ASSESSMENT OF CORTISOL HORMONE LEVELS AMONG CAPTIVE GORILLA USING SALIVA AND URINE CORTISOL

BY

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DECLARATION

I, **Namirimu Solome**, do hereby declare that this dissertation is original and has never been submitted to any other university/institution for any degree award.

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DEDICATION

This work is dedicated to my lovely mother Mrs. Edith Sekiwano and to my late father Mr. Nelson Sekiwano.

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LIST OF ABBREVIATIONS

ACTH	Adrenocorticotropic Hormone
AVP	Arginine Vasopressin
BINP	Bwindi Impenetrable National Park
CBC	Corticosteroid Binding Globulin
CRF	Corticotrophin Realizing Factor
CRH	Corticotrophin-Realizing Hormone
DRC	Democratic Republic of Congo
DSL	Diagnostic Systems Laboratories
EIA	Enzyme Immuno Assay
ELISA	Enzyme-Linked ImmunoSorbent Assay
HPA	Hypothalamic-Pituitary- Adrenal Axis
IUCN	International Union for Conservation of Nature
ml	millitre
OD	Optic Density
rpm	Revolutions per minute
RUFORUM	Regional Universities Forum for Capacity Building in Agriculture
TMB	TetraMethylBenzidine

ABSTRACT

Mountain gorillas (*Gorilla berengei berengei*) and Lowland gorillas (*Gorilla berengei graueri)* are the world's most endangered ape species. Both species have experienced poaching of adults and illegal trade. Confiscation of infants from illegal holders resulted in gorillas being kept in captivity at Kinigi in Rwanda, thus changing their natural environment. In captivity, gorillas have to cope with changes as a result of the design of enclosure, changes in food presentation, and social structures. Cortisol concentrations may rise in situation of stress and could be used as an indicator of an animal's welfare. This study examined cortisol levels by quantifying cortisol in saliva and urine among captive gorillas at Kinigi. Saliva and urine samples were collected from eight orphan gorillas in the quarantine facility at Kinigi. The orphans were composed of six female and two males, aged between 4-8 years. Saliva samples were collected by swabbing the mouth with cotton swabs. Urine samples were collected from the ground or leaves using a syringe, immediately after urination. A total of ten urine and eight saliva samples were collected from each gorilla in the morning and evening and analysed using competitive ELISA. There was a significantly higher ($p=0.001$) saliva cortisol levels in female than male in the morning, meanwhile there was no significant difference ($p=0.05$) between female and male saliva cortisol levels in the evening samples. There was also a significantly higher ($p=0.036$) urine cortisol levels in the male than female in the morning, while there was no significant difference ($p=0.05$) between female and male urine cortisol levels in the evening. In conclusion female saliva cortisol was significantly higher in the morning and declined in the evening, and for male cortisol was low in the morning and significantly higher in the evening. For urine cortisol both male and female cortisol levels were high in morning and declined in the evening. No correlation between the urine and saliva samples in all samples collected in both morning and evening.

CHAPTER ONE

INTRODUCTION

1.1 Background

Mountain gorillas (*Gorilla berengei berengei*) are the world's most endangered ape species (Harcourt, *et al.,* 1983). Mountain gorillas are considered to be vulnerable; a category of the degree of threat assigned to the members of the species *Gorilla gorilla* (Lee *et al.,* 1988). According to the new International Union for Conservation of Nature (IUCN) species categorization of threat version 2.2 (Mace 1984 and Harcourt, 1996), the species is presently considered to be endangered. There are two populations of *Gorilla beringei beringei*, one among the volcanoes of the Virunga Massif at the border of the Democratic Republic of the Congo (DRC), Rwanda and Uganda, and the other in Bwindi Impenetrable National Park in Southwest Uganda on the border with DRC. The Bwindi Mountain Gorilla could be a third subspecies, *Gorilla beringei bwindi* (Sarmiento *et al*., 1996), but the taxonomic status of the populations is as yet unclear (McNeilage *et al*., 2001). The Mountain Gorilla (*Gorilla beringei beringei*) belongs to the eastern gorilla species, which also includes the Eastern Lowland Gorilla (*Gorilla beringei graueri*). In 1990, it was estimated that only 650 mountain gorillas remained in the wild (Sholley, 1991). However, a survey of the Bwindi population carried out in 2002 showed that the population had increased since the previous census in 1997, by approximately 7%, to 320 individuals (McNeilage *et al.,* 2006). The census of the Virunga mountain gorilla population which was conducted in 2003 (Gray *et al*.,2009) the population was estimated to contain 380 individuals. The current estimated population size of 480 gorillas was done between March and April 2010 represents a 26.3% this translates into a 3.7% annual growth rate, which is higher than the projected growth rate calculated from Leslie matrix models using birth rates and age-specific survivorship values (Robbins *et al*., 2011). The increase in population is most likely due to continuous monitoring and veterinary interventions for the habituated groups (Robbins *et al*., 2011). Until recently, no mountain gorillas were known to exist in captivity. However the species has experienced poaching of adults and subsequent confiscation of infants from illegal holders, resulting in some gorilla being kept in captivity at Kinigi in Rwanda. These gorillas in captivity have to cope with changes in their physical and social

environment as a result of the design of enclosure, changes in food presentation, and changes to their social structures. Stress induces behaviour changes that are related to prolonged adrenal activity and long term welfare problems (Dierauf, 1990; Waples & Gales, 2002). Stress embodies almost any factor or threat that induces physiological or psychological tension. It is more appropriately defined as the sum of biological reaction to extrinsic stimuli that results in a perturbation of the homeostasis (Chrousos, 1998), causing negative effects on the system by eliciting a bodily response perceived as unrest or one that causes anxiety (Morley 1991). The coping stage involves the release of glucocorticoids and/or catecholamines by adrenal glands (Bauer *et al.,* 2001; Huber *et al.,* 2003). The mechanism of this response begins with release of epinephrine from the adrenal medulla and norepinephrine from sympathetic nervous system in response to perceived stressor (Nelson, 2000). When an animal is stressed, the hypothalamic-pituitary-adrenal axis (HPA) is activated as corticotropin releasing factor (CRF) is released from hypothalamus. The anterior pituitary gland then releases adrenocorticotropic hormone (ACTH) and Prolactin. Adrenocorticotropic hormone (ACTH) stimulates the adrenal cortex to release glucocorticoids, primarily cortisol and corticosterone in mammals' life. Cortisol concentrations therefore rise in situation of stress and this parameter is considered to be an indicator of an animal's welfare (Cook *et al*., 1996; Ekkel *et al*., 1997). Although traditionally cortisol concentrations have been determined in plasma or serum, their determination in other fluids or organic tissues (saliva, milk, muscle, urine, faeces and hair) can be of interest in animal welfare studies of different species (Cooper *et al*., 1989; Fritche and Steinhart, 1998; Verkerk *et al*., 1998; Cirimele *et al*., 1999; Fritsche *et al*., 1999; Antignac *et al*., 2000; Morrow, 2000; Palme *et al*., 2000). Cortisol levels in saliva corresponds to free fraction of cortisol in plasma, which is the only biologically active fraction in the organism, owing to it being able to bind to cell receptors (Vining *et al*., 1983). Protein-bound cortisol acts as a reserve and can be converted to free cortisol when production is reduced (Rijnberk and Mol, 1989).

