Measurement of faecal corticoid metabolites in domestic dogs*

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Summary

In the present study we established a method for the determination of faecal glucocorticoid metabolites in dogs and then used the assay to evaluate the adrenocortical activity in 12 dogs divided into two groups. In group A faecal samples were collected at their domestic setting. In group B, faecal samples were collected at home prior to transport to a boarding kennel, where faecal samples were then collected. In faecal samples most of the steroids were extracted with methanol and determined using a radioimmunoassay with an anti-cortisol antibody. Dogs in group A did not show any statistically significant inter-day variations in the basal levels of faecal corticoid metabolites. Faecal corticoid metabolites in dogs in group B were significantly higher on the first day at the kennel compared to animals kept at home. The peak concentration was found after 24 hours and followed by a slow decline. These results suggest that extraction with methanol and dosage with an anti-cortisol antibody by radioimmunoassay represents a valid approach technique for determination of faecal glucocorticoid metabolites and accurately reflects adrenocortical activity.

Keywords: dog, faeces, glucocorticoids, RIA, stress

Bestimmung von Corticoid-Metaboliten im Kot beim Hund

In vorliegender Arbeit wurde eine Methode zur Bestimmung von Glucocorticoid-Metaboliten im Kot von Hunden entwickelt und anschliessend bei 12 Tieren zur Überprüfung der Nebennierenrinden(NNR)-Aktivität eingesetzt. Bei Hunden der Gruppe A (n=6) wurde der Kot zu Hause in vertrauter Umgebung gesammelt, während die Hunde der Gruppe B (n=6) nach dem ersten Tag zur weiteren Untersuchung in ein Hundeheim gebracht wurden. Die Extraktion der Steroide aus dem Kot erfolgte mittels Methanol und zur radioimmunologischen (RIA) Bestimmung wurde ein gegen Corisol gerichteten Antikörper eingesetzt. Der Gehalt der Corticoid-Metaboliten zwischen den einzelnen Tagen war bei Hunden der Gruppe A nicht signifikant verschieden. Bei Hunden der Gruppe B war die Konzentration der Metaboliten am ersten Tag im Hundeheim signifikant höher als bei Hunden der Gruppe A und fiel anschliessend langsam ab. Diese Ergebnisse zeigen, dass die Extraktion mit Methanol und den Einsatz eines Anti-Cortisol Antikörpers im RIA-System geeignet sind, den Gehalt an Glucocorticoid-Metaboliten im Kot des Hundes und damit auch den Aktivitätszustand der NNR zu bestimmen.

Schlüsselwörter: Hund, Kot, Glucocorticoide, RIA, Stress

Introduction

During recent years animal welfare has become increasingly important and is also reflected in new European laws. However, the assessment of animal well-being is a complex matter (Moberg, 1987). Definitions of animal welfare and assays for its quantification are still under debate. One potential indicator of animal welfare is the absence of stress, although there is no standard definition of stress or a single biochemical assay system or endocrine parameter with which to evaluate it (Moberg, 1999). Thus, there is an objective need for additional biochemical or endocrine parameters for detection of animal disturbances. Transportation, unsuitable housing and handling are situations that are associated with poor animal welfare and can cause neuroendocrine stress responses (Clark et al., 1997). These stress responses mirror undesirable feelings and may have a negative impact on resistance to disease, behaviour, libido and fertility (Breazile, 1987; Rivier and Rivest, 1991). The magnitude of neuroendocrine stress responses provides information on the

