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Non-invasive Sampling Strategy for Monitoring Free-ranging Mountain Gorilla (*Gorilla berengi berengi*) Fecal Corticoid Excretion in Bwindi Impenetrable National Park, South-Western Uganda

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Abstract: Health monitoring of the critically endangered mountain gorilla (*Gorilla berengei berengei*) as one of the strategies for their conservation requires establishment of non-invasive methods that do not interfere with their natural behaviors. Therefore a fecal sampling strategy was established for use in assessing their adreno-cortical activity non-invasively by investigating corticoid distribution along the fecal strand, possible existence of fecal corticoid excretion rhythms and environmental effect. Fecal samples were collected from nests and along the trails, preserved in 99.7% ethanol and analyzed using validated ICN RIA. The results revealed that gorilla fecal corticoid concentrations increased till 72 h after defecation and thereafter began to decline towards the original levels. At 60 h the corticoid levels began showing significant difference ($p<0.001$) from the concentrations measured in samples collected at the time of defecation (0 h). There was no difference in fecal corticoid concentrations measured from Nest, Morning Trail and Afternoon Trail samples (S; $p>0.05$, SC; $p = 0.65$). There was a strong similarity (SC = 0.79 and ICC = 0.75) between corticoid concentrations of previous night nest and morning trail fecals; supporting the assumption among field workers that gorillas defecate in the nests early morning just before they leave their nests. No difference (S; $p>0.05$) in corticoid levels among the fecal strand sections was observed. Thus corticoids measured from any section of the firm fecal strand up to 60 h post-defecation effectively represent the non rhythmic fecal corticoid excretory profile, offering the best non-invasive and non-intrusive sampling strategy for study of corticoids in this species.

Key words: Corticoid degeneration, distribution along fecal strand, excretion rhythm, ICN RIA, nests, trails

INTRODUCTION

The Bwindi gorilla population is estimated to be ~324 individuals (Gray *et al*., 2007) and inhabit 330 km^2 Bwindi Impenetrable National Park (BINP) in southwestern Uganda. Until 1992, the Bwindi gorillas were considered to be Eastern lowland gorillas (*Gorilla gorilla graurer*) (Cousins, 1990). Although Sarmiento *et al*. (1995) supported this classification based on morphological traits, this population was till recently grouped with the Virunga Massif Mt. gorillas (*Gorilla gorilla beringei* population for the purposes of conservation management based on the phylogenetic study of Karen *et al.* (1996). Since then, the conservation management strategies which had been developed for the Mt. gorillas inhabiting the Virunga Massif were adopted, and the current on-going reclassification of the Virunga and Bwindi gorillas as *Gorilla beringei beringei* and *Gorilla beringei* "unnamed" respectively (Groves, 2001) has not changed the status quo in Bwindi. Consequently many conservation programs and activities including intense habitat management, legalization of access to resources by local people in multiple-use zones, gorilla habituation and controlled eco-tourism, increased research and veterinary interventions have been developed. Despite the intensification of management strategies illegal activities also persist in this habitat. On the other hand the small size of the park coupled with loss of fear for man

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due to habituation makes it easy for gorillas to frequent crop fields and other areas close to human habitations (Goldsmith, 2000), raising the need to intensively manage human-gorilla (HUGO) conflicts. Though natural; the recent stead increase in gorilla population (Gray *et al*., 2007) has the potential to increase intra-specific conflicts and changing of foraging patterns by families. Taken together all these issues and activities render the Bwindi gorilla ecosystem extremely dynamic, raising the necessity to establish intense long-term health monitoring programs in order to ensure the survival of this critically endangered species whose total population number only 750 individuals.

The general health and stability of a population is reflected in its capacity to reproduce as reduced reproductive capacity is a non-life threatening aspect of adaptive physiology which is usually the first and almost only physiologic loss resulting from severe stress (Moberg *et al*., 1985; Wasser *et al*., 1993). For this reason methods for monitoring and assessing reproductive status have been developed to allow both captive and free-living wildlife to be evaluated while avoiding capture technology. There exist sufficient amount of data to suggest that in general the excretion profiles of steroid hormones reflect gonadal activity and provide an accurate assessment of over all endocrine status. There is a broad application of these methods to a wide range of species and of recent has been broadened to include monitoring of adrenal activity in various species.