1.2 Statement of the problem

Gorilla are highly endangered and threatened with extinction, so monitoring cortisol hormones is important for the gorilla's welfare in captivity. Invasive method have been used like collection of blood from the fragile endangered gorillas for the

purpose of evaluating circulating cortisol hormones which requires physical restraint or anaesthesia, and usually is stressful, dangerous, costly and ultimately impractical. In contrast, non-invasive endocrine methods allow repeated sampling thereby permitting longitudinal studies while enhancing statistical accuracy (Monfort 2001). Documentation of cortisol hormone level in captive gorillas of Kinigi using saliva and urine, as a non-invasive method has not as yet been done.

1.3 Justification/significance of the study

The mountain gorilla and lowland gorillas are regarded as the most endangered of the great apes and they form the biggest chunk of wildlife tourism in many countries. Gorilla tourism therefore contributes considerable revenue to the economy. The current study was aimed at quantifying cortisol levels in urine and saliva among the eight captive gorillas of Kinigi in Rwanda. Efforts to re-introduce these gorillas in some families in the wild have so far failed they are rejected and being raised and living in captive, they easily access food not free to make own choose its all managed by humans. These individuals are therefore bound to stay longer in captive facilities than what was anticipated. So there is need to monitor the individual's welfare and non invasive techniques to monitor cortisol would be useful. Results from this study could help in designing effective management strategies that will help ensure long-term survival of gorillas in captivity.

1.4 Overall objective

Assessment of cortisol hormone levels among captive gorillas by quantifying cortisol levels in urine and saliva.

1.5 Specific Objectives

- To establish the sexual relationship of urine and saliva cortisol levels in gorilla

  To establish the relationship between saliva and urine cortisol levels in gorillas

1.6 Research questions

i. Can urine and saliva cortisol be used to monitor stress hormones in captive gorillas?
ii. Is there a relationship between cortisol levels in urine and saliva of the captive gorillas?

CHAPTER TWO

LITERATURE REVIEW

2.1 Taxonomy of gorilla

Gorillas have traditionally been classified as three sub-species; western lowland gorilla (*Gorilla gorilla gorilla*), eastern lowland gorilla (*Gorilla gorilla graueri*), and mountain gorilla (*Gorilla gorilla beringei*). In the recent taxonomic reclassification, gorillas were grouped into two species and four sub-species: (1) western gorillas: western lowland gorillas (*Gorilla gorilla gorilla*) and Cross River gorillas (*Gorilla gorilla diehli*); and (2) eastern lowland gorilla or Grauer's gorilla (*Gorilla beringei graueri*) and mountain gorilla (*Gorilla beringei beringei*) (Groves, 2001). The taxonomy currently followed by Wilson & Reeder (2005) recognises two species and including both the eastern lowland (*Gorilla beringei graueri)* and the mountain gorilla (*Gorilla beringei beringei*). The Mountain Gorilla (*Gorilla beringei beringei*) belongs to the eastern gorilla species, which also includes the Eastern Lowland Gorilla (*Gorilla beringei graueri).* There are two populations of *Gorilla beringei beringei;* one among the volcanoes of the Virunga Massif at the border of the Democratic Republic of the Congo (DRC), Rwanda and Uganda, and the other in Bwindi Impenetrable National Park in Southwest Uganda on the border with DRC. The Bwindi Mountain Gorilla could be a third subspecies, *Gorilla beringei bwindi* (Sarmiento *et al*., 1996), but the taxonomic status of the populations is as yet unclear (McNeilage *et al*., 2001).

2.2 Social structure of mountain gorillas

The primary social group consists of one dominant adult or "silverback" male, an average of three adult females and their offspring (Harcourt, 1981; Parnell, 2002). Groups may also consist of multiple often related adult males such as brothers or father/son pairs (Robbins, 1999). On average most groups contain 5-10 individuals though groups of 29 or more have been reported (Gatti, *et al.*, 2004; Magliocca, *et al*., 1999, Parnell, 2002; Robbins, *et al.*, 2004). Most male offspring remain with their natal troop until roughly 6-8 years of age at which time they emigrate from their natal group to all-male groups or range as a solitary males. Male offspring may also remain in their natal group, and "sneak" copulations with unrelated females within the group.

Female offspring also emigrate around 6-8 years of age from their natal group directly to another adult male to form a breeding group, or transfer into an existing group (Stokes and Parnell, 2003). Gorillas, as with most primates, are gregarious. They live in relatively stable bisexual social groups (Watts, 1990).

2.3 Habitat of mountain gorillas

The habitat of *Gorilla gorilla beringei* consists of subtropical/tropical moist forest (IUCN, 2002). Forest edges and regenerating or secondary forest are favoured gorilla habitat (IUCN, 1982). A number of vegetation zones have been identified in the mountain gorilla habitat of the central Virunga Volcano region, which mostly consist of *Hagenia-Hypericum* woodland with a relatively open canopy and extremely dense herbaceous under storey (Watts, 1997). Mountain gorillas habitant range up to 3,400 metres in altitude with occasional forays even higher (IUCN, 1982). Bwindi gorillas tend to live in lower elevations, warmer temperatures and are more arboreal than Virunga gorillas (Sarmiento *et al*., 1996). The area of habitat occupied by the mountain gorilla in the Virungas is approximately 375 Km^2 and the Bwindi gorillas occupy an area of approximately 215 Km^2 (Butynski, 2001).

2.4 Actual and potential threats to gorillas

The major threats to gorillas are habitat loss or modification e.g. through infrastructure development, wood extraction, human settlement and agricultural crops and forest encroachment hunting or poaching, disease transmission from humans and war or political unrest (Plumptre *et al*., 2003; Muruthi *et al*., 2000; IUCN, 2002). Other threats include the risk of inbreeding (Muruthi *et al*., 2000) and ongoing disturbance from tourism (IUCN, 2002). The two mountain gorilla populations are separated by a 45 Km stretch of densely populated land and intense human land use is putting intense pressure on both populations (GROMS, 2002). Ongoing harvesting, hunting and gathering for food is a threat to the mountain gorilla (IUCN, 2002). In the Virunga and Volcano National Parks of DRC and Rwanda, respectively, infant gorillas may be captured for sale. Adults may also be killed in order to gain access to the infants. An infant can reportedly fetch as much as £86,000 on the black market in DRC and Rwanda (Vesperini, 2002).

2.5 Feeding habits of gorillas

In general gorilla diet is primarily frugivorous supplemented with foliage during seasons when fruits are less plentiful (Doran, *et al*., 2002; Remis, 1997). The mountain gorilla is highly folivorous with the rest of their diet contributed by shoots and pith of terrestrial herbs, which are highly distributed throughout their habitat (Watts, 1990). The diet of the gorilla may be supplemented by stems of herbs, bark and wood that are often abundant and are higher in fibre content, less digestive and lower in protein content (Fossey and Harcourt, 1997, Watts, 1984). Zoological institutions typically feed primates on fruit purchased from local markets. It is suspected, however that human cultivated fruits differ significantly when compared to wild fruits (Crissey *et al.*, 1997, Milton, 1999). Wild fruits have higher contents of fibres, minerals, proteins, and vitamins as well as a lower content of total sugar (Milton 1999, .Baker *et al*., 1998). The average captive gorilla diet consists of cultivated fruits, leafy greens, other vegetables, natural browse items, cereals, grains, and "nutritionally complete" commercial primate biscuits, which function to fortify gorilla nutritional needs as well as to provide the necessary quantities of fiber (Lukas *et al.*, 1999).

