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Salivary cortisol: a marker of the adaptive response of the organism to environmental stimuli

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The increasing attention to animal well-being has stimulated the study of biomarkers of an organism's adaptive response to the environment, with cortisol emerging as one of the most interesting. Saliva is a biological fluid that is easy to collect and has the advantage that its cortisol content parallels that in blood with a 20-30 minutes delay, thus "photographing" the adaptive response to a past stimulus without interference due to handling while taking the sample. Recent studies have shown that salivary cortisol can be used as a biomarker for some diseases and behavioural modifications, as well as in support of canine activities and sports. The new point-of-care devices for assaying salivary cortisol concentration in dogs provide practitioners with a useful method for evaluating the degree of activation of the hypothalamic-pituitary-adrenal axis in response to central or peripheral stimuli.

Key words - Cortisol, Saliva, Pleiotropic role, Adaptive response, Environmental stimuli.

Cortisol is a glucocorticoid (GC) that is often used to evaluate the activity of the hypothalamic-pituitary-adrenal (HPA) axis and the adaptive response of the body at a central level, in reaction to environmental stimuli to the nervous system, and peripherally, in reaction to metabolic variations, trauma or immune system responses of various tissues and organs. Besides being secreted by the HPA axis, in some cases, the secretion of GC can also be stimulated by higher brain centres, both in normal conditions (sleep-wake cycle in humans) and in unfavourable circumstances (fear, anxiety, pain, cold, etc.), thus promoting the recovery of homeostasis.[1,2]

Cortisol is a glucocorticoid that is produced predominantly by the hypothalamic-pituitary-adrenal axis as part of the adaptive response to various endogenous and exogenous stimuli.

THE PLEIOTROPIC ROLE OF CORTISOL

The multiple effects of cortisol on metabolism and the adaptive response require a complex mechanism of regulation which is not yet completely understood. Adrenocorticotropic hormone (ACTH) stimulates the release of cortisol which, once in the circulation, has a half-life that varies between 70 and 120 minutes in humans.[3,4]

The main metabolic effects of cortisol are gluconeogenesis in the liver, deposition of fat, and regulation in the brain of the hypothalamic neuropeptides involved in appetite control. Furthermore, in these tissues, cortisol can be locally inactivated to cortisone through the

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action of isoforms 1 and 2 of 11β-hydroxysteroid dehydrogenase or regenerated starting from the same hormone, thereby bypassing secretion of the hormone by the adrenal glands.[5]

In circumstances of acute stress ("fight or flight") high concentrations of cortisol increase the release of glucose into the blood and the feeling of hunger, thus facilitating the response to the stress itself. In adipose tissue, on the other hand, cortisol has an anabolic effect that can potentially promote the deposition of new tissue in metabolically disadvantaged areas. This feature has made cortisol a focus of attention in the strive for better understanding of the endocrine mechanisms related to obesity.[5]

The pleiotropic effects of cortisol are fundamental for the adaptive response of the body and strict regulation, through negative feedback on the HPA axis, avoids overexposure of target tissues, thus preventing the typical negative effects of cortisol, such as glucose intolerance, immunosuppression, increased blood pressure, osteoporosis, insulin resistance, altered growth and tissue repair and even Cushing's syndrome.[5]

Cortisol has pleiotropic effects on both metabolism and the body's adaptive response. Its action at a cellular level is mediated by activation of glucocorticoid receptors.

At a cellular level, the effects of cortisol are mediated by activation of GC receptors (GR), of which there are five different isoforms with specific functions, not yet completely understood, but probably responsible for the pleotropic activity of the hormone.[6] The complexity of the effects also derives from the ability of cortisol to regulate the transduction of signals in various tissues and systems through binding to both cytosolic receptors (non-genomic control) and nuclear receptors (genomic control) (Figure 1).

As far as concerns **non-genomic control**, GC have an activating/deactivating role in the cytosol by interacting with the cell membranes in a GR-independent manner, acting directly on protein kinases (MAPKs) or by binding to membrane GR, which can lead to rapid activation of anti-inflammatory signals.[7,8] Furthermore, at a central level, GR, together with receptors for mineralocorticoids, play a critical role in coordinating a rapid adaptive response to stress, with the involvement of pre-receptors that are still under investigation.[9,10]

With regards to **genomic control**, on the other hand, the GR act directly on the nucleus. The GR are initially present in the cytosol in association with large, multiprotein complexes with chaperone functions. After binding to the ligand, the complex breaks down, exposing nuclear localisation sequences enabling translocation to the nucleus, where the GR can cause positive transcriptional modifications (transactivation) or negatives ones (transrepression).[6]

