Faecal cortisol metabolites in Bengal (*Panthera tigris tigris*) and Sumatran tigers (*Panthera tigris sumatrae*)

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Abstract

The tiger (*Panthera tigris*) faces a great risk of extinction as its wild numbers have plummeted due to poaching and habitat destruction so *ex-situ* conservation programs are becoming ever more necessary. Reliable non-invasive biomarkers of the stress hormone (cortisol) are necessary for assessing the health and welfare of tigers in captivity. To our knowledge, non-invasive stress endocrinology methods have not been tested as widely in tigers. The first aim of this study was to describe and validate a faecal cortisol metabolite enzyme-immmunoassay (FCM EIA) for two tiger sub-species, the Bengal tiger (*Panthera tigris tigris*) and the Sumatran tiger (*Panthera tigris sumatrae*). Individual tigers (n = 22) were studied in two large Zoos in Queensland, Australia (Dreamworld Theme Park and Australia Zoo). Fresh faecal samples (< 12 h old) were collected each morning from both Zoos over a study period of 21 days. Biological validation was conducted separately by collecting feces 5 days before and 5 days after blood was taken from four male and five female tigers. Results showed that mean FCM levels increased by 138 % and 285 % in the male and female tigers within 1 day after bloods were taken, returning to baseline in 5 days. Laboratory validations of the FCM EIA were done using an extraction efficiency test and parallelism. Results showed > 89 % recovery of the cortisol standard that was added to tiger faecal extract. We also obtained parallel displacement of the serially diluted cortisol standard against serially diluted tiger faecal extract. Our second aim was to determine whether the FCM levels were significantly different between tiger sub-species and sex. Results showed no significant difference in mean FCM levels between the Bengal and Sumatran tiger sub-species. Mean levels of FCMs were significantly higher in females than in male tigers. Those male and female tigers with reported health issues during the study period expressed higher FCM levels than the reportedly healthy tigers. Interestingly, those tigers that took part in some activity (such as walks, photos, presentations and guest feeds) expressed moderately

higher FCM levels at Dreamworld and lower FCM levels at Australia Zoo in comparison to those tigers that did not take part in such activities. These results indicate potential habituation in some tigers for routine activity through specialized training and pre-conditioning. In conclusion, the FCM EIA described in this study provides a reliable non-invasive method for evaluating the stress status of tigers in Zoos.

Key words: Tiger (*Panthera tigris*); Stress; Conservation Physiology; Feces; Cortisol; Sumatran; Bengal; Health; Zoo

1.0 Introduction

As the global population of tigers (*Panthera tigris*) existing in the wild today continues to decline, captive breeding programmes have become a fundamental component for conservation and biological research, and for the maintenance of genetic variability "genetic reservoir" that is representative of the remaining wild populations [25]. Tigers are considered a flagship species for wildlife conservation due to their charismatic nature and alluring features, such as vibrant coat colours, that draw great public concern [44]. As with many of the worlds threatened species, Zoos are playing an increasingly important role in preventing extinction of tigers through the initiation of captive breeding programmes [25; 44]. For captive facilities to achieve success in the management and maintenance of tigers, it is important that welfare and health are the focus of their efforts; which involves minimising and reducing stressful stimuli facing tigers in the captive environment. Studies that use rapid and reliable measures of stress hormones, rather than relying on behavioural indicators alone, are likely to provide for a more precise assessment of health and conditioning of tigers in captivity [18]. Being able to monitor and manage stress in captive tigers will allow for a successful and self-sustainable captive population, as the reduction of stress can enhance

overall physical and psychological wellbeing as well as improve the reproductive performance in felids [46; 50]. To achieve this, stress hormone levels need to be quantified and measured in a manner that is non-invasive and also provides reliable measurement of stress hormones.

Over recent years, there has been an increasing demand to focus efforts on the development of conservation physiology tools that can quantify stress and reproductive hormones in captive and managed wildlife populations [9; 35]. Non-invasive analysis of the stress hormone cortisol through faecal samples, has provided a powerful method for assessing the status of the stress endocrine system, the hypothalamic-pituitary-adrenal (HPA) axis, in mammalian species with respect to management interventions, such as captive husbandry [24; 42]. In mammals, stressful stimuli induce the release of adrenocorticotropic hormone (ACTH) through activation of the HPA axis, which in turn stimulates the synthesis and secretion of glucocorticoids (GCs) from the adrenal cortices [2; 50]. In feline species, cortisol has been identified as the major naturally occurring GC [5]. Non-invasive measurement of GCs is a key component of this emerging science of conservation physiology [9; 35], as it enables the rapid assessment of the well-being of threatened wildlife under wild, semi-wild and captive environments [9]. Recent developments of non-invasive techniques have allowed for the measurement of GCs in different biological samples from wildlife, such as using urine [26], saliva [22], hair [11] or faecal samples [36; 49]. The majority of recent studies have employed broad-spectrum (polyclonal antibody based) enzyme-immunoassay (EIA) or radio-immunoassay (RIA) to measure the metabolites of biologically active ("free cortisol") in mammalian feces [8]. For examples; felids in general [5], Grevy's zebra (*Equus grevyi*) [13], spotted hyena (*Crocuta crocuta*) [15], African elephant (*Loxodonta sp.*) [14], southern white rhinoceros (*Ceratotherium simum simum*) [27], greater bilby (*Macrotis lagotis*) [34], koala,

phascolarctos cinereus) [36], mountain gorilla (*Gorilla beringei beringei*) [37], cats (*Felis catus*) and dogs (*Canis lupus familiaris*) [41], cheetah (*Acinonyx jubatus*) [46], clouded leopard (*Neofelis nebulosa*) [49] and carnivores in general [50]. These studies on diverse mammals highlight that faecal analysis of cortisol metabolites is a valuable method for evaluating status of the HPA-axis under basal conditions as well as useful for quantifying cortisol response to potential acute stimuli (such as injuries and activities), which in turn, can aid in optimizing environmental and management conditions of captive wildlife.

Despite the usefulness and practicality of non-invasive stress endocrine methods, only a handful of studies to date have focused on quantifying fecal cortisol metabolites in the tiger [32; 40]. In this study, we measured cortisol metabolites in tigers from two large Australian Zoos. The overall aim of this study was to describe and validate a faecal cortisol metabolite enzyme-immunoassay (FCM EIA) for assessing cortisol metabolite levels in tigers. We biologically validated the FCM EIA by demonstrating raise and return to basal patterns of FCM in male and female tigers in relation to a blood sampling event. We also validated the FCM EIA using standard laboratory methods, including accuracy-recovery checks and parallelism. Furthermore, we aimed to test the null hypothesis that FCM levels will not be different between the sexes or tiger sub-species, the Bengal tiger (*Panthera tigris tigris*) and the Sumatran tiger (*Panthera tigris sumatrae*) within each Zoo.

