FAECAL CORTISOL METABOLITES AS AN INDICATOR OF STRESS IN CAPTIVE SPOTTED DEER (*Axis axis*) AND BLACKBUCK (*Antilope cervicapra*) IN INDIA.

BY

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DECLARATION

This thesis is my original work and has not been presented for any degree or other certificates at any other University.

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DEDICATION

I dedicate this thesis to my beloved mother, Mrs Suman Sopan Bangar and my father Mr Sopan Bhayappa Bangar for always being supportive and encouraging me to be who I am today.

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ABBREVIATIONS

ACTH: Adrenocorticotropic Hormone

AVP: Arginine-Vasopressin

BARC: Bhabha Atomic Research Centre

CCTV: Closed Circuit Television

CNS: Central Nervous System

CRH: Corticotropin Releasing Hormone

DF: Degree of Freedom

DNA: Deoxyribonucleic Acid

EIA: Enzyme-Immunoassay

FC: Faecal Cortisol

FGM: Faecal Glucocorticoid Metabolites

GC: Glucocorticoid

GR: Glucocorticoid Receptor

HPA: Hypothalamic Pituitary Adrenal axis

IUCN: International Union for Conservation of Nature

LC: Locus Coeruleus

NE: Noradrenergic

POMC: Pro-opiomelanocortin

PVN: Paraventricular Nuclei

RGZP: Rajiv Gandhi Zoological Park

RH: Relative Humidity

RIA: Radioimmunoassay

RNA: Ribonucleic Acid

SD: Standard Deviation

SEF: Steroid Extracted from Faeces

THI: Temperature Humidity Index

ABSTRACT

Wildlife is currently facing a lot of challenges in their natural ecosystem such as negative effect of anthropogenic activities and climate change. Zoos play an important role in conservation and protection of wildlife through education, captive breeding and research. However, while in the zoo's wildlife is exposed to a different environment from their natural habitats and disturbances from human audience and climate variations. The aim of this study was to determine the effect of zoo visitor numbers, environmental factors (variation in Temperature Humidity Index during the October heat and winter season) and sex on faecal cortisol concentrations (ng/gm), as an indicator of stress levels in a captive population of blackbuck *(Antilope cervicapra)* and spotted deer *(Axis axis)* at Rajiv Gandhi zoological park in Pune, India. Assessment of adrenocortical activity through measurement of faecal glucocorticoid levels in faeces has significantly enabled data collection from wildlife, owing to its non-invasive nature. The blackbuck and spotted deer each consisted of six (6) adult males and six (6) adult females with an average age of 5.4 ± 0.55 and 5.5 ± 0.45 years respectively. The study animals were born and raised in the zoo. Each study animal was marked with ear tags for easy identification. The study was conducted in two (2) seasons during October heat (October-November) and winter (December-January). Visitor numbers was taken from the zoo records and temperature and humidity were measured every day using automatic hygrometer located in the zoo. Faecal sample collection was carried out between 12 noon to 6 pm on Wednesday (Low/Zero visitor category), Friday (Medium visitor category) and Sunday (High visitor category) per week for three (3) weeks in each season. The faecal cortisol metabolites were determined using Radioimmunoassay method. The blackbuck faecal cortisol con-

centration was within a range of 0.18 ng/gm to 2.62 ng/gm while that of spotted deer was within a range of 0.18 ng/gm to 3.07 ng/gm. The faecal cortisol concentration in adult males and females were not significantly different in both seasons. Visitor numbers significantly affected faecal cortisol concentrations (ng/gm) of blackbuck during winter but not during October heat. Temperature Humidity Index (THI) significantly affected the faecal cortisol metabolites in both blackbuck and spotted deer during winter but not during October heat. The observed differences in faecal cortisol concentrations (ng/gm) in blackbuck and spotted deer during winter are significant with negative correlation. The results of this study can be useful in informing management of captive wildlife and designing captive facility. Understanding the stress response of wildlife in captivity especially endangered ones is essential when planning for their effective conservation programmes.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information

