement is a non-invasive way to get more relevant information about the activity of the HPA-axis of the animal in the past. This pilot study showed that it is also possible and reliable in alive canine puppies. Many parameters affect, however, cortisol concentration in canine hair what makes interpreting difficult, also in canine puppies. This study should therefore enlarge in the near future with testing more canine puppies to expand the knowledge about this topic and to further standardize sampling methods. With regard to evaluating prenatal stress, more hormones in the hair should be considered to be tested. Prenatal stress can also affect sexual differentiation by influencing androgens and oestrogens. Kapoor et al. (2016) and Meise et al. (2016) found for example significant differences in hair testosterone levels from neonates born to mothers from control or stressed pregnancies. Further research on this topic is clearly promising.

Acknowledgements

I want to thank my supervisors Claudia Vinke and Vivian Goerlich-Jansson for the opportunity to perform this research at the department of Animal in Science and Society. We worked together at a very nice level, I learned a lot and had a great time during the past three months. I also want to thank Susanne Kirchhoff for the great assistance in the laboratory. This was also a wonderful and instructive period.

Very important for this research were the puppy owners who gave consent to collect hair from their puppies. So I also really want to thank them: Maaike, Geranda, Nelleke, Lianne, Niels, Danielle and Sabine. Special thanks to Marjo and Wim Reurik who were very open and helpful and who gave us the opportunity to collect hair from a Dutch canine litter at several points during their life.

References

Accorsi, P. A., Carloni, E., Valsecchi, P., Viggiani, R., Gamberoni, M., Tamanini, C., & Seren, E. (2008). Cortisol determination in hair and faeces from domestic cats and dogs. *General and Comparative Endocrinology, 155 (2), 398-402*.

Arslan, S. H. I., Mackenzie, C. P., Brown, R., & Baxter, J. T. (1983). The phase of hair growth in hypothyroidism in the dog. *Veterinary Research Communications, 6(1), 51-8*.

Ashley, N. T., Barboza, P. S., Macbeth, B. J., Janz, D. M., Cattet, M. R. L., Booth, R. K., & Wasser, S. K. (2011). Glucocorticosteroid concentrations in feces and hair of captive caribou and reindeer following adrenocorticotropic hormone challenge. *General and Comparative Endocrinology*, *172*(3), 382–391. https://doi.org/10.1016/j.ygcen.2011.03.029

Bartlett, J. W., & Frost, C. (2008). Reliability, repeatability and reproducibility: analysis of measurement errors in continuous variables. *Ultrasound in Obstetrics and Gynecology*, *31*(4), 466–475.

Bechshøft, T. Ø., Sonne, C., Rigét, F. F., Letcher, R. J., Novak, M. A., Henchey, E., … Dietz, R. (2013). Polar bear stress hormone cortisol fluctuates with the North Atlantic Oscillation climate index. *Polar Biology*, *36*(10), 1525–1529.

Bennett, A., & Hayssen, V. (2010). Measuring cortisol in hair and saliva from dogs: Coat color and pigment differences. *Domestic Animal Endocrinology*, *39*(3), 171–180.

Binz, T. M., Braun, U., Baumgartner, M. R., & Kraemer, T. (2016). Development of an LC-MS/MS method for the determination of endogenous cortisol in hair using (13)C3-labeled cortisol as surrogate analyte. *Journal of Chromatography. B, Analytical Technologies in the Biomedical and Life Sciences*, *1033–1034*, 65–72.

Bortolotti, G. R., Marchant, T. A., Blas, J., & German, T. (2008). Corticosterone in feathers is a long-term, integrated measure of avian stress physiology. *Functional Ecology*, *22*(3), 494–500.

Bryan, H. M., Adams, A. G., Invik, R. M., Wynne-Edwards, K. E., & Smits, J. E. G. (2013). Hair as a meaningful measure of baseline cortisol levels over time in dogs. *Journal of the American Association for Laboratory Animal Science : JAALAS*, *52*(2), 189–96.

Carpenter, T., Grecian, S. M., & Reynolds, R. M. (2017). Sex differences in early-life programming of the hypothalamic–pituitary–adrenal axis in humans suggest increased vulnerability in females: a systematic review. *Journal of Developmental Origins of Health and Disease*, 1–12.

Charmandari, E., Tsigos, C., & Chrousos, G. (2005). Endocrinology of the stress response 1. *Annu. Rev. Physiol*, *67*, 259–84.