2.6 Parental care among gorillas

Gorillas live in stable, cohesive social groups called troops led by the adult, dominant silverback male (> 12 years), who provides protection, mediates disputes, determines the home range, has exclusive breeding rights to the females, and basically regulates what time they wake up, eat, and go to sleep (Dian Fossey Gorilla Fund International, 2010b). Other members of the troop can be several young males called black backs (8-12 years), adult females (> 8 years), sub adults (6-8 years), juveniles (3.5-6 years), and infants (0-3.5 years) (Robbins, 2007). The social structure is clearly defined in gorillas and seems to be an important factor in the stability of the troop.

Since mortality is as high as 38% in mountain gorillas during the infancy period (from birth to three years), adult females are acutely important in the survival of their infants (Watts, 1989). The juvenile period is from three to six years and is characterized by a decrease in maternal grooming, no longer sharing a sleeping nest with the mother and eventually weaning (Stewart, 2001). Infants are dependent on their mothers for food,

suckling at least once per hour and sleep at night in their mothers' nests (Stewart, 1988). After five months, mother-infant pairs break body contact, but only for a few seconds and by 12 months, infants venture up but not more than five meters (16.4 ft) away from their mothers. By 18 to 21 months, this distance between the pair is irregular and increases (Fletcher, 2001). Concurrent with this decrease in proximity is a decrease in nursing frequency, with infants only nursing once every two hours (Stewart, 1988).

2.7 Stress in gorillas

The term “stress” has a multitude of definitions, depending upon the context (Broom and Johnson, 1993; Moberg, 1987). Stress can be precisely defined as the biological process by which an individual attempts to cope with a real or perceived threat to physiological or psychological integrity (Broom and Johnson, 1993; McEwen, 2000). The real or perceived threatening situation that elicits this biological process, or stress response, is referred to as a "stressor”. When a stressor is sensed by the brain, it triggers the hypothalamus to release two hormones: Corticotrophin-Releasing Hormone (CRH) and Arginine Vasopressin (AVP). These hormones cause the anterior pituitary gland to release adrenocorticotrophic hormone (ACTH), which in turn stimulates the adrenal cortex to secrete corticosteroids, namely cortisol/corticosterone (Buckingham, 2000).

During a stress response, the corticosteroids divert energy from normal biological functions and concentrate energy on coping with the stress. Cortisol is one of the corticosteroids that have been recognized as useful in indicating stress (Carlstead *et al.*, 1992; Thomas and McCann, 1997; Wielebnowski *et al*., 2002). Patterns of cortisol excretion have successfully been measured in blood (Boinski, *et al.*, 1999), urine (Czekala, *et al.*, 1994), feces (Wasser, *et al.*, 2000), and saliva (Kuhar, *et al*., 2005) from various animal species. Conversely, repeated acute stress or prolonged, chronic stress has been shown to suppress reproduction, reduce immune effectiveness, and cause the development of aberrant behaviors (Moberg, 1985).

Although there is no single definition or physiological measure of stress, glucocorticoid hormones, especially cortisol secretion has been considered reflective of adrenal status in many species held *ex situ* as well as those living *in situ* (Hofer and

East, 1998). External stressors are known to activate the hypothalamic pituitary-adrenal (HPA) axis resulting in release of glucocorticoids (Bradley *et al*., 1980; Sapolsky, 1985; Alexander and Irvine, 1998). Assessing stress in animals, particularly chronic stress, is crucial in fields like animal welfare and conservation biology (Vleck *et al*., 2000). There is good evidence, mostly from captive studies, that physiological stress responses in primate and other mammals can vary with sex (Boonstra *et al*., 2001; Von der Ohe & Servheen, 2002), age (Gust *et al*., 2000; Stoinski *et al*., 2002; Erwin *et al*., 2004), sexual activity , reproductive status (Tilbrook *et al*., 2000; Boonstra *et al*., 2001; Lynch *et al*., 2002), and social environment (Ziegler *et al*., 1995; Shively *et al*., 1997).

Monitoring the health status is important for effective management of captive and free-ranging wildlife; non-invasive methods have been developed to facilitate assessment of reproduction and stress physiology without the adverse effects of instituting chemical or physical restraints. The broad application of these methods to a wide range of species is related to conservation of the parent steroid hormone molecule during metabolism across taxa and the stability of resultant steroid hormone metabolites in excreta (Lasley *et al.*, 1991). Dominance hierarchies, and the effects to subordinate individuals, have been examined in orangutan *Pongo pygmaeus* (Maggioncaida, *et al*., 2002), rhesus macaques, *Macaca mulatta* (Bercovitch and Clarke, 1995), and primates in general (Abbott, *et al*., 2003). Changes to group social dynamics, such as removal of individuals from a group, addition of individuals to a group, high population density due to space restriction or social isolation of an individual has been shown to be stressful events. These stressful events consequently result in increased aggression (Burks, *et al.*, 2001; Hoff, *et al.*, 1996), decreased affiliate behaviours (Aureli *et al.*, 1995; Aureli and de Waal, 1997; Perloe, 1986), increased stereotypic behaviours (Bowen, 1980; Schmid, *et al.*, 2001), and increased levels of cortisol (Kuhar, *et al.*, 2005; Stoinski, *et al*., 2002; Ziegler, *et al*., 1995). In western lowland gorillas, (Kuhar, *et al*., 2005) monitored behavioural and cortisol changes in an all-male group (Stoinski, *et al*., 2002) examined differences in cortisol levels in males of different ages and males living in different social settings. In addition to capture and restraint, recent studies have demonstrated that animals are also significantly stressed by other routine husbandry procedures, palpation,

pregnancy examination, and weighing (Graham and Brown, 1996; Mellen, 1991; Saco *et.al.,* 2008).

2.7.1 Cortisol level in saliva

As a biological medium, saliva is a variable and complex fluid and is mostly produced by three pairs of salivary glands (paratoid, submandibular and sublingual) with a small contribution from the buccal glands which line the mouth. Hormones can enter saliva by a variety of mechanisms, but for the natural steroids the most common route is rapid diffusion through the acinar cells and their concentration is independent of the rate of saliva flow (Vining *et al*., 1983). Whereas hormones enter saliva within 20-30 minutes after their appearance in blood, salivary cortisol levels are particularly suitable for assessing plasma cortisol levels because they accurately reflect the level of the biologically active free fraction of the hormone in the plasma (Haeckel, 1989). Sampling of saliva can be performed frequently, which is particularly important as it allows acute stress to be monitored by measuring short-term changes in cortisol levels.

The first assays for salivary steroids were described by Selye (1959), but this method did not gain widespread acceptance until researchers at the Tenovus Institute in Wales developed reliable assays for steroids in small volumes of whole saliva (Riad-Fahmy *at el.,* 1982; Walker *et al.,* 1978). The advantages of salivary sampling for cortisol analysis compared to traditional procedures for blood sampling have been summarized in several reviews (Kirschbaum & Hellhammer, 1989, 1994; Vining *et al*., 1983). The ease and non-invasive nature of sample collection; and the fact that salivary cortisol is “free”, unbound by Corticosteroid-binding globulin (CBG) or other carriers, is advantageous, as free cortisol represents the biologically active fraction of the hormone (Mendel, 1989). Saliva samples can be obtained with no pain or discomfort (Queyra and Carosi, 2004) in animals, samples can be collected while the animal is in its social group. Non-invasive measurement of glucorticoids in saliva may be an ideal means of assessing on-going levels of stress and acute responses to experimental stressors.

