

The effect of ACTH upon faecal glucocorticoid excretion in the koala

Nicole Davies, Amber Gillett¹, Clive McAlpine², Leonie Seabrook², Greg Baxter², Daniel Lunney^{3,4} and Adrian Bradley

School of Biomedical Sciences, The University of Queensland, St Lucia, Queensland 4072, Australia

¹Australia Zoo Wildlife Hospital, Beerwah, Queensland 4519, Australia

²Landscape Ecology and Conservation Group, School of Geography, Planning and Environmental Management, The University of Queensland, St Lucia, Queensland 4072, Australia

³Office of Environment and Heritage NSW, PO Box 1967, Hurstville, New South Wales 2220, Australia

⁴School of Biology and Biotechnology, Murdoch University, Perth, Western Australia, Australia

Correspondence should be addressed to N Davies
Email
 n.davies1@uq.edu.au

Abstract

Environmental changes result in physiological responses of organisms, which can adversely affect population dynamics and reduce resistance to disease. These changes are expressed in chronic levels of stress. The measurement of glucocorticoid (GC) concentrations in faeces is a non-invasive method for monitoring stress in wildlife. The metabolism and excretion of steroids differ significantly between species and, as a consequence, non-invasive methods must be physiologically validated for each species. Koalas (*Phascolarctos cinereus*) are declining in numbers through much of their range. The role of chronic stress in koala populations has not been identified. Prior to the assessment of faecal GC concentrations in wild koala populations, the excretion timing and concentrations of GCs need to be determined. In this study, we assessed a method for identifying and measuring the concentrations of GC metabolites in faecal pellets of captive koalas following ACTH treatment. The results show that an elevation of plasma cortisol concentrations, using sustained release of ACTH, results in elevated concentrations of faecal cortisol/cortisol metabolites. Taking into account the excretion time lag, an increase in faecal cortisol metabolite concentrations corresponds to the release of GCs from the adrenal cortex as early as 36 h before faecal pellet collection. The calculations of steroid partitioning of plasma cortisol showed that the ACTH-stimulated values were significantly different from the control values for the concentrations of free, corticosteroid-binding globulin-bound and albumin-bound cortisol. This study validates the use of faecal cortisol analysis to assess the activity of the hypothalamo–pituitary–adrenocortical axis in freshly collected koala faecal pellets and indicates that the method should be suitable to assess the adrenocortical status of koalas in wild populations.

Key Words

- ▶ ACTH challenge
- ▶ koala
- ▶ glucocorticoid
- ▶ metabolites
- ▶ faecal pellets

Journal of Endocrinology
 (2013) 219, 1–12

Introduction

Understanding the physiological responses of organisms to changes in their environment is of great importance to conservation biologists as stress can alter behaviour, reduce resistance to disease and adversely affect

population performance (including impacting survival and reproduction; Munck *et al.* 1984, Wingfield & Farner 1993, Millspaugh & Washburn 2004). Vertebrates cope with unfavourable conditions by initiating a stress

response that is generally characterised by the release of glucocorticoid (GC) hormones (mainly cortisol or corticosterone in mammals) by the adrenal cortex (Seyle 1935, Axelrod & Reisine 1984, Mostl & Palme 2002, Stewart 2003). Short-term GC secretions, resulting from acute stress (e.g. predator attack), are beneficial, as they assist an animal in coping with a stressor by mobilising energy reserves during the stressful situation (Lee & Cockburn 1985, Wingfield *et al.* 1998, Sapolsky 2002, Mateo 2006). However, if the stress persists over a long period (i.e. chronic stress), the sustained high levels of GCs can lead to deleterious effects on the physiology of the animal and greater susceptibility to disease and reduced fecundity and survivorship (Munck *et al.* 1984, Sapolsky *et al.* 2000, Mostl & Palme 2002, Stewart 2003, Lane 2006). The release of GCs is routinely used as a measure of the stress response in ecological and conservation studies to assess the health of animals (Romero 2004). The measurement of GC concentrations can reveal how stressors affect the survival and reproductive success of wild animals and how natural environmental challenges (e.g. predators, conspecifics and weather), climate change and habitat disturbance have an impact on wild populations (Boonstra & Singleton 1993, Wasser *et al.* 1997, Creel *et al.* 2002, Thiel *et al.* 2008, Sheriff *et al.* 2009).

In plasma, cortisol exists primarily in three states: unbound, bound to albumin and bound to corticosteroid-binding globulin (CBG; Smith *et al.* 1980). CBG binds to cortisol with a high affinity and facilitates transport in the blood, while cortisol binds to albumin with a lower binding affinity (Hiramatsu 1983, Chu & Ekins 1988). Under normal conditions, ~70–85% of the secreted cortisol is bound to CBG (Hiramatsu 1983) and 10–15% to albumin (Chu & Ekins 1988), and the remaining 5–10% of cortisol is unbound and free to diffuse across cell membranes and bind to intracellular GC and mineralocorticoid receptors (Mendel 1989). Steroid hormones bound to carrier proteins (CBG and albumin) are considered to be biologically inactive. They provide a reservoir of inactive circulating hormones and regulate the amount of free hormones available for diffusion into tissues (Mendel 1989). The unbound cortisol reflects the biologically active concentration of the hormone that is capable of interacting with intracellular steroid receptors (Willcox *et al.* 1985). It is important to investigate the partitioning of steroids because changes in the plasma concentration of biologically active GCs are the foundation of physiological action, and when in excess (e.g. chronic stress), it can cause deleterious effects on the health of an animal.

Traditionally, GC concentrations have been measured in blood plasma. However, this procedure is invasive and blood GC levels are 'point' samples that are highly variable and are strongly affected by the time of day, food intake and any stressful situation or environmental disturbance that may have occurred shortly before sampling (including capture and handling techniques; Sapolsky 1982, Wingfield *et al.* 1992, Beerda *et al.* 1996). Therefore, specific point measurements may be unrepresentative of long-term hormone exposure levels (Harper & Austad 2000, Davenport *et al.* 2006). Furthermore, blood sampling can be difficult to carry out under field conditions. More recently, non-invasive methods for measuring GC metabolite concentrations in faeces and hair have become widely accepted tools for gaining important information about an animal's endocrine status (Mostl & Palme 2002, Young *et al.* 2004, Palme *et al.* 2005, Davenport *et al.* 2006, Bosson *et al.* 2009, Sheriff *et al.* 2009). The employment of non-invasive techniques to assess the physiological status of wild populations can be advantageous for both practical and ethical reasons (Lane 2006).

Faecal GC measures provide an integrated measure of fluctuating blood concentrations during the time the product was being formed and stored (usually a good indicator of GC levels of the previous 24–48 h), thus providing a more representative measure of an individual's average hormone secretion (Goymann *et al.* 1999, Harper & Austad 2000, 2001). Furthermore, faecal GC measures can be obtained easily without handling or observing the animals (Mostl *et al.* 1999, Palme *et al.* 1999, Harper & Austad 2000, Mostl & Palme 2002, Millsbaugh & Washburn 2003). The metabolism and excretion of steroids differ significantly between species and sexes (Touma *et al.* 2003, Palme 2005, Palme *et al.* 2005, Touma & Palme 2005); therefore, non-invasive methods must be physiologically validated for each species (Rehnus *et al.* 2009). This validation is typically carried out using an adrenocorticotrophic hormone (ACTH) challenge, which involves pharmacologically inducing physiological changes in circulating GC levels to evaluate how these changes are reflected in the concentrations of faecal GC metabolites (Touma & Palme 2005). Although a large proportion of the conjugated GC (cortisol in marsupials) is known to be excreted in urine, in this study, we used faecal and plasma samples to assess the short-term elevation of cortisol levels in the blood plasma and the long-term delayed elevation of cortisol metabolite levels in faeces.