Monitoring an animal's stress physiology is essential to understanding and improvement of the well-being and reproduction in captivity, as well as monitoring impacts of social and environmental stressors on behavior, health and reproduction in free-ranging populations (Bahr *et al*., 2000). While the necessary restraint techniques employed in invasive methods can affect physiological parameters in animals; non-invasive methods provide an alternative for assessing endocrine status in undisturbed individuals. Since the adrenal gland plays a major physiological role to an animal's response to stressors, it is important to have in place a non-invasive tool with which to investigate the gorilla adrenal activity; as a step towards establishing a program to monitor the response and impact of intra-specific conflicts and environmental stressors on this endangered species' dynamic habitat.

Two steroid hormone characteristics are currently being exploited to develop general evaluative strategies applicable to almost all mammalian species. The first is the common trait that steroids have the same molecular structure through the animal kingdom. Secondly, the steroid hormones are secreted into the vascular space and then cleared within minutes after undergoing only modest physical change. The resulting metabolites are concentrated either in the urine and/or feces and are relatively stable for a long time. The excreta containing the metabolites can be collected and preserved and measured in the excreta by utilizing a relatively small number of similar assays (Lasley *et al*., 1991). This strategy was first applied to humans and domestic species when most of the modern analytical methods were still insufficiently sensitive to detect steroid hormones circulating in the vascular bed. With the advent of Immuno-assays, methods became more sensitive and measuring the active substance in circulation became more important and excretion profile monitoring was abandoned.

Of recent there is recognition of advantages to collecting and analyzing excreted steroid metabolites if the non-invasive sampling strategy allows identification of samples to individual level. Human epidemiologists have their subjects participate in collection of their own samples and temporarily store them in the freezers; while zoo keepers adapt their daily routines to accommodate urine or fecal sampling needs (Loskutoff *et al*., 1982; Loskutoff *et al*., 1986) or can train the subject to give the sample at a command (Walker *et al*., 1998). However the scientists working in the field must follow closely behind their subject and collect samples opportunistically from identified individuals (Poole *et al*., 1984; Kirkpatrick *et al*., 1988). The samples can be stored with preservative or simply frozen without preservatives until assayed; and at the current rates of development, assays will soon be field based, eliminating the need for sample storage and transportation costs (Lasley *et al*., 1991). The application of non invasive endocrine assessment to free-ranging wildlife are numerous and increased emphasis on understanding the biology of free-ranging endangered species such as Mt. gorillas also provides a useful application for this non capture technology and collection and analysis of fecal samples stand a good chance to be employed in adrenal activity monitoring as a long-term health management tool of gorillas in range countries.

Quantifying corticoids in feces has got challenges which should be addressed before wide application in the field. Fecal steroids can appear un-conjugated in excreta; in part because bacterial enzymes hydrolyze or de-conjugate steroid conjugates in the large intestine. Bacterial-derived desmolase in humans and rats has been shown to remove side chains from some C-21 steroids (Cerone-Mclernon *et al*., 1981; MacDonald *et al*., 1983). Similarly this enzyme has been shown to impact the quantification of steroid metabolites in cows when feces were maintained at room temperature (Möstl *et al*., 1999) who also demonstrated that enzymatic activity was still active after freezing, but that enzyme activity was abolished by heating at (95^{o}C) and once enzymatic activity was abolished steroid metabolites were quite stable. Clearly, the stability of steroid metabolites is an

important consideration for practicability of the assay techniques in wildlife where the time interval from defecation to sample collection is unknown or variable (Möstl *et al*., 1999). Adopting standardized collection and storage methods can minimize the potential impact of bacterial degradation on steroid metabolites which continues after defecation. It is always advisable to collect fecal samples as quickly as possible after defecation, followed immediately by treatment to minimize continued bacterial degradation. In situations where fecal samples cannot be collected within 1-2 h post-defecation, the impact of "time from defecation to sample collection" on hormonal metabolite concentrations should be tested systematically.

Fecal storage and preservation methods range from simple freezing (liquid nitrogen tank or household freezer), drying in a portable oven, preservation in alcohol, or using simple field extraction methods (Whitten *et al*., 1998). Surprisingly there were no systematic comparisons among preservation methods that had been published by the conception of this study 1in 1999. Though Khan *et al*. (2002) evaluated the storage of baboon fecal samples; there is still no consensus on the recommendation for field use. Bwindi gorillas are in a very remote area; far from electricity and liquid nitrogen production plants thus freezing samples as the most preferred preservation choice is not a possibility. In short, fecal preservation methods should be thoughtfully considered and these methods should be validated for each species and specific hormone metabolites of interest before large-scale application to a field setting (Khan *et al*., 2002).

