



CHRONIC STRESS INDICATORS IN CANINES

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ABSTRACT

With a growing number of dogs abandoned, living in shelters, and being rehomed, it is important to distinguish behavioural responses due to stress in our domestic companions. Cortisol is involved in the stress responses in animals which generally enters the individual's body into a "state of emergency". Prolonged stress can lead to exhaustion, disease, and death. Chronic stress can be detected by evaluating cortisol concentration in hair. Most domesticated dogs respond well to hair collection, thus avoiding further stressors. The method is simple, relatively inexpensive, and non-invasive. Our experiment focused on assessing multiple parameters using a modified Canine Behavioural Assessment and Research Questionnaire to evaluate their significance with cortisol in hair samples from a diverse range of dogs. Each stress parameter was tested against cortisol concentration using a t-Test, i.e., the Paired Two Sample for Means. The effect of weight on cortisol levels was statistically significant ($P=0.03$). This

fact revealed that an increase in body weight correlated with an increase in cortisol levels.

Keywords: canines; cortisol; hair; stress; welfare

INTRODUCTION

The body's response to a whole range of stressors is very similar, involving the release of certain hormones. Moberg [36] described the physiological changes elicited by these hormones as attempts to restore the balance of the body's metabolism to homeostasis. Stressors include physical trauma, pain, surgery, excessive physical effort, etc. The main hormonal mechanism of the stress reaction is the activation of the axis hypothalamus-sympathetic-adrenomedullary system which immediately induces the release of catecholamines and glucocorticoids. Catecholamines have been termed the hormones of "fight or flight" because of their effects on the heart, blood vessels, smooth muscle, and metabolism that assist the organ-

ism in responding to stress. They induce hyperglycaemia by inhibiting insulin secretion, increasing the absorption of glucose from the intestine, and glycogenolysis. They also mobilize energy sources and increase the energy uptake to the nervous system and skeletal muscles. Glucocorticoids, on the other hand, aid the animal in adapting to adverse situations and surviving. They stimulate protein catabolism, encourage the liver to take up amino acids, increase gluconeogenesis and inhibit glucose uptake by many cells, excluding the brain. Stress can also cause responses such as an increase in epinephrine or glucocorticoid release. A chronic response to these stressors acts via the amygdala to release corticotropin-releasing factor (CRF) from the hypothalamus which then increases adrenocorticotropin (ACTH) secretion from the anterior pituitary. ACTH increases the activity of both the adrenal medulla and the cortex. It enhances glucose and glycogen synthesis at the expense of lipid and protein catabolism, pain suppression, and the anti-inflammatory response. Selye [43] proposed the General Adaptation Syndrome (GAS) which states that a long-term response to stress acts through increased adrenal function and consists of three phases: alarm, resistance, and exhaustion. These responses disrupt normal physiological mechanisms and trigger an array of diseases. If untreated, it always leads to infection, illness, and eventually death [20].

In vertebrates, an important mechanism for coping with stressors begins with adrenally derived glucocorticoid hormones (cortisol). As previously discussed, these molecules drive gluconeogenesis, however they also: suppress the reproductive processes, alter movement and feeding rates, impact immune functions, and generally help an individual enter a "state of emergency" [8]. Cortisol acts as an immunosuppressant by suppressing protein synthesis, including the synthesis of immunoglobulin. Cortisol also reduces the peripheral blood concentrations of eosinophils, lymphocytes, and macrophages. Large doses of cortisol are known to promote the atrophy of lymphoid tissue in the thymus, spleen, and lymph nodes. Therefore, this action could account for lymphoid atrophy. Cortisol directly influences the immune response to antibodies. The mechanisms of inhibition of the immune response are multifactorial. When an antigen intrudes into the body, it is picked up by a macrophage [11]. The macrophage then presents the antigen to thymus-derived lymphocytes (T cells) and simultaneously produces and releases interleukin-1 (IL-1).