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degree of stress that an animal experiences. Such information may help to prevent stress and its negative consequences (Mormede, 1988).
A physiological response to stress involves the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary and subsequent secretion of glucocorticoids by the adrenal cortex (Axelrod and Reisine, 1984). Elevated levels of glucocorticoids resulting from chronic stress may also cause depression, hypertension, gastrointestinal ulceration, electrolyte imbalances, calcium loss and bone mass reduction and inhibition of growth (Breazile, 1987; Young et al., 2004). As a result, levels of glucocorticoids or their metabolites are widely used as an indicator of stress (Beerda et al., 1996; Graham and Brown, 1996) and can be measured in blood, urine, saliva, milk or faeces (Vincent and Michell, 1992; Verkerk et al., 1998; Morrow et al., 2002). However, sampling of blood, urine and saliva may itself be difficult and stressful since they require restraint or immobilisation with consequently unreliable results. Moreover, it is dangerous or even impossible to collect samples in zoos and/or wildlife species (Schwarzenberger et al., 1996; Palme et al., 1999; Stead et al., 2000; Teskey-Gerstl et al., 2000; Goymann et al., 2001; Mashburn and Atkinson, 2004). Conversely, faecal sampling is easy to perform without disturbing the animals and results in an integrated assessment of cortisol production over a period of time (Schatz and Palme, 2001).
Measurement of faecal glucocorticoids has been used to investigate adrenocortical activity during exposure to stressful stimuli, such as exposure to new environments, transportation, social tension and aggression, human disturbances and exposure to predators in various species, including felids (Graham and Brown, 1996; Terio et al., 1999; Wielebnowski et al., 2002), canids (Creel et al., 1997; Monfort et al., 1998), hyenas (Goymann et al., 1999), primates (Whitten et al., 1998), lagomorphs (Teskey-Gerstl et al., 2000), cervids and bovids (Schwarzenberger et al., 1998; Palme et al., 2000; Dehnhard et al., 2001; Morrow et al., 2002). In some studies, high performance liquid chromatography (HPLC) analysis of faecal extracts has revealed the presence of multiple immunoreactive glucocorticoid metabolites in a variety of carnivore species (Schatz and Palme, 2001; Young et al., 2004). Radioimmunoassay (RIA) and enzyme immunoassay (EIA) have been used to quantify these metabolites. In particular, a cortisol EIA has been used for monitoring the adrenocortical activity by analysis of faecal glucocorticoids in domestic dogs (Palme et al., 2001; Schatz and Palme, 2001).
The main objective of this study was to establish a suitable RIA method for determination of faecal glucocorticoid metabolites in dogs. The validated method was then used to evaluate the concentration of corticoid metabolites in the faeces of dogs in their domestic setting compared to dogs transferred to a new environment.

Animals, Materials and Methods

Animals

A total of 12 pet healthy dogs were studied with an age between 1 and 10 years.
Five dogs (3 males: Siberian Huscky, German Shepherd, mixed-breed; 2 females mixed-breed) were studied at their domestic setting (group A). Seven dogs (4 males: Belgian Shepherd, Golden Retrivier, Boxer, German Shepherd; 3 females: Maremma Sheepdog and two mixed-breed) were moved by car for approximately 40 km to a boarding kennel (group B) where they were kept for one week in individual pens (2.4 x 1.5 m) and able to maintain olfactory, vocal and visual contacts with each other. The dogs were walked three times a day and were moved to an enclosed place daily for socialization for one hour. This was the first visit to that kennel for all dogs.
The health status of the animals was checked daily by clinical assessment of pulse, body temperature and respiratory rate. All animals remained healthy throughout the study. All dogs received moist dog food twice daily (morning and afternoon) and water was provided ad libitum.

Collection of faecal samples

All faeces was immediately collected in plastic bags. For dogs in group B, faecal samples were collected at home the day before transport and then for seven consecutive days after transfer to the kennel. The faeces of the dogs of group A were collected only in their domestic setting for five consecutive days. The collection of samples of group A was done by owner and the samples of group B were collected by the author. All faecal samples were taken immediately after the dogs passed them and directly stored at –20°C until processing.