Due consideration should be paid to examining the possible existence of diurnal rhythms for steroid hormone excretion and this is even more crucial under field conditions where sampling is often conducted in an "opportunistic" fashion (Sousa and Ziegler, 1998). Previous work with captive gorillas suggested that adrenal status is best evaluated from samples collected during the later afternoon to evening because cortisol excretion during the early part of the day is highly variable (Czekala *et al*., 1994). In humans, research work conducted under controlled conditions have concluded that single urine samples collected from 22:00-23:00 hours reflect corticosteroid excretion at its nadir (Contreras *et al*., 1986; Zis *et al*., 1989). In related studies, diurnal fecal glucocorticoid fluctuations in common marmosets revealed that sampling should be limited to either morning or afternoon in this species (Sousa and Ziegler, 1998). However Goyman *et al*. (1999) suggest that usual hormonal fluctuations due to secretory patterns are attenuated in feces.

Most of the time Mt. Gorilla fecal material is deposited as a strand of tri-lobed segments joined together by undigested plant material. This is especially so during the dry season when their diet is less in succulent plants. This characteristic presents problems when attempting to mix the feces from which to pick a representative field sample. However during the fruiting season when their diet is rich in fruits, the fecal material is mixable and at times lacks form. Sometimes gorillas let out diarrheic fecal material, which are also easy to mix. Nonetheless there is need to investigate presence of hot spots in an attempt to find out whether fecal corticoid metabolites are evenly distributed in well formed firm fecal strands.

Permission to follow Mt. gorillas closely in their habitat is granted only for research groups. For groups used in eco-tourism, regulations that restrict the number of visitors and the one-hour admissible to stay with the gorilla family makes it hard for scientists to evaluate their health status on long-term basis without compromising the park management regulations. This demands that non-invasive field sample and data collection techniques be validated and training given to park staffs who come in touch with these animals on daily basis. Such staffs in Protected Areas are increasingly becoming adaptive to generating field data which is of scientific value when using standardized protocols and portable field technologies.

The aim of the current study was thus to establish a fecal sampling strategy to non-invasively monitor the Mt. gorilla adrenal corticoid metabolites by investigating:

- The hormonal distribution along the fecal strand
- The effect of the environment on the fecal adrenal corticoid concentrations in order to establish the appropriate sampling time frame
- The fecal corticoid excretion rhythms and comparing two preservation methods namely 99.7% ethanol at ambient temperature and 99.7% ethanol at sub-zero temperatures

Materials and methods: Permission was received from Uganda Wildlife Authority (UWA) in 2000 to carry out the study on identifiable individuals in two habituated gorilla families freely ranging in Bwindi Impenetrable National Park (BINP). These included Nkuringo family; then undergoing habituation process, this was used to study the fecal corticoid distribution and degradation. The second was Mubale; a tourist family whose samples were used to investigate the fecal corticoid diurnal excretory rhythms and comparison of fecal sample preservation techniques. In addition samples were collected for over a year from both families and used to investigate the possible existence of any seasonal variation in fecal corticoid excretion.

In general gorilla tracking began at 8.00 am from the park office or ranger post to the area where the family was left feeding the previous day. The gorilla trail was followed till the nesting site was reached, there after the morning trail was followed till contact was made with the gorilla family. Before sampling, the whole fecal material

was observed; and the consistence noted. The fecal location was noted as either being on the trail (T) or in the nest (N). Firm fecal material was dissected using two pieces of wooden spatula and /or loose feces mixed and there after samples picked. Five-ml cryogenic vials (# 430663; Corning Incorporated, Corning, NY 14831) were filled to three-quarter mark. A thinly split tongue depressor was used to create a narrow canal in the sample material from top to the bottom of the vial to facilitate perfusion and immediately 99.7 % Ethanol (BDH lab supplies, Poole, BH15 1TD, England) was added up to the 5 mL mark. The vials were capped, labeled and left at ambient temperature till they were transported to the Smithsonian Institution's Centre for Conservation and Research (CRC, Front Royal,Virginia USA) for laboratory processing and analysis.

Formally validated Munro cortisol EIA (In house Assay Services Unit, National Primate Research Centre, University of Wisconsin, Madison) and ICN corticosterone RIA (ICN Biomedicals, Inc., Diagnostics Division, 3300 Hyland Avenue, Costa Mesa, CA 92626) kits were used to quantify the Fecal Glucocorticoid Metabolites in a preliminary and final study, respectively.