Specialized techniques make it possible to extract cortisol from smaller sample volumes, which may be a great advantage in studies of infants (de Weerth *et al.,*

2003). Saliva has been used in stressed captive gorilla (Bettinger *et al.,* 1998). There is evidence that saliva levels reflect unbound concentrations in plasma (Umeda *et al*., 1981). Stress is known to induce behavioural changes (Dierauf 1990; Waples & Gales 2002) that could be related to prolonged adrenal activity and long-term welfare problems, making these individuals more susceptible to health problems (Broom & Johnson, 1993). Animals that cannot afford adaptive responses to stress and are unable to maintain homeostasis will develop pathological problems that impact the immune and reproductive systems (Sapolsky *et al.,* 2000; Chrousos 1998; Reeder & Kramer 2005).

Direct measurement of cortisol in saliva may be comparable to extraction, but extraction has the advantage of allowing the analysis of low volume of saliva samples. This can avoid the use of stimulants and the loss of data due to insufficient sample volume (de Weerth *et al.,* 2003). Salivary cortisol determinations have proved popular in psychobiology and sports medicine studies (Abercrombie *et al.,* 2006; Lindahl *et al.,* 2005; Alehagen *et al.,* 2005; Paccotti *et al.,* 2005; McGuigan *et al.,* 2005). There are number of biological factors that can modify cortisol levels (Honess *et al*., 2005; Moberg, 2000) which include time of day (Honess and Marin, 2006a; McCallister *et al.,* 2004; Raminelli *et al.,* 2001; Smith and French 1997a), genetics (Kulberg *et al.,* 2002), temperament (Anestic and Bribiescas, 2004; De palma *et al.,* 2005; Ray and Sapolsky, 1992; Sapolsky, 2005), social status (Sapolsky, 2005), reproductive condition (Owen *et al*., 2004; Raminelli *et al.*,2001; Wingfield *et al.*,1994), developmental history (Dettling *et al*., 2002; Paulk *et al.,* 1997; and age (Pryce *et al.,* 2002). Other factors that modify cortisol levels are physical health (Health and Dufty, 1998; Smith *et al.*,1994), social environment (Ahola *et al.,* 2006), diet (Erwin *et al*., 1973), season (Mckenzie and Deane, 2003; Owen *et al.,* 2005; Reyes *et al.,* 1997) and climate (Marai *et al.,* 2003).

Saliva samples for cortisol assay can be stored at room temperature or in refrigerator or freezer until they are delivered to the laboratory. Estimates of how long cortisol is stable at room temperature range from seven days (Groschl *et al*., 2001) to at least four weeks (Kirschbaum & Hellhammer, 2000). Centrifuging samples before storage appears to prolong the stable period (Groschl *et al*., 2001). However, increasing variance as well as decreasing levels over time indicate that storage at room

temperature for more than two to maximally four weeks should be avoided (Garde & Hansen, 2005). Salivette samples develop mould and a bad odour after about four days at room temperature though this does not affect the cortisol concentrations, but makes laboratory handling unpleasant. Freezing clearly prolongs the stability of saliva cortisol. In samples frozen at either –20ºC or –80ºC, cortisol concentrations remained stable for nine months (Aardal & Holm, 1995) to one year (Garde & Hansen, 2005); and freezing for as long as two years is probably possible. Salivary cortisol levels are relatively insensitive to repeated thawing and refreezing. In recent studies, cortisol levels remained stable in samples undergoing up to three (Groschl *et al*., 2001) or four (Garde & Hansen ,2005) freeze/thaw cycles prior to assay.

Studies done in salivary cortisol levels obtained in baboons were comparable to published plasma cortisol levels within this genus at the 10–15% fraction secreted in saliva (Negrao *et al.,* 2004; Queyras & Carosi, 2004). For example, in restrained and sedated male savannah baboons *(Papio cynocephalus/anubis)*, Bentson *et al.*,2003) reported plasma concentrations of 232 ± 8ng/ml, which were approximately 10–15 times higher than the salivary cortisol levels obtained. Similarly, among restrained adult male *hamadryas* baboons, Taranov and Goncharov (1981) reported average plasma cortisol levels ranging from 150 to 500ng/ml depending upon the time of day and degree of habituation to restraint.

The indistinct diurnal pattern seen in the African elephants was mainly lowered cortisol levels in the evening found in other species that are provided with food throughout the day including cows (Wagner and Oxenreider, 1972) and horses (Irvine and Alexander, 1994).

The means found in three African elephants (*Loxodonta Africana)* were 0.054, 0.034 and 0.030µg/dl are lower than Asian elephants (Dathe,Kuckelkon and Minnermann 1972).

2.7.2 Cortisol levels in urine

Circulating adrenal steroids are metabolized in the liver or kidney before excretion into urine or bile. Generally, biologically potent steroids (cortisol and corticosterone) are rendered impotent during metabolism through subtle molecular changes or through conjugation to highly charged, side chain moietes (e.g. glucuronide) before

excretion. Conjugated steroids have increased molecular polarity that improves solubility in the aqueous environments of urine. The proportion of steroids excreted in urine usually is species or taxon specific. For example baboons excrete >80% of gonadal steroids into urine (Wasser *et al.*, 1991). Steroids are known to vary in the extent to which they are metabolized before excretion, both within and among individuals.

Assay of urine cortisol levels directly reflects the blood levels of cortisol. Urinary cortisol is not bound to proteins, but its levels are dependent on glomerular and tubular function. Elevations in urinary cortisol have been shown to reflect the cumulative stress response in humans (Burke and Beardwell, 1993; Contreas *et al.*, 1986; Barton *et al*., 1993). Similar methods have also been employed in non-human primates for example (Mason 1972; Byrne and Suoni, 1991; Crockett *et al.*, 1993; Smith and French, 1997; Bahr *et al*., 1998). Urine cortisol evaluations have also been investigated in free-living male mountain gorillas (Robbins and Czekala, 1997), rhesus monkey (Boyce *et al.*, 1995); squirrel monkey (Fuchs *et al.*, 1997) and tree shrew (Ohl *et al.*, 1999). Assays for measuring stress hormone metabolites in urine have also been validated in sheep, cattle (Palme,. *et al*., 1999) and rats (Bamberg, *et al.*, 2001). Assay of stress hormone metabolites in urine have been used to monitor stress in farm animals e.g. cattle. (Palme *et al*, 2000), domestic and non-domestic felids (Carlstead *et al.*,1992), and dogs (Beerda *et al.*, 1996; and Beerda *et al.*, 1999). In primates, there is a long history of monitoring stress with the non-invasive method of measuring both adrenocortical responses (Mason, 1972; Whitten *et al.*, 1998; Mason *et al.*, 1973).

Urinary steroids are excreted relatively rapidly (Ziegler *et al.*, 1989; Mohle *et al.*, 2002) compared to faecal steroids, and thus are more conducive for identifying hormonal correlates of singular stressful events. In urine the time lag is usually about 2-8 hours and generally consistent across species (Moh¨le, 2002; Wasser *et al*., 1994). Cortisol concentrations in gorilla urine are highest in the early morning hours and lower as the afternoon and evening progress (Czekala *et al.*, 1994, Muller and Lipson, 2003; van Eekelen *et al.*, 2003). This is a circadian rhythm is endogenously driven by central nervous system (Liotta & Krieger,1990), and its synchronization is influenced by both light/ dark and sleep wake cycles (Morin&Dark, 1992). Baseline urine

cortisol values for captive lowland gorilla have been reported. Czekala *et al.* (1994) reported a range from approximately 10 – 140 ng/mg with a mean of 63.0±7.9 ng/mg in captive lowland gorillas, and approximately 10 – 80 ng/mg, mean = 26.7±4.1 ng/mg in wild mountain gorillas. Urine samples can be kept at room temperature, without preservatives, for at least 24 hours without degradation of glucocorticoids (Gouarne *et al*., 2004).