Likewise, T helper cells secrete interleukin 2 (IL-2), a protein that stimulates the proliferation of still more T cells. T cells can either activate or suppress bursa-derived lymphocytes (B cells). B cells then produce antibodies directed against the original invading antigen. Cortisol inhibits the production of both IL-1 and macrophages and IL-2 by helper T cells, thus decreasing T cells responses and the generation of fever. The diminished helper T cells cause a decrease in B cells and antibody production. Once antibodies are present, neither their degeneration nor their specific reaction with antigen molecules is affected by cortisol [16].

The interaction between stress hormones and immune functions is important in the understanding of disease-coping mechanisms in wild animals [27]. For example, many urban bird populations regulate stress hormones differently than their rural or suburban counterparts [7, 14, 40]. One study reported that the presence of an introduced species impacted glucocorticoid regulation in a native species [5]. In this study, neither immunological nor disease consequences of stress hormone variation were considered, but the possibility exists that introduced species may have negative impacts on stress hormones in native species [21]. There is now evidence in both human and other animal studies that the magnitude of stress-associated immune dysregulation is large enough to have health implications [11, 38]. Lymphocytes, macrophages, and granulocytes exhibit receptors for many neuroendocrine products of the hypothalamic-pituitary-adrenal (HPA) and sympathetic-adrenal medullary (SAM) axes [42]. These HPA and SAM, such as cortisol and catecholamines, can cause changes in cellular proliferation, cytokine secretion, antibody production, and cytolytic activity [28]. This can cause a shift from cell-mediated immune activities to a shift in antibody production [31]. This stress-induced decrease of immune-mediated cytokines results in dysregulation of cell-mediated immune responses [38]. A study conducted by Borcel et al. [9] investigated the spatial-cognitive abilities and the survival of new-born hippocampal cells in aging animals. Male Wistar rats were subjected to chronic unpredictable stress at 12 months old and then re-exposed to stress each week for a set time period. Subsequently, they were evaluated in a water maze during the early stages of aging. Chronic unpredictable stress seemed to exacerbate the spatial-cognitive decline. This was accompanied by a reduction in the survival of new-born cells and

in the number of adult granular cells in the hippocampus. Porter, Landfield [41] and McEwen [32] also showed that continuous exposure to stressors could accelerate cognitive decline during ageing.

The stress responses that lead to pathology and death are clearly indicative of poor animal welfare [18]. Stress indicators in animals are a valuable tool for assessing their welfare and overall health. However, as it may be difficult to define the stress of an animal, it is necessary to combine physiological and behavioural indicators of acute or chronic stress in individuals [6]. The physiological indicators of stress include the various hormonal measures mentioned above, such as: catecholamines, glucocorticoids, prolactin, gonadotrophins, thyroid-stimulating hormone, and insulin. It can also include the measures of: heart rate, blood pressure, respiratory rate, body temperature, evaluations of the immune status, and of disease incidence [6]. The serum catecholamines and glucocorticoids levels are most commonly measured in dogs as they correlate with the level of stress, however, it can be difficult to obtain accurate reading. The anticipation and immediate stress invoked by the blood sampling procedure triggers a response that may increase the plasma levels of these hormones, complicating the interpretation of the results. It can be ethically difficult to measure these physiological indicators, so it is important to choose the least invasive procedure. Thus, urine, saliva, and faeces are the most frequently used in sampling [6, 15]. Most recently, hair has been proven to be a reliable source of measuring chronic cortisol. Except for blood serum sampling of hormones, other sampling methods are focused on measuring acute stressors. However, for evaluating the long-term effects of stressors, such as kennelling, and their effects on the body and welfare of the animal, hair sampling provides a clearer picture of the stress levels. This is due to a number of reasons, including that blood-borne hormones are incorporated into the shaft during the growth phase; it is stable, and can be transported at room temperature [34]. Lastly, if the action of removing hair (e.g., in wild animals) is stressful, the stress during this event will not affect the cortisol concentration in the sample. Cortisol enters the hair shaft through two main mechanisms. Free cortisol is incorporated during the growth phase of the follicle [10]. Additionally, sweat and sebum excreted by skin glands contain cortisol which can also be incorporated into the shaft [2].