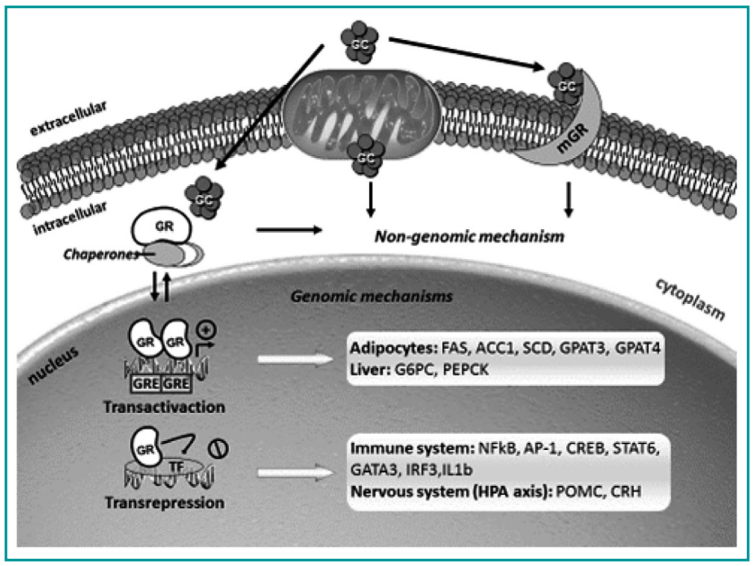


Figure 1 - Genomic and non-genomic mechanisms of GC signal transduction (modified[6]).
mGR: membrane glucocorticoid receptor; **GR:** glucocorticoid receptor; **GC:** glucocorticoid; **GRE:** glucocorticoid response element; **TF:** transcription factor.

ASSAYING CORTISOL IN BIOLOGICAL MATRICES

Cortisol is involved in various metabolic, immune and nervous system processes and variations in its levels therefore reflect the adaptive response to the environment in the broadest sense of the term, taking into account all the actions in which this hormone is involved. The cortisol assay is a method that, together with other diagnostic information, has the advantage of providing the most objective and complete evaluation of an organism's biological response, thus enabling data collected in different contexts to be compared with reference values.

Cortisol can be assayed in various biological substrates, of which the least invasive to obtain are hair, urine and saliva.

Sampling

Cortisol can be assayed in various biological matrices including blood, saliva, urine and hair. The potential applications of cortisol assays have stimulated research into non-invasive methods of sampling that do not affect the secretion of the hormone, but that accurately reflect the activation of the HPA axis.[11]

Blood was one of the first reference substrates in

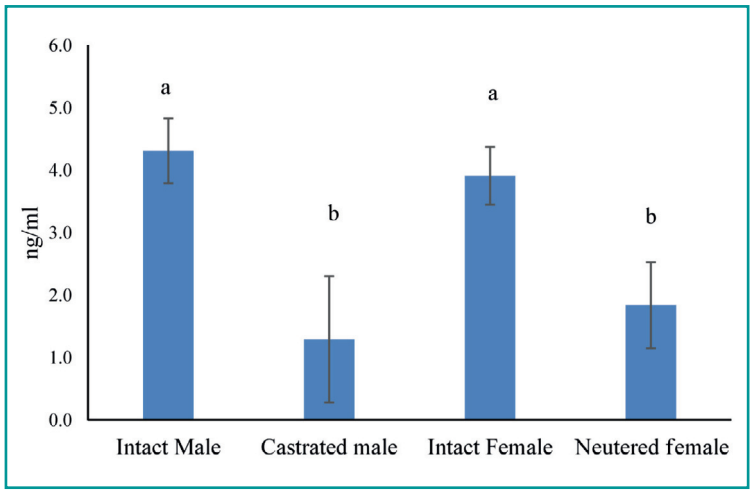
which cortisol concentrations were evaluated to make a diagnosis of behavioural problems or diseases and, subsequently, to determine the efficacy of treatments.
A blood sample provides a snapshot of cortisol levels. The secretion of cortisol is very sensitive to both internal and external stimuli, with these latter including handling during sample collection. Indeed, manipulation for more than 2 minutes can cause significant changes in blood cortisol concentrations[12,13,14,15] with the risk of artefacts when evaluating activation of the HPA axis.
An alternative to blood is hair, which does not provide information on acute stress, including that occurring during the sampling. So far, research into the mechanism by which steroids accumulate in growing hair indicates that this occurs through the blood vessels that supply the follicle and, to a lesser degree, also through the sebaceous sweat glands and surrounding eccrine glands which, once the hair has emerged from the scalp, coat it with sebum and sweat, respectively. However, it seems that the hair follicle also produces cortisol locally in response to more widespread systemic stress, to localised skin irritation or as part of its normal function. The relationship between cortisol and the hair fibre within the follicle is complex and probably involves both melanin and keratin.[16,17] The amount of cortisol in the hair therefore reflects the endocrine secretion of the hormone over a period of months and is useful as a sensitive marker of chronic stress, without detectable changes related to short-lasting events.