2.7.3 Effect of age on saliva cortisol levels

Although an individual's age may modify its levels of cortisol, there is controversy regarding the effect of age on the activity of the HPA system (Sapolsky, 1991). Age effect in cortisol levels have been found in several primate species, including common marmosets, *Callithrix jacchus* (Pryce *et al.,* 2002), rhesus macaques, *Macaca mulata* (Capitanio *et al.,* 2005), mountain gorilla (*Gorilla beringei)* and Western lowland gorillas (*Gorilla, gorilla gorilla)* (Robbins and Czekala, 1997; Stoinski *et al.,* 2002). In all of the apes, younger animals had higher cortisol concentration levels which may be explained by the role of cortisol in energy metabolism (Sapolsky, 1991). Younger animals often have higher levels of activity or energy expenditure than older animals (Robbins and Czekala, 1997; Stoinski *et al.,* 2002). It is also possible that in some cases younger animals find certain events more stressful than older animals due to lack of experience or their rearing history. Capitanio *et al*. (2005) and Stoinski *et al. (*2002) found an increase in cortisol levels within different age groups of young individuals from sub-adult to young adult males of western low gorillas. This difference may reflect an increased stress levels in captive young males. Aging is hypothesised to alter the function of the HPA-axis in both men and women with increasing cortisol levels as a result, especially regarding nocturnal levels (Van Cauter *et al.,* 1996; Ferrari *et al.,* 2001). Research suggested that age (Capitanio *et al*., 2005) and sex (Ahola *et al.,* 2005) may have an effect on modifying cortisol levels with several species. Cortisol is secreted in a specific diurnal pattern with a normal curve presenting a sharp peak in the early morning to then gradually decrease over the day and end up very low in the evening and at night.

2.7.4 Effect of sex on saliva cortisol levels

Cortisol is a potent stress hormone and the secretion is regulated by the Hypothalamic-Pituitary-Adrenal-axis (HPA-axis). Previous studies in human have also indicated that cortisol levels differ between men and women (Van Cauter *et al.,* 1996; Zhao *et al.,* 2003; Laughlin *et al.,* 2000; Gusenoff *et al.,* 2001). However, while several of the studies have found men to have higher levels than women there are inconsistencies regarding what age-groups these findings were related to (Van Cauter *et al.,* 1996; Zhao *et al.,* 2003). For example reproductive hormones influence HPA activity and cortisol production (Honess and Marin, 2006a) in both male and female animals (Rivier and Rivest, 1991). For example cortisol levels in oestrus female savannah baboons *(Papio hamadryas ursinus*) are lower than levels found in individuals at other reproductive stages (Wengrill *et al*., 2004). Female show a higher cortisol response than males to various stressors in long-tailed macaques, *Macaca fascicularis* (Crockete *et al*., 1993), rhesus macaques (Capitanio *et al.,* 2005) and marmosets, *callithrix kuhli* (Smith and French, 1997a). The opposite was found in squirrel monkeys where males showed a higher response than female (Coe *et al.,* 1978). Sex variation may also reflect underlying differences in steroid metabolism, excretion routes and pituitary responsiveness. (Handa and McGivern, 2000).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study area and population

Kinigi gorilla quarantine facility is found in Musanze district, Northern Province of Rwanda. The facility holds endangered mountain gorilla infants rescued from poachers. It is a joint effort striving to rehabilitate confiscated gorillas and return them to their wild home. It is supported by partnership of government institutions and non-governmental conservation organisations: Office Rwandese du Tourisme et des Parc,Nationaux, L'institut congolais pour la conservation de la Nature, Mountain Gorilla Veterinary Project & Diana Fossey Gorilla Fund International. There were eight gorillas in the quarantine facility. Two mountain gorillas and six are Eastern lowlands, all between the ages 4-8 years. Six of the gorillas are female and two are males that were rescued after being kidnapped by poachers for the live animal trade. These gorillas live in a group and socialize together in enclosed outdoor yard of about 6000 square metres with climbing structure, shaded areas, and natural foliage. Caretakers provided them with a diet including natural forest food from morning at 8:00hours up to evening at 18:00hours, within an interval of 2 hours, mountain gorilla veterinary doctor are always there to intervene in case of anything. They tend to the needs of the gorillas 12 hours a day. This includes collecting and preparing their daily food and watching over their development both as individuals and as a group, by playing the role of surrogate mother and helping them to socialize with each other and break up fights. They have three sleeping rooms which are shared by the same animal each animal has a partner to share a room. They sleep on forest vegetation, hammocks and wooden platforms.

3.2 Study design

This was a cross-sectional study in which captive gorillas at the quarantine facility in Kinigi were sampled. Samples were collected during the day 8:00 hours to 18 hours and were classified according to sex, age and time. Immediately the gorilla urinated on the ground the care takers collected the urine using a syringe and drew it in the eppendoff tube and put in the cool box to be transported to the freezer, then the gorilla was swabbed the mouth to collect saliva sample the swabs were also but in the ice

box. This was done to ensure that urine collection and saliva sample is done at the same time under similar conditions.

Sample size estimation, total population sampling a type of purposive sampling technique that involves examining the entire population.All the gorillas in the facility, were sampled thus, the whole population of eight gorillas was studied**.** The study involved the entire population of eight individuals living under similar captive conditions of age and sex structure as summarised in table 1 below:

Table 1: Structure of gorilla composition by a sex, species and age

Name of Gorillas, sex and species	**Initial age into facility**	**Age when sampled 2010**
Ntabwoba Male lowland	1year	7years
Maisha female mountain gorilla	3years	8years
Itebero female low land	1 year	7 years
Kaboko male (RIP) 2012 mountain gorilla	3years	6years
Serufuri lowland female	2years	6years
Dunia Lowland female	1year	5years
Pinga lowland female	3years	8years
Tumaini lowland female	3month	4years

3.3 Saliva and urine samples collection and handling

According to Boyce *et al*.,(1995),gorillas were swabbed the mouth with Salivette® Cotton Swab and after the swab was put in the cool box. According to (Czekala *et al*., 1994; Robbins& Czekala, 1997) when the gorilla urinated the urine sample was collected and immediately put in the eppendoff tube and put in the ice box. Samples were collected during the day in the month of February and March 2010.All samples were labeled with the name of the gorilla, date and time of sampling. A total of sixty two swab samples were collected, eight samples were collected from each gorilla. For urine eighty samples were collected and ten urine samples were collected from each animal.

All samples were transported to MGVP Musanze Rwanda laboratory and stored at -20ºC. Then flown in to Uganda in the ice box by the Mountain Gorilla Veterinary staff and were stored at -20ºC until extraction of cortisol.

3.5 Laboratory analyses

Both the urine and saliva analysis was undertaken in the Enzymology Laboratory in the Department of Anatomy, School of Veterinary Medicine, Makerere University.

3.5.1 Extraction of cortisol from saliva and urine samples

Cortisol was extracted from the cotton swab as previously described (Thijssen *et al.*, 1980). Briefly, by opening the tube on one side and rinsing the cotton roll in the tube with 1 ml of 960 ml/L ethanol in deionised water at a ratio of 24:1, followed by centrifugation using Labofuge 200 (Heraeus) Germany at 1500 rpm for 5 minutes. The specially designed centrifuge tubes had an inside container where the swab was placed and the outer tube where the supernant was collected. The supernant was evaporated and the residue dissolved in 100 µl of 0.01 mol/l phosphate-buffered saline (pH 7.0) containing 2 g/l bovine serum albumin (BSA). After repeated mixing using a vortex-mixer for 15 minutes, 25 µL was used in the ELISA.