Specific study design: To investigate whether Mt. Gorilla fecal corticoid concentrations are evenly distributed along firm fecal strands, the night nest of the Silverback was identified with help of guides and trackers, confirmation was made by finding silver hair and measurement of fecal lobe diameter corresponding to Silverback age group (7.5 cm). Fecal strand lay out was observed to earmark the Proximal (P), Middle (M) and Distal (D) sections so named according to their location from the animal at the time of defecation (P: The part that was the last to exit the anal orifice, identified by its pointed end due to an impression formed by the closure of the anal sphincter muscles; D: The part that was the first to exit the anal orifice, identified by its convex end due to an impression formed by forceful opening of the anal sphincter by the abdominal pressure; M: The mid region between P and D). A total of 48 triplets representing 16 samples were taken from the three sections, preserved and analyzed with Munro Cortisol EIA in a preliminary study and a total of 94 samples in duplicates of D and P were taken and analyzed using ICN RIA in the final study following validation of the two assays for this species. Analysis of Variance was run to find out whether the corticoid concentrations were significantly different between the sections.

To investigate whether there is a diurnal rhythm in Mt. Gorilla fecal corticoid excretion, comparison was made between corticoid levels of samples collected from night nest, morning and afternoon day-trails.

- Permission to carry out minimally intrusive sampling before, during and after tourist visits was granted by UWA
- Mubale gorilla family was tracked and samples collected from night nest of the silverback
- The family was then contacted and followed closely in the morning (07:00-11:00 h) and effort was made to collect fresh feces when the study animal was observed defecating and time noted
- Similarly trail samples were collected in the afternoon (12:00-15:00 h). Sampling was not done beyond 15:00 hours in order to minimize disturbance on gorilla daily activities
- All samples were preserved, processed and analyzed using ICN RIA kit, and results were analyzed by Analysis of variance for differences using Wilcoxin Signed Rank (S), Spearman's Correlation Coefficient (SC), and Intra Class Correlation Coefficient (ICC)

To investigate the time range in which effect of the environment on free-ranging Mt. gorilla fecal glucocorticoid metabolites degeneration is minimal:

- Minimally intrusive sampling was carried out in Nkuringo gorilla family
- Identifiable individuals were followed and observed for defecation at a distance of not less than five meters
- Immediately upon defecation, the identity of the animal and time of defecation were noted, a fresh sample was taken and preserved in 99.7% ethanol , and the fecal age (hours after defecation) was noted as 0 h (also called first sampling)
- A polythene bag was used to collect and carry the rest of the fecal material and deposited back in the forest habitat about 20 m from camp to facilitate re-sampling
- Subsequent samples were taken and preserved every after one hour for 12 h, and thereafter every 6 h for 7 days. All samples were processed and assayed with ICN RIA. One Way Analysis of Variance was run to investigate the differences, and All Pairs Multiple Comparison Procedures (Turkeys Test) was used to identify the critical time period when fecal corticoid concentrations significantly differed from the original concentrations

To investigate whether there is an underlying seasonality in Mt. Gorilla fecal corticoid excretion: Fecal samples were collected for a year from nests of silverback, adult nursing female and its infant in Mubale family, and from two silverbacks of Nkuringo family. Daily weather data was recorded for the Buhoma station where Mubale ranges and for Nkuringo ranger station where Nkuringo ranges. Sample extracts were assayed using ICN RIA. Correlation analysis was run to investigate relationship between individual's monthly mean fecal glucocorticoid levels and means of rainfall and temperatures.

RESULTS

Field fecal preservation methods: Results comparing fecal samples preserved in 99.7% Ethanol at ambient temperatures versus duplicate samples preserved in 99.7% Ethanol but stored under sub zero temperatures revealed that there was no difference in fecal corticoid concentrations ($p>0.05$ n = 30). For the rest of the study, samples were preserved in 99.7% Ethanol at ambient temperature because it was difficult to maintain a cold chain for many samples due to the remote location of BINP.

Fecal corticoid distribution along firm fecal strands: In a preliminary study, corticoid concentrations in the proximal (P), middle (M) and distal (D) sections of well formed fecal strands were compared. Analysis of Variance indicated that there was no significant difference in the corticoid concentrations between the 3 fecal segments (df = 15, p = 0.5399). For a more elaborate study, samples from two sections of the fecal strands (D and P) which are farther apart were used. Data was generated from 45 paired samples from silverback Nkuringo, and the average standard deviation from the means and percentage Coefficient of Variation (%CV) were 12.90 and 14.65, respectively. For the second silverback Safari data was generated from 49 paired samples and the average standard deviation from the means and %CV were 25.28 and 12.76, respectively. All data was subjected to SAS analytical software and matrices of the p values of the different tests generated (Table 1).