Extraction and determination of metabolites

Briefly, frozen faeces were dried in an incubator at 37°C for 72 h, pulverized and thoroughly mixed. Faecal samples from the four dogs used to validate the assay system were extracted with either diethyl ether (Miller et al., 1991) or methanol as described by Palme et al., 1996 with slight modifications. For dogs in groups A and B faecal samples were extracted only with methanol. The determination of faecal glucocorticoid metabolites levels was performed using a RIA assay. Two RIA kits were initially tested: one with an anti-cortisol antibody (Cortisol Cat # 2466, Immunotech, Marseille, France) and the other with an

anti-corticosterone as antibody (ICN07-120102 corticosterone-ICN, Costamesa, USA).
The efficiency of steroid extraction from faeces was evaluated by measuring known amounts of labelled cortisol added to faecal samples prior to extraction (5.3-10.6 ng labelled cortisol/ml).

Extraction with diethyl ether
Pulverized faecal samples (0.5 g) was placed in a 16 x 100 mm glass tube and rehydrated with 5 ml deionised water. The tubes were shook for 1 h and then centrifuged at 3000 x g for 15 min. The supernatant was collected and frozen at –20°C until assayed. An aliquot (500 µl) of each faecal supernatant was placed in a 16 x 150 mm glass tube and anhydrous ethyl ether (5 ml) was added. The mixtures were vortexed for 1 min and the tubes were rapidly frozen in liquid nitrogen for few minutes. After freezing, the organic phase containing the steroids was poured into a fresh glass tube containing 500 µl of distilled water for washing. The tubes were again vortexed and rapidly frozen. The washed organic phase was then poured into a clean glass tube. This extraction process was again repeated and the final washed organic phases were added to the first and dried in an incubator overnight at 37°C. The dried samples were reconstituted in 1 ml of gel-PBSS (0.1% gelatine in a phosphate-buffered saline solution [0.01 M phosphate, 0.14 M sodium chloride, pH 7]), vortexed, incubated for about 18 h at 22°C, vortexed again and used to measure the concentration of corticoid metabolites.

Extraction with methanol
Pulverised samples (0.5 g) were placed in glass tubes and methanol (5 ml) was added. Instead Palme et al., 1996 used first a mixture of distilled water- methanol (2 ml + 3 ml) and then methanol (5 ml) for a further extraction step. After shaking (30 min) and centrifugation (2500 x g for 15 min) the supernatant was removed and used to measure the concentration of corticoid metabolites.

Radioimmunoassay 1
The determination of faecal glucocorticoid metabolites levels was performed using a commercial RIA kit (Cortisol Cat # 2466, Immunotech, Marseille, France). According to the manufacturer's protocol, duplicate 50 µl aliquots of faecal extract were added to 500 µl of ^{125}I-labelled cortisol in tubes coated with an anti-cortisol antibody. The tubes were incubated for 1 hour at 18-25°C. After shaking, the contents have been aspirated and the radioactivity was counted for one minute in a Á-counter (Perkin Elmer, Cobra II, Monza, Italy). A standard curve was prepared using known concentrations of cortisol (18-2269 ng/ml). The assay sensitivity was 20 nM. The inter-assay coefficient of variation was 9% for a high-cortisol control and 12.5% for a low-cortisol control; the intra-assay coefficient of variation was less than 10%.

Radioimmunoassay 2
Duplicate 100 Ìl aliquots of the faecal supernatants in gel-PBSS were placed in glass tubes containing 500 µl of rabbit antiserum against corticosterone (ICN07-120102 corticosterone-ICN, Costamesa, USA) diluted 1:10 in 0.1% bovine serum-albumin in 0.005M Tris-HCl, 0.1M NaCl, 0.1% NaN_3, pH 8. The tubes were incubated for 30 min at room temperature. Next, 100 µl of ^{3}H-corticosterone (Perkin Elmer, Monza, Italy) (about 15 pg/tube tritiated corticosterone with a specif activity of approximately 70 Ci/mM) was added and the tubes were incubated for 1 h at 37°C and then for 15 min at 4°C. Following this, 200 µl of charcoal (0.5%) dextran (0.05%) solution, pH 7.4, was added and the tubes were vortexed and incubated for 15 min at 4°C. Finally, tubes were centrifuged at 2000 x g for 15 min at 4°C. Radioactivity was counted in a liquid-phase scintillation counter (Canberra Packard, Tricarb 1600, Zurich, Switzerland). Standard curves were prepared using known concentrations of corticosterone between 1.25 and 40 ng/ml. The assay sensitivity was 30 pg/ml. The inter-assay coefficient of variation was 10% for a high-corticosterone control and 14% for a low-corticosterone control; the intra-assay coefficient of variation was less than 10%.