The first study examining cortisol concentration in hair

was performed by Koren et al. [23] using hair from wild hyraxes. They determined the cortisol concentrations using a modified salivary ELISA assay. This sampling method found a positive correlation between hair cortisol and social ranking, thus supporting the hypothesis that domination is a stressful event. Other studies, such as Accorsi et al. [1] compared cortisol in faecal samples with that in hair from domestic cats and dogs. Similarly, Benet, Hayse n [3] showed that distal hair segments in dogs have a higher concentration of cortisol metabolites. In terms of assessing disease, hair cortisol levels have proven reliable. Coradini et al. [12] showed significantly higher cortisol levels in dogs with Cushing's syndrome. Park et al. [39] have also illustrated that dogs with atopic dermatitis have increased cortisol concentrations in hair samples [34].

MATERIALS AND METHODS

Study Design and Sampling

The experiment aimed to analyse the cortisol levels in dogs who live in various home environments and originate from different backgrounds. For the study purpose, familiar dog owners were contacted via social media/phone and asked to participate. Animals were chosen at random to include a diverse range of pedigree breeds, mixed breeds, dogs adopted from a shelter, and dogs bought from a breeder. The dogs were also randomly selected for different coat colours, home environments, size categories, and weight categories. This was in an attempt to identify possibly different stress parameters in a dogs' life. In total, there were 54 subjects (animals) analysed. A survey was made based on the Canine Behaviour and Research Questionnaire (<https://vetapps.vet.upenn.edu/cbarq/>) to assess individual dogs' behaviour and possible stress-related behavioural manifestations. This C-BARQ survey was modified (hereafter MCBARQ) to focus on behavioural manifestations of stress. Due to COVID-19 pandemic restrictions, it was not possible to collect the samples in person by the research team members. All participants were given the same instructions regarding collecting samples and completing the individual survey. They were instructed to shave a section of hair, close to the skin without plucking (as this could contaminate the sample with follicles and blood). It was highly recommended to shave the hair sample from the

abdominal/inguinal region or the inner hind thigh. The reasoning was to perverse cosmetic appearances of the animal and to try to have a similar region for analysis. This should have amounted to approximately 5 grams of hair. The sample was then sealed in a plastic, airtight bag and sent to the laboratory to be stored before further processing.

Sample processing and analysis

200 mg of each hair sample were weighed and washed three times with 5 ml of isopropanol using a vortex mixer. After washing the isopropanol, it was discarded and the samples were left to dry at room temperature for three days. Dry hair samples were cut into 1 mm pieces. For further processing, 100 mg of cut hair were dipped in 3 ml of ethanol, mixed on vortex, and left to be shaken at room temperature for 24 hours on a laboratory shaker. After 24 hours, 2 ml of the supernatant were transferred into a clean tube and left to dry at room temperature. Before the analysis of the samples were reconstituted by adding 200 μ l of PBS (phosphate buffer) they were shaken on a vortex mixer. The samples were then placed into the ELISA well for further analysis according to the manufacturer's instructions. A DRG cortisol ELISA testing kit was used to measure the cortisol in each hair sample. The DRG Cortisol ELISA Kit is a solid phase enzyme-linked immunosorbent assay (ELISA), based on the principle of competitive binding. The microtitre wells are coated with a monoclonal antibody directed towards an antigenic site on the cortisol molecule. The endogenous cortisol of a sample competes with a cortisol-horseradish peroxidase conjugate for binding to the coated antibody. After incubation, the unbound conjugate is washed off. The amount of bound peroxidase conjugate is inversely proportional to the concentration of cortisol in the sample. After the addition of the substrate solution, the intensity of colour developed is inversely proportional to the concentration of cortisol in the sample.