Cobb, M. L., Iskandarani, K., Chinchilli, V. M., & Dreschel, N. A. (2016). A systematic review and meta-analysis of salivary cortisol measurement in domestic canines. *Domestic Animal Endocrinology*, *57*, 31–42.

Corradini, S., Accorsi, P. A., Boari, A., Beghelli, V., Mattioli, M., Famigli-Bergamini, P., & Fracassi, F. (2013). Evaluation of hair cortisol in the diagnosis of hypercortisolism in dogs. *Journal of Veterinary Internal Medicine*, *27*(5), 1268–1272.

Davenport, M. D., Tiefenbacher, S., Lutz, C. K., Novak, M. A., & Meyer, J. S. (2006). Analysis of endogenous cortisol concentrations in the hair of rhesus macaques. *General and Comparative Endocrinology, 147 (3), 255-61*.

Dettmer, A. M., Novak, M. A., Suomi, S. J., & Meyer, J. S. (2012). Physiological and behavioral adaptation to relocation stress in differentially reared rhesus monkeys: Hair cortisol as a biomarker for anxiety-related responses. *Psychoneuroendocrinology*, *37*(2), 191–199.

Dettmer, A. M., Novak, M. F. S. X., Novak, M. A., Meyer, J. S., & Suomi, S. J. (2009). Hair

cortisol predicts object permanence performance in infant rhesus macaques (Macaca mulatta). *Developmental Psychobiology*, *51*(8), 706–13.

Diaz, S. F. (2004). The effect of body region on the canine hair cycle as defined by unit area trichogram. *Veterinary Dermatology*, *15*(4), 225–229.

Diaz, S. F., Torres, S. M. F., Dunstan, R. W., & Lekcharoensuk, C. M. (2004). An analysis of canine hair re-growth after clipping for a surgical procedure, (1), 25–30.

Egliston, K.-A., McMahon, C., & Austin, M.-P. (2007). Stress in pregnancy and infant HPA axis function: Conceptual and methodological issues relating to the use of salivary cortisol as an outcome measure. *Psychoneuroendocrinology*, *32*(1), 1–13.

Favarato, E. S., & Conceição, L. G. (2008). Hair cycle in dogs with different hair types in a tropical region of Brazil. *Veterinary Dermatology*, *19*(1), 15–20.

Fraser, R., Ingram, M. C., Anderson, N. H., Morrison, C., Davies, E., & Connell, J. M. (1999). Cortisol effects on body mass, blood pressure, and cholesterol in the general population. *Hypertension*, *33*(6), 1364–8.

Freedman, D. G., King, J. A., & Elliot, O. (1961). Critical period in the social development of dogs. *Science*, *133*(3457), 1016–7.

Galeandro, L., Sieber-Ruckstuhl, N. S., Riond, B., Hartnack, S., Hofmann-Lehmann, R., Reusch, C. E., & Boretti, F. S. (2014). Urinary corticoid concentrations measured by 5 different immunoassays and gas chromatography-mass spectrometry in healthy dogs and dogs with hypercortisolism at home and in the hospital. *Journal of Veterinary Internal Medicine*, *28*(5), 1433–41.

Gow, R., Thomson, S., Rieder, M., Van Uum, S., & Koren, G. (2010). An assessment of cortisol analysis in hair and its clinical applications. *Forensic Science International*, *196*(1–3), 32–37.

Hayssen, V., Harper, J. M., & Defina, R. (2002). Fecal corticosteroids in agouti and non-agouti deer mice (Peromyscus maniculatus). *Comparative Biochemistry and Physiology Part A*, *132*, 439–446.

Hyttel, P., Sinowatz, F., & Vejlsted, M. (2010). *Essentials of Domestic Animals Embryology*. *Climate Change 2013 - The Physical Science Basis*.

Kaiser, S. Sachser, N. (2005). The effects of prenatal social stress on behaviour: mechanisms and function. *Neuroscience and Biobehavioral Reviews*, *29*, 283–294.

Kapoor, Lubach, Ziegler, & C. (2016). Hormone levels in neonatal hair reflect prior maternal stress exposure during pregnancy. *Psychoneuroendocrinology*, *66*, 111–117.

Keckeis, K., Lepschy, M., Schöpper, H., Moser, L., Troxler, J., & Palme, R. (2012). Hair cortisol: A parameter of chronic stress? Insights from a radiometabolism study in guinea pigs. *Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology*, *182*(7), 985–996.