The concentration of cortisol in the hair reflects long-term changes in the levels of the hormone (chronic response), while that in the saliva reflects short-term variations (acute response).

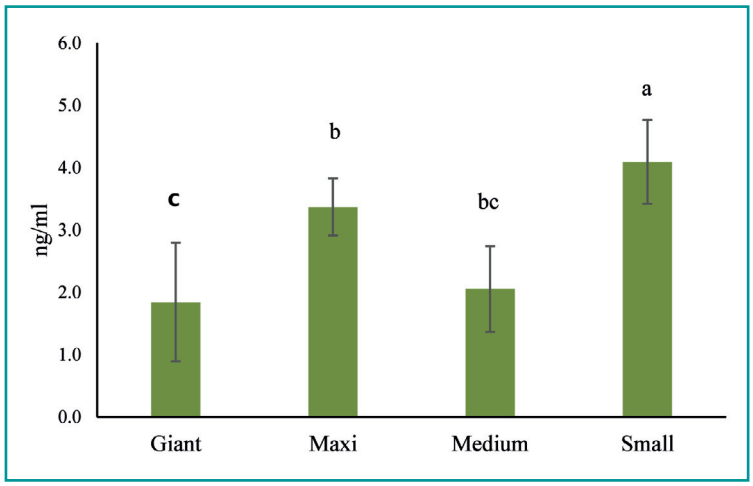
The accumulation of cortisol in hair does, however, vary in relation to the characteristics of the animal's coat, because, in mammals, there are physiological and biochemical similarities between the production of glucocorticoids and that of hair pigments. Indeed, pigment production is regulated by melanocyte-stimulating hormone (MSH), which is derived from pro-opiomelanocortin, which is also the precursor of ACTH, the hormone that stimulates the secretion of cortisol. Furthermore, the product of the Agouti genes, one of the main genes involved in coat colour, has a competitive antagonistic effect on the melanocortin receptors of MSH. Thus, genetic characteristics of the coat can also influence the accumulation of cortisol in the hair. Indeed, the concentration of cortisol is lower in black hairs than in non-black hairs, since the former contain more pigment that occupies the 'space' available for the GC, while the concentrations are higher in the hair of animals with lighter coloured coats.[15]
The levels of cortisol in the saliva accurately reflect those in the blood,[18] with the variations occurring about 20-30 minutes later.[19,20] For this reason salivary cortisol levels are measured to assess the acute response to stress, being useful in dogs for evaluating the adaptive response, such as immediate reactions to threats and man-animal interactions. The slight delay in the variations avoids spurious values due to handling while taking the sample. In fact, salivary values simultaneously reflect the activity of the sympathetic nervous system (acute stress) and the HPA axis.[20] Furthermore, salivary sampling is a non-invasive method[21,22] and repeating the assay over time enables evaluation of the adaptive response in the mid- and long-term.
Since the concentration of cortisol in the saliva is about 7-12% that in plasma[21,23] the assay for the former must be more sensitive than that used for plasma, and care must be taken to ensure that materials which interact with the analyte are not present either in the sampling phase or during storage of the sample.
Contamination of the blood, haemolysis, pH, excessive residual food and material used to collect the sample are all factors that can influence the analysis of cortisol in the saliva, while plasma proteins, present only in trace amounts in the saliva, are not a problem.[21]
It should be remembered that the secretion of saliva is under the control of the nervous system and that sympathetic nerve stimulation can cause vasoconstriction and a decrease in salivary flow, thus reducing the possibility of collecting a sample sufficient for analysis. For this reason, it is always useful to stimulate the dog's salivation, for example by giving it food to sniff for a few seconds, thus inducing secretion of saliva without causing stress.[15]

Factors that influence salivary cortisol levels in the dog

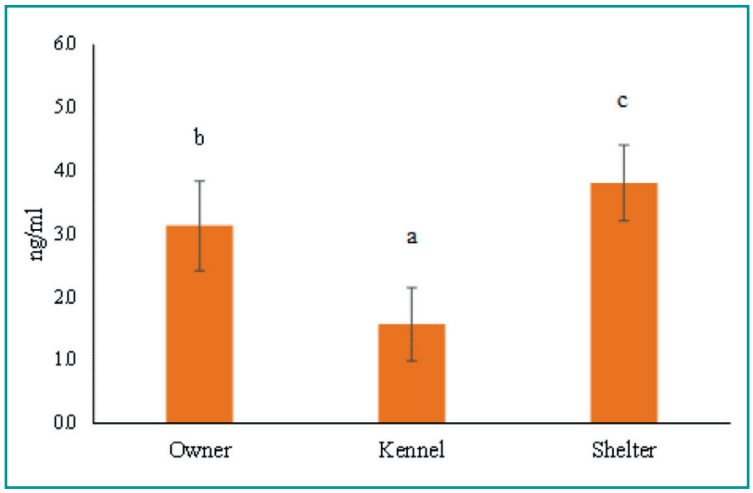
In studies carried out in clinically healthy, adult dogs[24,25] it was found that sexual status and gender have significant effects on salivary cortisol levels (Graph 1): sterilised animals have lower levels than sexually intact ones, while intact females and males do not have significantly different values Furthermore, there are relationships between salivary cortisol levels and dogs' weight, size and breed[24,25,26,27] (Graph 2)[25].
These findings are consistent with those of another study[28] showing significantly greater activity and impulsiveness in small dogs than in large ones.
Living environment also influences cortisol concentrations, with levels of the hormone being higher in animals living in shelters than in animals living in ken-