Cortisol was extracted from urine samples according to Lewis (1985), a micro pipette was used to draw 250 µl of the sample and placed in a marked 12 x 75 mm glass tube. 1 ml of Dichloromethane was added and then vortexed for 30 seconds to ensure thorough mixing. The mixture was allowed to stand for 30 minutes and separated into two layers; the upper layer and lower layer. The upper layer was discarded while 100 µl of the lower layer was put in duplicate plastic tubes and evaporated to dryness by leaving the tubes to stand at room temperature. A 25 µl of cortisol diluents (100 µl of 0.01 mol/l Phosphate buffered saline (pH 7.0) containing 2 g/l BSA was added in each tube of dried urine extract.

3.5.2 Quantification of cortisol from saliva and urine samples

Cortisol concentrations in saliva sample were determined using Cortisol Enzyme Immunoassay Kit Catalog No. DSL-10-67100 (DSL-10-67100 ACTIVE®, Diagnostic Systems Laboratories, Webster, Texas USA). This kit that has been validated by

Smulders *et al*.,(2006) and uses specific rabbit anti-cortisol antibody. The antibody has high affinity for cortisol and low cross-reactivity to other naturally occurring steroids. Following the manufacturer's instructions, the reagents were brought to room temperature and gently mixed by repeated inversion before use. Standard controls and samples were assayed in duplicates.

Cortisol concentration in saliva was measured by pippeting 25 µl of the sample extract on a goat anti-rabbit globulin serum pre-coated microplate then 100 µl of the enzyme conjugate solution was added to all wells. This was followed by adding 100 µl of the cortisol antiserum to all wells and then incubated at room temperature for 45 minutes. The wells were washed thoroughly with a wash buffer and blotted until they were dry. Then to each well 100 µl of the tetramethylbenzidine (TMB) chromogen solution was added and incubated for 10 minutes at room temperature. After waiting for 30 minutes, 100 µl of the stopping solution was added. Light absorbance was read at 450 nm using Elisa Reader (SIRIOS®, SEAC-RADIM, Italy) and the results were expressed as µg/dl.

Cortisol concentration in the urine sample extracts was determined using a cortisol EIA kit, Catalog No.DSL-10-2000 (Active® cortisol EIA, Diagnostic systems laboratories Webster, Texas USA). This kit has been validated for baboons (Hodge *et al*., 2005). The cortisol concentration was measured by pippeting 25 µl of sample extract on a pre-coated microplate the plates containing goat anti-rabbit globulin serum and 100 µl of the enzyme conjugate solution was added to all wells. This was followed by adding 100 µl of the cortisol antiserum and incubated at room temperature for 45 minutes. After incubation, the plates were thoroughly washed with a wash buffer then blotted to dryness. Then 100 µl of the tetramethylbenzidine (TMB) chromogen solution was added to each well and incubated for 10 minutes at room temperature. Following incubation, 100 µl of the stopping solution was added at room temperature and after 30 minutes the light absorbance was read at 450 nm using Elisa Reader (SIRIO S®, SEAC-RADIM, Italy) and results were expressed as µg/dl.

3.6 Ethical issues

Since this study involved sampling the endangered species which were rescued from poachers, permission was sought from the Office Rwandais Du Tourisme Et Des

Parcs Nationaux through the Mountain Gorilla Veterinary Project. The Office Rwandais Du Tourisme Et Des Parcs Nationaux is responsible for wild animals in Rwanda. Mountain Gorilla Veterinary Project is a certified organization which is cleared to work on wild animal health in Rwanda, Uganda and DRC.

3.7 Data management and statistical analysis

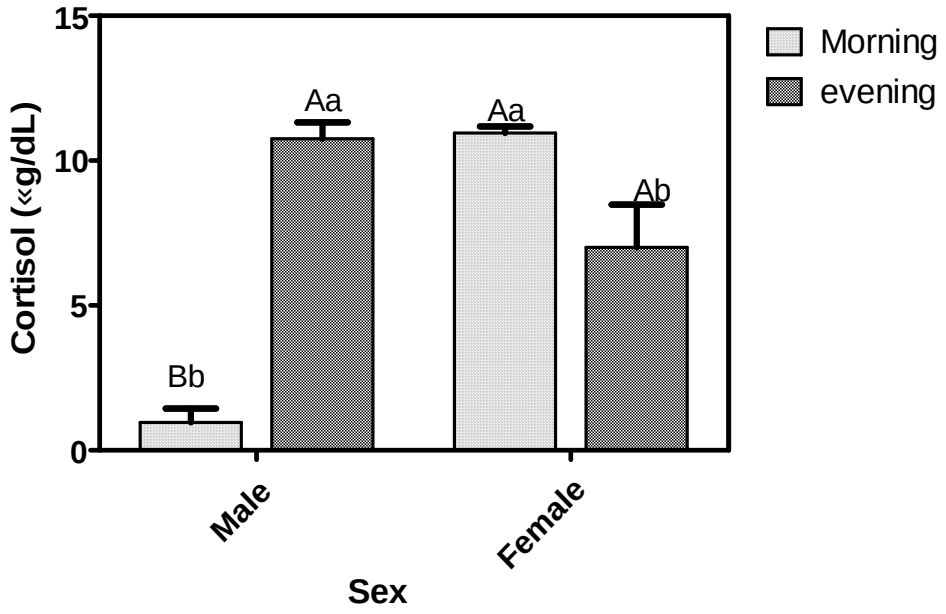
Optic density readings (OD) were entered in duplicate in Microsoft office Excel and means were calculated. Then data analysis was done using Graph Pad 5.0 statistical package from the standard curve (Appendix I). Comparison of cortisol levels among gorilla sex, age and collecting time was done using unpaired t-test set at a significant level of 5 percent. Correlation studies between cortisol levels in urine and saliva were done using Pearson correlation matrix at 5 per cent significant levels.

CHAPTER FOUR

RESULTS

4.1 Effect of sampling time and sex on saliva cortisol levels

This study showed variable levels of cortisol in saliva in all the sampled gorillas (Fig.1). Female saliva cortisol ranged from 9.6 to 12.7 µg/dl, and in males saliva cortisol ranged from 0.12 to 3.9 µg/dl in the morning. Evening cortisol levels in females and males ranged from 0.12 to 12.2 µg/dl and 9.6 to 12.2 µg/dl, respectively. The morning saliva cortisol levels were significantly higher ($p < 0.05$) in females than in the males. Meanwhile, there was no significant difference ($p > 0.05$) between female and male evening saliva cortisol levels. However, cortisol levels in the males were significantly higher ($p < 0.001$) in the evening than morning, but in females cortisol levels were significantly higher ($p < 0.001$) in the morning than in the evening (Fig.1).

