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Non-invasive evaluation of physiological stress in an iconic Australian marsupial: the Koala (*Phascolarctos cinereus*)

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Abstract

Koalas (*Phascolarctos cinereus*) are the only extant representatives of Australia's unique marsupial family Phascolarctidae and were listed as nationally Vulnerable in 2012. Causes of mortality are diverse, although the disease chlamydiosis, dog attacks, collisions with cars, and loss of habitat represent the principal reasons for the continued species decline. Koala breeding facilities in Queensland and New South Wales, Australia have been established for conservation and tourism. Non-invasive monitoring of physiological stress is important for determining the sub-lethal effects of environmental stressors on the well-being, reproduction and survival of Koalas in Zoos and also in the wild. In this study, we developed a faecal cortisol metabolite (FCM) enzyme-immunoassay (EIA) for monitoring physiological stress in Koalas from two established Zoos in Australia and also within a free-living sub-population from Queensland. Biological validation of the FCM EIA was done using an adrenocorticotropic hormone (ACTH) challenge. We discovered excretory lag-times of FCM of 24 h in females (n = 2) and 48 h in male (n = 2) Koalas in response to the ACTH challenge. FCM levels showed an episodic and delayed peak response lasting up to 9 days post ACTH challenge. This finding should be taken into consideration when designing future experiments to study the impacts of short-term (acute) and chronic stressors on the Koalas. Laboratory validations were done using parallelism and recovery checks (extraction efficiency) of the cortisol standard against pooled Koala faecal extracts. Greater than 99 % recovery of the cortisol standard was obtained as well as a parallel displacement curve against Koala faecal extracts. FCM levels of the captive Koalas (n = 10 males and 13 females) significantly differed by sex, reproductive condition (lactating versus non-lactating Koalas) and the handling groups. Handled male Koalas had 200 % higher FCM levels than their non-handled counterparts, while females were not affected by handling as long they were not undergoing lactation. There was no significant difference in FCM levels between the captive and wild Koalas (n = 9 males and 7 females). Overall, these results provide foundation knowledge on non-invasive FCM analysis in this iconic Australian marsupial. Non-invasive stress endocrinology opens up opportunities for

evaluating the sub-lethal physiological effects of management activities (including caging, translocation) on the nutritional status, reproductive behaviors and disease status of captive and managed *in-situ* Koala populations.

Key words: Conservation Physiology; Stress; Captive Management; Handling; Koala (*Phascolarctos cinereus*); Faecal Cortisol Metabolites; Cortisol; Wild Population; Zoo Visitors

1. Introduction

Stress is one of the major issues facing Zoos around the world today and stress maintenance should be a key consideration for breeding and recovery programmes for threatened wildlife. Animals often fail to breed successfully in captivity due to numerous problems associated with the captive environment that lead to increased physiological stress, such as inadequate space, limited food, lack of environmental enrichment and continuous human interventions (See [25] for a detailed review of the various types of emotional and physical stressors that are faced by animals in captivity). Therefore, stress monitoring should be a key component of captive breeding programs at Zoos. Stress modulates the activities of the hypothalamo-pituitary-adrenal (HPA) axis and sympathoadrenal axis, releasing a variety of hormones to counter adverse stimuli [52]. Activation of the HPA axis during stress enhances the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland, stimulating the synthesis and secretion of glucocorticoids (cortisol and/or corticosterone) from the adrenal cortices [42]. While blood glucocorticoid concentrations are accepted indices of stress, their usefulness in long-term studies with wildlife is limited due to the circadian rhythm and the pulsatile nature of glucocorticoid secretion and the possible induction of a physiological stress hormone response during sampling procedures [53]. Conversely, the excretion of metabolized blood plasma steroids in feces permits the monitoring of physiological stress without disturbance to wildlife. For this study, we term this as "faecal cortisol metabolites" [FCM], which are the metabolites of cortisol that were formerly circulating in the blood. Analysis

of FCM generally also provides a more representative measure of adrenocortical activity over time because the pooling of metabolites occurs during excretion rather than the episodic secretion of blood glucocorticoids [53]. Physiological validation of the assay is achieved through experimental challenge with a precursor hormone, such as ACTH for glucocorticoids, that also enables the excretory lag-time (time between hormone secretion in blood, metabolism in the liver and its first appearance in excreta) to be determined [29; 45; 48]. Non-invasive stress endocrinology (NISE) is a recent methodological advancement under this emerging field of conservation physiology that enables the quantification of stress hormones from excretory metabolites in wildlife with minimal disturbance [30]. Many comprehensive reviews are available on the technical aspects of non-invasive FCM analysis that also discusses these issues and contrasts the pros and cons of each matrix to measure stress hormones [1; 26; 39].

The Koala (*Phascolarctos cinereus*) is a unique Australian marsupial; it is the only extant representative of the family Phascolarctidae, order Diprotodontia. Koalas have a habitat range extending from northeast to southeast Australia, from near Adelaide to the southern part of Cape York Peninsula. The Koala is nocturnal to crepuscular and is one of the largest arboreal mammals, resting in trees without building nests [43]. Koalas are listed nationally as a Vulnerable species (even considered rare in New South Wales and South Australia) and its distribution range is restricted to the east coast of Australia [21]. Causes of mortality are diverse, although the disease (essentially chlamydiosis, a reproductive disease caused by a genus of bacteria known as *Chlamydia* [*C. pneumoniae* and *C. pecorum*]), collisions with cars, attacks by dogs and loss of habitat represent the principal reasons for the species continuous decline. Among various management approaches, conservation centres have been established around Australia that aims to simultaneously protect Koala habitat and educate the public. In these sanctuaries, the natural habitat of the Koala is re-created and, in some instances, boardwalks crossing the canopy of the Eucalyptus forests used by the Koalas allow tourists to view animals from a close range. Due to their iconic status, Koalas are

a good educational tool for increasing public awareness of conservation for both young and adults [12]. As with all native mammals that have been taken into Zoo care, minimising stress is always a major consideration. Choosing suitable housing can help to create a stress-free environment. Common stresses in captivity include handling, disruption of feeding times, and disruption of sleeping times, overcrowding, and separation of the sexes, controlled mating and controlled weaning [21]. Recently, [37] used a minimally invasive electrocardiograph (ECG) technique to assess resting heart rates (during periods of inactivity) of the Koalas exposed to tourists with the tourist-free Koala. Results showed higher resting heart rates in tourist exposed exhibit areas. In their study, [37] also highlighted the importance of studies investigating the physiological reaction of animals to tourists and that measurement of the stress hormone cortisol would help in quantifying the stress imposed by tourist proximity.

It has been suggested that stress may be immunosuppressive and increase the risk of infections and the likelihood of chlamydiosis in Koalas. In captivity, chronic stress and heightened activity of HPA axis in larger vertebrates (i.e. glucocorticoids, ACTH, and corticotropin releasing hormone) can have detrimental effects on reproductive function, suppression of the immune-system and atrophy of tissues [52]. Thus, improving the health and general well-being of wildlife in captivity requires identifying stressful environmental conditions or management practices and developing mitigating strategies. Earlier studies on the cortisol profiles of other marsupials were performed largely using blood plasma samples [4; 15]. In Koalas, there is some information on the blood plasma concentrations of cortisol in captive and wild Koalas [6; 15] and profiles of blood plasma concentrations of testosterone in males, and progesterone (P4), estradiol-17b (E2), luteinizing hormone (LH), and prolactin in females [19]. In practice, it may be difficult to collect blood periodically and longitudinally to investigate their stress endocrinology. Successful endocrine monitoring of marsupials by a non-invasive technique using feces has been reported in two species of wombats, the common wombat (*Vombatus ursinus*) and the southern hairy-nosed wombat

(*Lasiorhinus latifrons*) [34], and androgen values in male southern hairy nosed wombats indicated a positive significant correlation between blood plasma and faecal metabolite levels [16]. Most recently, FCM analysis was successfully used to assess physiological stress in captive male and female greater bilbies (*Macrotis lagotis*) [30]. Also recently, a non-invasive endocrine monitoring technique for reproductive physiology of female Koalas was developed [19]. Faecal progestagen profiles were correlated with the observed behaviour in non-pregnant and pregnant (copulated) females. Fecal progestagen analysis was reported as a helpful and non-invasive tool to monitor ovulatory activity in northern and southern Koalas [19].