Kirschbaum, C., Tietze, A., Skoluda, N., & Dettenborn, L. (2009). Hair as a retrospective calendar of cortisol production-Increased cortisol incorporation into hair in the third trimester of pregnancy. *Psychoneuroendocrinology, 34 (1), 32-7*.

Macbeth, B. J., Cattet, M. R. L., Stenhouse, G. B., Gibeau, M. L., & Janz, D. M. (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),935-949*

Mack, Z., & Fokidis, H. B. (2017). A novel method for assessing chronic cortisol concentrations in dogs using the nail as a source. *Domestic Animal Endocrinology*, *59*, 53–57.

Meise, K., von Engelhardt, N., Forcada, J., Hoffman, J. I., Barker, D., Eriksson, J., … Franco-Trecu, V. (2016). Offspring Hormones Reflect the Maternal Prenatal Social Environment: Potential for Foetal Programming? *PLOS ONE*, *11*(1).

Meyer, J., Novak, M., Hamel, A., & Rosenberg, K. (2014). Extraction and Analysis of Cortisol from Human and Monkey Hair. *Journal of Visualized Experiments*, (83).

Mongillo, P., Prana, E., Gabai, G., Bertotto, D., & Marinelli, L. (2014). Effect of age and sex on plasma cortisol and dehydroepiandrosterone concentrations in the dog (Canis familiaris). *Research in Veterinary Science*, *96*(1), 33–38.

Montillo, M., Comin, A., Corazzin, M., Peric, T., Faustini, M., Veronesi, M. C., … Prandi, A. (2014). The Effect of Temperature, Rainfall, and Light Conditions on Hair Cortisol Concentrations in Newborn Foals. *Journal of Equine Veterinary Science*, *34*(6), 774–778.

Mormede, P. Andanson, S. Auperin, B. Beerda, B. Guemene, D. Malmkvist, J. (2007). Exploration of the hypothalamic-pituitary-adrenal function as a tool to evaluate animal welfare. *Physiology & Behavior* , *92*, 317–339.

Morrow, M., Ottobre, J., Ottobre, A., Neville, P., St-Pierre, N., Dreschel, N., & Pate, J. L. (2015). Breed-dependent differences in the onset of fear-related avoidance behavior in puppies. *Journal of Veterinary Behavior: Clinical Applications and Research*, *10*(4), 286–294.

Nagasawa, M., Shibata, Y., Yonezawa, A., Morita, T., Kanai, M., Mogi, K., & Kikusui, T. (2014). The behavioral and endocrinological development of stress response in dogs. *Developmental Psychobiology*, *56*(4), 726–733.

Nicholson, S. L., & Meredith, J. E. (2015). Should stress management be part of the clinical care provided to chronically ill dogs? *Journal of Veterinary Behavior: Clinical Applications and Research*, *10*, 489–495.

Noppe, G., Rossum, E. F. C. Van, Koper, J. W., Manenschijn, L., Bruining, G. J., De Rijke, Y. B., & Van Den Akker, E. L. T. (2014). Validation and Reference Ranges of Hair Cortisol Measurement in Healthy Children. *Horm Res Paediatr*, *82*, 97–102.

Ouschan, C., Kuchar, A., & Möstl, E. (2013). Measurement of cortisol in dog hair: A noninvasive tool for the diagnosis of hypercortisolism. *Veterinary Dermatology*, *24*(4).

Palazzolo, D. L., & Quadri, S. K. (1987). The effects of aging on the circadian rhythm of serum cortisol in the dog. *Experimental Gerontology*, *22*(6), 379–387.

Park, S.-H., Kim, S.-A., Shin, N.-S., & Hwang, C.-Y. (2016). Elevated cortisol content in dog hair with atopic dermatitis. *The Japanese Journal of Veterinary Research*, *64*(2), 123–9.

Pretzer, S. D. (2008). Canine embryonic and fetal development: a review. *Theriogenology*, *70*, 300–303.

Protopopova, A. (2016). Effects of sheltering on physiology, immune function, behavior, and the welfare of dogs. *Physiology & Behavior*, *159*, 95–103.

Renard, G. M., Rivarola, M. A., & Suárez, M. M. (2007). Sexual dimorphism in rats: effects of early maternal separation and variable chronic stress on pituitary-adrenal axis and behavior. *International Journal of Developmental Neuroscience*, *25*(6), 373–379.