Wild animals inhabit every one of the Earth's ecosystems including grasslands, forests, plains and deserts among others (Morrison *et al*., 2016). Although these animals live in their natural environment, research has shown that finding an unaltered wildlife natural environment is rare mainly due to increased anthropogenic activities; and this has greatly contributed to the global decrease of wildlife (Baird *et al*., 2009; Chapman *et al*., 2018). Continued loss of these species in the wild has accelerated the need for wildlife management outside their natural environment, in captivity, as an effort for conservation (Russello and Jensen, 2018). Present day zoos are crucial in the conservation of wild animals (Majie *et al*., 2018).

Wild animals either in the wild or in captivity habitually experience antagonistic stimuli which triggers a physiological stress response (Fourie *et al.*, 2015). To survive such stressors, adrenocorticotropic hormone from the pituitary gland stimulates glucocorticoid production from the adrenal gland and its action on the digestive system (Touma et al., 2005). When produced in connection with short-term stress responses, adrenocorticotropic hormones have beneficial effects on the individual (Munck et al., 1984) and thus, can positively influence specific life-history stages, survival and reproduction (Wingfield and Sapolsky, 2003). Yet, when production of glucocorticoid hormones is induced for prolonged periods of time, the hormone may harmfully distress some physiological functions (Munck et al., 1984; Sapolsky et al., 2000; McEwen 1998; Smith and Dobson, 2002), which can result in reduced survival probability (Pride, 2005). Consequently,

assessment of glucocorticoid levels has become common practice for monitoring welfare of wild and captive animals (Lane and McDonald, 2010; Hill and Broom, 2009), particularly in threatened or endangered species.

Zoos play a significant role in the conservation and protection of species through education, captive breeding and research (Hutchins et al., 2003). However, many zoos all over the world are not able to simulate the captive surroundings to mimic the extensive wild habitat of the species that are kept in captivity (Mellor et al., 2018). Due to these restrictions, captive habitats have to struggle in providing the idyllic setting for the natural ideal behaviour of captive animals resulting in welfare issues among captive animals (Morgan and Tromborg, 2007). In these new habitats, captive animals are exposed to a variety of external and internal stressors including seasonal variations, human presence, type of food among others which affect their dynamic constancy (Courtney *et al*., 2018). In a number of animal species, environmental elements, for instance humidity and temperature, have been confirmed as stressors (Huynh *et al.*, 2005; Dikmen and Hansen, 2009; Smitha *et al.,* 2011). However, few studies have been carried out or published regarding the impact of these elements on animals in captivity but the probable impact cannot be underestimated (Hosey., 2000; Rajagopal *et al*., 2011).

Since the beginning of this millennium, observational pragmatic studies on the behavioural and physiological patterns on captive animals have increased (Hosey, 1997; MacLeod *et al.*, 2018). The core of these studies has been applied in experimental research that estimates negative animal welfare in captivity as an influence of human visitors (Hosey, 1997; Dancer and Burn, 2019). Animal welfare science is a developing discipline with boundless potential over which

elementary behavioural scientific studies are unified alongside physiology, pathology and immunology to permit pristine understanding to improve lives of captive animals (Marchant-Forde, 2015). Captive animals display anomalous behaviour due to poor well-being, since behaviour is used as the first line of defence towards undesirable environmental changes (Mench, 1998; Vaz *et al.*, 2017). Manifestation of negative animal welfare in captive wild animals may involve a change in animal behaviour such as increased hostility, frustration, anxiety, decreased natural exploration and foraging among others. This is simply defined as stress in these animals (Bracke and Hopster, 2006, Dancer and Burn, 2019).