2.0 Methods

2.1 Experimental design and study animals

We sampled a total of 22 individual tigers from Dreamworld Theme Park and Australia Zoo, which are two of the largest tiger captive breeding facilities in Australia. We had access to

two sub-species; the Bengal tiger (*P. tigris tigris*) and the Sumatran tiger *(P. tigris sumatrae*). The Dreamworld Theme Park manages 13 tigers; 7 male and 1 female individual of the Bengal sub-species; 1 male and 4 female individual of the Sumatran sub-species. Australia Zoo manages 9 tigers; 1 male and 2 female individual of the Bengal sub-species; 3 male and 3 female individual of the Sumatran sub-species. We highlight the fact that the sexes were unbalanced in Dreamworld within each sub-species thus we analysed and interpreted the results with caution. All intact females were reportedly in oestrous state of their reproductive cycle during the study. Each Zoos database was used to obtain information such as sex, birth date, reproductive status and health condition of each tiger during the sampling period (see Table 1 and Table 2 for summary). Both Zoos have a very similar management program, enclosure sizes and substrates (general quality) are fairly similar. At both locations, all of the sampled individuals were housed alternately between on exhibit areas (in view of public) and off exhibit (with no public exposure) which ensures that all tigers were exposed to the same environmental conditions throughout the duration of the sampling period.

2.2 Faecal sample collection and storage

Collection of fresh tiger faeces took place daily at each Zoo over a period of 21 days, commencing in August, 2012. Samples from known individual tiger were collected early in the morning at 0600 h during routine husbandry. Faecal samples were stored in sealed plastic bags, which recorded the collection date as well as individuals ID and sex (written clearly with permanent marker on the outside of each bag). Due care was taken to avoid sampling feces that were contaminated with urine. Immediately after collection, faecal samples were frozen (-20^{o}C). Samples were processed (extracted and assayed) within 20 days.

151 *2.3 Biological validation*

152 Tigers at each Zoo underwent blood collection during routine veterinary checks so we took
153 this opportunity to biologically validate the FCM EIA. This was done separately (1 month)
154 prior to the 21 days sampling period began. We used this as an alternative to conducting an
155 Adrenocorticotropic hormone (ACTH) challenge since the Zoos did not permit any
156 exogenous chemical treatment on this endangered and precious animal. For blood sampling,
157 at both Dreamworld and Australia Zoo, the keepers use their training commands to have the
158 tiger sit on a marker, then just use treats like milk, and keep the cats distracted while the
159 blood is taken. Blood (2-5 ml) is drawn by venipuncture of the tail vein through 18 gauge
160 disposal syringe in a tube containing Ethylenediaminetetraacetate acid (EDTA at 2 mg/ml of
161 blood) as the anticoagulant. Where a blood sample could be obtained, faecal samples were
162 collected daily for 5 days before to the blood sampling event and daily for up to 5 days post
163 blood collection, so that any effect that the 'jab' of the needle would have had on stimulating
164 the HPA axis and plasma cortisol secretion could be detected in the FCM.

165

166 *2.4 Faecal hormone extraction*

167 Methods for extraction followed that previously described for the clouded leopard (*Neofelis*
168 *nebulosa*) [49]. In preparation for hormone extraction, all faecal samples were freeze-dried at
169 -80^{o}C for a period of 5 days in a lyopholizer that enabled the samples to completely
170 dehydrate. Any hair, bone or fibrous matter was carefully sifted and sieved out from the
171 dried samples, leaving only dried faecal matter that was able to be ground down to a fine
172 powdery consistency using a mortar and pestle. FCMs were extracted from the powdered
173 samples (0.2 g per sample) in a 90 % ethanol solution (diluted in deionized water) in a glass
174 tube. This process helped to attain maximum binding of the faecal steroids from the solid
175 phase (feces) to the liquid phase (90 % ethanol). Catalyzation of this steroid-ethanol binding

process was completed by placing the prepared tubes in a hot water bath (approximately 100 °C) for a period of 20 minutes. The faecal extract (1 mL) was then transferred into an Eppendorf tube for centrifugation for 5 min at 5000rpm. The liquid portion was vortexed into a new Eppendorf tube (0.5 mL) and then dried under warm air in a fume hood for 3-5 days. After this process, the solid phase (steroids attached to the wall of the test tube) was scrapped off using a fine spatula. The faecal extract was then reconstituted into an EIA buffer (39mM $NaH_2PO_4.H_2O$, 61mM $NaHPO_4$, 15mM NaCl and 0.1% bovine serum albumin, pH 7.0, re-centrifuged for 5 min at 5000rpm and the supernatant was collected in an Eppendorf tube and stored at -20 ^oC until the EIAs were performed.

2.5 Laboratory validation

We followed the detailed guidelines provided by [3] for validating the FCM EIA. Laboratory validation of the FCM EIA was achieved using two methods: 1) parallelism between serial dilutions of pooled tiger faecal extracts and the respective serially diluted cortisol standard curve, 2) significant recovery of exogenous steroid standard added to faecal extracts. Confirmation of parallelism helped in determining the dilution factor to use for sample extracts, which was based on the 50 % binding point on the parallelism curve (1:16 for tiger faecal extract). Extraction efficiency was calculated as the amount of hormone observed relative to the amount expected, and was expressed as a percentage (mean ± standard error of the mean [SEM]). Extraction efficiency was presented as a linear regression equation: $y = mx + b$, where y is the concentration of the hormone observed, x is the concentration of the hormone expected, b is the y intercept, and m is the slope of the line that was expressed as a percentage representing extraction efficiency [33]. The resulting equation was $y = 0.894x - 6.217$, $r^2 = 0.9913$; $n = 6$. Thus, extraction efficiency of > 89 % was achieved. The sensitivity of the FCM EIA was 0.4 ± 0.1 pg $well^{-1}$ (n = 50 plates analysed). The intra- and