The koala (*Phascolarctos cinereus*) is a widely distributed, arboreal marsupial folivore, with its range extending across

30 bioregions from tropical Queensland to temperate Victoria and South Australia. Koalas are declining in numbers through much of their range. The drivers of these declines include habitat loss and fragmentation, thermal stress, fodder quality reduction, and water availability and disease. To appropriately quantify the impact of environmental and physiological stressors on koala populations, the validation of non-invasive techniques and assays is a necessary first step.

The aims of this study were to examine the adrenocortical status of wild koalas held captive under controlled conditions and to compare this with the adrenocortical status following a 24-h ACTH challenge. This was achieved by measuring cortisol concentrations in plasma and faeces as well as by employing glucuronidase cleavage of polar bonds to liberate unconjugated cortisol (Mostl & Palme 2002). The establishment of the temporal relationship between ACTH-stimulated GC concentration elevation in plasma and that in faeces would validate the use of fresh faecal pellets from wild koalas to enable an estimate of their adrenocortical status to be made. We did not collect urine sample since this would be impractical for any studies on koalas in the wild.

Materials and methods

Animals

The research was conducted at the Australia Zoo Wildlife Hospital (Beerwah, Queensland). The koalas examined in this study were wild animals being rehabilitated as part of the hospital's standard procedures for koalas prior to release. The koalas were at the later stages of rehabilitation when any injuries had healed and they no longer appeared to be disturbed by the presence of veterinary and nursing staff. The use of animals in an unstressed condition eliminated any influence of past trauma or impacts of treatment on GC concentrations.

The five koalas (four males and one female) used in this study were aged between 2 and 12 years. The daily diet of these koalas was fresh leaves of various *Eucalyptus* species and was the same for each koala. The study was conducted between March and May 2012. Each koala was held in a large wire mesh cage (~3×3×3 m) and provided with 2–3 solid tree trunks and connecting horizontal poles. One to two experienced animal handlers lightly restrained each koala, enabling ACTH or saline (control) injections or blood sample collection for this study.

ACTH challenge experiment

Each experiment was carried out over a 7-day period, and each koala was subjected to both a control treatment (injection of saline) and an ACTH challenge treatment (ACTH in saline), with repeat treatments separated by 1 month to allow for the recovery of physiological parameters.

For the control treatment, the koalas were injected intramuscularly with 0.5 ml of saline on the morning of the first day (0 h (T₀)). Faeces (one pellet from pellets voided during the collection period) and a blood sample taken by venepuncture of the cephalic vein were collected at 0, 12, 24 and 36 h. Faecal pellets were also collected daily (in the morning) from day 0 to 7 of the experiment. The samples were stored at –20 °C until analysis. The weights and body condition scores of the koalas were also recorded.

For the ACTH challenge, the koalas were treated in the same way, but injected intramuscularly with 25 IU 0.25 ml of depot ACTH (Novartis Pharmaceuticals Australia Pty Ltd.) in 0.5 ml of 0.9% saline at 0 h to increase circulating GC concentrations and again at 12 h. The Synacthen Depot is supplied as a suspension in which the active hormone is adsorbed onto an inorganic zinc complex that enables protracted release. This was used to elevate plasma GC concentrations for a 24–36-h period to allow for the appearance of GC metabolites in the faecal samples. In all the studies on marsupials in which it has been used, the long-acting synthetic β -1–24 amino acid form of ACTH has been shown to be potent in stimulating cortisol secretion from the adrenal cortex.

Blood samples were collected in lithium heparin vials, and plasma was immediately separated by centrifugation for 5 min at 1500 g. Faecal pellets and plasma samples were stored at –20 °C until analysis.

Faeces: extraction and analysis

Faecal pellets from individual koalas were oven-dried at 70 °C for 10 h. The dried samples were then crushed between two 10 mm PVC plates, passed through a fine sieve (1 mm) to remove leaf particles and then collected in 5 ml flat-bottomed polypropylene screw-capped vials. For each sample, 200 mg (\pm 1 mg accuracy) of a dry faecal powder were weighed into 16×100 glass test tubes. Subsequently, 2 ml of borate buffer (pH 6.5; 0.1 M) were added to the dry powder and vortex-mixed, and then 50 μ l of β -glucuronidase (β -D-glucuronoside glucuronosohydrolase, EC 3.2.1.31, Sigma Chemical Co.) containing ~4000 units were added to each test tube. β -Glucuronidase

is derived from *Helix pomatia* and is active in cleaving both glucuronidase and sulphatase. The test tubes were then incubated for 4 h at 37 °C on an orbital mixer. Then, 3 ml of redistilled diethyl ether were added to each test tube and vortex-mixed for 2 min, after which the test tubes were allowed to stand for 2 min. The lower aqueous phase was frozen in liquid nitrogen and the supernatant ether was decanted into 12×75 mm glass test tubes and evaporated to dryness at 40 °C in a hot block evaporator in a fume hood. The residue containing the extracted steroid was re-dissolved in 200 µl of a diluted zero cortisol calibration solution (Demeditec Saliva Free Cortisol Kit; diluted 1:10) and placed on an orbital mixer at 37 °C for 60 min, followed by short, high-speed vortex (20 s). Then, 100 µl of test samples, standards and controls were pipetted into wells of a 96-well Saliva Free Cortisol Kit (Demeditec DES6611, Demeditec Diagnostics GmbH, Kiel, Germany) and run as per kit directions. The efficiency of the extraction process was progressively tracked by addition of 30 000 d.p.m. ³H-cortisol (1,2,6,7 ³H-cortisol 160 Ci/mmol, PerkinElmer, Melbourne, Victoria, Australia), and the final assay concentration for cortisol was corrected for this efficiency.

The specificity of the assay was as follows: 100%, cortisol; 0.38%, corticosterone; 1.85%, cortisone; 0.88%, 11-deoxycortisol and 9.89%, prednisolone; with all other steroids of a similar structure having specificity <0.20%. Inter-assay variation was 5.4% coefficient of variation (CV), while intra-assay variation was 5.5% CV. The analytical sensitivity of the assay was 0.014 ng/ml or 4.2 pg per loaded sample.

Serial dilutions of glucuronidase-treated koala faecal extracts run against the Demeditec assay kit calibrator standards gave a satisfactory degree of parallelism for the assay.

Assay data were analysed employing a four-parameter logistic fit using MyAssays Analysis Software Solutions (www.myassays.com).

To assess the effect of enzymatic cleavage of glucuronide and sulphate polar bonds using β-glucuronidase, the pellets collected 36 h after ACTH injection were pooled from four koalas and cortisol was extracted as described above. From the common pool of dry faecal powders, 16 extracts were prepared. Eight samples were extracted without enzymatic treatment and eight after enzymatic treatment.

Plasma: extraction and cortisol RIA

A commercially available cortisol antiserum (product number: C8409-100TST, Sigma Chemical Co.) was diluted with buffer G (0.05 M phosphate buffer containing

0.1% porcine gelatin and 0.1% sodium azide, pH 7.4) to result in ~50% of the total ³H-cortisol being bound in the absence of competing unlabelled steroid. The optimal titre was determined to be 1/30 000.

Plasma samples of 10 µl were combined with 100 µl of phosphate buffer (0.05 M, pH 7.4) in 16×100 mm glass test tubes and the steroid was extracted by vortex mixing for 2 min using 2 ml of redistilled analytical reagent-grade AR dichloromethane. The test tubes were left standing for 2 min to allow separation of the aqueous and organic phases. The aqueous phase was then frozen to the walls of the test tubes by dipping the test tubes into liquid nitrogen for 10 s, and the dichloromethane lower phase was decanted into 12×75 mm glass test tubes and dried in a hot block evaporator at 45 °C in a fume hood. The dried residue was re-dissolved in 100 µl of phosphate buffer (0.05 M phosphate buffer containing 0.1% porcine gelatin and 0.1% sodium azide, pH 7.4).