Reynolds, R. M. (2013). Glucocorticoid excess and the developmental origins of disease: Two decades of testing the hypothesis - 2012 Curt Richter Award Winner., *Psychoneuroendocrinology*, *38*, 1–11.

Rijnberk, A., & Kooistra, H. S. (2010). *Clinical Endocrinology of Dogs and Cats*.

Roth, L. S. V., Faresjö, Å., Theodorsson, E., & Jensen, P. (2016). Hair cortisol varies with season and lifestyle and relates to human interactions in German shepherd dogs. *Scientific Reports*, *6*(6), 19631.

Russell, E., Kirschbaum, C., Laudenslager, M. L., Stalder, T., de Rijke, Y., van Rossum, E. F. C., … Koren, G. (2015). Toward standardization of hair cortisol measurement: results of the first international interlaboratory round robin. *Therapeutic Drug Monitoring*, *37*(1), 71–5.

Salimetrics. (n.d.). Expanded Range High Sensitivity.

Sandri, M., Colussi, A., Perrotta, M. G., & Stefanon, B. (2015). Salivary cortisol concentration in healthy dogs is affected by size, sex, and housing context. *Journal of Veterinary Behavior: Clinical Applications and Research*, *10*, 302–306.

Sapolsky, R. M., & Meaney, M. J. (1986). Maturation of the adrenocortical stress response: neuroendocrine control mechanisms and the stress hyporesponsive period. *Brain Research*, *396*(1), 64–76.

Schats, S., & Palme, R. (2001). Measurement of Faecal Cortisol Metabolites in Cats and Dogs: a Non-invasive Method for Evaluating Adrenocortical Function. *Veterinary Research Communications*, *25*(4), 271–287.

Schoeman, J. P., Goddard, A., & Herrtage, M. E. (2007). Serum cortisol and thyroxine concentrations as predictors of death in critically ill puppies with parvoviral diarrhea. *Journal of the American Veterinary Medical Association*, *10*, 1534–1539.

Scott, J. P., & Fuller, J. L. (1965). *Genetics and the social behavior of the dog*.

Scott, W. M., Miller, W. H., & Griffin, C. H. (2001). Structure and function of the skin. *Muller & Kirk's Small Animal Dermatology (Sixth Edition)*, 1–70.

Sharpley, C. F., McFarlane, J. R., & Slominski, A. (2011). Stress-linked cortisol concentrations in hair: what we know and what we need to know. *Reviews in the Neurosciences*, *23*(1), 111–21.

Slominski, R., Rovnaghi, C. R., & Anand, K. J. S. (2015). Methodological Considerations for Hair Cortisol Measurements in Children. *Therapeutic Drug Monitoring*, *37*(6), 812–820.

Terwissen, C. V., Mastromonaco, G. F., & Murray, D. L. (2013). Influence of adrenocorticotrophin hormone challenge and external factors (age, sex, and body region) on hair cortisol concentration in Canada lynx (Lynx canadensis). *General and Comparative Endocrinology*, *194*, *162-7*.

Veronesi, M. C., Comin, A., Meloni, T., Faustini, M., Rota, A., & Prandi, A. (2015). Coat and claws as new matrices for noninvasive long-term cortisol assessment in dogs from birth up to 30 days of age. *Theriogenology*, *84*(5), 791–6.

Wester, V. L., & van Rossum, E. F. C. (2015). Clinical applications of cortisol measurements in hair. *European Journal of Endocrinology*, *173*(4), M1–M10.

Yamanashi, Y., Teramoto, M., Morimura, N., Hirata, S., Suzuki, J., Hayashi, M., … Idani, G. (2016). Analysis of hair cortisol levels in captive chimpanzees: Effect of various methods on cortisol stability and variability. *MethodsX*, *3*, 110–117.