Statistical analysis

Data were analysed by using GraphPad Instat (ANOVA test one way, Tukey test, Paired t test and t-student test, GraphPad Software Inc., San Diego, CA, USA). Gaussian distribution of data was tested using the method Kolmogorov and Smirnov.

Results

The RIA using an anti-corticosterone antibody did not give any results and was unable to quantify the glucocorticoid metabolites in samples extracted with either diethyl ether or methanol because the immunoreactive material was below the limit of detection. In contrast, the cortisol RIA kit provided good results. The recovery was 23.2% ± 2 and 89% ± 6 for ether and methanol extracts, respectively.
Detailed faecal corticoid metabolites levels in dogs of group A and B are shown in Tables 1 and 2. The data are sampled from populations that follow Gaussian distributions. For group A the mean values of faecal corticoid metabolites in the five days were compared to each other. No significant inter-day variations were observed. In group B a statistically significant increase

(P < 0.05) was observed when comparing the mean value (835.4 ± 265.9 ng/g) at home (Fig. 1) versus the mean value at kennel-day 1 (2523.6 ± 595.0 ng/g). A statistically significant (P < 0.05) diminution was found comparing the mean values in kennel-days 4, 5, 6 and 7 versus kennel-day 1. Statistically significant differences were not found on days 1, 2 or 3. The highest concentrations of faecal corticoid metabolites in the kennel were found in faecal samples collected on days 1 and 2.
The mean values at home and in the kennel in dogs of group B were also compared with mean values of dogs of group A, although no statistically significant differences were found. Marked individual differences were observed between the amounts of faecal corticoid metabolites in faecal samples of dogs in group A

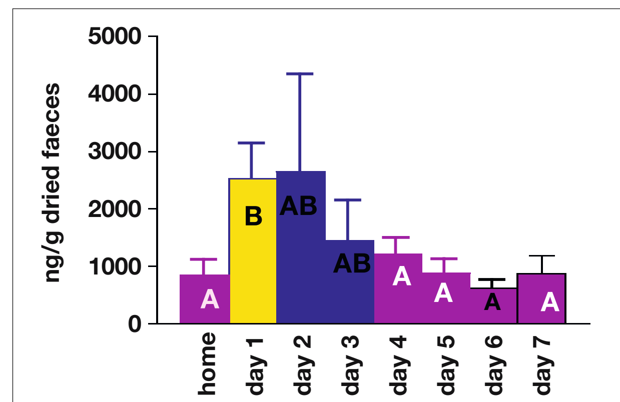

Figure 1: Faecal glucocorticoid metabolites in Group B (Kennel). Mean (m ± SEM) values of samples collected at home and daily collections at the kennel are shown. Different letters (A, B) indicate significant differences (P<0.05).

Table 1: Faecal glucocorticoid metabolite concentrations in dogs maintained in a domestic setting (group A). Values are expressed in ng/g of dried faeces.

Dogs	1	2	3	4	5
S_1	279	2767	1482	1350	1771
S_2	591	2086	1042	2747	2656
S_3	229	902	1989	1774	2164
S_4	355	4397	1985	1293	1838
S_5	331	1786	1756	1426	579
Mean±SEM	357±62.4	2387±584.9	1650.8±178.4	1718±270.5	1802±343.4

S_{1-5} = faecal samples of each day ($_{1-5}$).