In this study, we validated a FCM enzyme-immunoassay (EIA) for monitoring the physiological stress hormone response to routine husbandry in the Koala as part of a broader program aimed at improving their captive management and breeding. There is recent controversy surrounding the handling of Koalas during public displays especially for taking photos. It is imperative that Koalas are chosen based on based on their temperament and are conditioned for handling. It is also important that constant attention is paid to the Koalas, particularly if being held by the visitor, to assess the level of stress in each animal. Therefore, we also compared FCM levels of Koalas that were handled by the public visitors versus non-handled male and female (lactating versus non-lactating) Koalas. We also quantified the FCM levels in a sub-population of wild male and female Koalas from the Gold Coast in Southeast Queensland.

2. Methods

2.1 Study animals and sampling design

All animal handling procedures were approved by the Animal Ethics Committee of Griffith University (AEC Permit No. ENV/17/11/AEC). Captive Koalas (10 adult male and 13 adult female Koalas) from each of the following reproductive groups (lactating or female with pouched young [n

= 6], non-lactating female [n = 7] and adult male) were used for this study from the Dreamworld Koala Country houses in Queensland, Australia. Koalas were kept in an indoor viewing area. From the Koalas, 5–10 fresh-looking, grain-shaped feces were collected from the ground in the viewing area on a daily basis during routine husbandry of the enclosure. Sampling was done early (0600 – 0900 h) in the mornings by the Zoo keepers who collected feces from the ground immediately after noticing excretion by known individual Koalas. Faecal samples from each Koala were immediately transferred into labelled Zip Lock© bags and frozen on-site. The area was checked in the evening at 2000 h and cleaned of all feces and then checked at 0600 h the next day so that the samples were deposited up to 10 hours previous day. The Koala database was used to obtain information on activities that each Koala performed in captivity. The main activity involved photographing of Koalas as part of daily tourist interactions. Time taken for each handling event was reported as between 5 min – 30 min for each Koala. Faecal samples from known handled and non-handled Koalas (from each of the above groups, including lactating females that were never handled) were collected daily for a period of 20 days in November 2011. All Koalas used for the tourist interactions were reported as healthy during the study period.

Fresh faecal samples were collected (identified based on their *Eucalyptus* smell) from 7 adult females and 9 adult male wild Koalas between the periods from the 3/11/11 to the 2/2/12 from a forest reserve in the Gold Coast in Southeast Queensland. Koalas were radio-collared for tracking and re-capture by the Gold Coast City Council. Six out of the seven female Koalas had a joey each (ranging from 3 – 5 weeks old) in their pouch during the study period and the male Koalas were reported as either healthy (4 male Koalas), in poor body condition (1 male Koala) or with signs of disease, such as cystitis and conjunctivitis (4 male Koalas). Only single and up to 4 faecal samples could be collected from these Koalas hence detailed comparisons between their health status and levels of FCM were beyond the scope of this study. Samples from the captive and wild Koalas

were transported to the laboratory in an ice box and kept at -20 ^{o}C for a period of less than 1 month until assays.

2.2 Biological Validation

An adrenocorticotropic hormone (ACTH) challenge was performed on four Koalas (2 male and 2 female) that were available at the Taronga Zoo, New South Wales, Australia in November 2011. Each Koala was anaesthetised by a Veterinary professional. During sampling, each Koala was physically restrained in a catch bag and a mask venting isoflurane (2% v/v) and oxygen (100%) was placed over the entire muzzle and retained until the animal was sufficiently sedated for intubation. Once sedated, a blood sample was collected at time = 0 and ACTH was injected intramuscularly (Synacthen, 500ug in 2ml saline; Provet Pty Ltd, Australia) at this time. Blood samples (2-3 ml) were then collected at 15 minute intervals to a maximum of 6 samples for each individual. Blood was centrifuged on-site for collection of plasma that was later used for the assays. A general health evaluation was conducted concurrently and anaesthesia was reversed at the conclusion of the procedure. No reversal agent was used and recovery was quick. Daily faecal samples were collected for 3 days prior and 7-10 days after the procedures (samples were collected at the same times each day for all four Koalas) and were analysed in conjunction with the plasma samples for total cortisol concentrations. All four individuals were returned to their enclosures upon recovery from anaesthesia for the ACTH challenge. The age of the four Koalas at the time of ACTH challenge was as follows; Arthur = 2.5 yrs, Irwin = 3 yrs, Carrie = 9 yrs and Erna = 4 yrs.

2.3 Laboratory Validation

Laboratory validation of the FCM EIA was based on similar procedure undertaken recently in the Greater Bilby (*M. lagotis*) [30]. It was achieved by demonstrating: a) parallelism between serial dilutions of pooled faecal extracts and the respective cortisol standard curves; b) significant recovery of exogenous cortisol standard (#27840; Sigma-Aldrich) added to the Koala faecal

extracts. Validations were performed separately on both captive and wild Koala samples. The sensitivity of the faecal cortisol FCM was calculated as the value 2 standard deviations (SD) from the mean response of the blank samples. Intra- and Inter-assay coefficients of variation were determined from high- (approximately 70%) and low- (approximately 30%) binding internal controls run on all assays. The possible limitation of utilizing only a one available polyclonal antibody (R4866) for this study is noted and we recommend that future studies perform additional validations using radio-metabolism to show that the actual plasma hormones were being excreted in the feces and also use High-performance liquid chromatography (HPLC) to show that the R4866 antibody used does in fact detect the hormone metabolites of interest (See the latest review on these topics by [32]).

2.4 Faecal Cortisol Metabolite Extractions

FCM was extracted from Koala faecal samples using the methods as previously described for other mammals [23; 30; 49]. Briefly, samples were freeze-dried or lyophilized for 7 days to eliminate all water content, ground, and 0.2 g of well-mixed faecal powder was boiled in 2.5 mL aqueous ethanol (90 % vol) for 15 minutes. After centrifuging at 1,500*g* for 20 minutes, the supernatant was recovered and the pellet resuspended in 2.5 mL aqueous ethanol, vortexed for 1 minute, and re-centrifuged at 1,500*g* for 20 minutes. Both supernatants were combined, taken to dryness (under warm air in a fume cupboard), and extract particles adhering to the vessel wall were reconstituted in 1ml assay buffer (39mM $NaH_2PO_4.H_2O$, 61mM $NaHPO_4$, 15mM NaCl and 0.1 % bovine serum albumin, pH 7.0) before analysis by the EIA.