201 inter-assay coefficients of variation (CV) were 2.1 % and 6.5 % for the high-binding internal
202 control and 1.5 % and 10.3 % for the low-binding internal control (n = 50 plates analysed).
203
204 *2.6 Faecal cortisol metabolite (FCM) enzyme-immunoassay*
205 Concentrations of FCM were determined using a polyclonal anti-cortisol antiserum (R4866,
206 procured from the University of California-Davis, USA) diluted 1: 15,000, horseradish
207 peroxidase conjugated cortisol label diluted 1: 80,000 and cortisol standards (1.56–400
208 $pgwell^{-1}$). Cross reactivity of the R4866 anti-cortisol antiserum is reported as 100 % with
209 cortisol and less than 10 % with other steroids tested [31]. The same reagents were validated
210 recently for assessing FCM for diverse mammals [30; 34; 36]. Samples were assayed on
211 Nunc Maxi-Sorp plates (96 wells) and in duplicate. For each EIA, the Nunc Maxi-sorp plates
212 were coated with 50µL of cortisol antibody (R4866) diluted to the appropriate concentration
213 in a coating buffer ($50mmolL^{-1}$ bicarbonate buffer, pH 9.6) and incubated for at least 12h at 4
214 ^{o}C. Plates were washed using an automated plate washer supplied with phosphate buffered
215 saline containing 0.5 mlL^{-1} Tween-20 to rinse away any unbound antibody. Stocks of the
216 standards, high- and low- binding internal controls, faecal extracts and horseradish
217 peroxidase labels were diluted to the appropriate concentration in the EIA buffer. For each
218 EIA, 50µL of cortisol standard, internal control and diluted faecal extract was added to each
219 well. For all assays, 50µL of the corresponding horseradish peroxidase label was then added
220 to each well and the plates were incubated at room temperature for 2 h. Plates were then
221 washed and 50µL of a substrate buffer (0.01 % tetramethyl-benzidine and 0.004 % H_2O_2 in
222 0.1M acetate citrate acid buffer, pH 6.0) were added to each well. Stop solution (50µL of 0.5
223 $molL^{-1}$ H_2SO_4) was added based on the visual inspection of plates so that the optical density
224 of the zero wells read between 0.7 and 1.0 usually after 7 – 10 min incubation at room

225 temperature. Plates were then read at 450 nm (reference 630 nm) using an EL800 (BioTek)
226 microplate reader.
227
228 *2.7 Statistical analysis*
229 Data were tested for normality and equal variances and FCM data were log-transformed to
230 ensure these statistical assumptions were met. Repeated Measures Analysis of Variance
231 (ANOVA) was used to compare the level of significant difference in mean FCM within and
232 between the tigers by time, sex and sub-species. A Repeated Measures ANOVA was also
233 used to compare the level of significance difference in mean FCM (taken before and after
234 blood sampling) by time, sex and sub-species. Multiple comparisons were done using a
235 Kruskal-Wallis Test. All faecal data are expressed as (ng/g) net dry feces. $P < 0.05$ was
236 considered as significant.
237
238 **3.0 Results**
239
240 *3.1 Biological validation*
241 Between tigers, there was a significant effect of sex [$F_{1,56} = 73.932$, $p < 0.001$] and sub-
242 species [$F_{1,56} = 8.381$, $p = 0.005$] on the FCM levels of the tigers during the blood sampling
243 event. There were mild increases in FCM levels of two male tigers from Dreamworld (M1,
244 Bengal sub-species and M2, Sumatran sub-species; Fig. 1A) after the blood sampling event
245 while two male tigers from Australia Zoo (M1 and M2, both Sumatran sub-species; Fig. 1A)
246 showed moderate increases in FCM after the blood sampling event. As shown in Fig. 1C,
247 mean FCM in all male tigers returned to baseline levels within the 5 days after blood
248 sampling. In females (n = 5 from Australia Zoo only, see Table 2.0 for sub-species), FCM
249 increased in four female tigers, except (F4, Fig.1B) after blood sampling. Mean FCM in

females changed significantly by time [$F_{1,56}$ = 17.90, p < 0.001], 5 days before and 5 days after the day of blood sampling (Fig. 1C). Mean FCM increased by 138 % and 285 % in the male and female tigers respectively within 1 day after blood sampling (Fig. 1C).

3.2 Comparison of FCM between and within tigers by sex, time and sub-species

Between tigers, there was a significant effect of sex [$F_{1,161}$ = 7.559, p = 0.006] on FCM levels of the tigers. There was no significant effect of sampling days [$F_{1,161}$ = 3.836, p = 0.051] or sub-species [$F_{1,250}$ = 2.768, p = 0.091]. Female tigers had higher mean FCM levels than the male tigers (63.49 $\pm$ 4.98 c.f. 70.46 $\pm$ 7.30 ng/g; Fig. 2) at Dreamworld. Likewise, female tigers at Australia Zoo had higher mean FCM levels than the male tigers (99.61 $\pm$ 14.20 c.f. 153.40 $\pm$ 20.23 ng/g; Fig. 2). Overall, female tigers at the Australia Zoo had the highest coefficient of variation (CV = 103 %) in mean FCM (Fig. 2). Male tigers at Dreamworld had the least variation in mean FCM (CV = 73 %; Fig. 2). Mean FCM levels were similar between the Bengal and Sumatran sub-species at each Zoo. Mean FCM levels ranged from 57.41 $\pm$ 12.66 c.f 58.79 $\pm$ 13.91 ng/g respectively for Sumatran (n = 5) and Bengal (n = 8) tigers at Dreamworld. Mean FCM levels ranged from 145.50 $\pm$ 15.75 c.f 144.10 $\pm$ 65.68 ng/g respectively for Sumatran (n = 6) and Bengal (n = 5) tigers at Australia Zoo.

Within tigers, there was a significant effect of time [$F_{1,250}$ = 7.37, p = 0.007] on mean FCM levels, significant interactions between time*sex [$F_{1,250}$ = 8.075, p = 0.003], however the interaction between time*sub-species was not significant [$F_{1,250}$ = 2.203, p > 0.05]. Male Bengal tiger (no. 1) from Dreamworld showed the highest mean FCM level in comparison to the other male tigers at Dreamworld, (Fig. 3A). The 4 year old female Sumatran tiger (no.1) from Dreamworld showed the highest mean FCM out of all females at Dreamworld (Fig. 3B). Male Bengal tiger (no.6) and female Sumatran tiger (no.5) showed the highest CV in their

mean FCM (103 % and 96 % respectively) out of all tigers at the Dreamworld (Fig. 3A-B). The 14 year old Bengal male tiger (no.1) and male Sumatran tiger (no.2) showed similar levels of mean FCM (Fig. 3A). The 14 year old Bengal female tiger (no. 2) from Dreamworld had slightly lower mean FCM in comparison to the FCM levels of the 4 year old female Sumatran tiger (no.1; Fig. 3B).