Appendices

Appendix 1

Authors	*Dogs used (number, age, breed, gender, neutering-status)*	*Sample site, amount of hair and collection method*	*Hair preparation and cortisol extraction*	*Cortisol determination*	*Cortisol concentration (mean ± standard deviation)*
Veronesi et al. (2015)	165 dead puppies - 25 premature - 97 born-dead - 43 dead 1-30 days of age 80 ♀ 85♂ 32 breeds - 60 <10 kg - 105 >10 kg	Back and dorsal neck ≥ 20 mg hair Shaving Dark & Light hair	Comin et al. (2014)	RIA	65.2 ± 52.23 pg/mg With increasing age: - 123 pg/mg - 72 pg/mg - 48 pg/mg Light coat: 67.6 ± 55.81 pg/mg Dark coat: 63.0 ± 50.06 pg/mg
Bennet & Hayssen (2010)	48 dogs 9 mo-11 years 28 ♀ 20 ♂ 23 Labrador Retrievers 25 German Shepherds May - October	Ischiatic region 35 cm^2 Shave and re-shave Old & New hair Proximal & Distal hair Black & Agouti & Yellow hair	Davenport et al. (2006) + Powdering	EIA	Old hair: 12.63 ± 5.45 pg/mg New hair: 10.88 ± 3.85 pg/mg
Accorsi et al. (2008)	29 dogs 1 – 7 years 8 ♀ 21 ♂ 14 Mixed breeds 15 German Shepherds March- December	Ischiatic region Shaving and clipping	Koren et al. (2002)	RIA	2.10 ± 0.22 pg/mg
Bryan et al. (2013)	7 dogs Adult 2 ♀ 5 ♂ Mixed breeds August - November	Shoulder 10 x 10 cm Electric clippers Right & Left shoulder 1 monthly hair in 3 months (right). New hair after 3 months (left)	Davenport et al. (2006) and Koren et al. (2002) + Powdering	LC-MS/MS	11.6 ± 0.8 pg/mg
Roth et al. (2016)	59 dogs - 17 companion - 16 competition - 14 working - 12 unspecified Mean age: 3.4 years 31♀ 18 ♂ German Shepherds January, May, September	Chest and neck 0.5 gram Cutting Companion & Competiting & Working dogs Guard & Wool hair January & May & September	Karlen et al. (2011) and Morelius et al. (2004)	RIA	Different dog types in different months: Companion dog: - 18.0 pg/mg - 9.0 pg/mg - 9.5 pg/mg Competing dog: - 40.0 pg/mg - 12 pg/mg - 15 pg/mg Working dog: - 11 pg/mg - 9 pg/mg - 12 pg/mg
Ouschan et al. (2013)	22 dogs - 12 Cushing - 10 healthy 6 – 14 years 10 ♀ 12 ♂ Different breeds	V. Cephalica region Shaving Cushing dogs & Healthy dogs		EIA	Cushing group: 5.6 ng/g (=pg/mg) Healthy group: 1.5 ng/g (=pg/mg)
Corradini et al. (2013)	90 dogs - 22 Cushing - 28 sick control - 40 healthy 6 – 16 years 41 ♀ 49♂ Different breeds	Xiphoid region 9-10 cm^2 Shaving Cushing & Healthy dogs	Accorsi et al. (2008)	RIA	Cushing: 4.53 pg/mg Sick control: 1.49 pg/mg Healthy: 1.28 pg/mg
Siniscalchi et al. (2013)	14 dogs 2 – 13 years 8 ♀ 6♂ Different breeds	Ischiatic region 300 mg 9:00 and 17:00 o'clock	Sharpley et al. (2009)	RIA	Variation between 89.5 pM/g (= 32.44 pg/mg) and 567.5 pM/g (= 205.73 pg/mg) 09:00 o'clock: 180.8 pM/g 17:00 o'clock: 264.89 pM/g
Park et al. (2016)	26 dogs - 21 atopic dermatitis - 5 healthy 3 – 7 years 10 ♀ 16 ♂ Different breeds	Ischiatic region 25-150 mg Shaving within 5 mm of the skin Atopic Dermatitis & Healthy dogs		EIA	Atopic dermatitis: 16.25 ± 2.86 pg/mg Healthy: 5.65 ± 1.28 pg/mg
Nicholson & Meredith (2015)	33 dogs - 16 chronically ill - 17 healthy 2 – 15 years 19 ♀ 14♂ Different breeds	300 mg Brushing and trimming Chronically Ill & Healthy dogs Black hair & Non-black hair	Bennet & Hayssen (2010) and Davenport et al. (2006) + Powdering	EIA	Chronically ill: 1.77 pg/mg – 25.22 pg/mg Healthy control: 1.70 pg/mg – 28.79 pg/mg

Appendix 2
Protocol for hair collection of a canine puppy to extract cortisol

Appendix 3
Protocol laboratory work to extract cortisol from canine puppies' hair