Table 2: Faecal glucocorticoid metabolite concentrations in dogs transferred to a kennel (group B). Values are expressed in ng/g of dried faeces.

Dogs	6	7	8	9	10	11	12	Mean ± SEM
S_0	324	287	180	1673	1389	320	1675	835.4±265.9
S_1	4705	856	1340	3695	2694	721	3654	2523.6±595.0
S_2	2863	249	1009	1246	434	218	12501	2645.7±1678.6
S_3	2794	391	280	308	1132	190	4981	1439.4±685.9
S_4	1338	1066	735	770	1913	282	2361	1209.3±273.3
S_5	2052	808	371	841	827	181	1100	882.9±227.8
S_6	689	963	330	820	313	187	1010	616.00±127.3
S_7	2486	254	271	890	600	253	1355	872.7±310.1

S_0 = faecal samples at home; S_{1-7} = faecal samples of days $_{1-7}$ at the kennel.

(range: 357–2903 ng/g faeces) and B (287–1675 ng/g faeces). No significant differences were found between males and females.

Discussion

Glucocorticoids are subject to extensive metabolism. Steroids circulating in blood are catabolised in the liver before excretion in urine and bile. Additional changes to steroid metabolites are facilitated by the enzymatic activities of bacterial flora during transit through the intestinal tract. In agreement with a previous report (Schatz and Palme, 2001), the majority of faecal metabolites are extracted with methanol. In contrast to domestic livestock (Palme et al., 1996; Palme and Mostl, 1997), which excrete mainly unconjugated ether-extractable metabolites, the faecal metabolites in dogs were only poorly extracted with diethyl ether indicating a predominance of conjugated or polar unconjugated metabolites (Schatz and Palme, 2001). The RIA assay using an anti-corticosterone antibody was not able to quantify glucocorticoids metabolites in the dog. Nonetheless, many authors have found the highest responses in their studies with radioimmunoassays utilizing this anti-corticos-

terone antibody in cheetah (Terio et al., 1999), clouded leopard (Wielebnowski et al., 2002), African wild dog (Creel et al., 1997), spotted hyena (Goymann et al., 1999), cat (Graham and Brown, 1996), other carnivores (Young et al., 2004) and nondomestic mammalian and avian species (Wasser et al., 2000). This difference might be due to cross-reactions by the antibodies used in comparison with different metabolites. In fact, the selection of the antibody and label used in immunoassays for measuring steroids is of primary concern because there are a large number of steroid metabolites and only small amounts, if any, of the respective blood hormones in faeces (Schatz and Palme, 2001). The cortisol RIA kit was used to successfully quantify corticoid metabolites in faecal samples from dogs. The antibody against cortisol probably acts as a group-specific antibody with cross-reactivities to several of the glucocorticoid metabolites excreted in faeces. In the study by Schatz and Palme (2001), analysis of canine faecal extracts after intravenous administration of [^{14}C] cortisol HPLC revealed the presence of a large number of radioactive metabolites with elution times similar to cortisol. The injected [^{14}C] cortisol was present only in small quantities. In the two studies that have measured faecal glucocorticoid metabolites in dogs, a cortisol-EIA was used, while a corticosterone-EIA gave inconsistent results (Palme et al., 2001; Schatz and Palme, 2001).

The results obtained here indicate that this technique may be useful as a measure of stress under different conditions as the mean value of faecal corticoid metabolites on the first day in the kennel was significantly higher compared with the mean value before transport. We found the highest concentration of faecal corticoid metabolites was present around 24 hours after stress stimuli and background levels were reached within 3-4 days. In accordance with these results, a previous study (Schatz and Palme, 2001) reported high concentrations of faecal corticoid metabolites around 24 ± 4 hours after iatrogenic administration of [^{14}C] cortisol with a slow decline in radioactivity. The delay prior to the peak faecal concentration reflects the time required for chyme to pass from the duodenum to the rectum (Graham and Brown, 1996; Schatz and Palme, 2001).