Graph 1 - Differences in salivary cortisol levels (ng/ml) in relation to sexual status.
a, b, c: the letters indicate statistically significant differences (P<0.05) between the means.



Graph 2 - Differences in salivary cortisol levels (ng/ml) in relation to size.
a, b, c: the letters indicate statistically significant differences (P<0.05) between the means.



Graph 3 - Differences in salivary cortisol levels (ng/ml) in relation to living environment.
a, b, c: the letters indicate statistically significant differences (P<0.05) between the means.

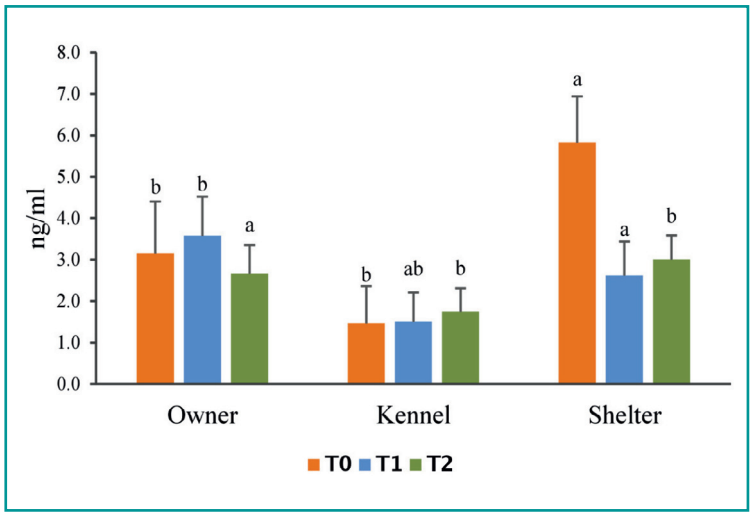
nels or private homes (Graph 3).[24] In this regard, some researchers[29] showed that admission of dogs into a shelter caused prolonged activation of the HPA axis. However, although this represented a condition of chronic stress, the animals gradually adapted to their new environment, as reflected by their cortisol concentrations.

Furthermore, the dogs living in shelters showed changes of cortisol levels during the course of the day, with a decrease following interactions with humans (Graph 4).[25] The samples of saliva taken before and after interaction with a human were both collected in the morning, about 30 minutes apart, to avoid any effects of circadian rhythm on the levels.[30,25] The results appear to show that a positive interaction with a human improves social behaviour and physiological wellbeing of dogs living in shelters, giving the animals a greater possibility of adoption.[31] Other researchers also observed variations in cortisol concentrations in dogs in relation to the sex of the person with which the animals interacted, with there being lower concentrations of cortisol in the saliva following interactions with a female person.[29]

Concentrations of cortisol in canine saliva are influenced by the sex, size and living environment of the animal.

In another study, a significant increase in salivary cortisol concentration was found in dogs following their admission to hospital, with there being a statistically significant correlation between high concentrations and 'anomalous' behaviours such as panting and lip-licking.[32] In a previous study,[20] salivary cortisol concentrations increased from baseline values of between 2.16 and 4.68 ng/ml to a mean value of 6.01 ng/ml in dogs exposed to external stimuli. Similar results were obtained comparing dogs of the same breed subjected for 6 weeks to segregated housing in spaces more restricted than those to which they were accustomed. The salivary cortisol concentrations corresponded with the manifestation of abnormal behaviours, with subsequent development of depressed responsiveness of the HPA to acute stimuli.[33,11]

In conclusion, the ease and non-invasiveness of salivary sampling make it possible for an owner to collect a sample at a particular time and in different circumstances from the canonical ones such as the veterinary clinic (Figures 2, 3, 4, 5). Furthermore, the delay of about 20-30 minutes in the increase of cortisol levels in the saliva compared to those in the blood means that the values in the saliva following an event of interest are not affected by handling the animal to collect the sam-



Graph 4 - Differences in salivary cortisol levels (ng/ml) in relation to environment and time of day.
T0: sample taken in the morning during the first interaction with a human, just before a meal (6:00-8:00 a.m.).
T1: sample taken 30 minutes after the first interaction with the human in the morning.
T2: sample taken in the evening, 30 minutes after the last interaction with a human.
a, b, c: the letters indicate statistically significant differences (P<0.05) between the means.