Extraction efficiency was tracked using 30 000 d.p.m. ³H-cortisol added to the initial extraction tubes. Extraction efficiency was determined to be 90%.

From each sample, 25 µl were transferred into 12×75 mm polypropylene test tubes. To all the test tubes, 25 µl of ³H-cortisol (30 000 d.p.m./25 µl of 0.05 M phosphate buffer containing 0.1% porcine gelatin and 0.1% sodium azide, pH 7.4) were added. To the test tubes containing calibration standards, only 25 µl of buffer were added. Then, 50 µl of steroid-specific antibody were added to all the test tubes, which were then vortex-mixed for 2 min. The samples were then incubated at 0 °C using an ice-water slurry for 4 h. Into each test tube, 500 µl of dextran-coated charcoal (500 mg activated charcoal (fines removed) (Sigma), 50 mg Dextran T70 (Pharmacia), 400 ml phosphate buffer (0.05 M, pH 7.4) and 20 mg Thimerosal (Sigma Chemical Co.)) were pipetted. The test tubes were incubated at 4 °C for 15 min and were then centrifuged at 4 °C in a Beckman GPR refrigerated centrifuge for 10 min at 1500 g. Then, 100 µl of the supernatant containing the antibody-bound steroid were added to 1 ml of Ultima Gold scintillation fluid (PerkinElmer) in a scintillation vial, vortex-mixed and counted using a liquid scintillation spectrometer (Beckman LS 6000 TA, Beckman Coulter, Lane Cove, NSW, Australia). The concentrations of unknown samples were determined using the AssayZap Software (version 2.32, AssayZap, Cambridge, UK).

Plasma cortisol partitioning

Plasma steroid partitioning was determined using methods adapted from the works of Tait & Burstein

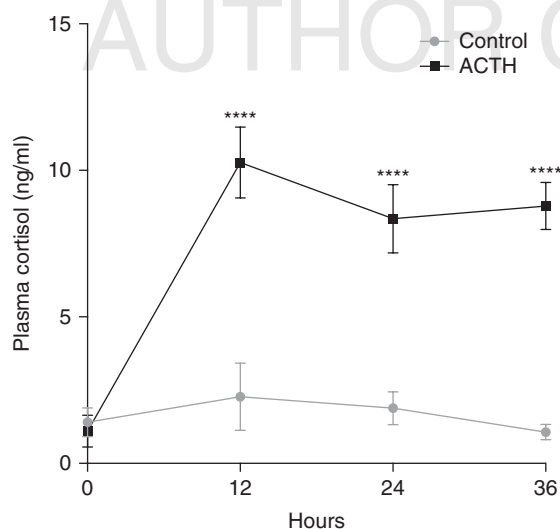


Figure 1

Mean (\pm s.e.m.) plasma cortisol concentrations for the control and ACTH treatments at each collection time. *Post hoc* tests indicate a significant difference (**** signifies $P < 0.001$) between 0 h and all other collection times for the ACTH treatment and no difference between 0 h and all other collection times for the control treatment.

(1964) and Paterson & Hills (1967) for the determination of the CBG-bound, albumin-bound and free compartments within the plasma. Microequilibrium dialysis has been used to describe the high-affinity and low-affinity binding of cortisol to plasma proteins (Bradley *et al.* 1980, Bradley 1990). The high-affinity binding constant employed (K_a) was $3.5 \times 10^7 \text{ M}^{-1}$ and the low-affinity binding constant used was 0.275 for a 1% (w/v) koala albumin solution. These constants were approximately in the middle of the range of published values for marsupials (Bradley *et al.* 1976, Sernia *et al.* 1979, Bradley 1982).

Statistical analysis

Baseline concentrations for the control and ACTH treatments were defined as the concentrations at 0 h (T0) of the first morning before injection of saline or depot ACTH. Where necessary, data were logarithmically transformed to satisfy the assumption of normal distribution and homogeneity of variances for the parametric tests. A two-way repeated-measures ANOVA was used to examine the effects of collection time and treatments (ACTH and control) for both plasma (including the partitioning of cortisol into plasma compartments (free, CBG bound and albumin bound)) and faecal samples (Lèche *et al.* 2011, Legagneux *et al.* 2011, Fanson *et al.* 2012). Treatment (ACTH and control) was the main factor and time was the repeated measure. Dunnett's multiple comparison tests

were performed for *post hoc* analysis to test for differences between baseline (T0) and all other times (Fanson *et al.* 2012). The concentrations of saline-injected controls provided a relative baseline for comparisons with the ACTH treatment. *t*-Tests were used to examine whether the concentrations for ACTH treatment were higher than those for the control treatment for both plasma and faecal samples after injection (i.e. all times after T0).

Results

Plasma GC levels

There was a significant relationship between treatment (ACTH or control) and time of sample collection for plasma cortisol levels. The effect of the treatment varied with collection times (two-way repeated-measures ANOVA: $P \leq 0.0001$, $F_{3, 21} = 12.93$). *Post hoc* tests indicated that for the ACTH treatment, plasma cortisol levels measured after the challenge (12, 24 and 36 h) were significantly higher compared with the pre-injection levels (T0), while for the control treatment, there was no significant difference between the pre-injection and post-injection levels (Fig. 1).

Faecal GC metabolite levels

There was a significant relationship between treatment (ACTH or control) and time of sample collection for faecal GC metabolite levels (two-way repeated-measures

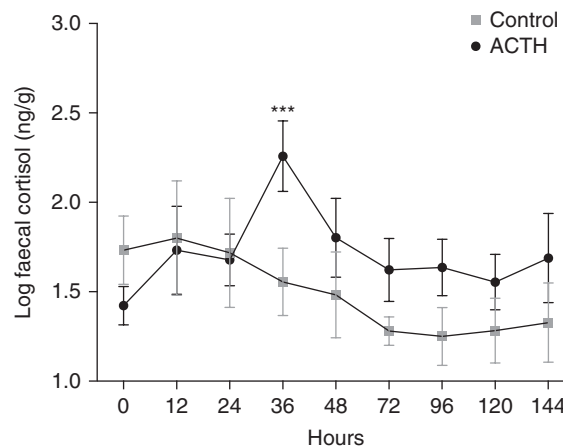


Figure 2

Mean (\pm s.e.m.) faecal cortisol concentrations for the control and ACTH treatments at each collection time. *Post hoc* tests indicate a significant difference between samples collected at 36 h and those collected at 0 h (***signifies $P < 0.01$) for the ACTH treatment and no difference between 0 h and all other collection times for the control treatment.

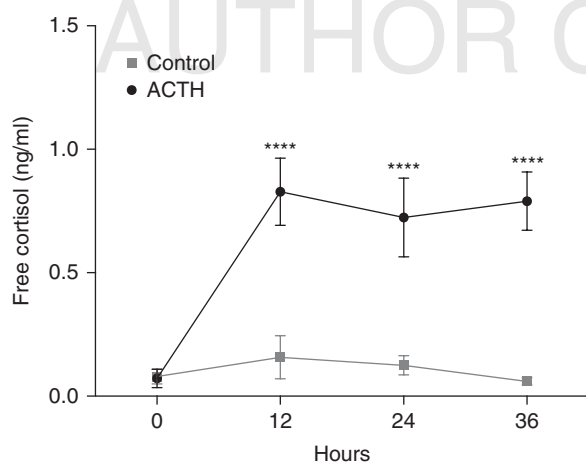


Figure 3

The difference in the mean (\pm s.e.m.) concentration of free cortisol between the control and ACTH treatments over time. It highlights the increase in free cortisol concentrations after injection (T0) for the ACTH treatment compared with no difference in free cortisol concentrations after injection for the control treatment. ****signifies $P < 0.001$.