2.5 Faecal Cortisol Metabolite and Total Plasma Cortisol Enzyme-immunoassay

We followed the detailed guidelines provided by [5] for validating the FCM and total plasma cortisol enzyme-immunoassay. Concentrations of FCM were determined using a polyclonal anticortisol antiserum (R4866) diluted 1: 15 000, horseradish peroxidase conjugated cortisol label

diluted 1: 80 000 and cortisol standards (1.56–400 pgwell^{-1}). Cross reactivity of the R4866 anticortisol antiserum is reported as 100 % with cortisol and less than 10 % with other steroids tested [28; 29]. The same reagents (supplied by Coralie Munro at the University of California-Davis, USA) were used recently for assessing FCM for other mammals [27; 30]. Samples were assayed on Nunc MaxiSorp plates (96 wells) and in duplicate. For each EIA, the NuncMaxisorp plates were coated with 50 µL of cortisol antibody diluted to the appropriate concentration in a coating buffer (50 mmol L^{-1} bicarbonate buffer, pH 9.6) and incubated for at least 12 hours at 4ºC. Plates were washed using an automated plate washer supplied with phosphate-buffered saline containing 0.5 ml L^{-1} Tween 20 to rinse away any unbound antibody. Stocks of standards, high- and low-binding internal controls, faecal extracts, and horseradish peroxidase labels were diluted to the appropriate concentration in assay buffer. For each EIA, 50 µL of cortisol standard, internal control and diluted faecal extract was added to each well. Dilution rates for Koala faecal extracts were based on the concentration of pooled samples that resulted in 50 % binding on the parallelism curve (See Fig. 1A for captive and wild Koala faecal extracts and Fig. 1B for Koala plasma dilution factors). For all assays, 50 µL of the corresponding horseradish peroxidase label was then added to each well and the plates were incubated at room temperature for 2 h. Plates were then washed and 50 µL of a substrate buffer (0.01% tetramethylbenzidine and 0.004% H_2O_2 in 0.1 M acetate citrate acid buffer, pH 6.0) were added to each well. Stop solution (50 µL of 0.5 mol L^{-1} H_2SO_4) was added based on the visual inspection of plates so that the optical density of the zero wells reads between 0.7 and 1.0 usually after 7–10 min incubation at room temperature. Plates were then read at 450 nm (reference 630 nm) on an EL800 (Bio Tek) microplate reader. All faecal data is expressed (ng/g) net dry feces weight basis. Assay sensitivity for FCM EIA was 2.04 $\pm$ 0.39 pg well^{-1} (n = 15). Intra- and inter- assay coefficients of variation were determined from internal control samples (~30% and 70% bound) included in all assays. Intra-assay coefficients of variation were 5.9% and 3.9% for low- and high- percentage-bound controls, and inter-assay coefficients of variation were 12.3% and 1.9% respectively. Koala plasma samples from the ACTH challenge

were assayed using the similar methods as described above for determining whether the ACTH treatment led to increased levels of total plasma cortisol (expressed as ng/ml plasma).

2.6 Statistical analysis

Statistical analyses were performed using Systat (Systat Software Inc.) and Prism (Graphpad Software Inc.). Faecal cortisol metabolite (FCM) concentrations were transformed to logarithms, and Levene's and Bartletts's tests used to check homogeneity of variances. Data are presented as individual or mean (± SEM) values.

2.6.1 ACTH challenge

The sample sizes for the ACTH challenge (n = 4 Koalas) were too small for robust statistical analysis. Therefore, the individual data and the mean (± S.E) were plotted by days before and after the ACTH challenge to facilitate comparisons. The percentage (%) rise in FCM levels of the four Koalas was also calculated.

2.6.2 Captive and Wild Koalas

A General Linear Model (GLM) univariate repeated measures ANOVA was used as assess the level of significant difference in FCM levels between the handling groups (handled versus non-handled Koalas) with sex and reproductive condition as the factors. The interaction terms included sex*handling and sex*reproductive condition. Post-hoc comparisons between sex, handling groups and reproductive condition were done using Wilcoxon Signed-Rank Test. Furthermore, a Spearman Rank Correlation (*r*) was used for comparing the mean FCM concentrations of the handled Koalas by time (min). A General Linear Model (GLM) was used to determine the level of significant difference in FCM levels between reproductive condition, sex and the environment as the factors. Post-hoc comparisons were done using Wilcoxon Signed-Rank Test with sex*environment and sex*reproductive condition as the interaction terms.

3.0 Results

3.1 Laboratory validation

Serial dilutions of the pooled Koala faecal extracts (for the ACTH challenge, captive and wild Koala samples; Fig. 1A) and pooled Koala plasma samples (for the ACTH challenge samples; Fig. 1B) showed parallel displacement with the cortisol standard curves. The quantitative recovery of cortisol standard was measured by adding different amounts of cortisol standards to pooled Koala faecal extracts. Recovery of cortisol standard (15 – 30 ng) that was added to the Koala faecal extract was $y = 0.957x - 6.112$, $r^2 = 0.993$ ($n = 6$), where y is the concentration observed and x is the concentration expected.

3.2 Biological validation

Total plasma cortisol levels of the Koalas increased from time (0 min) after ACTH injection. The individual plasma cortisol patterns were different for the male and female Koalas (Fig. 2A) and the peak responses were also different between male and female Koalas (Fig. 2A). The two male Koalas (Author and Irwin) had a similar pattern of plasma cortisol secretion in response to the ACTH challenge. Of the four Koalas, Carrie had the highest baseline plasma cortisol level so she also had the highest peak response at 15 min (Fig. 2A). The percentage rise in plasma cortisol from baseline (t = 0 min) was the highest for Erna (1175 % rise at time = 15 min). The mean plasma cortisol level of the four Koalas showed a pattern of increasing plasma cortisol beyond t = 0 min (Fig. 2B) and percentage rise in plasma cortisol for each time period (15, 45, 60 or 75 min) from baseline (t = 0 min) ranged from 217 – 267 %. Total plasma cortisol levels of the Koalas ranged from 0.08 ng/ml to 2.9 ng/ml.

There was an increase in FCM concentrations within 24 – 48 h of administration of the synthetic ACTH in all four Koalas that were subjected to the challenge (Fig. 3). ACTH injection increased

faecal cortisol levels (compared to baseline) within 1 day after injection for the two female koalas injected with ACTH but not for the two male koalas (Fig 3). Fecal cortisol metabolite levels in the two male koalas were higher than baseline 2 days after the ACTH injection (Fig. 3). An episodic pattern of excretion of faecal cortisol metabolites was observed in three Koalas (Arthur, Irwin and Erna) with a delayed second peak occurring after day 3 (Fig. 3). Mean faecal cortisol metabolite concentrations in the four Koalas before the ACTH challenge were as follows: Arthur (5.3 $\pm$ 0.31 ng/g dry feces, n = 3 days), Irwin (7.1 $\pm$ 1.98 ng/g dry feces, n = 2 days), Carrie (2.9 $\pm$ 0.45 ng/g dry feces, n = 6 days) and Erna (3.3 $\pm$ 0.46 ng/g dry feces, n = 2 days). Mean faecal cortisol metabolite concentrations in the four Koalas after the ACTH challenge were as follows: Arthur (8.2 $\pm$ 1.21 ng/g dry feces, n = 10 days), Irwin (9.9 $\pm$ 1.24 ng/g dry feces, n = 7 days), Carrie (3.6 $\pm$ 0.40 ng/g dry feces, n = 10 days) and Erna (10.7 $\pm$ 2.15 ng/g dry feces, n = 8 days). Mean faecal cortisol metabolite concentrations in the males and females before the ACTH challenge were as follows: males (7.1 $\pm$ 1.29 ng/g dry feces, n = 6; Fig. 4) and females (3.9 $\pm$ 0.51 ng/g dry feces, n = 18; Fig. 4). Mean faecal cortisol metabolite concentrations in the males and females after the ACTH challenge were as follows: Males (8.9 $\pm$ 0.80 ng/g dry feces, n = 19; Fig. 4) and females (6.7 $\pm$ 0.47 ng/g dry feces, n = 12; Fig. 4). The percentage rise in FCM concentrations between all days before and all days post ACTH challenge for each Koala was as follows: 54.7 % (Arthur), 39.43 % (Irwin), 24.13 % (Carrie), and 224 % (Erna). Percentage rise in faecal cortisol metabolite levels of the males and females after the ACTH challenge were 25.53 % and 71.79 % respectively.