Potential applications of salivary cortisol assays

The studies reported show the usefulness of assaying salivary cortisol levels in dogs in order to determine the state of activation of the HPA axis in relation to physiological conditions and various types of environmental stimuli. The interest in this information and its diagnostic utility are demonstrated by the growing number of scientific publications on salivary cortisol in the dog over the last 3-5 years. In the first place, cortisol assays are useful for supporting a diagnosis of behavioural disorders, but the pleiotropic role of GC, and cortisol in particular, lends them to numerous other uses, as already occurs in human biomedicine and animal livestock sciences.

Canine exercise and activities

There has been considerable interest recently in the relationship between salivary

ple. In practice, in the case of a suspected behavioural problem related to a particular environmental situation, the sample of saliva can be collected 20-30 minutes after the dog has manifested the abnormal behaviour, in order to compare the concentration of cortisol with that found in a period of no stress.

Figure 2 - Stimulation of salivation in the dog. Stimulating salivation in the dog by presenting the animal with food, but not allowing the dog to eat it before having taken the saliva sample.

Figure 3 - Sampling saliva in a dog. Placing the swab in the dog's mouth; to ensure that the swab becomes thoroughly imbibed with saliva, it should be put in the mouth two or three times and left in place for 15-20 seconds.

Figure 4 - Placing the imbibed swab in the test-tube.

Figure 5 - Closing the test-tube with its cap.

cortisol and exercise in humans, both with regards to the response to intensity of the activity and the body's recovery capacity,[34,35,36] as well as to determine the efficacy of various functional supplements during physical activity.[37]

Similarly, in canine activities, during which a dog is subjected to a variety of physical and psychological stimuli, variations in cortisol levels enable comparisons of the level of fitness between individuals during a working session and within the same animal during an exercise and training programme. The literature about changes in cortisol levels in dogs undergoing physical activity is still very limited. One study[38] evaluated the level of stress caused by agility competitions in purebred dogs (Border collie, Australian kelpie, Boxer, Belgian shepherd dog, German shepherd dog, Pit bull, Pinscher, Poodles) and mixed breed animals. The results showed an increase in the concentrations of cortisol following the competition, as well as some stress-referable behaviours.

Salivary cortisol levels may be a good marker for evaluating the effects of physical and/or mental exertion, such as training, canine activities and animal-assisted activities.

With regards to the effects of different workloads on cortisol concentrations, some researchers have investigated the effects of low and high intensity exercise on dogs. The animals were made to exercise on a treadmill at different speeds for about 90 minutes.[39] Blood cortisol concentrations increased during the exercise, at a rate reflecting the intensity of the exercise. The release of cortisol appeared to be related to both the duration and the intensity of the physical activity.

In another preliminary study[25] changes in salivary cortisol levels were measured during Pointing Hunting (English Setter), Blood Tracking (Bavarian mountain hound and Hannoverian scenthound) and Tracking for Ungulate Hunting (Istrian short-haired hound, Griffon nivernais, Italian short-haired hound). The duration and intensity of the mental and physical energy expended differed depending on the type of hunting in which the animals were engaged. There were significant increases in the concentrations of cortisol, which were particularly evident in the pointers, undergoing more intense physical effort ($P<0.05$).[25] The changes in salivary cortisol levels were, however, modest in the dogs employed in Blood Tracking and Tracking for Ungulate Hunting, confirming previous observations in German shepherd dogs involved in a track contest during an IPO competition, i.e., less activation of the HPA axis during activities in which greater mental concentration is required.[40]

Animal-assisted activities

Mental exertion in suitably trained animals does not, therefore, seem to lead to stress phenomena such as to alter homeostasis substantially. This appears to confirm findings in dogs involved in Animal-Assisted Activities (AAA), in which salivary cortisol levels did not change significantly following interaction with the pa-

tients.[41] Similar results were obtained in a preliminary study[25] in which salivary cortisol levels in dogs involved in AAA were significantly ($P<0.05$) lower 20 minutes after completion of the session (1.47+0.15 ng/ml) compared to the levels before starting the activity (2.07+0.16 ng/ml). Furthermore, it should be noted that the AAA took place between 10:00 a.m. and 11:45 a.m., a period of the day in which an increase in salivary cortisol could be expected as a result of circadian rhythm.[25,30] Measuring the concentration of cortisol in saliva could therefore offer a way of identifying animals less well adapted to AAA, through the detection of high levels of cortisol, and also a way of monitoring an animal's acceptance of such activities over time and picking up any changes early.