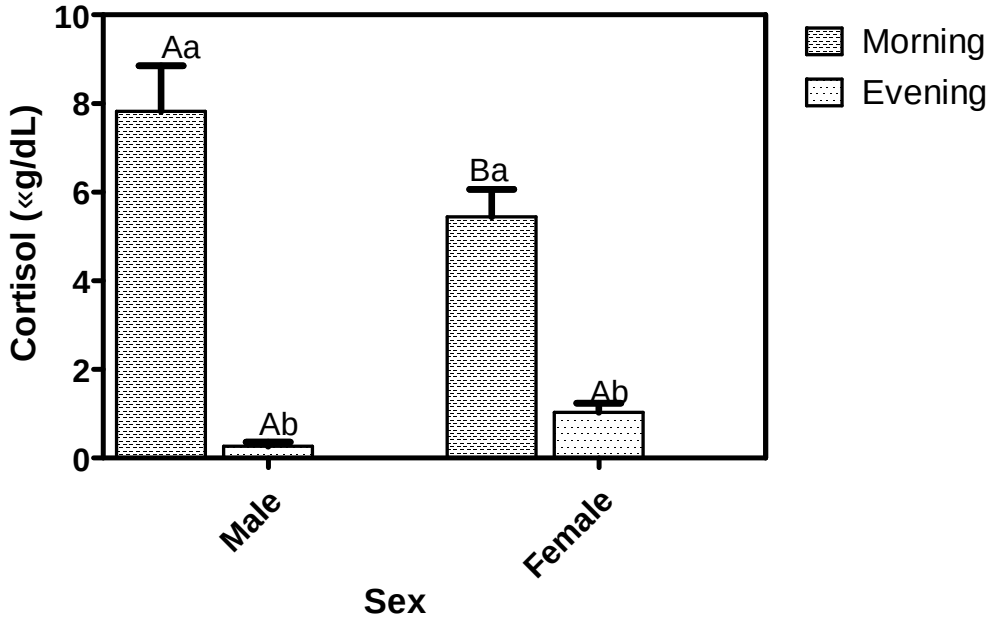


Different capital letters above the bars indicate significant difference between sexes; Different small letters indicate significant differences in sampling time.

Fig 1: Comparison of morning and evening saliva cortisol levels in female and male gorilla

4.2 Effect of sampling time and sex on urine cortisol levels

Cortisol levels in urine varied with time of sampling and sex in all the sampled gorillas (Fig. 2). For urine cortisol levels in female ranged from 3.3 to 10.2 µg/dl) and in males it ranged from 3.2 to 11.14 µg/dl in the morning. In evenings, female and male cortisol levels ranged from 0.1 to 1.64 µg/dl, and from 0.1 to 0.5 µg/dl, respectively. There was a significantly higher ($p < 0.036$) urine cortisol levels in the male than female in the morning, but no significant difference ($p > 0.05$) in the female and male urine cortisol levels in the evening. Cortisol levels were significantly higher ($p < 0.001$) in the morning than evening in the males. Like for saliva, the females' cortisol levels were significantly higher ($p < 0.001$) in the morning than in the evening.



Different capital letters above the bars indicate significant difference between sexes. Different small letters indicate significant difference between sampling time

Fig:2 Comparison of morning and evening urine cortisol levels in females and males .

4.3 Correlation between male saliva and urine cortisol in the morning

There was no correlation between saliva and urine cortisol in males in the morning r^2=0.387 p=0.448. (Fig. 3)

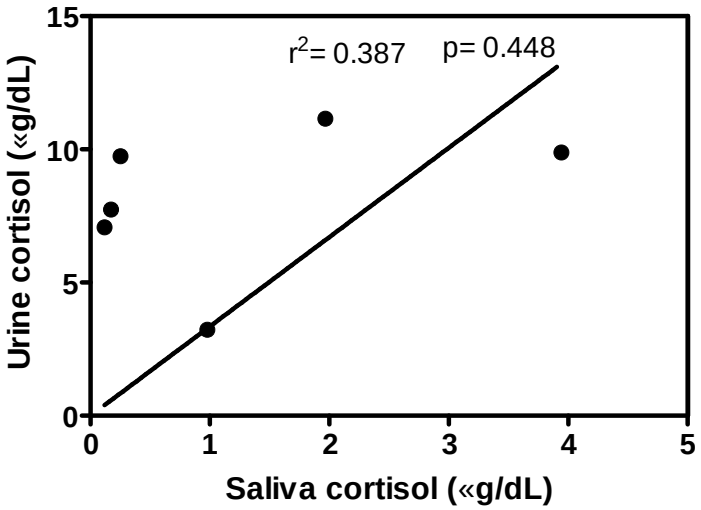


Fig 3: Correlation of urine and saliva cortisol levels in male in the morning.

4.4 Correlation between male saliva and urine cortisol in the evening

There was no correlation between saliva and urine cortisol in male in the evening r^2=0.01 and p=0.889.(Fig 4).

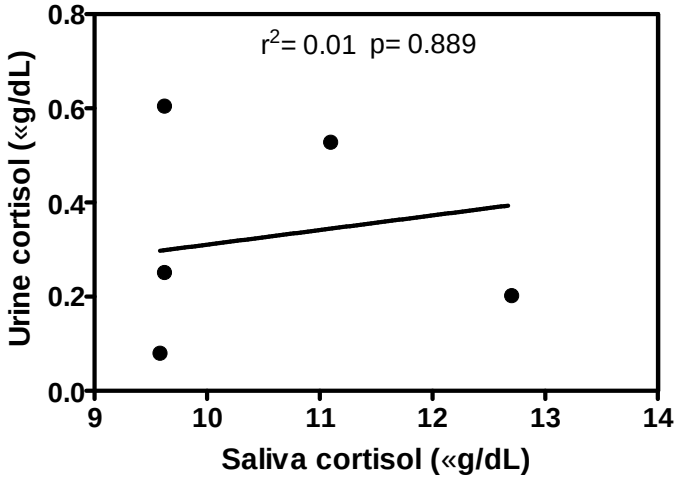


Fig 4: Correlation of urine and saliva cortisol levels in males in the evening.

4.5 Correlation between female saliva and urine cortisol in the morning

There was no correlation between saliva and urine cortisol in females in the morning r^2=0.03 and p=0.436 (Fig. 5).

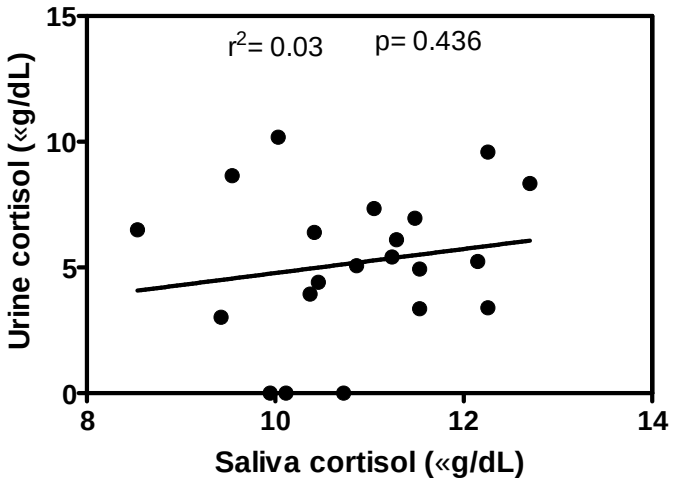


Fig 5: Correlation of urine and saliva cortisol in females in the morning

4.6 Correlation between female saliva and urine collected in the evening

There was no correlation between saliva and urine cortisol in female evening collected samples r^2=0.387 and p=0.06. Correlation analysed found urine cortisol to be uncorrelated with time (morning) of collection and also salivary cortisol was negatively correlated with urine cortisol in the evening for both female and male gorillas. (Fig. 6).