ANOVA: $P = 0.0161$, $F_{8, 56} = 2.630$). *Post hoc* tests indicated that for the ACTH treatment, faecal cortisol levels that reached a peak 36 h after the challenge were significantly higher compared with the pre-injection levels (T0), while for the control treatment, there was no significant difference between the pre-injection and post-injection levels (Fig. 2). Furthermore, there was a significant difference between the ACTH and control treatments for all faecal cortisol concentrations measured after injection (i.e. after T0; *t*-test: $P = 0.0091$, $t = 2.684$, $df = 70$; Fig. 2).

After the assessment of the effect of enzymatic cleavage of glucuronide and sulphate polar bonds using β -glucuronidase, it was found that faecal samples extracted without and with enzymatic pre-treatment had faecal cortisol concentrations of 17.50 ± 5.13 and 223.50 ± 18.20 ng/g (mean \pm s.d.; *t*-test: $P < 0.00$, $t = 30.82$, $df = 14$).

Steroid partitioning in plasma

The mean values for free cortisol, CBG-bound and albumin-bound concentrations between the treatments highlight a significant difference in the ACTH treatment compared with the control treatment (two-way repeated-measures ANOVA: free: $P = 0.0011$, $F_{3, 21} = 7.865$; CBG bound: $P = 0.0001$, $F_{3, 21} = 11.41$ and albumin bound: $P = 0.0002$, $F_{3, 21} = 10.68$; Figs 3, 4 and 5 respectively). In all the tests, the mean values in the *post hoc* tests indicated a significant increase from the pre-injection (T0) and post-injection collection times for the ACTH treatment

compared with control treatment, for which the values did not increase after injection (Figs 3, 4 and 5).

The mean values for cortisol partitioning (free, CBG bound and albumin bound) were converted to percentages of the total excreted cortisol for each treatment (control and ACTH) (Fig. 6). There was a significant interaction between treatments (ACTH and control) and the partitioning of cortisol (free, CBG-bound and albumin-bound cortisol) (two-way repeated-measures ANOVA: $P \leq 0.0001$, $F_{2, 102} = 17.54$). *Post hoc* tests indicated that when compared with those during the control treatment, the percentage of albumin-bound cortisol increased and that of CBG-bound cortisol decreased during the ACTH treatment, while that of overall free cortisol increased marginally but not significantly between the treatments (Fig. 6).

Discussion

This study shows that the elevation of plasma cortisol concentrations in koalas is reflected in the cortisol/cortisol metabolites of faecal pellets. The intestinal passage causes a time delay between the circulation of steroids in plasma and their appearance in faeces, and this delay in faecal GC excretion is correlated with gut passage time (confluence of the bile fluid to the rectum; Schwarzenberger *et al.* 1997, Mostl *et al.* 2005, Palme *et al.* 2005). This time delay can range from 30 min to more than 1 day depending on the species and activity of the animal, which can affect the rate

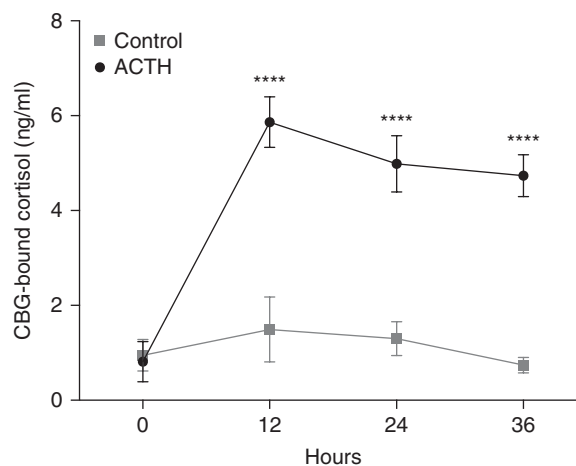


Figure 4

The difference in the mean (\pm s.e.m.) concentration of CBG-bound cortisol between the control and ACTH treatments over time. It highlights the increase in CBG-bound cortisol concentrations after injection (T0) for the ACTH treatment compared with no difference in CBG-bound cortisol concentrations after injection for the control treatment. ****signifies $P < 0.001$.

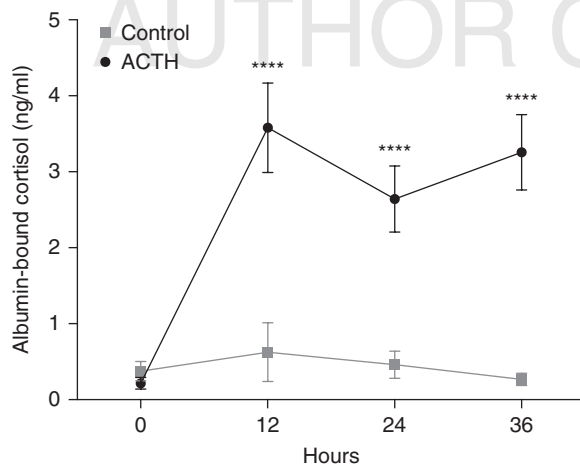


Figure 5

The difference in the mean (\pm s.e.m.) concentration of albumin-bound cortisol between the control and ACTH treatments over time. It highlights the increase in albumin-bound cortisol concentrations after injection (T0) for the ACTH treatment compared with no difference in albumin-bound cortisol concentrations after injection for the control treatment. ****signifies $P < 0.001$.

of gut passage (Young *et al.* 2004, Palme *et al.* 2005, Thiel *et al.* 2005, Touma & Palme 2005, Wasser & Hunt 2005). The 36-h delay between the elevation of plasma cortisol concentrations and the appearance of cortisol and its metabolites in the faecal pellets is similar to the findings reported for some vertebrates, but longer than that reported by other studies (Table 1). This is probably a result of the slow gut passage time of koalas. The mean retention time for particle markers for the koala has been recorded between 39 h (Krockenberger & Hume 2007) and 100 h (Cork & Warner 1983), while the mean retention time for solute markers has been recorded between 110 h (Krockenberger & Hume 2007) and 213 h (Cork & Warner 1983). The time delay of 36 h in the appearance of cortisol metabolites in faecal pellets is close to the average mean retention time of particle markers of 39 h reported by Krockenberger & Hume (2007). While the results of this study show a pattern of steroid excretion that is similar to that reported for several eutherian species, a study by Ellis (1997) using injected ^{14}C -cortisol in koalas showed that while gut passage time is slow in koalas, some individuals did show an elevation of faecal cortisol levels as early as 20 h after injection. This timing is similar to the appearance of ^{14}C -cortisol in urine (Ellis 1997), and it may be related to an obligate release of some urine into the cloaca during defaecation, resulting in the contamination of pellets with a small quantity of urine. In females, the urethral opening is deep within the cloaca, which would allow the faecal pellets more contact with any residual or

freshly voided urine than is the case with males. Although only one female was included in the study, it is interesting to note that the faecal cortisol concentration detected in pellets from the female was higher than that detected for males at all collection times after 24 h. Consistently, Narayan *et al.* (2013) found sex-related differences in faecal cortisol metabolite (FCM) levels in captive koalas. The percentage rise in FCM levels of the males and females after the ACTH challenge was 25.53 and 71.79% respectively. These observations suggest that future studies of faecal steroid metabolite excretion in the koala, and indeed in all marsupials, should consider this possible sex difference. However, the faecal pellet collectors in our study ensured that only freshly deposited pellets that were not near any area containing voided urine were collected for later analysis. Urine contamination is still feasible (the pellets may always have a trace of urine on them from the cloaca), but all possible measures were taken to reduce its impact and this contamination is unlikely to have confounded our results.