The inter-animal variation in faecal corticoid metabolites concentration was high in both groups and an elevated inter-day variation in the same animal was also observed in dogs in a domestic setting. Breed, age and temper might influence the glucocorticoid concentration. Thus, it is difficult to compare the faecal corticoid metabolite concentrations of different animals or animals in different situations. To overcome this problem, baseline periods must be included in the experimental design so that each animal can serve as its own control (Mostl and Palme, 2002). No gender differences in the amounts of excreted steroid metabolites were observed in this study, although the significance of this observation needs further evaluation. In conclusion, extraction with methanol and dosage with a ^{3}H-cortisol RIA kit can be considered suitable for the determination of faecal glucocorticoid metabolites in dogs. Moreover, we consider this determination useful in evaluating adrenal function and in demonstrating acute stress situations. The stress arising from transport and new housing conditions caused an increase in the concentration of faecal corticoid metabolites. Further studies are needed to determine the potential effects of sex, age, diet, reproductive status, type of stressor agents on faecal corticoid concentrations, but elevations in faecal glucocorticoids may be regarded as indicative of a physiological stress response. The assay is non-invasive and has potential utility for a wide variety of conservation, management and biomedical investigations.

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Mesures des métabolites de corticoïdes dans les selles de chiens

Dans le présent travail, une méthode visant à déterminer les métabolites de glucocorticoïdes dans les selles de chiens à été développée et utilisée ensuite sur 12 animaux pour tester l'activité du cortex surrénal. Chez les chiens du groupe A (N=6), les selles ont été collectées à la maison dans un environnement habituel alors que chez les chiens du groupe B (N=6) les chiens ont été placés en chenil après le

Determinazione dei metaboliti fecali dei glicocorticoidi in cani sottoposti a stress

Nel presente studio abbiamo stabilito un metodo per la determinazione dei metaboliti fecali dei glicocorticoidi nei cani e poi lo abbiamo applicato per valutare l'attività adrenocorticale di 12 cani divisi in due gruppi. I cani del gruppo A sono stati tenuti nel proprio ambiente abituale, mentre quelli del gruppo B sono stati portati in una pensione per animali. La maggior parte degli steroidi sono stati

premier jour pour la suite de l'examen. L'extraction de stéroïdes des selles a été faite au moyen de méthanol et des anticorps dirigés contre le cortisol ont été utilisés pour la détermination radio-immunologique. Le taux de métabolites de corticoïdes ne variait pas d'un jour à l'autre de façon significative chez les chiens du groupe A. Chez les chiens du groupe B, la concentration de ces métabolites était significativement plus élevée au premier jour de la mise en chenil par rapport aux les chiens du groupe A et elle s'abaissait ensuite lentement. Ces résultats montrent que l'extraction au méthanol et l'utilisation d'anticorps anti-cortisol en mesure radio-immunologique permettent de déterminer la quantité de métabolites de glucocorticoïdes dans les selles des chiens et ainsi l'activité du cortex surrénal.

estratti dalle feci con il metanolo e quantificati con una metodica RIA attraverso l'uso di un anticorpo anti-cortisolo. I cani del gruppo A non hanno mostrato alcuna variazione statisticamente significativa nei livelli basali dei metaboliti fecali dei glicocorticoidi, mentre nei cani del gruppo B tali metaboliti sono significativamente più alti nei campioni fecali raccolti il primo giorno in pensione rispetto ai livelli presenti nello stesso animale prima del trasporto. Il picco massimo di escrezione è stato raggiunto circa 24 ore dopo l'arrivo in pensione, seguito da un lento declino. Questi risultati suggeriscono che l'estrazione con metanolo seguita da un dosaggio RIA che impiega un anticorpo anti-cortisolo rappresentano una utile metodica per la determinazione dei metaboliti fecali dei glicocorticoidi nel cane.

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