Analysis of faecal hormone metabolites is a widely used tool to assess adrenocortical activity in many species (Schwarzenberger and Brown, 2013). This non-invasive method can easily be performed in the absence of the target animal, which provides an evaluation of hormone levels without the bias of capture or disturbance-induced increases in stress hormones (e.g. faecal cortisol hormones) due to restraint and handling by the researcher (Harper and Austad, 2004; Millspaugh et al. 2004).

1.2 Statement of the Problem

Many animals are kept in captivity or zoos for various reasons such as amusement, educational purposes, research, protection of endangered species and for conservational purposes like breeding programs (Hutchins *et al.*, 2003; Curtin and Green, 2018). The desire to safeguard and preserve the integrity of the biosphere alongside all species within it while exhibiting compassion towards wildlife has become a popular concept in the current world (Kazarov, 2008; Gross, 2015). It is for this reason that zoos have become popular as they draw a lot of visitors. This

spiked global interest has provided zoo professionals with additional theoretical and practical roles of ensuring that animals are kept in conditions that mimic the wild (Mazur and Clark, 2001).

This study investigated the impact of humidity, temperature and visitor numbers on a captive group of blackbucks (*Antilope cervicapra*) and spotted deer (*Axis axis*) at the Rajiv Gandhi Zoological Park in Pune, India by assessing the levels of faecal glucocorticoid metabolites with a focus on cortisol levels as a physiological response to various stressors. There is very limited information on the effect of temperature humidity index on cortisol hormone (Silanikove and Koluman, 2015). Information on the effect of visitor numbers on faecal cortisol metabolites in captive animals is also scanty (Vaz *et al.*, 2017). Findings of this study have imperative inferences for a well-organized management of wild and captive animals for their restorations, which has as yet been accorded little attention. Stress in an animal is defined as an interruption of homeostasis due to extrinsic and/or intrinsic factors termed as stressors which in turn mobilizes adaptive hormonal, psychological and behavioural responses to an allostatic state (Tilbrook and Ralph, 2018).

1.3 Objectives

1.3.1 Overall objective

To investigate the Faecal cortisol metabolites as a measure of stress in captive spotted deer (*Axis axis*) and blackbuck (*Antilope cervicapra*) in Rajiv Gandhi Zoological Park, Pune, India.

1.3.2 Specific objectives

1. To determine the effect of visitor number on Faecal Cortisol Metabolites in spotted deer *(Axis axis)* and blackbuck *(Antilope cervicapra)* at Rajiv Gandhi Zoological Park and Wildlife Research Centre (RGZP) in Pune, India.
2. To evaluate Faecal Cortisol Metabolites due to variation in temperature-humidity index in spotted deer *(Axis axis)* and blackbuck *(Antilope cervicapra)* at Rajiv Gandhi Zoological Park and Wildlife Research Centre (RGZP) in Pune, India.
3. To assess the effects of sex on Faecal Cortisol Metabolites of captive adult males and females in spotted deer *(Axis axis)* and blackbuck *(Antilope cervicapra)* at Rajiv Gandhi Zoological Park and Wildlife Research Centre (RGZP) in Pune, India.

1.4 Hypothesis

1. Visitor numbers has no effect on faecal cortisol metabolites of Spotted deer *(Axis axis)* and Blackbuck *(Antilope cervicapra)* of RGZP.
2. Variation in Temperature humidity index in Spotted deer *(Axis axis)* and Blackbuck *(Antilope cervicapra)* of RGZP has no effect on their faecal cortisol metabolites.
3. There is no significance difference in faecal cortisol metabolites of adult males, females of Spotted deer *(Axis axis)* and Blackbuck *(Antilope cervicapra)* of RGZP.