The Signed Rank test (S) p values in both Nkuringo and Safari silverback indicated that there was no significant difference between the corticoid concentrations from the proximal and distal sections of the fecal strands. For each silverback the Spearman's Correlation Coefficient ($p>0.5$) indicated that patterns were maintained whether corticoids were only measured from either the proximal or distal sections of the fecal strands. Finally the Intra Class Correlation Coefficient ($p>0.5$) indicated that the fecal corticoid levels in the paired samples were equivalent and values could be used interchangeably. Taken together, the results showed that for firm gorilla fecal material, a sample taken from any part of the fecal strand or fecal lobe will generate data which is a representation of the individual's fecal corticoid excretion profile.

Fecal corticoid excretory rhythm: During the study period of 15 months, 473 samples from Mubale Silverback were collected, only 186 (39.32 %) were morning trail(TAM) samples which were recovered only in 30 sampling days out of 244 sampling days. Only 8 sampling days had 3 TAM samples each and 2 days had up 4 TAM samples each. There were only 32 (6.77%) afternoon trail samples (TPM), out of which 10 sampling days had 2 samples each, while 3 sampling days had up to 3 samples each. This shows that the rate of recovering trail samples in free-ranging Mt. gorillas is low compared to recovery of nest samples.

Table 1: Matrix of p-values for comparison of corticoid concentration in fecal strand sections

	Silverback Nkuringo			Silverback Safari	
	Test	P	D	P	D
Proximal (P)	SC	1.0		1.0	
	ICC	1.0		1.0	
Distal (D)	SC	0.73	1.0	0.82	1.0
	ICC	0.69	1.0	0.81	1.0
	S test (p = 0.41) N = 45			S test (p = 0.90) N = 49	

SC: Spearman's Correlation Coefficient to test whether measured concentrations maintain a pattern between the paired samples; ICC: Intra Class Correlation Coefficient to test how far paired values are from each other (p = 1.00 representing a mirror image of each other); S: A non-parametric Wilcoxin Signed Rank test to test whether there are significant differences between concentrations measured in each pair

Table 2: Matrix of p values for comparison of diurnal fecal corticoid excretion by Mubale Silverback

	Test	N	TAM	TPM
Nest (N)	SC	1.0	S (p = 0.50)	S (p = 0.99)
	ICC	1.0		
Morning trail	SC	0.79(n =124)	1.0	S (p = 0.64)
(TAM)	ICC	0.75	1.0	
Afternoon trail	SC	0.45 (n = 32)	0.75 (n = 15)	1.0
(TPM)	ICC	0.61	0.53	1.0

S: Wilcoxin Signed Rank; SC: Spearman's Correlation Coefficient; ICC: Intra Class Correlation Coefficient

Analytical tests were used to compare fecal corticoid concentrations from nest, morning trail and afternoon trail samples and the p values are presented in Table 2. The Signed Rank test (S) indicated that there were no differences between the fecal corticoid concentrations measured in samples collected from nests and trails, and between concentrations measured from morning trail (TAM) and afternoon trail (TPM) samples ($p\geq0.50$). The Spearman's Correlation Coefficient (SC) indicated that measured concentrations maintained patterns between nest and morning trail samples, and between morning and afternoon samples ($p\geq0.50$), but maintenance of the pattern was less evident between nest and afternoon trail samples (p = 0.45). Finally the Intra Class Correlation Coefficient (ICC) indicated that the fecal corticoid levels measured in samples collected from the three locations were similar and values could be used interchangeably ($p\geq0.50$). The mirror image of each of the paired data under investigation would yield an ICC's p value of 1.0. The SC and ICC of 0.79 and 0.75, respectively (Table 2) were the highest for paired data comparisons and these indicated a stronger similarity/association between fecal corticoid levels in nest and morning trail samples. Taken together, the results showed that samples taken from any

