Measurement of faecal corticoid metabolites in domestic dogs*

A.M. Farca1, P. Cavana1, P. Badino2, R. Barbero2, R. Odore2, P. Pollicino1

1Section of Clinical Science and 2Section of Pharmacology and Toxicology, Department of Animal Pathology, University of Turin, Italy

**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

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**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 (Beerd 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), hynas (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 Husky, German Shepherd, mixed-breed; 2 females mixed-breed) were studied at their domestic setting (group A). Seven dogs (4 males: Belgian Shepherd, Golden Retriever, 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 125I-labelled cortisol in tubes coated with an anti-cortisone 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 Æ-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 µl 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% NaN3, pH 8. The tubes were incubated for 30 min at room temperature. Next, 100 µl of 3H-corticosterone (Perkin Elmer, Monza, Italy) (about 15 pg/tube tritiated corticosterone with a specific 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
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(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 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-corticos-terone 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-

(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-corticos-terone 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-

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

<table>
<thead>
<tr>
<th>Dogs</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>S0</td>
<td>324</td>
<td>287</td>
<td>180</td>
<td>1673</td>
<td>1389</td>
</tr>
<tr>
<td>S1</td>
<td>4705</td>
<td>856</td>
<td>1340</td>
<td>3695</td>
<td>2694</td>
</tr>
<tr>
<td>S2</td>
<td>2863</td>
<td>249</td>
<td>1009</td>
<td>1246</td>
<td>434</td>
</tr>
<tr>
<td>S3</td>
<td>2794</td>
<td>391</td>
<td>280</td>
<td>308</td>
<td>1132</td>
</tr>
<tr>
<td>S4</td>
<td>1338</td>
<td>1066</td>
<td>735</td>
<td>770</td>
<td>1913</td>
</tr>
<tr>
<td>S5</td>
<td>2052</td>
<td>808</td>
<td>371</td>
<td>841</td>
<td>827</td>
</tr>
<tr>
<td>S6</td>
<td>689</td>
<td>963</td>
<td>330</td>
<td>820</td>
<td>313</td>
</tr>
<tr>
<td>S7</td>
<td>2486</td>
<td>254</td>
<td>271</td>
<td>890</td>
<td>600</td>
</tr>
</tbody>
</table>

S0 = 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.

<table>
<thead>
<tr>
<th>Dogs</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>S0</td>
<td>384</td>
<td>287</td>
<td>180</td>
<td>1673</td>
<td>1389</td>
<td>320</td>
<td>1675</td>
<td>835.4±265.9</td>
</tr>
<tr>
<td>S1</td>
<td>4705</td>
<td>856</td>
<td>1340</td>
<td>3695</td>
<td>2694</td>
<td>721</td>
<td>3634</td>
<td>2523±659.0</td>
</tr>
<tr>
<td>S2</td>
<td>2863</td>
<td>249</td>
<td>1009</td>
<td>1246</td>
<td>434</td>
<td>218</td>
<td>12501</td>
<td>2645.7±1678.6</td>
</tr>
<tr>
<td>S3</td>
<td>2794</td>
<td>391</td>
<td>280</td>
<td>308</td>
<td>1132</td>
<td>190</td>
<td>4981</td>
<td>1439.4±685.9</td>
</tr>
<tr>
<td>S4</td>
<td>1338</td>
<td>1066</td>
<td>735</td>
<td>770</td>
<td>1913</td>
<td>282</td>
<td>2361</td>
<td>1209.3±273.3</td>
</tr>
<tr>
<td>S5</td>
<td>2052</td>
<td>808</td>
<td>371</td>
<td>841</td>
<td>827</td>
<td>811</td>
<td>1100</td>
<td>882.9±227.8</td>
</tr>
<tr>
<td>S6</td>
<td>689</td>
<td>963</td>
<td>330</td>
<td>820</td>
<td>313</td>
<td>187</td>
<td>1010</td>
<td>616.00±127.3</td>
</tr>
<tr>
<td>S7</td>
<td>2486</td>
<td>254</td>
<td>271</td>
<td>890</td>
<td>600</td>
<td>253</td>
<td>1355</td>
<td>872.7±310.1</td>
</tr>
</tbody>
</table>

S0 = faecal samples at home; S 1–7 = faecal samples of days 1–7 at the kennel.
terone antibody in cheetah (Tério 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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References


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Corresponding author

Prof. Anna Maria Farca, Via Leonardo da Vinci 44, 10095 Grugliasco (TO) – Italy, Phone number: ++39116709070, Fax number: ++390116709083, E-mail: anna.farca@unito.it

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