1.5 Study Justification

Wild animals experience new environments in captive facilities such as zoos. Additionally, they are invariably exposed to new sceneries, odours and human audience in these new living arrangements which might result in negative animal welfare (Bracke and Hopster, 2006). A variety of studies have demonstrated how different wild species in captivity respond to human spectators (Hosey, 2005; Fernandez *et al.*, 2009). However, these studies were conducted under different circumstances and locations. In addition, there is a dearth of empirical evidence on how a combination of factors (stressors) influence faecal glucocorticoid metabolites and on diverse species. Thus, this study will help to understand how visitor numbers affects the faecal cortisol metabolites in both Spotted deer (*Axis axis*) and Blackbuck (*Antelope cervicapra*). This will contribute to the welfare of the animals and provide important information to improve the animal welfare conservation programs in the country and further ensure the animals are well-managed and conserved.

Furthermore, knowing how variation in temperature humidity index in spotted deer and blackbuck may affects their faecal cortisol levels will help to understand the kind of enrichment activities to put in place for the sustenance of the animals in captive environments. This will also contribute to the animals' welfare and conservation breeding programs.

Stress has an undesirable influence on the animal as it greatly contributes to immunosuppression and low productivity (Wheeler *et al.*, 2013). Finding out about variation in faecal cortisol metabolites with respect to the gender of both species can lead us to find out how there may be coexistence between captive adult males and females' weather in breeding and non-breeding seasons.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1. Animals in Captivity

With the escalating human population and reduction of animal habitats, there is urgent need to assess the anthropogenic effect on wildlife and ecosystems. An estimated six billion wild animals consisting of about 10,000 species all over the world have been confined in zoological parks, breeding centres for conservation, laboratories and as pets (Mason, 2010). This has resulted from increased habitat loss due to human activity that has led to increase biodiversity loss (Baird *et al*., 2009). Progressively, the preservation of captive populations has become recognized as an important component in the conservation strategies for species. Wild animals kept in captivity have been reported to live longer, are healthier and more productive compared to free ranging wild animals (Young, 2013). Furthermore, they are available for the public eye, a move meant to encourage collective responsibility in terms of public education and engagement in conserving animals (Russello and Jensen, 2018). It is for these reasons that animals such as blackbucks *(Antilope cervicapra)* and spotted deer's *(Axis axis)* are kept in RGZP, Pune.

While animals within zoological institutions are protected from the many challenges that threaten their wild counterparts, for instance effects or reduced habitats, these animals are occasionally placed into typical social situations and unfavourable climatic conditions (Morgan and Tromborg, 2007; Wolfensohn *et al.*, 2018). Some of them are negatively affected in these artificial settings by environmental factors such as temperature and humidity, and human interaction among others

(Mason *et al.*, 2007; Hing *et al.*, 2014). This has necessitated behavioural research in zoos to properly understand the dynamics and to offer solutions that would enhance the positive conditions of animals in captivity (Armstrong and Botzler, 2016; Merrick and Koprowski, 2017). Psychologists trained in animal behaviour analysis have for a long time been conducting basic and applied research in animal behaviour in diverse animals in captivity (Ward *et al*., 2018). Behaviour is the most striking characteristic of all living organisms (Marchant-Forde, 2015). Many behavioural problems in zoos are an indication of poor welfare. Behavioural analysis is a significant tool for bettering welfare and attaining satisfactory levels of psychological comfort in zoo and aquarium animals (Marchant-Forde, 2015; Ward *et al*., 2018).

Despite assessment of stress via behavioural studies being a useful tool in ensuring a better welfare for animal captivity, it comes with its own set of challenges. Behavioural studies are limited to subjective opinions and are not essentially measurable and or quantifiable (Barber, 2009; Fraser, 2009). Whereas general evaluations of animals' behavioural responses can still have their benefits, a scientific approach to these issues is required (Hill and Broom, 2009).

2.1.1. Black buck *(Antilope cervicapra)*

Figure 2.1: Blackbuck antelope (Image by Dr Nikhil Bangar at RGZP, Pune).