In this study, following i.m. injection of ACTH, plasma cortisol concentrations were elevated at about 36 h, a delay expected with the administration of ACTH via this route. A recent study by Narayan *et al.* (2013) investigating non-invasive stress assessment methods in koalas has reported lower ACTH-stimulated plasma cortisol concentrations than those found in our study. This difference may be due to the use of the non-depot form of Synacthen by Narayan *et al.* (2013) rather than the long-acting Synacthen Depot that we employed. It is also interesting to note that FCM concentrations reported by Narayan *et al.* (2013) following ACTH stimulation were considerably lower than those that we found. While Narayan *et al.* (2013) used an assay for FCMs that employed a polyclonal antibody, they did not use enzymatic cleavage using glucuronidase/sulphatase prior to the extraction. Our study employed enzymatic cleavage prior to steroid extraction, which should have liberated

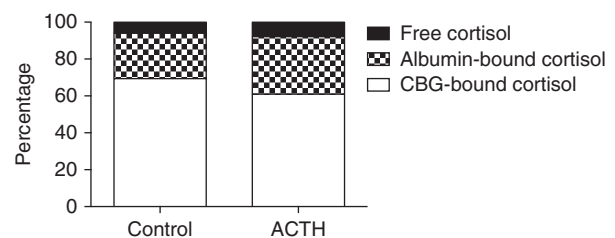


Figure 6

The mean (\pm s.e.m.) percentage of free and bound (CBG and albumin) cortisol for each treatment (ACTH and control).

Table 1 Comparison of faecal cortisol concentrations and different delay times of cortisol metabolite appearance in faeces for different species reported in the literature

Species	Faecal cortisol (basal/control/ pre-treatment)	Faecal cortisol (ACTH/peak/ post-treatment)	Delay time (h)	References
Columbian ground squirrels (<i>Spermophilus columbianus</i>)	263.03 ± 61.10 ng/g	416.87 ± 57.78 ng/g	7 ± 0.53	Bosson <i>et al.</i> (2009)
Snowshoe hares (<i>Lepus americanus</i>)	1054 ± 151 ng/g	11 130 ± 3317 ng/g	10	Sheriff <i>et al.</i> (2009)
African elephants (<i>Loxodonta africana</i>)	21–168 nmol/kg (median: 48)	–	20–25.5	Stead <i>et al.</i> (2000)
Spotted hyenas (<i>Crocuta crocuta</i>)	–	–	26 ± 5	Goymann <i>et al.</i> (1999)
	Male: 50.2 ± 20.4 ng/g Female: 37.3 ± 13.7 ng/g	Male: 576.0 ng/g Female: 913.9 ng/g	Male: 16 Female: 32	Benhaïem <i>et al.</i> (2012)
African wild dogs (<i>Lycaon pictus</i>)	–	1200–8000 ng/g	24–30	Monfort <i>et al.</i> (1998)
Himalayan black bear (<i>Ursus thibetanus</i>)	107.9 ± 8.0 ng/g	432.6 ng/g	24	Young <i>et al.</i> (2004)
Koala (<i>Phascolarctos cinereus</i>)	29.74 ± 6.99 ng/g	261 ± 97.93 ng/g	36	This study
Cheetah (<i>Acinonyx jubatus</i>)	628.4 ± 95.3 ng/g	7193.2 ng/g	24–48	Young <i>et al.</i> (2004)
Black-footed ferret (<i>Mustela nigripes</i>)	220.2 ± 19.3 ng/g	548.6 ng/g	24–48	Young <i>et al.</i> (2004)

free (unconjugated) cortisol, making this available for solvent extraction followed by ELISA known to specifically target cortisol.

Quite clearly, as mentioned by Touma & Palme (2005) and acknowledged by Narayan *et al.* (2013), antibodies used in some assays might not be ideal because they are produced primarily to measure concentrations of the respective unmetabolised steroid in plasma. To fully answer this question, an exhaustive HPLC study would be required to identify the primary FCMs in koala faeces and to ensure that appropriate antibodies are used to target the FCMs present at the highest concentration. We note that many studies of faecal GC excretion have not included the enzymatic cleavage procedural step to account for the fate of a significant proportion of the free cortisol conjugated by hepatocytes and entering the small intestine in bile secretions. Prior to the extraction of cortisol from faecal pellets, we suggest that it is important to treat samples with glucuronidase, which cleaves polar bonds and liberates extractable unconjugated cortisol. This is important because paired assays using a common pool of faecal extracts with or without prior enzymatic cleavage reveal considerably higher cortisol concentrations in the former compared with the latter. It is likely that FCMs of different molecular structures may not interact equally with the antibody provided with ELISA kits or RIAs designed to sensitively measure cortisol concentrations. In his study, Ellis (1997) employed solid-phase trapping (Sep Pak C18 solid-phase cartridges) in conjunction with glucuronidase cleavage of polar cortisol metabolites to extract cortisol. Faecal ¹⁴C-cortisol/cortisol metabolites increased 24 h after injection, and he suggested that the analysis of faecal cortisol/cortisol

metabolites would be a very useful method to be employed for the assessment of stress in koalas.

We suggest that it is prudent to use enzymatic cleavage prior to solvent partitioning or solid-phase extraction of the target deconjugated steroid. For these reasons, we are confident that our assay system measured the concentrations of extracted cortisol.

The plasma cortisol concentrations found in this study confirm the finding of generally low plasma concentrations of this steroid in other studies of koalas (Weiss & Richards 1970, Scoggins 1978, McDonald *et al.* 1990, Ellis 1997) and relatively low concentrations compared with other mammals (e.g. koala: basal 1.101 ± 0.53 ng/ml, ACTH 9.133 ± 0.61 ng/ml (this study); koala: 2.86–7.17 ng/ml (range) (Hajduk *et al.* 1992); koala: 0.0002–0.18 ng/ml (range) (Ellis 1997); fruit bats: *Pteropus hypomelanus* 1269 ± 207 ng/ml (basal) and *P. vampyrus* 596 ± 67 ng/ml (basal) (Windmaier & Kunz 1993); Steller sea lion (*Eumetopias jubatus*): Russia 151.5 ± 3.2 ng/ml, Southeast Alaska 152.7 ± 2.7 ng/ml and Southwest Alaska 134.9 ± 2 ng/ml (Myers *et al.* 2010); and Tasmanian devil (*Sarcophilus harrisii*): male 49 ± 9.19 ng/ml (capture) and female 74.0 ± 3.24 ng/ml (capture) (Jones *et al.* 2005)).

Most studies have not extended the treatment of GC excretion by calculating the partitioning of the principal adrenocortical hormone into the three plasma compartments: free, CBG bound and albumin bound (Bradley *et al.* 1980, Bradley 2003, Breuner *et al.* 2013). This study has shown that plasma free cortisol concentrations are increased significantly in response to ACTH administration and that the extent of this increase is not adequately described by just measuring plasma total cortisol concentrations (Breuner *et al.* 2013). Sheriff *et al.* (2010), working

with snowshoe hares, reported that plasma free cortisol levels mirrored bile and FCM levels, but plasma total cortisol levels did not. Since it is the free hormone fraction that is removed by the hepatocytes during the passage of blood through the hepatic microcirculation, the free cortisol is clearly most relevant to the appearance of conjugated steroids in both urine and faeces.