3.3 Comparisons between sex, handling groups, reproductive condition and environment

For the captive Koalas, the GLM results showed that between individuals there was a significant effect of sex ($F_{1,45} = 78.20$, $p < 0.0001$; Fig. 5) and reproductive condition ($F_{1,45} = 26.98$, $p < 0.0001$; Fig. 5) on the FCM levels. Within individual Koalas, there was a significant effect of handling ($F_{1,45} = 51.88$, $p < 0.0001$; Fig. 5) and significant interaction between handling*sex ($F_{1,45} = 45.41$, $p < 0.0001$; Fig. 5) and handling*reproductive condition ($F_{1,45} = 15.07$, $p < 0.0001$; Fig. 5). Post-hoc

comparisons showed that FCM levels were significantly different between the sexes (p = 0.001; Fig. 5) and the handling groups (p < 0.0001; Fig. 5) but not significantly different between the reproductive conditions (p = 0.46; Fig. 5). The individual FCM levels of the captive Koalas ranged from 3.38 – 46.35 ng/g dry feces for the handled males (n = 8), 2.35 – 10.40 ng/g dry feces for the non-handled males (n = 10), 2.67 – 14.18 ng/g dry feces for the handled non-lactating females (n = 12), 1.64 – 16.24 ng/g dry feces for the non-handled non-lactating females (n = 11) and 2.15 – 13.08 ng/g dry feces for non-handled lactating females (n = 7). One of the male individuals (Simba) from the handled Koala group had an unusually high level of FCM level (46 ng/g dry feces), which led to a very high variation in the FCM levels amongst the handled male Koalas in comparison to the other four handling groups (handled male 98.79 % c.f. non-handled male 37.90 % c.f. handled non-lactating female 37.32 % c.f. non-handled non-lactating female 63.28 % c.f. non-handled lactating female 42.46 %). This intra-individual variation could account for the observed difference between the sexes in the FCM levels and also for the difference between the handled and non-handled males (See Fig. 5, handled males have FCM levels that are ~200 % higher than those that were unhandled whereas the FCM levels of females were unaffected by whether they were handled by a human or not). Mean ($\pm$ SEM) FCM level were as follows; captive males = 9.7 $\pm$ 2.37 ng/g dry feces (n = 18 samples); non-lactating females = 8.5 $\pm$ 0.88 ng/g dry feces (n = 23 samples) and lactating females = 8.4 $\pm$ 1.36 ng/g dry feces (n = 7 samples).

Results for the second GLM showed that there was no significant difference in FCM levels between the captive and wild Koala sub-populations (Fig. 6). Post-hoc comparisons showed that within each environment (captive or wild) there was a significant effect of sex but not reproductive condition on the levels of FCM (Fig. 6). The individual faecal cortisol metabolites of the Koalas ranged from 2.35 – 46.35 ng/g dry feces for captive males (n = 18), 2.15 – 46.44 ng/g dry feces for wild males (n = 17), 1.64 – 16.24 ng/g dry feces for captive females (n = 30), 2.47 – 17.99 ng/g dry feces for wild females (n = 12). Spearman rank correlation result showed no significant effect of handling times

on the levels of FCM. Mean (± SEM) FCM level were as follows; wild males = 12.3 ± 2.60 ng/g dry feces (n = 17 samples) and wild females = 6.2 ± 1.77 ng/g dry feces (n = 12 samples).

4.0 Discussion

We have shown that a faecal cortisol metabolite enzyme-immunoassay (EIA) can be reliably used to measure biologically relevant changes in FCM concentrations in Koala feces. We validated this EIA by conducting an adrenocorticotropic hormone (ACTH) challenge on four captive Koalas. The FCM EIA detected a rise in FCM in Koala faecal extracts within 24 h (for females) and 48 h (for males) since the ACTH challenge. As shown in the results, the complexity of faecal cortisol detection in the Koala is with the prolonged excretory lag-time of FCM (see delayed second peak for some Koalas; Fig. 3) due to its excessively long gut system [7]. It is known that the difference in intestinal passage rate affects the response of FCM in animals [45]. A clearer picture of the lag-time of cortisol metabolites in Koalas would be possible would be possible if individuals were injected with radioactive (labelled) cortisol and its rate of excretion in feces was measured. This method has been demonstrated successfully in other animals [31]. The technique would verify the time required for cortisol metabolism and excretion. Age can also affect physiological stress responses in vertebrates [2; 51] and it is possible that Koalas have higheran older agef circulating plasma cortisol at older age (See Fig. 2 showing the baseline total plasma cortisol value for Carrie (9 years old) was the highest out of all four Koalas used for the ACTH challenge).

Koalas belong to herbivorous marsupials that are hindgut fermenters [18]. The observed episodic pattern of excretion of FCM lasted over 9 days in some Koalas (Fig. 3) and this result could be explained by the theory explained earlier by [7] stating that digesta reaching the junction of the small intestine with the caecum and proximal colon is divided into two fractions; larger particles pass down the proximal colon and are excreted rapidly, while the solute fraction and small particles are retained in the caecum and proximal colon. In an earlier study, [46] actually quantified the

retention time of solute phase markers to be 9 days in Koalas. Therefore, it is most likely that FCM gets pooled together with the excreta hence resulting in an episodic excretion of FCM. According to [1], the difference in the first appearance of cortisol between plasma and faecal samples is mainly due to the conjugation of circulating hormones in the liver, before they are excreted via the bile, and sometimes further hydrolysis by bacteria during passage through the gut. However, the discovery of 9-day lag to responses in Koala FCM measures may limit the type of acute or short-term stress studies that could be undertaken on this marsupial in the future. Other animals that have a long gutter system also tend to have a long excretory lag-time of faecal cortisol such as up to 48 hours in the pig (*Sus domestica*) and the white rhinoceros (*Ceratotherium simum*) and a variety of primates, such as the cotton top tamarin (*Saguinus Oedipus*), macaques and the ring-tailed lemur, *Lemur catta* (See review by [38]).

We found sex related differences in faecal cortisol metabolite levels in the captive Koalas. One of the most widely published factors influencing natural variation in glucocorticoid profiles is sex. There is growing recognition that there are these sex-differences in the excretion of faecal hormone metabolites. Sex related variation in faecal cortisol metabolites may reflect underlying differences in steroid metabolism, excretion routes, and pituitary responsiveness (See the latest review by [14]). Furthermore, cyclic fluctuations of oestrogen and progesterone concentrations in females may influence the expression of FCM [33]. Variation in the physiological stress response is also commonly reported to reflect reproductive status or phase (i.e. oestrous, gestation, lactation). While fluctuations in reproductive hormones are believed to be the primary cause of such variation, it is also thought that the increased metabolic demands associated with reproduction may also drive FCM concentrations during reproductive phases [45]. Additionally, health status has also been recorded as a source of individual variability in responses to stress as demonstrated by [22], who recorded strong correlations between health status and individual faecal cortisol metabolite concentration fluctuations in the tammar wallaby. Sex differences in FCM concentrations in the

Eastern Greater Bilby (*Macrotis lagotis*) were documented over a 21 day period with females producing consistently higher average FCM concentrations than males [30]. Sex differences in FCM excretion have been documented in other mammals, including the brown hare *(Lepus europeaus*) [44] and the Canada Lynx [11]. Furthermore, it is also possible that sex related difference in cortisol levels could be detected in plasma samples (See Fig. 2 showing that males and females have quite different patterns of total plasma cortisol response to ACTH challenge). Thus, this difference in plasma glucocorticoid levels could easily be detected in faecal levels as highlighted in recent work by [8]. Our EIA measured value for mean baseline plasma cortisol in captive Koalas was 0.6 ng/ml in comparison to 1.5 ng/ml in captive Koalas and 7.0 ng/ml in wild Koalas that were reported previously by [6] and [15] respectively.