All male tigers at the Australia Zoo showed similar mean FCM levels during the study period (Fig. 4A). Male Sumatran tiger (no.2) showed the highest variation in mean FCM at 116 % (Fig. 4A). Female Sumatran tiger (no.4) showed the highest variation in mean FCM at 132 % (Fig. 4B). Female Bengal tiger (no.1) had the highest mean FCM out of all female tigers at Australia Zoo (Fig. 4B). Female Bengal tiger (no.5) showed the lowest mean FCM levels out of all tigers at Australia Zoo (Fig. 5B).

3.3 Comparison of FCM levels with Zoo Health Checks and Activities Database

No apparent pattern in FCM could be obtained with respect to the morphometrics data such as the age and weight of the tigers from each Zoo (Table 1.0). There were some medical conditions reported during the sampling period for the tigers at Dreamworld. For example, female Bengal tiger (no.2) had a minor cracked footpad and male Sumatran tiger (no.2) had bone chip and arthritis and was given mobic daily (Table 1.0). The FCM data showed that both of these tigers expressed high mean FCM (Fig. 3A and 3B) in comparison to the tigers that were reported as healthy during the study (Table 1.0). Unfortunately, male Bengal tiger (no.1) with the higher mean FCM at Dreamworld died within a few months after the study period due to some unexpected illness that not reported in this study. No abnormal activities or events were reported to have occurred during the study (e.g. transportation, non-routine events, vet checks, anaesthesia). These results suggest that higher levels FCM reported for

298 tigers with some health issue or even for reportedly healthy tigers (in comparison to tigers
299 reported as healthy and with lower FCM levels) could provide a sub-clinical sign of stress.

300 Female Bengal tiger (no.2) was the only female at Dreamworld that took part in some activity
301 (walks, photos and presentation; Table 1.0). This female tiger expressed slightly higher mean
302 FCMs than the other female tigers at Dreamworld (no.3, 4 and 5; Fig. 3B). Mean FCM of
303 female no.2 was comparable with mean FCM levels of female tiger no.1. Female tiger (no2)
304 did not take part in any activity nor had any health issue. The only major difference between
305 these females is their age (female no. 2 was 14 years old while female no. 1 was only four
306 years old during this study; Table 1.0).

307 Male tigers (no. 5 and no. 8) at Dreamworld expressed slightly lower FCM levels than the
308 other male tigers (no. 1, 2, 3 and 4; Fig. 3A) that took part in activities
309 (photos/presentations/guest feeds) [Table 1.0]. The male tigers (no. 6 and no. 7) were two
310 recently born tiger cubs at Dreamworld that also took part in walks/presentations. These two
311 tigers expressed nadir levels of FCM during the study period (Fig. 3A).

312 All tigers at the Australia Zoo were reported as healthy and having no medical issues (Table
313 2.0). Interestingly, the three male tigers at the Australia Zoo (no. 2, 3 and 4; Fig. 4A) that
314 took part in walks or photo (Table 2.0) expressed slightly lower mean FCM level than the
315 only male tiger (no.1; Fig. 4A) that did not take part in any activity during the sampling
316 period (Table 2.0). Likewise for females; all of whom did not take part in walks (no. 1, 2 and
317 3; Fig. 4A) expressed slightly higher mean FCM than the two other females that took part in
318 walks (no. 4 and 5; Table 2.0].

319

320

4.0 Discussion

We have described and validated a FCM EIA for quantifying cortisol metabolites in the tiger in captivity. We compared FCM levels in two sub-species (Bengal and Sumatran) in two Zoos from Queensland, Australia. We found no differences in FCM between sub-species, however sex related differences was significant. We also biologically validated the FCM EIA by demonstrating raise and return to baseline profiles of FCM in male and female tigers within 5 days with respect to blood sampling. Furthermore, the FCM EIA detected higher FCM levels in tigers that were reported healthy during the sampling period in comparison to the reportedly healthy tigers. Recently, [32] assessed FCM in tiger sub-species and found that an average FCM level in captive Siberian tigers (*Panthera tigris altaica*) at the Moscow Zoo in Russia varied over the year within a range of 363-783 ng/g of dry faeces. Although differences in the assay system employed for FCM analysis is possible, our results have provided first ever comparative analysis of FCM in Bengal and Sumatran tiger sub-species in captivity. A study by [50] reported a range of mean FCM concentrations in several carnivore species including; 234.1 ng/g dry faeces (± 11.1 S.E.M) for the domestic cat (*Felis catus*), 751.1 ng/g faeces (± 66.8 S.E.M) for the cheetah (*A. jubatus*) and 282.9 ng/g faeces (± 27.8 S.E.M) for the clouded leopard (*N. nebulosa*).

Naturally, there is a lag or delay between stimulation of the HPA axis and an elevation in FCM excreted in faeces following a biological challenge [7]. This time is equivalent to the time it takes for food to be digested and passed from the duodenum to the rectum. Assessments of this delay for various species have been widely documented in published literature, with the most relevant studies to tigers being conducted on the Siberian sub-species; which concluded an approximate delay period of 2 days [32]. Determination of this time is important in being able to identify what causes elevations or abnormally high FCM levels, and fluctuations can be pinned to specific events. A common method used to determine this

346 delay period is via an ACTH challenge [32; 40; 50]. An ACTH challenge has the potential to
347 provide useful information as to the effect that a maximal stimulus has on tigers stress levels;
348 in which if the activity proved to be stressful for the animal, there would be a peak in FCM
349 concentration following the event. Our results have shown that blood sampling initiated an
350 adrenocortical response as reflected from the results. Blood sampling most likely has had no
351 immediate or long-term consequences on the tiger's health and well-being and the methods
352 used for blood collection at both Zoos are "tiger-friendly". Our results support the existing
353 knowledge that collection of bloods from captive animals induces an stress hormone response
354 [20; 28; 41].