The blackbuck (*Antelope cervicapra*) is an elegant, gazelle-like animal and medium-sized antelope that is native to the south-east Asia (Figure 2.1, Figure 2.2) and is regarded as the most handsome member of the order 'Artiodactyla' (Jhala and Isvaran, 2016; Chaudhary and Maharjan, 2019). According to its colour, coat, length and the shape of the horn, there are four sub-species of *Antilope cervicapra* (Chaudhary and Maharjan, 2019). Its common habitats are savanna and diverse shortgrass with the greatest categories in semi-arid grasslands (Jhala and Isvaran, 2016). Blackbuck can subsist on feeds with low quality whereby they catabolize proteins, forage consumption during the summer and also decrease their body movement (Hummel *et al.*, 2015; Jhala and Isvaran, 2016). Blackbuck populations vary frequently due to ecological disasters, which in-

clude droughts and floods. They demonstrate fast-numerical reaction and high prolificacy to food accessibility and predation (Jhala and Isvaran, 2016).

Predation on calves and adults can theoretically control their population to levels of carrying capacity of their habitats (Jhala and Isvaran, 2016). Predation and sex metamorphoses in mating approaches seem to profile the population structure of blackbuck (Jhala and Isvaran, 2016). Male blackbuck have shorter life spans compared to females with increased mortality occurring at sexual maturity corresponding to mating competition (Jhala and Isvaran, 2016). Other than predation, the animal suffers from a variety of non-infectious and infectious diseases alongside habitat reduction (Chaudhary and Maharjan, 2019; Debata, 2017),

It is listed as a vulnerable in the IUCN list with a record of about 35000 individuals as at June 2016 (IUCN., 2017). The situation seems to have changed as it was classified as near threatened a few years ago a move which necessitated conservation of this species (Chaudhary and Maharjan, 2019). The species was considered extinct in some countries within the subcontinent (Chaudhary and Maharjan, 2019; Debata, 2017). The species is widely kept in zoos in India for conservation and breeding (Rajagopal *et al*., 2011). In addition, it is kept in zoos for public access as zoos are popular in India.

Figure 2.2: Known blackbuck distribution in the world. Grey shading shows the range where blackbucks are known to be resident. (Source: International Union for Conservation of Nature 2017 https://www.iucnredlist.org/species/1681/50181949).

2.1.2. Spotted deer *(Axis axis)*

Figure 2.3: Spotted deer (Image by Dr Nikhil Bangar at RGZP, Pune).

Chital or spotted deer (Axis axis) (Figure 2.3) is the 3rd largest deer dwelling in the plains and rippling terrain of India. An adult is estimated at 90 cm tall and weighs up to 85 kg (Duckworth et al., 2015). This popular species is a favourite with zoological parks around the world for their beautiful appearance and graceful gait (reddish coats with white spots and a white ventral surface (Sankar and Acharya, 2004). The species is endemic in south Asia, occurring in India, Sri Lanka, Nepal and Bangladesh (Figure 2.4) with the species occurring sporadically in the forested areas throughout the Indian peninsular (Sankar and Acharya, 2004; Duckworth et al., 2015). They are found in a variety of forests including deciduous, thorny and mangrove forests (Baral, 2015).

The population of the species has declined significantly throughout their habitat, with their current population limited to one hundred and twenty-three Protected Areas of India and some forest tracts (Sankar. and Acharya., 2004). It is listed as a least concern in the IUCN as at June 2016 (IUCN., 2017). Chital are essentially social animals often observed in groups of females with an adjacent group of males, they are rarely seen solitary. The population generally consists of female (Mohanty *et al.,* 2013).

The main causes of population decline are predation are wild animals as well as anthropogenic activities (Sankar and Acharya, 2004; Mohanty *et al.*,2013). Nevertheless, the species are susceptible to various diseases especially those of domestic livestock, they are widely hunted within their range by humans and some die in car accidents in protected areas (Sankar and Acharya, 2004). Coincidentally, male fights during breeding seasons have been known to cause deaths (Sankar and Acharya, 2004; Mohanty *et al.*,2013). Due to the threats on its survival, efforts have been placed to ensure their prosperity. Though the species has thrived within protected areas, the populations are vulnerable to poaching, forage competition with livestock, habitat destructions and livestock-borne diseases (Sankar and Acharya, 2004; Mohanty *et al.*,2013).