Genetics and breeding

The data available on changes in salivary cortisol levels in relation to AAA, training, educational programmes and psychomotor rehabilitation as well as activities to improve physical condition or coordination more generally, are still limited, but do offer a starting point for some important considerations not only from a physiological point of view, but also regarding ethology and breeding programmes.

In humans, the CORNET (CORtisol NETwork) consortium has recently published a genome-wide association study showing that there is a significant genetic component in plasma cortisol concentrations, related to mutations in genes involving the binding of cortisol to corticosteroid-binding globulin and alpha1-antitrypsin.[42] Mutations in DNA associated with plasma cortisol concentrations have also been identified in pigs.[43] According to CORNET, the hereditability of plasma cortisol in humans varies between 30 and 60%.

Besides being a diagnostic aid, salivary cortisol levels could also have an interesting use in breeding, for example to select for adaptive response characteristics.

As far as concerns the species *Canis lupus familiaris,* no association studies or estimates of hereditability are yet available, but analogies with other mammals[43,44] suggest that there is a strong genetic basis to the adaptive response to the environment. This could be used for breeding purposes, exploiting differences in temperament and aptitude between breeds and enabling the identification of blood lines better adapted to the typical activities of the breed. In this case, the level of cortisol, an accurate marker of an individual's physiological condition, could be a phenotype for selective breeding, once the assay protocol has been standardised.

Diseases

Analysis of cortisol in the saliva is also useful in all those situations of suspected hypercortisolism or di-

Table 1 - Applications of salivary cortisol measurements in exercise and training, education, relational responses, pathophysiology and psychological disorders.

Exercise and training • Individual predisposition to canine activities • Level of fitness during exercise and training • Monitoring stress during periods of intense canine activities and competitions • Evaluation of efficacy of functional and ergogenic compounds • Monitoring stress control in dogs working for voluntary civilian services, the military and police	**Pathophysiology** • Cushing's syndrome, hypercortisolism • Addison's disease, hypocortisolism • Diabetes mellitus • Metabolic syndrome and obesity • Cardiovascular diseases • Myocardial infarction • Chronic inflammatory bowel disease and irritable bowel syndrome
Education and Relational response • Monitoring stages of puppy socialisation • Animal assisted activities and therapies (AAA, AAT) • Quality of life in kennels and shelters • Emotional response to education and re-education programmes • Adaptation to adoption • Adaptation to new environments	**Psychopathology** • Anxiety • Obsessive-compulsive disorders/phobias • Depression • Hyperactivity/increased excitation and state of hypersensitivity-hyperactivity (HS-HA) • Post-traumatic stress • Anorexia nervosa • Food-related compulsive behaviours

sordered function of the adrenal glands, including secondary dysfunction.[23] In human medicine, measurement of salivary cortisol is a diagnostic aid that is being increasingly used in a number of disorders, including non-alcoholic fatty liver disease, metabolic syndrome, obesity and cardiovascular diseases,[45,46] as well as irritable bowel syndrome and inflammatory bowel disease.[47] Table 1 reports some of the potential applications of salivary cortisol assays in the dog, on the basis of present knowledge and prospective uses, borrowing on current practice in humans.[48,49]

CONCLUSIONS

The evaluation of cortisol concentrations in saliva can be considered a resource given the non-invasiveness of the sampling technique and the good parallelism between changes in this biological matrix and those in blood.

Furthermore, the possibility of determining the cortisol concentration immediately, with the now available point-of-care kits is valuable for both veterinarians, who can gain a full picture of the state of an animal's health, and for other professional figures such as dog-trainers and educators, providing them with a more complete assessment of the quality of psycho-educational and training programmes and the dog's response to such activities. As far as concerns genetics, measuring salivary cortisol concentrations, in predetermined conditions and using a standardised protocol, could become a useful instrument for breeders to assess the character and aptitude of a breed, which could be of value for the purpose of selective breeding.

KEY POINTS

- The pleiotropic effects of cortisol are fundamental for the organism's adaptive response and are mediated at the cellular level by the capacity of the hormone to regulate transduction signals through binding to both cytosolic and nuclear receptors.
- The growing interest in salivary cortisol level in dogs derives from the numerous practical applications of its assay, already exploited in human biomedicine and animal livestock sciences.
- Although there are still no gene association studies or estimates of hereditability, the analogies with other species indicate that it would be possible to use salivary cortisol level as a phenotype for selective breeding.
- Assaying salivary cortisol levels with a point-of-care kit could be a valid, non-invasive test, useful for various professional figures including veterinarians, trainers, educators and breeders.