355 We found sex related differences in FCM levels in the captive tigers. This result is in line
356 with the growing recognition that there are these sex-differences in the excretion of FCM in
357 wildlife [23; 51]. Sex differences in FCM excretion have been documented in felids
358 generally [4]. Sex related variation in FCM may highlight underlying differences in steroid
359 metabolism, excretion routes, and pituitary responsiveness (see the latest review on this topic
360 by [17]). Females showed higher mean FCM than male tigers at both Zoos. This could be
361 due to the female's reproductive hormone cycles since cyclic fluctuations of oestrogen and
362 progesterone could influence the expression of FCM [38]. The reproductive and stress
363 hormonal axes are tightly linked and the variation in corticosteroids could reflect the different
364 phases of reproduction such as oestrous, gestation, lactation [21]. Most likely, it is the
365 increased metabolic demands associated with reproduction that could drive the variation in
366 FCM concentrations in females since cortisol is a metabolic hormone [48]. Variation in FCM
367 levels is also commonly reported to reflect reproductive status or phase (i.e. oestrous,
368 gestation, lactation). While fluctuations in reproductive hormones are believed to be the
369 primary cause for such variation, it is also thought that the increased metabolic demands
370 associated with reproduction may also drive GC concentrations during reproductive phases

[15; 47]. [6] documented a three-fold increase in faecal cortisol levels during late gestation in ring-tailed lemurs. The relationship between reproductive hormonal cycle and FCM levels in tigers warrants future investigation. Similarly, [16] found that FCM concentrations in the spotted hyena increased significantly during lactation. Various sources of stimuli could trigger adrenocortical response in captive animals and result in daily variation and elevations in FCM [45]. It is important to be able to detect these stressful stimuli in order to prevent long-term or chronic factors that has long been attributed to ill-health and failed reproduction [1; 19; 49]. Throughout the sampling period, some of the tigers were involved in various activities on particular days (such as photos, presentations or walks). There were also no exceptional events during this period such as transportation or anaesthesia that may have presented a good case for tracking changes in FCM of these individuals. We suggest that new studies could improve the study design as follows; in future all potential stimulus (even if only minor) must be recorded throughout the study period. Detailed information relating to each individual's daily activities must be kept and documented; such as recent research conducted on the captive population of Greater Bilbies (*M. lagotis*) at the Dreamworld by [34]. This design would allow for the identification of specific stressful stimuli for tigers, and to evaluate the extent to which these events or activities cause increases in FCM in individuals. Studies on non-domestic felids have concluded that stress can be ensued as a result of inadequate housing conditions and degree of environmental enrichment [29; 49]. Other factors such as visitor numbers and diet [10; 39; 43] could affect the FCM levels of captive animals hence warrant future investigation. It is possible that the huge range of variability exhibited among the tigers assessed in this study could potentially reflect either unique intra-specific metabolic patterns, or variability of their capacity to cope with stressful stimuli [49]. As shown in Tables 1 and 2, majority of the tigers from both Zoos did not have any medical conditions during this study and showed lower FCM levels hence it most likely

396 that these tigers have habituated into captivity. Interesting results found here, such as mean
397 FCM were lower in tigers that took part in activities at the Australia Zoo, could be explored
398 further to tease out whether this could be due adaptation of the tigers to interaction with the
399 public [36] or potentially result of pre-conditioning of the tigers for taking part in routine
400 activities.

401 Overall, this study has been useful in contributing to the existing literature on tiger
402 conservation physiology, which supports non-invasive FCM EIA as a useful tool for
403 assessing adrenal activity in felids [7; 32; 40; 49; 50]. For tigers, urine collection is
404 impractical as they tend to void urine by spraying, and collection of saliva still involves some
405 handling of the animal [12]. Restraint and anaesthesia have been found to temporarily
406 disturb cortisol dynamics, as these procedures themselves prove quite stressful for animals
407 [5]. Faecal based immunoassays are now recognised as the most desirable method of
408 assessing stress in mammals, especially for endangered and managed populations as they
409 provide a non-invasive alternative for monitoring stress [2; 20; 41; 50]. Another major
410 benefit of faecal methods over blood sampling is that faecal analysis of FCM provides a
411 pooled value of GC activity, as the sample represents metabolites over a period of hours;
412 compared to a value derived from blood that only represents the nadir, peak or mid-point of a
413 pulsed secretion [15]. The achievement of a high extraction efficiency (>89 %) for FCM
414 adds to the integrity of the results obtained in this study. We also recommend that future
415 studies perform additional validations using radio-metabolism to demonstrate that the actual
416 plasma cortisol were being excreted in the feces and also use High-performance liquid
417 chromatography (HPLC) to show that the R4866 antibody used does in fact detect the
418 hormone metabolites of interest [38]. Further complications with the assessment of FCM
419 measures arise when considering a range of other variables including normal seasonal and
420 daily rhythms, body condition, sample storage and treatment techniques, assay selection,

social arrangements, reproductive status, sample age and condition, and sample mass [28]. These factors therefore need to be taken into account when interpreting and making conclusions on results; further iterating the importance of obtaining data for a large sample size, including more replicates over a longer time period.

In conclusion, FCM EIA provides a useful tool for the evaluation of cortisol metabolites in tigers in relation to current husbandry procedures and management practices in captivity. As outlined in the discussion, there are many uncertainties left remaining as to causes and explanations of variations (CV %) in individual's FCM. At both locations, all of the sampled individuals were housed alternately between on exhibit areas (in view of public) and off exhibit (with no public exposure) which ensures that all tigers were exposed to the same environmental conditions throughout the duration of the sampling period. Thus we postulate that the observed variation in FCM between sexes could be related to individual personality with respect to human interactions. We also use this study as a platform to raise caution regarding uses of terms "stress" and "stressor", which are often used incorrectly to highlight that animals with a high cortisol level are more stressed or "sick" when compared to their counterparts that show lower cortisol levels. Without, appropriate health index (e.g. information that we have provided in Table 1 and Table 2), we cannot extrapolate the significance of the cortisol titres in relation to the animal's well-being. Thus, closer monitoring of individual behaviour and husbandry parameters in combination with non-invasive FCM EIA will provide an invaluable tool for tiger captive breeding programs. By identifying stressful stimuli, efforts can be made to reduce their effect or prevent their occurrence; in order to better the general wellbeing of tigers and more importantly increase captive breeding success for this iconic endangered species that is at serious risk of becoming extinct in the future without successfully managed captive populations.