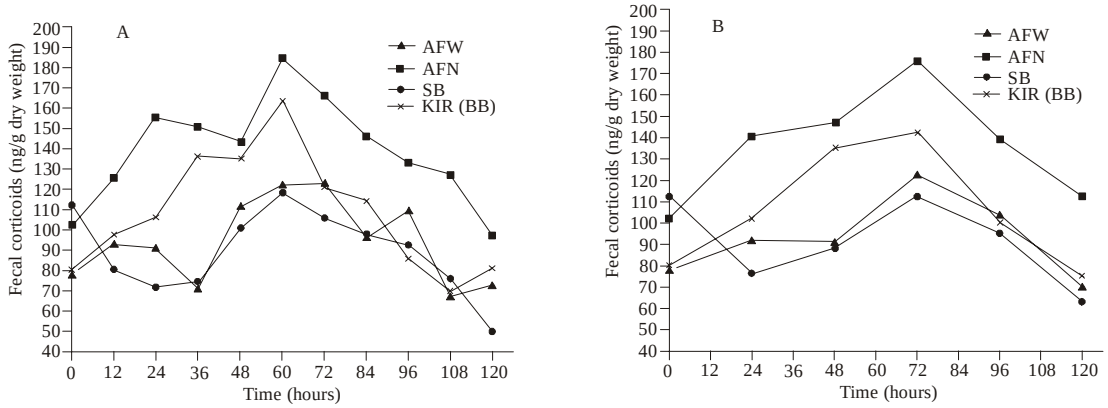

Fig. 1: Mt. gorilla fecal corticoid degeneration in the environment, a): Every 12 h corticoid means; b): Every 24 h corticoid means; AFW: Adult Female Wagen; AFN: Adult Female Nkuringo; SB: Silverback Nkuringo; KIR (BB): Black Back male KIR

Table 3: Seasonal mean fecal corticoid excretion of identifiable gorillas in Mubale and Nkuringo gorilla families

Months in 2002	Season	Buhoma Mean seasonal Rainfall (mm)	Mubale mean seasonal fecal corticoid excretion (ng/g)			Nkuringo mean seasonal rainfall (mm)	Nkuringo mean seasonal fecal corticoid excretion (ng/g)	
			S.B	AF	INF		NK	SAF
Jan-Feb.	Short dry	73.83	131.61	130.02	155.18	135.6	92.63	89.53
Mar-May.	Long wet	203.5	113.29	106.69	149.39	184.2	145.07	142.28
Jun- Sept.	Long dry	96.8	121.14	143.19	180.96	87.6	135.52	130.47
Oct- Dec.	Short wet	246.7	156.76	178.42	182.7	228.9	174.56	178.68

S.B: Mubale Silverback; AF: Adult female; INF: Infant; NK: Nkuringo Silverback; SAF: Safari Silverback

of the three locations representing the sampling time frame will yield the true representation of the fecal corticoid excretory profile in this species.

Mountain gorilla fecal corticoid degeneration in the environment: A time series study was carried out on fresh samples collected from four individuals of the same family and fig. 1 presents a summary of results grouped in 12 and 24 h mean concentrations. Generally after defecation, the fecal corticoids measured by the ICNRIA increased to maximum levels attained at 72 h before beginning to level down to the original concentrations (the slight exception noted with Silverback samples may be due to procedural issues). All raw data was analyzed with Sigma Stat, and for all the study subjects One Way Analysis of variance indicated that there was strong significant differences ($p<0.001$, $n = 32$) in the mean fecal corticoid metabolite concentrations measured in the samples taken from the same scat but varying on the time duration the fecal materials were exposed to the environment before being preserved. Similar results were obtained when data was grouped and analysis made between the mean concentrations of every 12 h ($n = 11$) or 24 h ($n = 6$). All Pairs Multiple Comparison Procedures (Turkey Test) was run to isolate which time period when concentrations were different from the original concentration (0 h). Results clearly indicated that gorilla fecal corticoid levels measured from samples collected every after 1 h for 12 h didn't differ from amounts measured at the time of defecation ($p>1.00$). The break-away point when the concentration of fecal corticoids significantly differed ($p<0.001$) from the amount measured in the same fecal material collected at the time of defecation (0 h) was at 60 h.

Seasonal variation in Mountain gorilla fecal corticoid excretion: The study subjects included 3 individuals from Mubale and 2 from Nkuringo families whose ranging areas experienced an annual rainfall of 1885.47 mm and 1860.25 mm respectively in 2002. There was no relationship observed between the mean monthly fecal corticoid excretion with either rainfall or temperatures in both families ($p>0.005$). In Mubale, the short-wet season was warmer with a maximum of 25ºC and an optimum temperature of 19.19ºC. In Nkuringo the Long-dry season was warmer reaching a maximum of 25.07ºC and an optimum temperature of 19.42ºC. The data was pooled and mean seasonal concentrations calculated. Table 3 shows that for all the animals investigated, the highest mean seasonal fecal corticoid excretion was recorded during the short-rain season. In Mubale, the individuals' highest mean seasonal fecal corticoid excretion was recorded in the short-wet season which corresponded to the highest mean seasonal rainfall and mean maximum temperatures, however in Nkuringo the individuals' highest mean fecal corticoid excretion corresponded only with the highest mean seasonal rainfall but not with highest mean seasonal temperature which was recorded in

the long dry season. It is important to note that the mean altitude for Nkuringo zone is higher than that of the Mubale zone with 1800, 1500 m a.s.l, respectively.