REFERENCES

1. Poli A. Fisiologia degli animali. Regolazione-diversità-adattamento. Edizione Zanichelli, 2006.
2. Swenson MJ, Reece WO. Duke's Fisiologia degli animali domestici. Edizione Idelso Gnocchi, 2002.
3. Wiebke A, Stewart PM. Adrenal corticosteroid biosynthesis, metabolism, and action. Endocrinology and Metabolism Clinics of North America 34:293-313, 2005.
4. Sjaastad ØV, Hove K, Sand O. Physiology of Domestic Animals. Scandinavian Veterinary Press, 2003.
5. Harno E, White A. Will treating diabetes with 11β-HSD1 inhibitors affect the HPA axis? Trends in Endocrinology and Metabolism 2:619-627, 2010.
6. Ratman D, Berghe WV, Dejager L, *et al.* How glucocorticoid receptors modulate the activity of other trascription factors: A scope beyond tethering. Molecular and Cellular Endocrinology 380:41-54, 2013.
7. Song IH, Buttgereit F. Non-genomic glucocorticoid effects to provide the basis for new drug developments. Molecular and Cellular Endocrinology 246:142-146, 2006.
8. Lowenberg M, Verhaar AP, van den Brink GR, *et al.* Glucocorticoid signaling: a nongenomic mechanism for T-cell immunosuppression. Trends in Molecular Medicine 13:158-163, 2007.
9. Strehl C, Gaber T, Lowenberg M, *et al.* Origin and functional activity of the membrane-bound glucocorticoid receptor. Arthritis Rheum 63:3779-3788, 2011.
10. Groeneweg, FL, Karst H, de Kloet ER, *et al.* Mineralocorticoid and glucocorticoid receptors at the neuronal membrane, regulators of nongenomic corticosteroid signalling. Molecular and Cellular Endocrinology 350:299-309, 2012.
11. Beerda B, Schilder MB, Van Hoff JA, *et al.* Chronic stress in dogs subjected to social and spatial restrictions. II. Hormonal and immunological responses. Physiology & Behavior 66(2):243-254, 1999b.
12. Kobelt AJ, Hemsworth PH, Barnett JL, *et al.* Sources of sampling vari-