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Figure Legends

Figure 1 Fig. 2A; Individual faecal cortisol metabolite (FCM) levels of male tigers during blood sampling. The pattern of FCMs of the two male tigers from Dreamworld with mild increases in FCM has been shown using dashed lines. Fig. 2B; Individual faecal cortisol metabolite levels of female tigers (all from Australia Zoo) during blood sampling. The pattern of FCMs of female tiger that showed decreasing level of FCM after day 0 has been depicted using dashed lines. Fig. 2C; Mean (± S.E.M) faecal cortisol metabolite levels of

619 male and female tigers 5 days before and 5 days after blood sampling. Day 0 denoted by
620 vertical line.
621
622 **Figure 2** Mean (± S.E.M) faecal cortisol metabolite (FCM) levels of tigers from Dreamworld
623 (n = 13) and Australia Zoo (n = 9). The symbols (circles, squares and triangles) represent
624 mean FCM value for individual tigers.
625
626 **Figure 3** Box Whisker plots with 5–95% percentile (represented by the whiskers) of faecal
627 cortisol metabolite levels of male (A) and female (B) tiger at Dreamworld. Variation (CV %)
628 for each tiger has been shown above each individual plot. Plots for male no.2 (only Sumatran
629 male) and female no.2 (only Bengal female) have been highlighted.
630
631 **Figure 4** Box Whisker plots with 5–95% percentile (represented by the whiskers) of faecal
632 cortisol metabolite levels of male (A) and female (B) tiger at Australia Zoo. Variation
633 (CV %) for each tiger has been shown above each individual plot. Plots for male no.4 (only
634 Bengal male) and female no.1 and no.5 (only Bengal females) have been highlighted.
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644 **Table Legends**

645

646 **Table 1.0** Dreamworld Tigers database showing individual tiger sex, date of birth (D.O.B),
647 sub- species, weight (kg), medical condition, activities and reproductive status recorded
648 during the study. n/a = not applicable. Individual males and females are numbered in
649 descending order of mean FCM as shown in Fig. 3A-B.

650

651 **Table 2.0** Australia Zoo Tigers database showing individual tiger sex, date of birth (D.O.B),
652 sub- species, weight (kg), medical condition, activities and reproductive status recorded
653 during the study. n/a = not applicable, - data not available. Individual males and females are
654 numbered in descending order of mean FCM as shown in Fig. 3A-B.

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669 **FIGURE 1**

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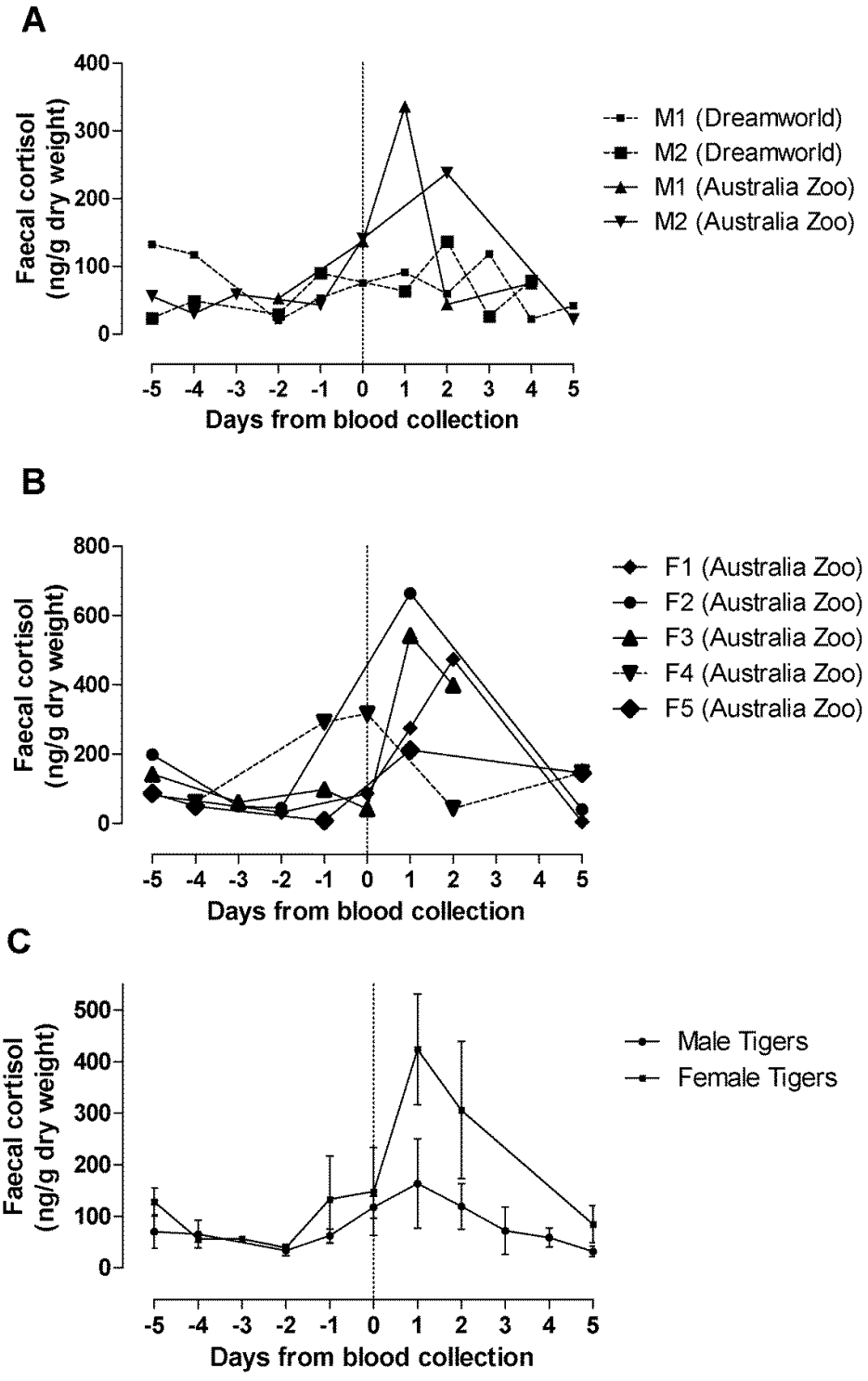