We found a significant difference in FCM levels between the handled and non-handled captive Koala groups. This result was evident mainly in the handled males, which were notably hyper-reactive to the handling in comparison to the non-handled males or handled females. This result could possibly be associated with the social behaviour of male Koalas (such as aggression). An earlier study by [40] found that Koalas hardly demonstrate social interactions in captivity, however aggression is more common during the breeding season than outside breeding. Social interaction is important so that males can maintain territoriality during breeding hence maintain high levels of testosterone. It is possible that high circulating levels of testosterone occurs during physical stressors, such as bleeding in male Koalas that are under breeding condition (see work done by [6]), which could potentially account for the high variability in FCM levels. Despite the highly FCM levels observed in the male captive Koalas it is highlighted that the Zoo Keepers certainly use Koalas with suitable temperaments for each photography session. It is also possible that the Koalas that were hand raised at Dreamworld were accustomed to human handling. Studies on other zoo animals have provided conflicting results with regard to the effects of handling, for example [10] found no chronic effects of moderate to high degrees of handling on the serum cortisol values in the

southern elephant seal (*Mirounga leonina*) mothers and pups. In another study, [47] studied the impacts of anthropogenic factors on the stress physiology of wild spotted hyenas (*Crocuta crocuta*). The study found that anthropogenic disturbance, such as pastoralist activity and increasing human population density along the edge of the group's home range caused an increase in glucocorticoid metabolite levels among adult male hyenas.

Most recently, [27] found the effect of tourist visitation on faecal glucocorticoid responses in the Pongo (*Pygmaeus morio*). The study found the individuals that were habituated for interaction with tourists generated short-term physiological stress responses a day after exposure to tourists, which highlighted that animals that were involved in tourist related activities were not chronically stressed (based on the assumption that chronic stress impairs the short-term physiological stress hormone response in animals [13]). A hypersensitive reaction of male wild Koalas could either be associated with reproductive condition and reproductive hormones, and this could also be affected by numerous other factors, such as health status and environmental condition. In the future, the seasonal changes in FCM should be studied in wild Koalas using latest radio-tracking methods [35] to better understand the relationships between environmental stress and the Koalas physiological sensitivity to the environment. Studies in other small mammals found that baseline faecal cortisol metabolite levels were lower in captive populations than in wild populations [36; 41], which was due to provision of suitable environment under captive conditions. Long-term monitoring of FCM levels in wild Koala populations should allow the discovery of new insights from Koalas into the relationships between ecological factors and their physiological stress response. Another important consideration for future studies is to investigate the possible effects of differences in diet between captive and wild Koalas on their FCM levels. Earlier [20] reported that there are marked local and seasonal preferences exhibited in the diet of koalas. Wild Koalas are sometimes found sitting in, and even feeding on, trees of genera other than *Eucalyptus* including *Pinus*, *Leptospermum*, *Melaleuca*, *Allocasuarina* and *Callitris glauca* [17]. Recently, [9] demonstrated in free-ranging

North American red squirrels (*Tamiasciurus hudsonicus*) that dietary fibre composition and even slight differences in diet could affect the faecal steroid hormone levels.

Overall, faecal cortisol metabolite enzyme-immunoassay could be used for assessing physiological stress in Koalas. The use of non-invasive stress endocrinology (NISE) in Zoo programs will be essential for increasing our understanding of the physiological stress hormone response of Koalas to the captive environment. Short-term physiological stress responses provide captive animals with the opportunity to increase vigilance, enhance learning, increase alertness and exploration (see review [50]). Chronic stress *via* long-term exposure to a stressor could have detrimental impacts on the animal's health and welfare in captive populations [24]. However, a recent review by [3] shows that chronic stress may not be maladaptive and actually evolves to support fitness in some free-living animals. An integrated approach through the use of behavioural and multiple physiological data will be required for effectively teasing out the influence of the captive environment and external factors such as tourists on Koala health and breeding success. NISE provides a perfect platform for tracking stress in diseased individuals as well as when animals are being treated (such as vaccination programs). Interactions with humans could affect Koala stress physiology and welfare; hence in the interim we recommend Koalas to be used for such programs, however only after careful selection (such as on the basis of reproductive condition, temperament, age and health). Overall, Zoos around Australian are vital for once again reviving the population of this iconic Australian marsupial.

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Figure Legends

Figure 1. Binding displacement curves of serially diluted Koala faecal extract for ACTH challenge, captive and wild samples (Fig. 1A) and ACTH challenge plasma samples (Fig. 1B) against the cortisol standard (R4866) used in the cortisol enzyme-immunoassay. The y-axis shows the % Hormone Bound/Total Binding measured at 450 nm (reference 630 nm). The 50 % binding point is denoted using a dashed line, which determined dilution factors for the unextracted Koala plasma samples and faecal extracts (dilution factor for each sample has been provided in parenthesis).

Figure 2. Individual (Fig. 2A) and mean (± SEM) [Fig. 2B] total plasma cortisol levels of Koalas (n = 4) after an adrenocorticotropic hormone (ACTH) challenge at time = 0 min.

Figure 3. Individual faecal cortisol metabolite levels of Koalas (n = 4) during an adrenocorticotropic hormone (ACTH) challenge (day 0 denoted by vertical dashed line).

Figure 4. Mean (± S.E.M) faecal cortisol metabolite levels of Koalas (n = 2 male and 2 female) during an ACTH challenge. Fig. 4A represents data for male Koalas and Fig. 4B represents data for female Koalas.

Figure 5. Box Whisker plots with 5-95 % percentile of faecal cortisol metabolite levels of handled males, non-handled males, non-lactating handled females, non-lactating non-handled females, lactating non-handled females, all captive males and all captive females. Sample size for each group ranges from n = 12 – 48.

Figure 6. Box Whisker plots with 5-95 % percentile of faecal cortisol metabolite levels of wild males, captive males, wild females, captive females, all captive Koalas and all wild Koalas. Outliers are shown using shaded dots. Sample size for each group ranges from n = 7 – 30.