While the plasma cortisol concentrations that occur in the koala are at the low end of the spectrum reported for other mammals, steroid partitioning within the blood plasma reveals a pattern that is quite similar to that of other mammals with most of the steroid being tightly bound to CBG and about 30% being bound more loosely to albumin, while about 10% appears as free or unbound hormone. However, it is clear that following ACTH stimulation, the relative sizes of the free and albumin compartments increase to a greater extent than does that of the CBG compartment. McDonald *et al.* (1990) described high-affinity binding between cortisol and CBG in koala plasma; however, they appear to have omitted an exponent from the stated affinity constant. Based upon the discussion in their book chapter in which a binding affinity that is much lower for the koala than for other marsupials is mentioned, we assume that the affinity constant stated should have been $0.90 \times 10^7 / \text{M}^{-1}$. If this is correct, then our chosen mid-range affinity constant of $3.5 \times 10^7 / \text{M}^{-1}$ would be too high and as a consequence the partitioning of cortisol within the blood plasma for koalas in our study would have resulted in much higher free and albumin-bound cortisol values than those appearing in our graphs. Clearly, dialysis experiments using koala plasma should be repeated to resolve this uncertainty.

It is apparent that there is a roughly tenfold increase in both plasma total and free cortisol concentrations from 12 to 36 h after ACTH injection. FCM levels increase significantly by 36 h after ACTH injection; however, the increase in FCM levels from time 0 is much smaller than changes in plasma cortisol concentrations, although FCM levels do remain elevated above FCM levels for controls for several days. This difference exists because hepatic clearance and excretion of polar GC metabolites in mammals via the renal route usually account for around 70% of all GC metabolites, excreted as β -glucuronides and sulphates (cortisol metabolites, CMLs), while the remaining 30% is excreted as polar conjugates in bile into the upper gastrointestinal tract (GIT). For this reason, FCMs appearing in the faecal pellets should represent only a small part of the total cortisol metabolites excreted. The FCMs are also, understandably, lower in concentration than the plasma cortisol.

At the commencement of the experiment, the FCM levels for the controls were higher than those for the ACTH-treated group. This observation, together with the gradual decrease in FCM levels for the controls during the experiment, is consistent with some of the control animals being exposed to some form of stressor 1 or 2 days prior to the commencement of the experiment. The FCM concentrations detected in this study following ACTH administration were considerably higher than those reported by Narayan *et al.* (2013), and this may, in part, be related to our use of the depot form of ACTH, which when administered via the i.m. route in other mammals is known to cause a significant elevation of plasma cortisol concentrations for 24–36 h (Novartis Pharmaceuticals Australia Pty Ltd.) after injection.

Passage time through the GIT of the koala is relatively slow (Krockenberger & Hume 2007), and this may allow time for some bacterial degradation of the CMLs to occur, resulting in the levels of extractable FCMs from faecal pellets being lower than might otherwise be found. When this FCM analysis is being considered as a stress assessment tool, it is important to note that 36 to 144 h after ACTH injection, there is a consistent elevation of FCM levels in faecal pellets.

We conclude that the results of the ACTH challenge in rehabilitating wild koalas validate this non-invasive technique for the monitoring of adrenocortical activity in free-ranging wild koalas. This study also validates the use of faecal cortisol analysis for the assessment of the activity of the hypothalamo–pituitary–adrenocortical axis of koalas using freshly collected koala faecal pellets.

Future studies could implement this non-invasive faecal sampling method to assess the physiological stress of koalas in wild populations that differ in exposure to various environmental and physiological (e.g. disease or injury) stressors. Further assessment should be made by suppressing endogenous GC concentrations using dexamethasone and examining the plasma and CML response to exposure of koalas to a predator such as a dog (Sheriff *et al.* 2009). This technique could also be extended to evaluate the impact of climate change, which is contributing to population declines, and how environmental stressors may affect the survival and reproductive success of koala populations throughout Australia.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by the Australian Koala Foundation, South West NRM, and the Australian Research Council (ARC Linkage Project 0882090).

Author contribution statement

N D and A B contributed to the design of the study, sample analysis, statistical analysis and interpretation and manuscript drafting. A G performed the ACTH challenge on the animals. A G, L S, G B, D L and C M participated in the conception and design of the study and helped to draft the manuscript. All authors read and approved the final manuscript.

Acknowledgements

The authors gratefully acknowledge the generous financial support provided by the Australian Koala Foundation, Australian Research Council (ARC Linkage Project 0882090) and South West NRM. They are extremely grateful for the assistance provided by all of the Australia Zoo Wildlife Hospital staff and volunteers while running the experiment. They also thank Jonathan Rhodes for his input. The approved protocol for this project was obtained from the Animal Ethics Committee at The University of Queensland (GPA/603/08/ARC) and the Environmental Protection Agency (now the Department of Environment and Resource Management) (Scientific Purposes Permit WISP10160211).