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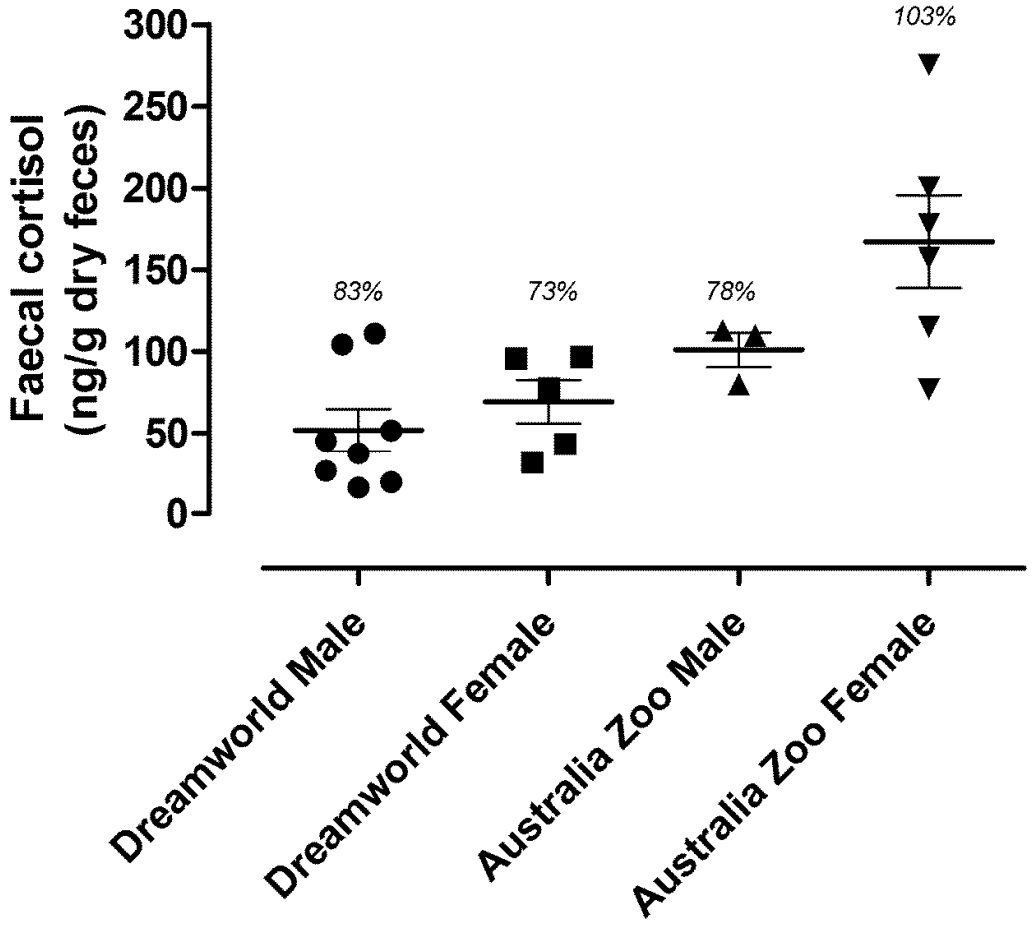
673 **FIGURE 2**