ETHICAL STATEMENT

Consent of owners was given for obtaining and examining hair samples from their animals and for publication of the results of this study.

The authors declare no conflict of interest.

Statistical analysis

In total, 16 categories were tested against the cortisol levels to look for significance. The categories that were analysed from the MCBARQ included: training, aggression, fear and anxiety, separation anxiety, excitability, general stressors, and miscellaneous. It was decided to evaluate other factors, not just those included in the MCBARQ. The analysed categories not relating to the MCBARQ included: breed, breed size, pedigree vs mixed breed, hair colour, and age. If the dog was adopted or bought from a breeder, also, the neutered status, sex and weight were considered. This information was taken from the first section of the MCBARQ "Section 1, General". Each breed involved in the experiment was given a numerical number and plotted against cortisol to evaluate if certain breeds are more predisposed to stress. Each breed was also entered into a size category. Small < 15 kg, medium 15–25 kg, and large > 25 kg to evaluate if certain size categories were more predisposed to stress. What is more, each subject was assigned to either 'adopted' or 'bought from a breeder' to evaluate a stress response. The cortisol levels from the hair samples were compared to the above categories by using a T-Test: Paired Two Sample for Means through Microsoft Excel. The T-Test was run for each of these categories against cortisol. The $P(T \leq t)$, one-tailed and two-tailed, was investigated for significance. If this number was above 0.05, the relationship between the two variables was deemed insignificant. Weight was the only category that was deemed significant with $P(\text{one-tailed}) = 0.019$ and $P(\text{two-tailed}) = 0.038$.

RESULTS AND DISCUSSION

Each subject was divided into certain groupings in order to assess breed significance on the cortisol levels of dogs. The subjects were divided into mixed or pedigree groups to evaluate if one group was more predisposed to cortisol. Some studies have reported that being adopted leads to an increased stress response in animals due to a less stable, overcrowded environment [4, 19, 33,]. Our results did not show any significant correlations.

After evaluating the MCBARQ, weight, and breed results, we explored some other possibilities of stress correlation and differences among the dogs. The results from the MCBARQ were analysed under 7 separate categories

in order to assess cortisol response to behavioural traits; specifically known stress behaviours. There were no significant results from the analysis. No statistically significant difference between the sexes, neuter status, or different age categories have been shown in previous studies as well [3, 34, 44]. Some studies have shown that some hair colours have a higher concentration of cortisol [3, 34], but our results did not comply with this finding.

The results have demonstrated that there was a correlation between weight and cortisol deposited in the hair strands ($P_{\text{two-tailed}} = 0.03$). It indicated that an increase in weight correlated with an increase in cortisol. A study by Hewagamage et al. [17] explained that sheep which can be categorised as high cortisol responders have a higher chance of weight gain and obesity than low-cortisol responders. They found that high cortisol responding ewes ate more in response to stress than others. This was due probably to the reduced feeling of feeling full after eating (or increased ghrelin hormone) in response to stress as well as reduced thermogenesis specifically in skeletal muscles. In our experiment, the dog with the highest peak of cortisol, $68.466 \text{ ng.ml}^{-1}$, was an overweight dog. As a German Shepherd weighing 37 kg, the weight was 5 kg over the normal weight range for this breed. A study by Luo et al. [26] investigated the relationship between cortisol and ghrelin and so-called “emotional or stress-related eating”. This theory supposed that there were changes in eating behaviour in response to negative stressors and suggested that it was a coping mechanism for stress. “Comfort foods” which are rich in energy, fat, or sugar are consumed to obtain a feeling of emotional well-being [13]. Another study by Luo et al. [25] conducted a survey that found that 82.7% of owners noticed their dogs showed signs of emotional eating in relation to behavioural problems.