FIGURE 1

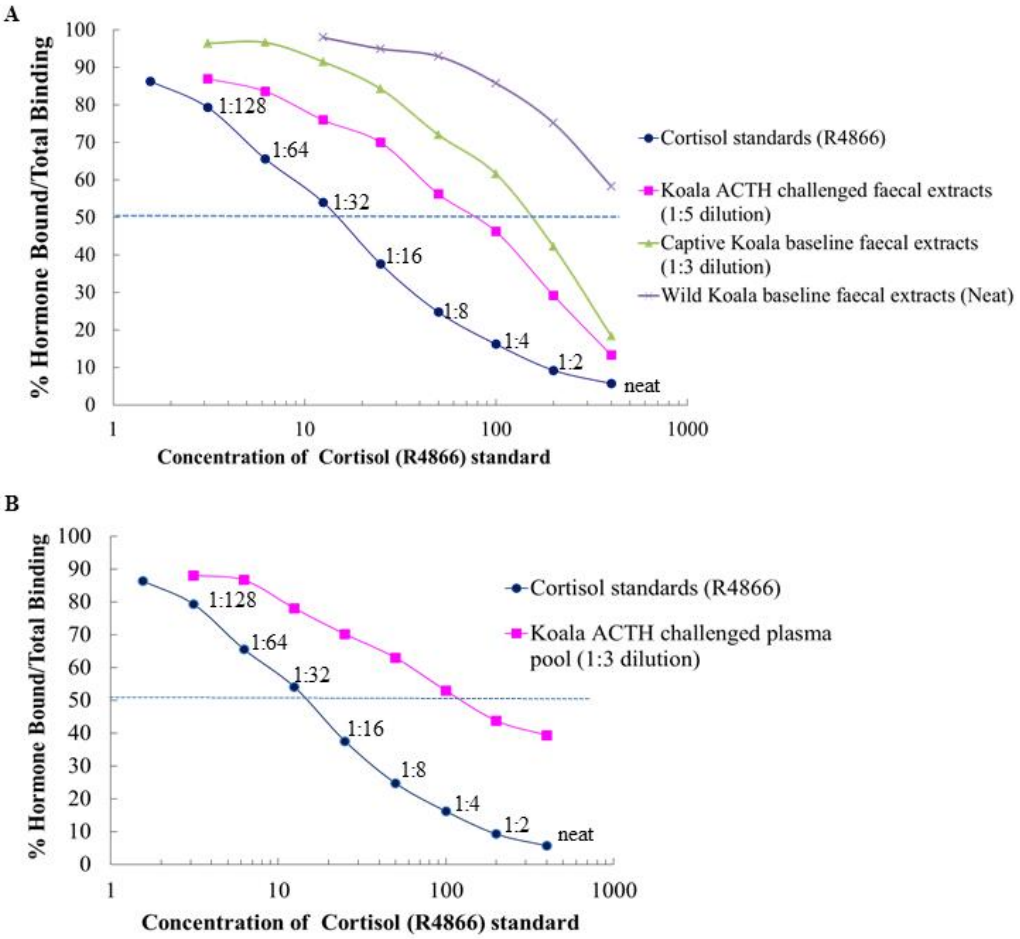

A
% Hormone Bound/Total Binding
100
90
80
70
60
50
40
30
20
10
0
1:128
1:64
1:32
1:16
1:8
1:4
1:2
neat
1
10
100
1000
Concentration of Cortisol (R4866) standard
Cortisol standards (R4866)
Koala ACTH challenged faecal extracts (1:5 dilution)
Captive Koala baseline faecal extracts (1:3 dilution)
Wild Koala baseline faecal extracts (Neat)
B
% Hormone Bound/Total Binding
1:128
1:64
1:32
1:16
1:8
1:4
1:2
neat
Concentration of Cortisol (R4866) standard
Cortisol standards (R4866)
Koala ACTH challenged plasma pool (1:3 dilution)

FIGURE 2

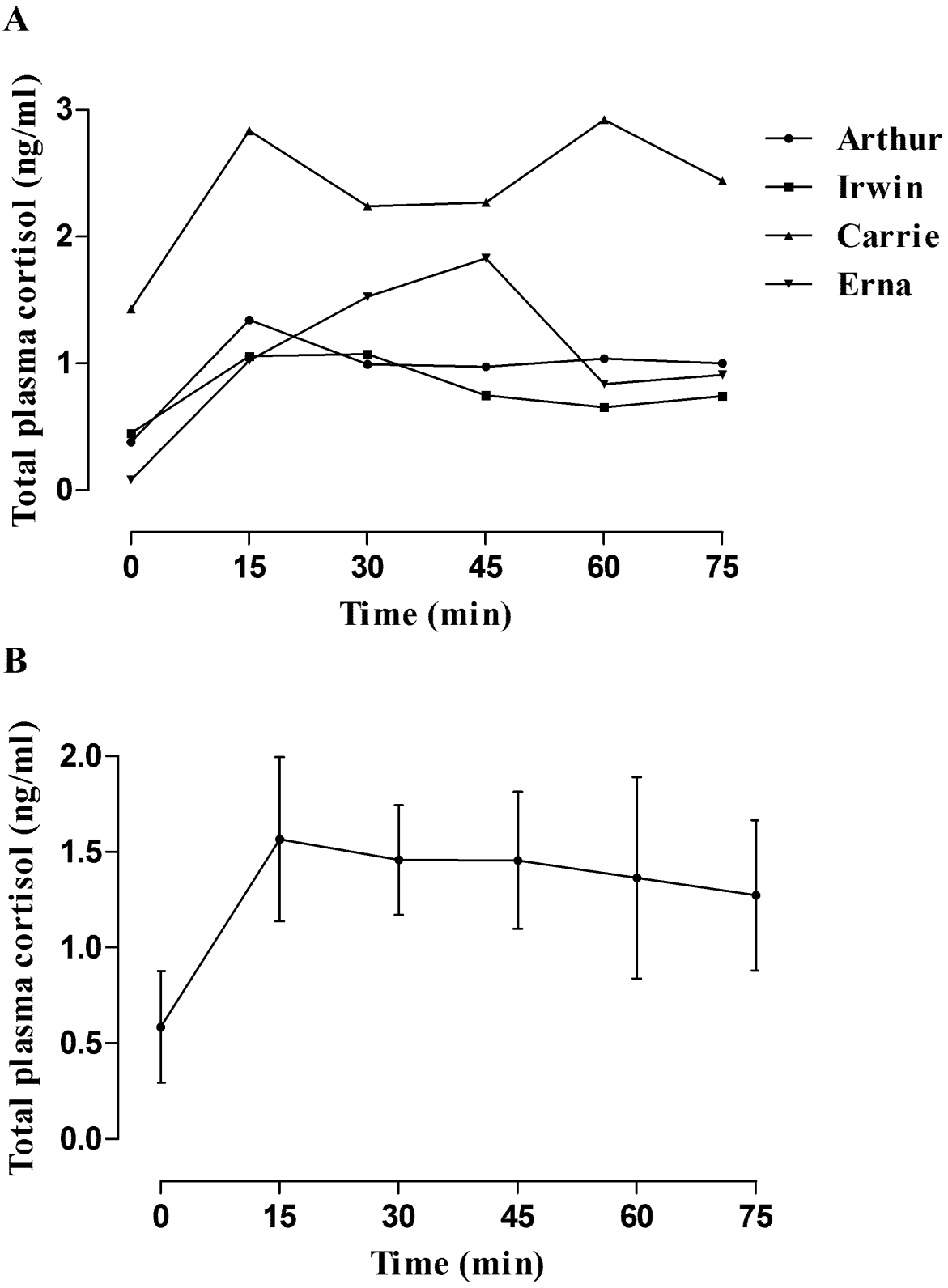

A
Total plasma cortisol (ng/ml)
3
2
1
0
0
15
30
45
60
75
Time (min)
Arthur
Irwin
Carrie
Erna
B
Total plasma cortisol (ng/ml)
2.0
1.5
1.0
0.5
0.0
0
15
30
45
60
75
Time (min)