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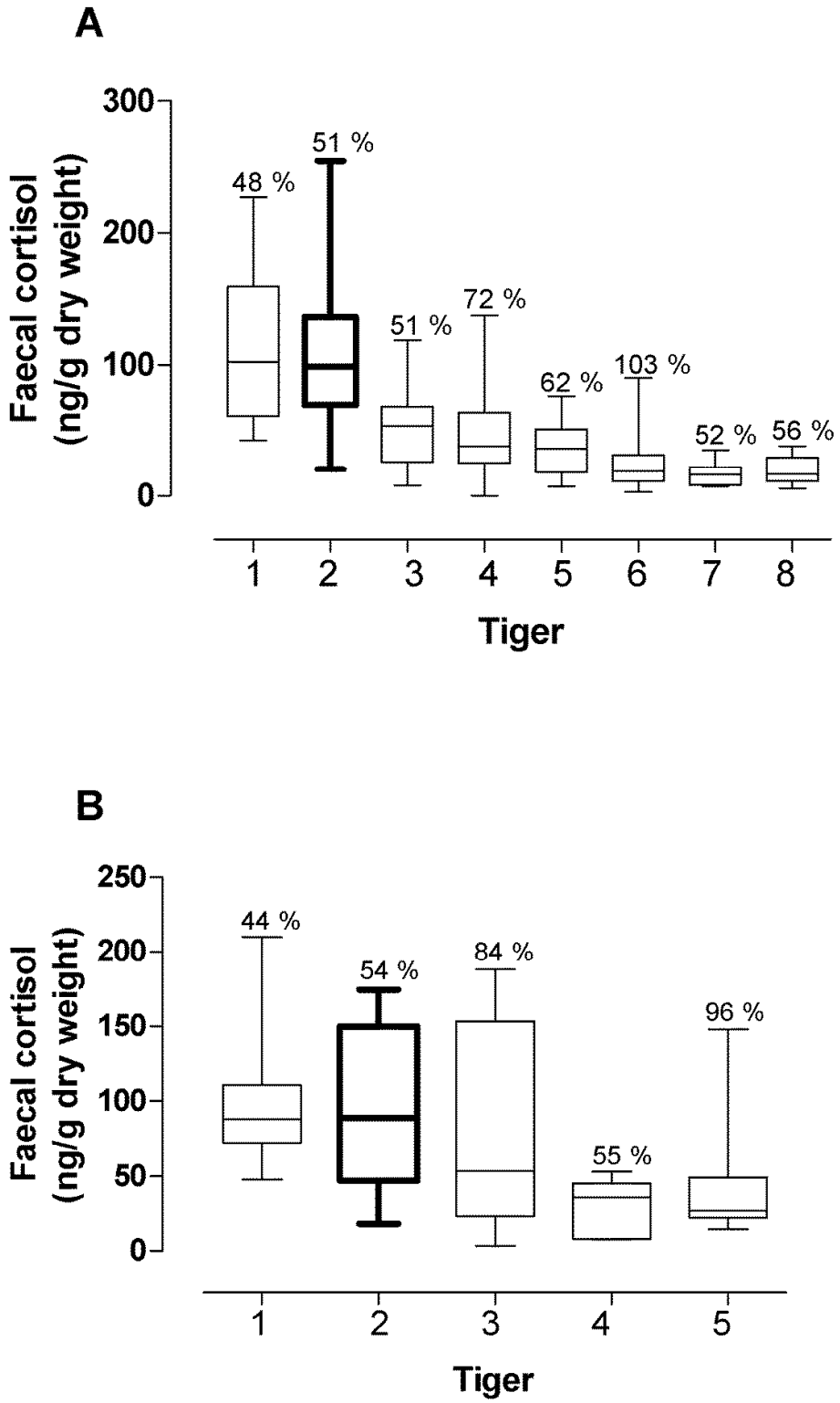
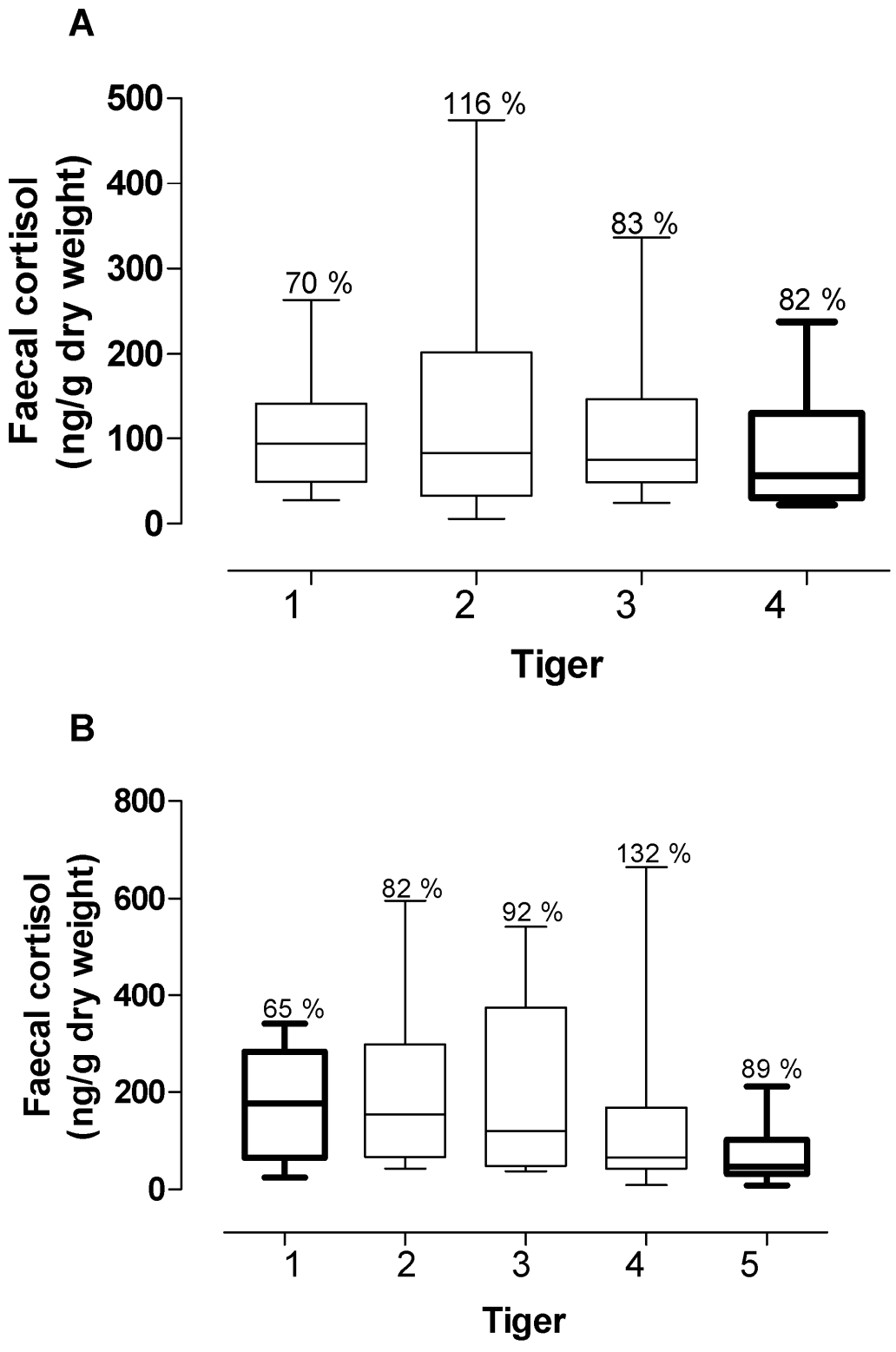
684 **FIGURE 3**



685

686 **FIGURE 4**



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Tiger	Sex	D.O.B	Sub-species	Weight (kg)	Medical condition	Activities	Reproductive status	Medications
1	F	9.6.08	Sumatran	81	Healthy	n/a	Intact	n/a
2	F	23.10.98	Bengal	145	Minor cracked foot pad	Walks, photos, presentations	De-sexed	n/a
3	F	9.6.08	Sumatran	89	Healthy	n/a	Intact	n/a
4	F	9.6.08	Sumatran	87	Healthy	n/a	Intact	n/a
5	F	31.3.07	Sumatran	88	Healthy	n/a	Intact	n/a
1	M	23.10.98	Bengal	183	Healthy	Presentations	Intact	Passed away 2012
2	M	23.10.98	Sumatran	181	Bone chip, arthritis	Photos, presentations	Intact	Mobic-daily
3	M	24.10.01	Bengal	150	Healthy	Guest feeds	Intact	n/a
4	M	23.10.98	Bengal	193	Healthy	Photos, presentations	Intact	n/a
5	M	25.12.03	Bengal	110	Healthy	n/a	Intact	n/a
6	M	2.3.12	Bengal	48	Healthy	Walks, cub experiences	Intact	n/a
7	M	2.3.12	Bengal	43	Healthy	Walks, cub experiences	Intact	n/a
8	M	26.7.10	Bengal	114	Healthy	n/a	Intact	n/a

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689 **TABLE 1**

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693 **TABLE 2**

Tiger	Sex	D.O.B	subspecies	Weight (kg)	Medical condition	Activities	Reproductive status	Medications
1	F	18.7.07	Bengal	91.5	Healthy	n/a	De-sexed	n/a
2	F	1.1.04	Sumatran	82.5	Healthy	n/a	Previously on contraceptive. Not recommended to breed	n/a
3	F	-	Sumatran	-	Healthy	n/a	n/a	n/a
4	F	4.12.07	Sumatran	78.5	Healthy	Walks, Photos	Intact	n/a
5	F	18.7.07	Bengal	89	Healthy	Walks, Photos	De-sexed	n/a
1	M	1.1.04	Sumatran	114.5	Healthy	n/a	Intact. Not breeding	n/a
2	M	1.1.04	Sumatran	127.5	Healthy	Walks	Intact. Not breeding	n/a
3	M	4.12.07	Sumatran	111.5	Healthy	Walks, Photos	Intact	n/a
4	M	18.7.07	Bengal	121.5	Healthy	Walks	De-Sexed	n/a

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