DISCUSSION

It is widely accepted that freezing of samples immediately after sampling is the most preferred method of preservation though only few studies have systematically compared field preservation methods. In a preliminary study which compared fecal corticoid concentrations in Mt. gorilla fecal samples preserved in 99.7% Ethanol at ambient temperatures versus duplicate samples preserved in 99.7% Ethanol stored at sub zero temperatures revealed that there was no difference in fecal corticoid concentrations. These results should be interpreted with caution as the study generated limited data due to the difficulties associated with maintaining a cold chain for many samples in the study area. In addition samples were stored for more than 6 months before analysis. Khan *et al*. (2002) suggested that ideally ethanol preserved samples at ambient temperature should not exceed 30 days and the ones for long term preservation at -20ºC should not exceed 90 to 120 days before being analyzed if the hormones of interest are fecal glucocorticoids. However the same authors recognized that their results from the baboon study tended to suggest that Ethanol preserved samples left at ambient temperature storage for 6 months or longer was a possibility because they observed that the final concentrations of fecal corticoids were approaching those of the initial concentration after 180 days of their experiment. Nonetheless they cautioned that validation was necessary for each species and for the corticoid metabolite of interest before such strategy is employed for long-term hormone monitoring. Bwindi Impenetrable National Park is about 150 km from a reliable source of electricity, and approximately 600 km from the nearest Liquid Nitrogen plant and such location presents difficulty in maintaining a cold chain. Ethanol preserved samples left at ambient temperatures was chosen for the rest of the samples analyzed in the current study, and a more thorough comparative study of the two preservation methods should be done on either Nyakagezi gorilla family in Mgahinga National Park (Uganda), or on gorillas in Parc Nationale des Volcans (Rwanda) where the source of electricity is nearer.

Through regulations permitting accessibility to Mt. gorillas for research; the park management agencies encourage development of non-invasive, non- intrusive research, while keeping opportunistic and minimally invasive research options open. It is essential that samples for hormonal studies be linked to the individual, so establishing an absolute non-invasive; non-intrusive field sampling technique has obstacles to overcome though the possibility exists in small gorilla families with a known family structure. In such family an absolute non-invasive design would employ night nest fecal samples, but has an inherent problem of not knowing the time when defecation takes place and therefore how long the fecal material has been exposed to the environment. During gorilla census exercise which takes place every 5 years, scientists back-track a gorilla family to three previous consecutive nesting sites (72 h) in order to establish the family composition. Fecal samples are often collected from such nest sites for analysis to answer particular research questions, and it was paramount in the current study to establish whether or not such samples can be viable for generation of true hormonal steroid profiles of the sampled animals. The current results have revealed that after defecation Mt. gorilla fecal corticoids will increase till 72 h and then begin to decline towards the original levels. The time period at which the concentrations began indicating a significant difference from the concentrations measured at the time of defecation was 60 h. In theory, this suggests that samples collected up to the 2 previous nesting sites (48 h) from identifiable individuals would be viable for fecal corticoid analysis. However for practical purposes, feces from the previous day's nest (12 h) should be sampled. This was support by the fact that there was no difference between corticoid levels measured every 1 h for 12 h and those measured from the samples collected at the time of defecation. In addition, there was a high similarity/ association between corticoid levels measured from nest and morning trail samples, the latter being the sampling design of choice if gorilla tracking regulations and management permitted intrusive sampling.

Samples that were analyzed in the time series study were collected in July 2001 and preserved in Ethanol at ambient temperatures up to March 2003 when they were processed and there after stored at sub-zero temperatures. The fact that such samples preserved for over 1 year could still reveal similar trends as documented in the baboon fecal preservation experiment carried out by Khan *et al*. (2002) suggests that Ethanol can be used for long-term storage of Mt. gorilla fecal samples as has also been demonstrated in the Ring-tailed lemurs (*Lemur catta*) by Cavigelli (1999), and in Mongoose lemurs (*Eulemur mongoz*) by Curtis *et al*. (2000).

In view of the suggestion that preservation can not stop enzymatic process of steroid hormone degradation other than slowing it down as advanced by Möstl *et al*. (1999) and also observed by other authors (Cerone-Mclernon *et al*., 1981; MacDonald *et al*., 1983), it would be interesting to find out the type of metabolites in Mt. gorilla fecal samples detected between 60 and 72 h of exposure to the environment which attained a rather maximum stability that could still be detected after over 1 year of storage in Ethanol. However since the ICN RIA

used is non-specific, it may be that in this species the assay kit cross-reacts with a family of metabolites originating from the same parent hormone molecule such as has been documented in other studies (Palme and Möstel, 1997; Schwarzenberger *et al*., 1997; Wasser *et al*., 2000). The trend observed in the current time series study could have been showing different sensitivities to changing fecal corticoid types as noted by Khan *et al*. (2002). Because of lack of field based assay kits, it was not possible to measure the fecal corticoid concentrations in non preserved fecals at each sampling time for comparison with the current data generated from preserved portions. Such design would have assisted in verifying whether the impact of ambient temperature on the pattern of quantified fecal corticoids in these preserved samples is similar or different from the pattern observed in quantifying steroid metabolites on non preserved samples such as has been documented by Möstl *et al*. (1999).