FIGURE 3

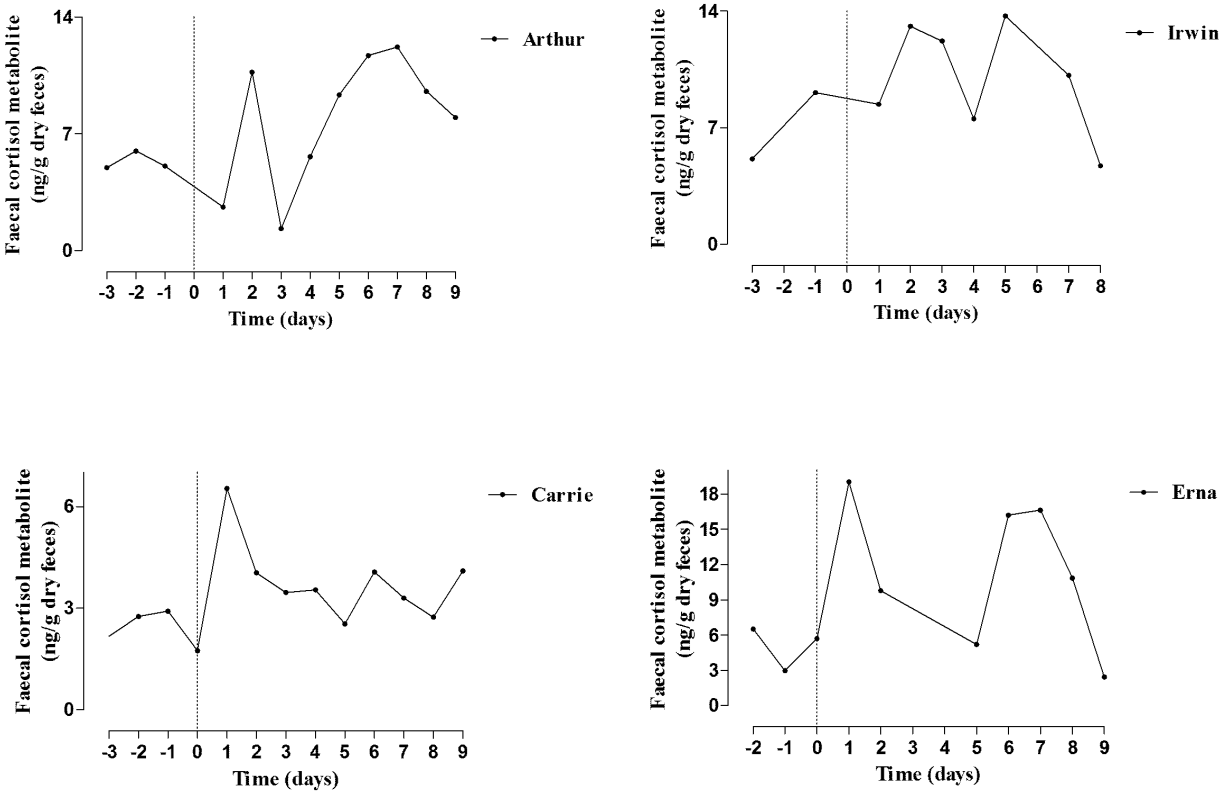

Faecal cortisol metabolite (ng/g dry feces)
Time (days)
Arthur
Irwin
Carrie
Erna

FIGURE 4

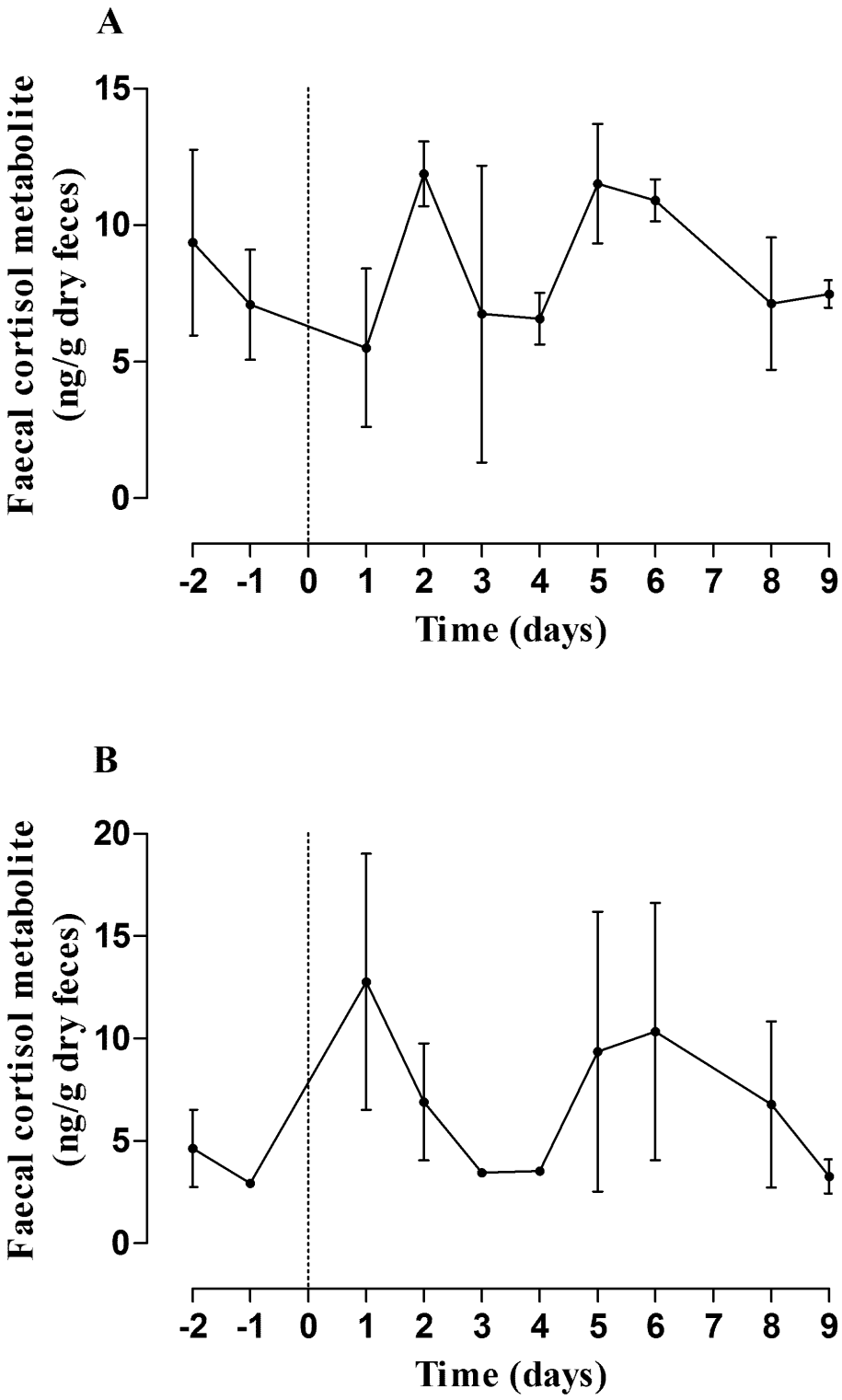
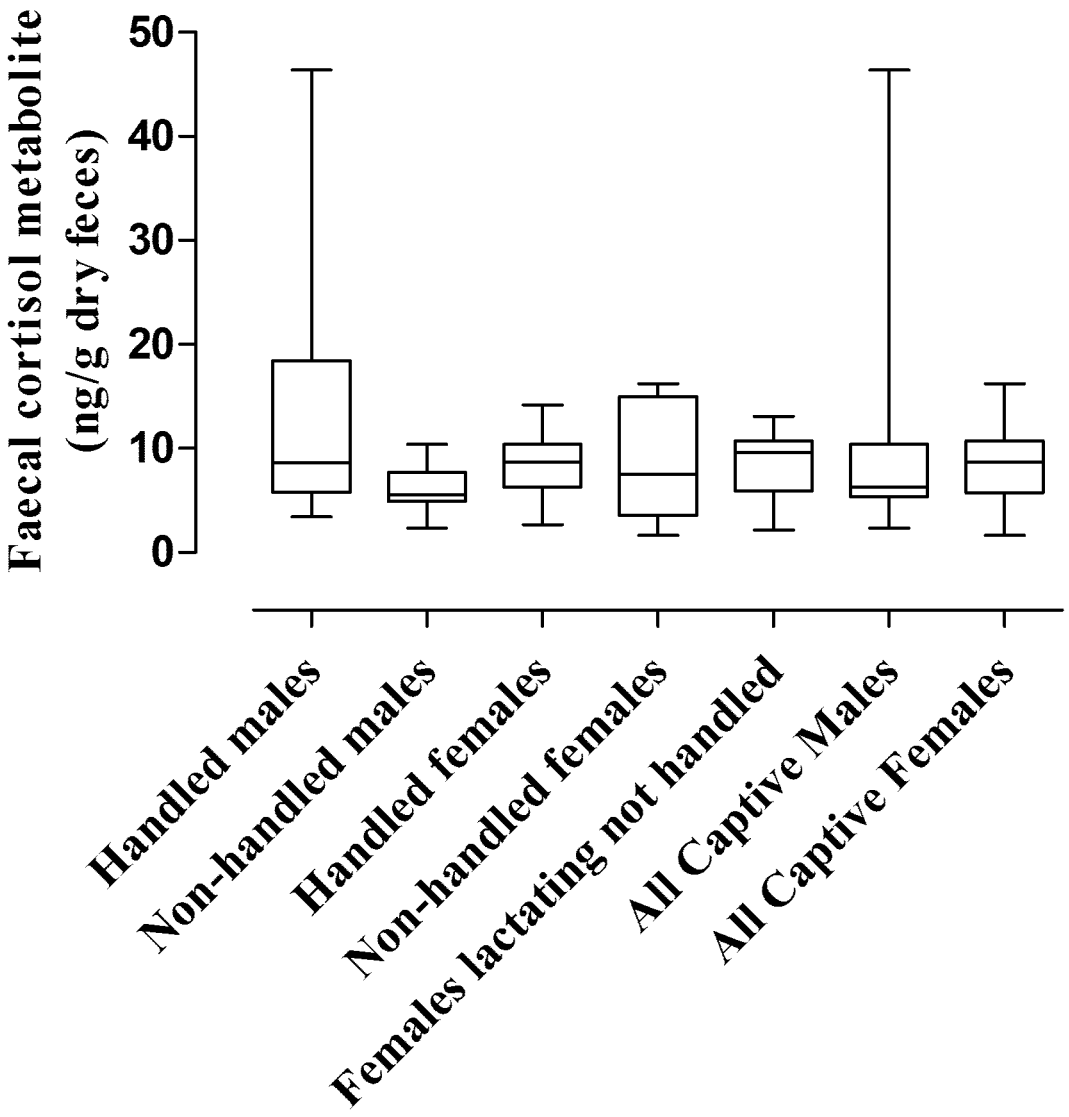
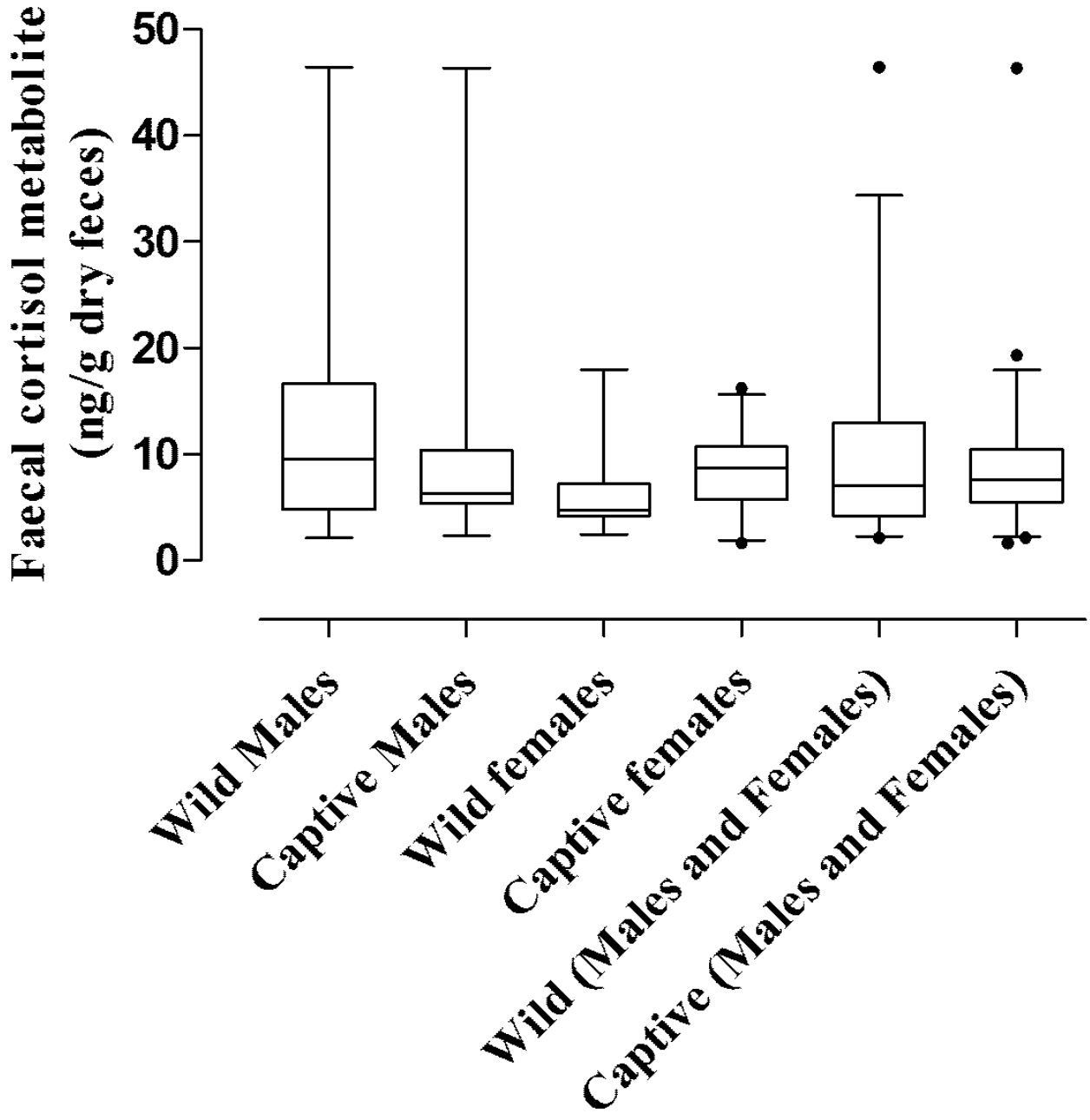

A
Faecal cortisol metabolite (ng/g dry feces)
15
10
5
0
-2 -1 0 1 2 3 4 5 6 7 8 9
Time (days)
B
Faecal cortisol metabolite (ng/g dry feces)
20
15
10
5
0
-2 -1 0 1 2 3 4 5 6 7 8 9
Time (days)

FIGURE 5


Faecal cortisol metabolite
(ng/g dry feces)
50
40
30
20
10
0
Handled males
Non-handled males
Handled females
Non-handled females
Females lactating not handled
All Captive Males
All Captive Females


Faecal cortisol metabolite
(ng/g dry feces)
50
40
30
20
10
0
Wild Males
Captive Males
Wild females
Captive females
Wild (Males and Females)
Captive (Males and Females)