Figure 2.4: Known spotted deer distribution in the world. Grey shading shows the range where spotted deer are known to be resident. (Source: International Union for Conservation of Nature 2017 https://www.iucnredlist.org/species/41783/22158006).

2.2 Stress in Captive Wild Animals

The validation of stress in animals using faecal glucocorticoid is challenging since the normal range of the hormone in animals are difficult to know and it may be gender- biased and discretely different (Goymann, 2005). Moreover, animals respond differently to various stressors (Laws *et al.*, 2007). Leading to varying measures of glucocorticoids. Furthermore, the final outcomes may be prejudiced due to procedural issues due to the uneven distribution of metabolites within faecal pellets (Millspaugh and Washburn, 2004). If small quantity of the faeces is obtained, there would be a resultant implication when interpreting the faecal cortisol metabolites

measurement. Also, samples may deteriorate as a result of bacterial enzymes since there is shortage of storage facilities in the field. Möstl and Palme (2002) reported that within few hours after defaecation, bacterial enzymes can metabolize steroids. Thus, it is evident that storing faecal samples can be challenging. There are existing literatures on numerous reviews and procedures on the biological and physiological assessment of hormonal researches (Touma and Palme, 2005; Goymann, 2005; Sheriff *et al.*, 2011). These factors requires further investigations in free- ranging populations studies (Sheriff *et al.*, 2011).

The zoo animals respond to captive environments with behavioural modification. These behaviour modifications are modulated by the adrenal cortex (Breed and Moore, 2015). Activation of the hypothalamic pituitary adrenal is thought to be related to the induced physiological stress (Westerink *et al.*, 2002; Aguilera, 2012; Franco *et al.*, 2016; Allen and Sharma, 2019). During stress, hypothalamic pituitary adrenal axis activation boosts the secretion of adrenocorticotropic hormone from the anterior pituitary (Franco *et al.*, 2016; Allen and Sharma, 2018). This stimulates the synthesis and release of glucocorticoids from the adrenal cortices (Seasholtz, 2000; Aguilera, 2012; Franco *et al.*, 2016; Allen and Sharma, 2018).