Comparison of fecal corticoid concentrations of triplicates in a preliminary study revealed no difference. When we considered results of duplicate samples of Proximal and Distal portions of the fecal strands, the Signed Rank test showed that there was no difference in corticoid concentrations between the two portions. Similarly the Spearman's correlation coefficient revealed that a pattern would be maintained if samples were taken consistently from one section of the fecal strand. Also a high Inter Class Correlation coefficient revealed that the corticoid concentrations in the two sections (P&D) of fecal strands were similar and could be used interchangeably. Therefore for the long-term fecal corticoid monitoring, a sample taken from any section of the firm fecal strand should be enough and is capable of yielding the true excretion profile of the fecal corticoids in this species. However there would be need for validation and verification if a different class of steroids was to be measured in Mt. gorilla feces.

Though results have shown that there was no difference in the corticoid levels measured in samples collected from nests and trails, and that a pattern was maintained if samples were taken consistently from each location, and that results could be used interchangeably between locations, the strong association between Nest and Morning trail samples revealed by the highest SC and ICC p-values of 0.79 and 0.75, respectively provides the first researched information which supports the long-standing assumption among field workers that gorillas defecate in the nest during early morning hours just before they leave their nests. Taken together, the study has shown that samples collected from either the nest or the trail, or taken at any time-frame consistently will yield the true representation of the fecal corticoid excretory profile in this species.

The free-ranging Mt. gorilla defecation rate has not been studied though the gut transit time is expected to fall within 24 to 72 h as it is for other non human primates. The Mt. gorillas seem to deposit much of the fecal material in their nests, consequently there was low rate of sample recovery from the trails. This led to generation of limited data that was not enough to investigate whether there exists great variability in corticoid concentrations during morning hours which was documented in Mt. gorilla urinary cortisol studies (Czekala *et al*., 1994; Robbins and Czekala, 1997). Nevertheless the data in the current study was adequate to demonstrate that there was no diurnal corticoid excretory rhythm expressed in the fecal medium in this species. However if at all there exists diurnal rhythms in corticoid secretion, the current study results could mean that it is fully attenuated by pooling of feces in the gastro-intestinal system; which is one of the major advantage of measuring excretion profiles.

The annual rainfall for Buhoma tourist zone and Nkuringo in 2002 were 1885.47 mm and 1860.25 m, respectively. This is within the expected climatic range for Bwindi as described by Howard (1991) and Uganda National Parks (1993); that is being tropical with two rainfall peaks from March to May and September to November, with an annual precipitation which ranges from 1,130 to 2,390 mm and annual mean temperature ranges from a minimum of 7-15ºC to a maximum of 20-28ºC, however our investigation found out that there was no relationship between excreted mean monthly fecal corticoid levels and rainfall. Though for all subjects the highest mean seasonal fecal corticoid excretion levels corresponded to the short rain season, there was no relationship between seasons and fecal corticoid excretion. Other factors in the environment could be responsible for the trends observed in the current results. On the hand seasonal changes in a tropical environment are not so dramatic and may not offer an effective stressor that elicits adrenal activity so strongly as to be reflected in the pooled fecal corticoid metabolites. In the north and southern hemisphere where seasons are quite distinct, studies of seasonal effects on steroid hormone secretion and excretion have concentrated on reproductive hormones due to their inherent association with behavioral evidence of the breeding season but not much has been done on stress hormones in this respect.

CONCLUSION

In conclusion the study has proven that Mt. gorillas defecate in the nests during early morning hours just before they leave their nests. There is no observable diurnal and seasonal fecal corticoid excretion and corticoid concentrations measured in any section of a firm fecal strand effectively represent the overall corticoid

excretory profile. Fecal samples are viable for corticoid analysis up to 60 h post defecation but for practical purposes overnight nest (~12 h) samples should be utilized as they yield similar corticoid excretory profile as the morning and afternoon trail samples; thus far offering the best non-invasive and non-intrusive sampling strategy for monitoring adrenal activity in this species.

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