References

- Axelrod J & Reisine TD 1984 Stress hormones: their interaction and regulation. *Science* **224** 452–459. (doi:10.1126/science.6143403)
- Beerda B, Schilder MB, Janssen NS & Mol JA 1996 The use of saliva cortisol, urinary cortisol, and catecholamine measurements for a noninvasive assessment of stress responses in dogs. *Hormones and Behavior* **30** 272–279. (doi:10.1006/hbeh.1996.0033)
- Benhaïem S, Dehnhard M, Bonanni R, Hofer H, Goymann W, Eulenberger K & East ML 2012 Validation of an enzyme immunoassay for the measurement of faecal glucocorticoid metabolites in spotted hyenas (*Crocuta crocuta*). *General and Comparative Endocrinology* **178** 265–271. (doi:10.1016/j.ygcen.2012.05.006)
- Boonstra R & Singleton GR 1993 Population declines in the snowshoe hare and the role of stress. *General and Comparative Endocrinology* **91** 126–143. (doi:10.1006/gcen.1993.1113)
- Bosson CO, Palme R & Boonstra R 2009 Assessment of the stress response in columbian ground squirrels: laboratory and field validation of an enzyme immunoassay for fecal cortisol metabolites. *Physiological and Biochemical Zoology* **82** 291–301. (doi:10.1086/597530)
- Bradley AJ 1982 Steroid binding proteins in the plasma of dasyurid marsupials. In *Carnivorous Marsupials*, pp 651–657. Ed. M Archer. Sydney, Australia: Surrey Beatty and Sons.
- Bradley AJ 1990 Failure of glucocorticoid feedback during breeding in the male red-tailed phascogale, *Phascogale calura* (Marsupialia: Dasyuridae). *Journal of Steroid Biochemistry and Molecular Biology* **37** 155–163. (doi:10.1016/0960-0760(90)90384-W)
- Bradley AJ 2003 Stress, hormones and mortality in small dasyurid marsupials. In *In Predators with Pouches: The Biology of Carnivorous Marsupials*, pp 254–267. Eds M Jones, C Dickman & M Archer. Sydney, Australia: CSIRO Press.
- Bradley AJ, McDonald IR & Lee AK 1976 Corticosteroid binding globulin and mortality in a dasyurid marsupial. *Journal of Endocrinology* **70** 323–324. (doi:10.1677/joe.0.0700323)
- Bradley AJ, McDonald IR & Lee AK 1980 Stress and mortality in a small marsupial (*Antechinus stuartii*, Macleay). *General and Comparative Endocrinology* **40** 188–200. (doi:10.1016/0016-6480(80)90122-7)
- Breuner CW, Delehanty B & Boonstra R 2013 Evaluating stress in natural populations of vertebrates: total CORT is not good enough. *Functional Ecology* **27** 24–36. (doi:10.1111/1365-2435.12016)
- Chu FW & Ekins RP 1988 Detection of corticosteroid binding globulin in parotid fluids – evidence for the presence of both protein-bound and non-protein-bound (free) steroids in uncontaminated saliva. *Acta Endocrinologica* **119** 56–60. (doi:10.1530/acta.0.1190056)
- Cork S & Warner ACI 1983 The passage of digesta markers through the gut of a folivorous marsupial, the koala (*Phascolarctos cinereus*). *Journal of Comparative Physiology B* **152** 43–52. (doi:10.1007/BF00689726)
- Creel S, Fox JE, Hardy A, Sands J, Garrott B & Peterson RO 2002 Snowmobile activity and glucocorticoid stress responses in wolves and elk. *Conservation Biology* **16** 809–814. (doi:10.1046/j.1523-1739.2002.00554.x)
- Davenport MD, Tiefenbacher S, Lutz CK, Novak MA & Meyer JS 2006 Analysis of endogenous cortisol concentrations in the hair of rhesus macaques. *General and Comparative Endocrinology* **147** 255–261. (doi:10.1016/j.ygcen.2006.01.005)
- Ellis WAH 1997 Stressors, prevalence of disease and cortisol concentrations in koalas (*Phascolarctos cinereus*). *PhD Thesis*. School of Biological Sciences, The University of Queensland, St Lucia.
- Fanson KV, Wielebnowski NC, Shenk TM & Lucas JR 2012 Comparative patterns of adrenal activity in captive and wild Canada lynx (*Lynx canadensis*). *Journal of Comparative Physiology. B, Biochemical, Systemic, and Environmental Physiology* **182** 157–165. (doi:10.1007/s00360-011-0597-8)
- Goymann W, Möstl E, Van't Hof T, East ML & Hofer H 1999 Noninvasive fecal monitoring of glucocorticoids in spotted hyenas, *Crocuta crocuta*. *General and Comparative Endocrinology* **114** 340–348. (doi:10.1006/gcen.1999.7268)
- Hajduk P, Copland MD & Schultz DA 1992 Effects of capture on haematological values and plasma cortisol levels of free-range koalas (*Phascolarctos cinereus*). *Journal of Wildlife Diseases* **28** 502–506.
- Harper JM & Austad SN 2000 Fecal glucocorticoids: a noninvasive method of measuring adrenal activity in wild and captive rodents. *Physiological and Biochemical Zoology* **73** 12–22. (doi:10.1086/316721)
- Harper JM & Austad SN 2001 Effect of capture and season on fecal glucocorticoid levels in deer mice (*Peromyscus maniculatus*) and red-backed voles (*Clethrionomys gapperi*). *General and Comparative Endocrinology* **123** 337–344. (doi:10.1006/gcen.2001.7682)
- Hiramatsu R 1983 Determination of cortisol concentration in human erythrocytes. *Clinica Chimica Acta* **134** 143–153. (doi:10.1016/0009-8981(83)90192-4)
- Jones SM, Lockhart TJ & Rose RW 2005 Adaptation of wild-caught Tasmanian devils (*Sarcophilus harrisii*) to captivity: evidence from physical parameters and plasma cortisol concentrations. *Australian Journal of Zoology* **53** 339–344. (doi:10.1071/ZO05043)
- Krockenberger AK & Hume ID 2007 A flexible digestive strategy accommodates the nutritional demands of reproduction in a free-living folivore, the koala (*Phascolarctos cinereus*). *Functional Ecology* **21** 748–756. (doi:10.1111/j.1365-2435.2007.01279.x)
- Lane J 2006 Can non-invasive glucocorticoid measures be used as reliable indicators of stress in animals? *Animal Welfare* **15** 331–342.
- Lêche A, Busso JM, Navarro JL, Hansen C, Marin RH & Martella MB 2011 Non-invasive monitoring of adrenocortical activity in greater Rhea (*Rhea americana*) by measuring fecal glucocorticoid metabolites. *Journal of Ornithology* **152** 839–847. (doi:10.1007/s10336-011-0661-x)
- Lee AK & Cockburn A 1985 In *Evolutionary Ecology of Marsupials*. Cambridge, UK: Cambridge University Press.
- Legagneux P, Gauthier G, Chastel O, Picard G & Bety J 2011 Do glucocorticoids in droppings reflect baseline level in birds captured in the wild? A case study in snow geese *General and Comparative Endocrinology* **172** 440–445. (doi:10.1016/j.ygcen.2011.04.009)
- Mateo JM 2006 Developmental and geographic variation in stress hormones in wild Belding's ground squirrels (*Spermophilus beldingi*). *Hormones and Behavior* **50** 718–725. (doi:10.1016/j.yhbeh.2006.05.008)