A study by Kuhn et al. [24] investigated dogs living in a human-dog relationship. They found that if the relationship to humans was unpleasant or if the environment was uncontrollable with unpredictable stimuli, the dog may develop chronic or recurrent stress. An unpleasant human-dog relationship was the leading cause of behaviour problems and the reason for dogs being brought to a shelter. However, previous studies have discovered that humans experience significant changes in blood pressure, heart rate (HR), oxytocin release, and immune defence as a result of petting a dog. This indicated that if the hu-

man-dog relationship is one of kindness and good welfare, the relationship can be mutually beneficial.

The experiment conducted had many parameters to compare, yet there was little significance in the results. This could have been due to several factors. Firstly, due to the COVID-19 pandemic restrictions, we were unable to collect the hair samples ourselves. This led to inconsistency in the amount and place on the body that the hair was removed from. Despite each person being given the same instructions on how to sample the hair inconsistencies occurred. It was recommended to shave the hair as close to the skin as possible on the inguinal region or inside of the thigh however there is no way to know if this was where the hair samples came from or how close to the skin the hair was removed from. If it was not cut close to the skin, there could be contaminants in the sample leading to an inaccurate cortisol value. Each hair shaft grows to a specific length, growth stops, and the final shedding cycle commences. Therefore, hair samples obtained without previous shaving will contain a mixture of hairs that have incorporated cortisol over different time periods. This can be resolved by shaving an area, then re-shaving approximately 3 months later, thus establishing a known timeline of cortisol incorporation [35]. Another possible explanation for the variations in cortisol levels may be differences in the moulting patterns and growth rate between hair from the neck and the ischiatic region [44].

Another possible issue is owner compliance. The survey comparison was a large part of the data comparison. Perhaps some owners misinterpret their animals' behaviour or perhaps they exaggerate some behaviours on the survey. This will lead to an inaccurate overall account and could account for some misevaluation of the data. Behavioural indicators of stress are rarely recognized by owners; thus only some specific situations will be noted [24, 30]. Ideally, a comprehensive evaluation of dogs' stress should be evaluated to define the human-dog relationship. Therefore, it is important to evaluate chronic stress levels and the behaviour of dogs both at home and at shelters to gain a full understanding of our pets' welfare, or indeed over a longer time period. Hair washing and shampooing could contribute to insignificant results in the effect of washing hair in dogs. Currently, there are only human studies for comparison however, damage done to human hair structure could have been caused by liquids (e.g., water and cosmetics) or the alcohol used to wash the samples before

analysis [22, 29]. The amount of cortisol after washing was decreased after a high number of shampoo washes [35]. It is yet unknown if dogs are similarly affected by shampoo washing, although dogs are usually shampooed less frequently than humans so the comparison would also have to include products and time of washing. A larger sample size would give a more accurate evaluation of the data and hopefully a clearer picture of significance. Measuring any parameters can be difficult to interpret accurately without comparative baseline values and without such a single diagnostic test; it should be remembered that the welfare of animals should be judged on how far measurements deviate from normal [37]. Nonetheless, a few studies have attempted to examine the behaviour of dogs under normal home conditions.

CONCLUSIONS

In our study, the only significant parameter was that an increase in weight (kg) led to an increase in cortisol ($\text{ng}\cdot\text{ml}^{-1}$). It is theorized to be related to emotional comfort eating as there are papers where owners reported that their dog showed signs of emotional eating relating to behavioural issues. This is an interesting theory to consider studying in terms of both human and companion animal lifestyles. For humans, there is an increasing global risk of obesity and for understanding the importance of nutrition in companion animals, especially understanding how much ‘human food’ and treats to reward the animals. Interestingly, there was no significance between dogs adopted from shelters and those bought from a breeder, despite reporting behavioural issues in the MCBARQ. We believe further investigations could be done as to time adopted and specific behavioural traits in order to obtain a fuller picture. In conclusion, the topic of animal welfare in dogs relating to stressors in their life is poorly understood. This paper has provided an insight into areas that still need to be documented with more time and resources to fully understand this topic sufficiently.

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