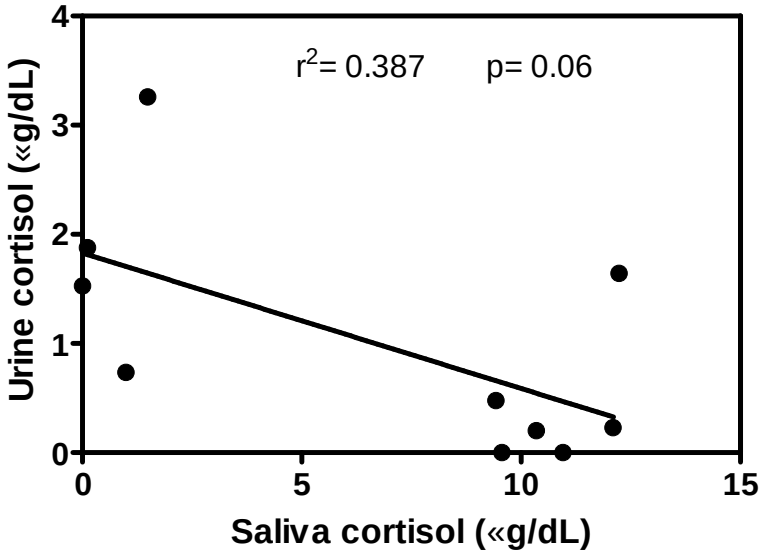


Fig. 6: Correlation of urine and saliva cortisol level collected from female in the evening.

CHAPTER FIVE

DISCUSSION

Cortisol is one of the corticosteroids that have been recognized as useful indicator of stress (Carlstead, *et al.,* 1992).

Female saliva cortisol ranged from 9.6 to 12.7 µg/dl, and in males saliva cortisol ranged from 0.12 to 3.9 µg/dl in the morning. Evening cortisol levels in females and males ranged from 0.12 to 12.2 µg/dl and 9.6 to 12.2 µg/dl, respectively. The morning saliva cortisol levels were significantly higher ($p < 0.05$) in females than in the males. But there was no significant difference ($p > 0.05$) between female and male evening saliva cortisol levels.

However, cortisol levels in the males were significantly higher ($p < 0.001$) in the evening than morning, but in females cortisol levels were significantly higher ($p < 0.001$) in the morning than in the evening. In previous studies the indistinct diurnal pattern seen in the African elephants was mainly lowered cortisol levels in the evening found in other species that are provided with food throughout the day including cows (Wagner and Oxenreider, 1972) and horses (Irvine and Alexander, 1994).

Female saliva cortisols were significantly higher in the morning and low in the evening, and for male cortisol was low in the morning and significantly higher in the evening. According to Czekakala *et al*., 1994 gorilla conform to the human pattern of diurnal cortisol production. Cortisol hormones diffuse into the saliva within 20-30 minutes after their appearance in blood as reported by Haeckel, 1989. It is also possible that the low cortisol in female evening might have been due to the gorilla's normal day is that they feed, play and groom in the morning but as the day goes on they tend to rest in the evenings.

For urine cortisol levels in female ranged from 3.3 to 10.2 µg/dl) and in males it ranged from 3.2 to 11.14 µg/dl in the morning. In evenings, female and male cortisol levels ranged from 0.1 to 1.64 µg/dl, and from 0.1 to 0.5 µg/dl, respectively. There was a significantly higher ($p < 0.036$) urine cortisol levels in the male than female in the morning, but no significant difference ($p > 0.05$) in the female and male urine

cortisol levels in the evening. Cortisol levels were significantly higher ($p < 0.001$) in the morning than evening in the males.

Both male and female cortisol levels were high in morning urine and declined in the evening. This is normal physiology where the circadian pattern of urinary cortisol excretion observed in the gorillas in this study is similar to the circadian pattern of plasma cortisol secretion observed in other mammalian species like non-primate (e.g. squirrel monkeys, *Saimiri sciures* Coe and Levine 1995, rhesus monkey, *macacaca mulata* Bercovitch and Clarke 1995, Smith and Norman 1987), and rodents Dallman *et al*., 1987. Similar results were reported by (Czekala *et al.*, 1994, Muller and Lipson, 2003; van Eekelen *et al.*, 2003) who stated that cortisol concentrations in gorilla urine is highest in the early morning hours and lower as the afternoon and evening progress. High cortisol in the morning probably serves to mobilize energy stores in preparation for activity (Sapolsky, 1992). The level of cortisol in the morning urine was a reflection of cortisol that had accumulated from early evening the previous day. The time lag for urine is usually about 2-8 hours and generally consistent across species (Moh¨le, 2002; Wasser *et al*., 1994). This study showed no significant difference on cortisol levels in both sexes in the evening, also similar results have been found in some studies on marmosets (Smith& French, 1997a) and rats (Van Eekelen *et al*., 1991).

No correlation between the urine and saliva samples in all samples collected in both morning and evening and these results are in agreement with studies done, three studies compared averaged cortisol values from multiple or integrated daytime salivary collection to urinary cortisol and reported little (Blackburn *et al.*, 1987) or no association (Kathol *et al*., 1995, Putignano *et al.*, 2001) between salivary and urinary indices. The lack of correlation may be due to the fact that diurnal changes in urine cortisol show a time lag during cortisol excretion, in saliva and urine cortisol.

The results of this study are valuable for future research how ever the study had limitations and assumptions. These are the assumptions and limitation; Assumed that the kits validated at the species level will work with the subspecies in the current study. Orphans were being rehabilitated and no experimental designs were allowed to be carried out like validation.

The cross- section nature of the study provides snapshot picture and time did not allow observation of any erratic manage mental issues or intra species interactions that would have enabled assessment of their stressful nature.

CHAPTER SIX

CONCLUSION AND RECOMMENDATION

6.1 Conclusion

- For saliva, female cortisol was significantly higher in the morning and low in the evening, and for male cortisol was low in the morning and significantly higher in the evening.
- For urine cortisol both male and female cortisol levels were high in morning and declined in the evening.
- No correlation between the urine and saliva samples in all samples collected in both morning and evening

6.2 Recommendation

- Longitudinal studies are required to get a more comprehensive view in relation to management events of the facility

- The non-invasive measure can be the method of choice in primatological research projects and routine programmes related to the well-being of these captive animals.

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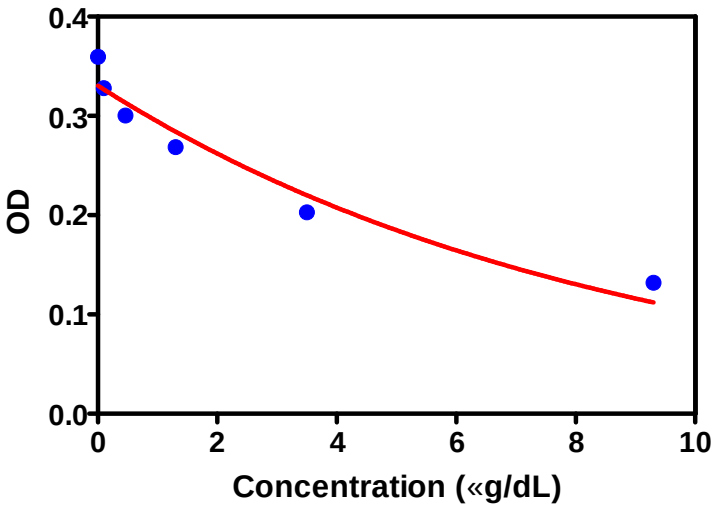
APPENDICES

STANDARD CURVES FOR SALIVA AND URINE

Appendix 1: Standard curve for saliva



Appendix 2: Standard curve for urine