ation in saliva cortisol in dogs. Research in Veterinary Science 75(2):157-161, 2003.
13. Hiby EF, Rooney NJ, Bradshaw JW. Behavioural and physiological responses of dogs entering re-homing kennels. Physiology & Behavior 89(3):385-391, 2006.
14. Accorsi PA, Carloni E, Valsecchi P, *et al.* Cortisol determination in hair and faeces from domestic cats and dogs. General And Comparative Endocrinology 155(2):398-402, 2007.
15. Bennett A, Hayssen V. Measuring cortisol in hair and saliva from dogs: coat color and pigment differences. Domestic Animal Endocrinology 39:171-180, 2010.
16. Meyer JS, Novak MA. Minireview: hair cortisol: a novel biomarker of hypothalamic-pituitary-adrenocortical activity. Endocrinology 153(9): 4120-4127, 2012.
17. Bryan HM, Adams AG, Invik RM, *et al.* Hair as a meaningful measure of baseline cortisol levels over time in dogs. Journal of the American Association for Laboratory Animal Science 52(2):189-196, 2013.
18. Oyama D, Hyodo M, Doi H, Kurachi T, *et al.* Saliva collection by using filter paper for measuring cortisol levels in dogs. Domestic Animal Endocrinology 46:20-25, 2013.
19. Vincent IC, Michell AR. Comparison of cortisol concentrations in saliva and plasma of dogs. Research in Veterinary Science 53(3):342-345, 1992.
20. Beerda B, Schilder MB, van Hooff JA, *et al.* Behavioural, saliva cortisol and heart rate responses to different types of stimuli in dogs. Applied Animal Behaviour Science 58:365-381, 1998.
21. Beerda B, Schilder MB, Janssen NS, *et al.* The use of saliva cortisol, urinary cortisol, and catecholamine measurements for a noninvasive assessment of stress responses in dogs. Hormones and Behavior 30(3):272-279, 1996.
22. Dreschel NA, Granger DA. Methods of collection for salivary cortisol measurement in dogs. Hormones and Behavior 55(1):163-168, 2009.
23. Wenger-Riggenbach B, Boretti FS, Quante S, *et al.* Salivary cortisol concentrations in healthy dogs and dogs with hypercortisolism. Journal of Veterinary Internal Medicine 24(3):551-556, 2010.
24. Sandri M, Colussi A, Perrotta MG, *et al.* 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, 2015.
25. Stefanon B, Sandri M, Bastiani E, *et al.* Misura del cortisolo salivare nel cane: Sviluppo, Validazione e Applicazioni di un sistema Point of Care. Congresso Internazionale Scivac, Rimini, 2015.
26. Houpt KA. Genetics of canine behavior. Acta Veterinaria Brno 76:431-444, 2007.
27. Spady TC, Ostrander EA. Canine behavioral genetics: pointing out the phenotypes and herding up the genes. American Journal of Human Biology 82:10-18, 2008.
28. Vas J, Topa J, Pech E, Miklosi A. Measuring attention deficit and activity in dogs: a new application and validation of a human ADHD questionnaire. Applied Animal Behaviour Science 103:105-117, 2007.
29. Hennessy MB, Davis HN, Williams MT, *et al.* Plasma cortisol levels of dogs at a county animal shelter. Physiology & Behavior 62(3):485-490, 1997.
30. Giannetto C, Fazio F, Assenza A, *et al.* Parallelism of circadian rhythmicity of salivary and serum cortisol concentration in normal dogs. Journal of applied biomedicine 12:229-233, 2014.
31. Luescher AU, Medlock RT. The effects of training and environmental alterations on adoption success of shelter dogs. Applied Animal Behaviour Science 117:63-69, 2009.
32. Hekman JP, Karas AZ, Dreschel NA. Salivary cortisol concentrations and behavior in a popolation of healthy dogs hospitalized for elective procedures. Applied Animal Behaviour Science 141:3-4, 2012.
33. Beerda B, Schilder MB, Van Hoff JA, *et al.* Chronic stress in dogs subjected to social and spatial restrictions. I. Behavioral responses. Physiology & Behavior 66(2):233-242, 1999.
34. McGuigan MR, Egan AD, Foster C. Salivary cortisol responses and perceived exertion during high intensity and low intensity bouts of resistance exercise. Journal of Sports Science and Medicine 3:8-15, 2003.
35. Gatti R, De Palo EF. An update: salivary hormones and physical exercise. Scandinavian Journal of Medicine & Science in Sports 21:157-169, 2011.
36. Powell J, DiLeo T, Roberge R, *et al.* Salivary and serum cortisol levels during recovery from intense exercise and prolonged, moderate exercise. Biology of Sport 32:91-95, 2015.
37. McNaughton L, Bentley DJ, Koeppel P. The effects of a nucleotide supplement on salivary IgA and cortisol after moderate endurance exercise. The Journal of Sports Medicine and Physical Fitness 46:84-89, 2006.
38. Pastore C, Pirrone F, Balzarotti F, *et al.* Evaluation of physiological and behavioral stress-dependent parameters in agility dogs. Journal of Veterinary Behavior: Clinical Applications and Research 6:188-194, 2011.
39. Radosevich PM, Nash JA, Lacy DB, *et al.* Effects of low- and high-intensity exercise on plasma and cerebrospinal fluid levels or ir-beta-endorphin, ACTH, cortisol, norepinephrine and glucose in the conscious dog. Brain Research 498(1):89-98, 1989.
40. Colussi A. Influenza delle componenti genetiche, fisiologiche ed ambientali sul cortisolo salivare del cane. Università degli studi di Udine. AA 2012/2013.
41. Glenk LM, Kothgassner OD, Stetina BU, *et al.* Salivary cortisol and behavior in therapy dogs during animal-assisted interventions: A pilot study. Journal of Veterinary Behavior 9:98-106, 2014.
42. Bolton JL, Hayward C, Direk N, *et al.* Genome wide association identifies common variants at the serpina6/serpina1 locus influencing plasma cortisol and corticosteroid binding globulin. PLoS Genetics 10(7): e1004474, 2014.
43. Muráni E, Ponsuksili S, D'Eath RB, Turner SP, *et al.* Association of HPA axis-related genetic variation with stress reactivity and aggressive behaviour in pigs. BMC Genetics 11:74, 2010.
44. Mormede P, Terenina E. Molecular genetics of the adrenocortical axis and breeding for robustness. Domestic Animal Endocrinology 43:116-131, 2012.
45. Woods CP, Hazlehurst JM, Tomlinson JW. Glucocorticoids and Non-Alcoholic Fatty Liver Disease. Journal of Steroid Biochemistry and Molecular Biology 154:94-103, 2015.
46. Baudrand R, Vaidya A. Cortisol dysregulation in obesity-related metabolic disorders. Current opinion in endocrinology, diabetes, and obesity 22(3):143-149, 2015.
47. Vanuytsel T, van Wanrooy S, Vanheel H. Psychological stress and corticotropin-releasing hormone increase intestinal permeability in humans by a mast cell-dependent mechanism. Gut 63:1293-1299, 2014.
48. Chrousos GP, Gold PW. The concepts of stress and stress system disorders. Overview of physical and behavioural homeostasis. Journal of American Medical Association 267(9):1244-1252, 1992.
49. Charmandari E, Tsigos C, Chrousos G. Endocrinology of the stress response. Annual Review of Physiology, 67:259-284, 2005.