- McDonald IR, Than KA, Handasyde KA & Michaelides J 1990 Factors affecting plasma adrenocortical hormone concentrations of koalas. In *In Biology of the Koala*, pp 289–294. Eds AK Lee, KA Handasyde & GD Sanson. Sydney, Australia: Surrey Beatty and Sons.
- Mendel CM 1989 The free hormone hypothesis – a physiologically based mathematical model. *Endocrine Reviews* **10** 232–274. (doi:10.1210/edrv-10-3-232)
- Millsbaugh JJ & Washburn BE 2003 Within-sample variation of fecal glucocorticoid measurements. *General and Comparative Endocrinology* **132** 21–26. (doi:10.1016/S0016-6480(03)00061-3)
- Millsbaugh JJ & Washburn BE 2004 Use of fecal glucocorticoid metabolite measures in conservation biology research: considerations for application and interpretation. *General and Comparative Endocrinology* **138** 189–199. (doi:10.1016/j.ygcen.2004.07.002)
- Monfort SL, Mashburn KL, Brewer BA & Creel SR 1998 Evaluating adrenal activity in African wild dogs (*Lycan pictus*) by fecal corticosteroid analysis. *Journal of Zoo and Wildlife Medicine* **29** 129–133.
- Mostl E & Palme R 2002 Hormones as indicators of stress. *Domestic Animal Endocrinology* **23** 67–74. (doi:10.1016/S0739-7240(02)00146-7)
- Mostl E, Messmann S, Bagu E, Robia C & Palme R 1999 Measurement of glucocorticoid metabolite concentrations in faeces of domestic livestock. *Journal of Veterinary Medicine A* **46** 621–632. (doi:10.1046/j.1439-0442.1999.00256.x)
- Mostl E, Rettenbacher S & Palme R 2005 Measurement of corticosterone metabolites in birds' droppings: an analytical approach. *Annals of the New York Academy of Sciences* **1046** 17–34. (doi:10.1196/annals.1343.004)
- Munck A, Guyre PM & Holbrook NJ 1984 Physiological functions of glucocorticoids in stress and their relation to pharmacological actions. *Endocrine Reviews* **5** 25–44. (doi:10.1210/edrv-5-1-25)
- Myers MJ, Litz B & Atkinson S 2010 The effects of age, sex, season and geographic region on circulating serum cortisol concentrations in threatened and endangered Steller sea lions (*Eumetopias jubatus*). *General and Comparative Endocrinology* **165** 72–77. (doi:10.1016/j.ygcen.2009.06.006)
- Narayan EJ, Webster K, Nicolson V, Mucci A & Hero J-M 2013 Non-invasive evaluation of physiological stress in an iconic Australian marsupial: the koala (*Phascolarctos cinereus*). *General and Comparative Endocrinology* **187** 39–47. (doi:10.1016/j.ygcen.2013.03.021)
- Palme R 2005 Measuring faecal steroids: guidelines for practical application. *Annals of the New York Academy of Sciences* **1046** 75–80. (doi:10.1196/annals.1343.007)
- Palme R, Robia C, Messmann S, Hofer J & Mostl E 1999 Measurement of fecal cortisol metabolites in ruminants: a non-invasive parameter of adrenocortical function. *Wiener Tierärztliche Monatsschrift* **86** 237–241.
- Palme R, Rettenbacher S, Touma C, El-Bahr S & Mostl E 2005 Stress hormones in mammals and birds: comparative aspects regarding metabolism, excretion, and noninvasive measurement in faecal samples. *Trends in Comparative Endocrinology and Neurobiology* **1040** 162–171.
- Paterson JY & Hills F 1967 Binding of cortisol by ovine plasma proteins. *Journal of Endocrinology* **37** 261. (doi:10.1677/joe.0.0370261)
- Rehnus M, Hacklander K & Palme R 2009 A non-invasive method for measuring glucocorticoid metabolites (GCM) in Mountain hares (*Lepus timidus*). *European Journal of Wildlife Research* **55** 615–620. (doi:10.1007/s10344-009-0297-9)
- Romero LM 2004 Physiological stress in ecology: lessons from biomedical research. *Trends in Ecology & Evolution* **19** 249–255. (doi:10.1016/j.tree.2004.03.008)
- Sapolsky RM 1982 The endocrine stress-response and social status in the wild baboon. *Hormones and Behavior* **16** 279–292. (doi:10.1016/0018-506X(82)90027-7)
- Sapolsky RM 2002 Endocrinology of the stress response. In *Behavioural Ecology*, pp 409–450. Eds JB Becker, SM Breedlove, D Crews & MM McCarthy. Cambridge, MA, USA: The MIT Press.
- Sapolsky RM, Romero LM & Munck AU 2000 How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions *Endocrine Reviews* **21** 55–89. (doi:10.1210/er.21.1.55)
- Schwarzenberger F, Palme R, Bamberg E & Mostl E 1997 A review of faecal progesterone metabolite analysis for non-invasive monitoring of reproductive function in mammals. *Journal of Mammalian Biology* **62** 214–221.
- Scoggins BA 1978 Adrenal steroid production by the koala. In *The Koala*, pp 198–199. Ed. TJ Bergin. Artarmon, New South Wales, Australia: John Sands Pty Ltd.
- Sernia C, Bradley AJ & McDonald IR 1979 High affinity binding of adrenocortical and gonadal steroids by plasma proteins of Australian marsupials. *General and Comparative Endocrinology* **38** 496–503. (doi:10.1016/0016-6480(79)90159-X)
- Seyle H 1935 A syndrome produced by diverse nocuous agents. *Nature* **138** 32–33. (doi:10.1038/138032a0)
- Sheriff MJ, Bosson CO, Krebs CJ & Boonstra R 2009 A non-invasive technique for measuring fecal cortisol metabolites in snowshoe hares (*Lepus americanus*). *Journal of Comparative Physiology. B, Biochemical, Systemic, and Environmental Physiology* **179** 305–313. (doi:10.1007/s00360-008-0314-4)
- Sheriff MJ, Krebs CJ & Boonstra R 2010 Assessing stress in animal populations. Do fecal and plasma glucocorticoids tell the same story? *General and Comparative Endocrinology* **166** 614–619. (doi:10.1016/j.ygcen.2009.12.017)
- Smith JB, Nolan G & Jubiz W 1980 The relationship between unbound and total cortisol: its usefulness in detecting CBG abnormalities. *Clinica Chimica Acta* **108** 435–445. (doi:10.1016/0009-8981(80)90351-4)
- Stead SK, Meltzer DGA & Palme R 2000 The measurement of glucocorticoid concentrations in the serum and faeces of captive African elephants (*Loxodonta africana*) after ACTH stimulation. *Journal of the South African Veterinary Association* **71** 192–196. (doi:10.4102/jsava.v71i3.712)
- Stewart PM 2003 The adrenal cortex. In *Williams Textbook of Endocrinology*, pp 491–551. Eds PR Larsen, HM Kronenberg, S Melmed & KS Polonsky. Philadelphia, PA, USA: Saunders.
- Tait JF & Burstein S 1964 *In vivo* studies of steroid dynamics. In *The Hormones*, pp 441–557. Eds G Pincus, K Thimann & EB Astwood. New York, NY, USA: Academic Press.
- Thiel D, Jenni-Eiermann S, Braunisch V, Palme R & Jenni L 2008 Ski tourism affects habitat use and evokes a physiological stress response in Capercaillie *Tetrao urogallus*: a new methodological approach. *Journal of Applied Ecology* **45** 845–853. (doi:10.1111/j.1365-2664.2008.01465.x)
- Thiel DS, Jenni-Eiermann S & Palme R 2005 Measuring corticosterone metabolites in droppings of Capercaillies (*Tetrao urogallus*). *Annals of the New York Academy of Sciences* **1046** 96–108. (doi:10.1196/annals.1343.009)
- Touma C & Palme R 2005 Measuring faecal glucocorticoid metabolites in mammals and birds: the importance of validation. *Annals of the New York Academy of Sciences* **1046** 54–74. (doi:10.1196/annals.1343.006)
- Touma C, Sachser N, Mostl E & Palme R 2003 Effects of sex and time of day on metabolism and excretion of corticosterone in urine and feces of mice. *General and Comparative Endocrinology* **130** 267–278. (doi:10.1016/S0016-6480(02)00620-2)
- Wasser SK & Hunt KE 2005 Noninvasive measures of reproductive function and disturbance in the barred owl, great horned owl, and northern spotted owl. *Annals of the New York Academy of Sciences* **1046** 109–137. (doi:10.1196/annals.1343.010)
- Wasser SK, Bevis K, King G & Hanson E 1997 Noninvasive physiological measures of disturbance in the northern spotted owl. *Conservation Biology* **11** 1019–1022. (doi:10.1046/j.1523-1739.1997.96240.x)
- Weiss M & Richards PG 1970 Adrenal steroid secretion in koala (*Phascolarctos cinereus*). *Journal of Endocrinology* **48** 145. (doi:10.1677/joe.0.0480145)

- Willcox DL, Yovich JL, McColm SC & Phillips JM 1985 Progesterone, cortisol and oestradiol-17 β in the initiation of human parturition: partitioning between free and bound hormone in plasma. *British Journal of Obstetrics and Gynaecology* **92** 65–71. (doi:10.1111/j.1471-0528.1985.tb01050.x)
- Windmaier EP & Kunz TH 1993 Basal, diurnal, and stress-induced levels of glucose and glucocorticoids in captive bats. *Journal of Experimental Zoology* **265** 533–540. (doi:10.1002/jez.1402650509)
- Wingfield JC & Farner DS 1993 Endocrinology of reproduction in wild species. In *Avian Biology*, pp 163–327. Eds DS Farner, JR King & KC Parkes. New York, NY, USA: Academic Press.
- Wingfield JC, Vleck CM & Moore MC 1992 Seasonal changes of the adrenocortical response to stress in birds of the Sonoran desert. *Journal of Experimental Zoology* **264** 419–428. (doi:10.1002/jez.1402640407)
- Wingfield JC, Maney DL, Breuner CW, Jacobs JD, Lynn S, Ramenofsky M & Richardson RD 1998 Ecological bases of hormone–behavior interactions: the “emergency life history stage”. *American Zoologist* **38** 191–206. (doi:10.1093/icb/38.1.191)
- Young KM, Walker SL, Lantheir C, Waddell WT, Monfort SL & Brown JL 2004 Noninvasive monitoring of adrenocortical activity in carnivores by fecal glucocorticoid analyses. *General and Comparative Endocrinology* **137** 148–165. (doi:10.1016/j.ygcen.2004.02.016)

Received in final form 20 June 2013

Accepted 5 July 2013

Accepted Preprint published online 9 July 2013