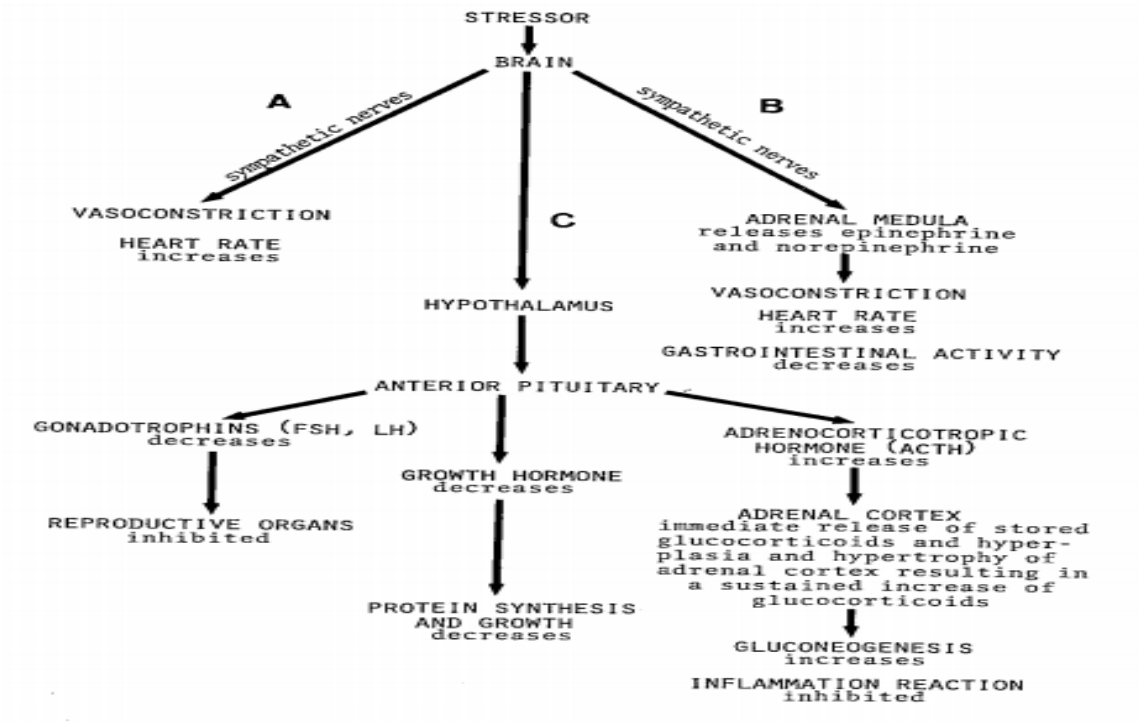

Figure 2.5: Major pathway on how an animal responds to stress as influenced by different conditions (Friend, 1980)

The hypothalamic-pituitary-adrenal (HPA) axis controls the release of Cortisol by the adrenal cortex. Activation of this axis begins with the release of hypothalamic corticotrophin releasing hormone (CRH) followed by pituitary pro-opiomelanocortin (POMC) gene activation and transcription as a feedback towards CRH secretion. The activation of POMC-encoded adrenocorticotropic hormone (ACTH) and the ACTH induces synthesis of cortisol from the adrenal gland (Malkoski and Dorin, 1999; Geerlings and Gerritsen, 2017). Cortisol in turn will impede the secretion and expression of CRH gene at the hypothalamic level and interfere with ACTH-secretion and POMC transcription, hence endowing a regulatory feedback cycle. Cortisol and other glucocorticoids (GCs) cause their physiologic effects at the cellular level by bonding to a specific in-

tracellular glucocorticoid receptor (GR) (Oyola and Handa, 2017). The GR is a hormone receptor which belongs to the nuclear receptor subclass and the transcription factors superfamily.

When cortisol binds to cytosol, it is then translocated by the glucocorticoid receptor (GR) and is released into the nucleus from a heat shock protein and while there, serves as a DNA sequence-specific transcriptional regulator of distinct cortisol-responsive target genes (van Bodegom *et al*., 2017). The GR will lay out unsuitable animals hence its importance in ensuring the survival of an animal in captivity (Geerlings and Gerritsen, 2017). Other than the DNA-binding dependent role, a majority of the GR actions involves its direct protein–protein interaction capabilities with other transcriptional regulators. It also controls a distinctive subgroups of target genes. A study by Pochigaeva *et al.*, (2017) proved this by demonstrating the survival of a mutant GR-carrier transgenic mice. The GR compounded the capability to bind to DNA but lacked the ability to bind to other classes of proteins.

The major biological roles of the GC–GR axis encompass annihilation of inflammation along with energy control of metabolism among others (Vegiopoulos and Herzig, 2007). Stress-induced glucocorticoid release has a wide range of additional physiological effects that aim to correct mobilization of resources so as to prevent extreme response of the different systems initially activated by stress and to prepare the organism for further stress (Pochigaeva *et al.*, 2017).

2.2.2 Neuroendocrine Effectors of the Stress Response

The stress system or response has two (2) components which are the peripheral and the central nervous system (CNS) components. According to Serrats *et al.*, (2017), these components are located in the brainstem and the hypothalamus. They include the parvocellular neurons of cortico-

trophin-releasing hormone (CRH), the CRH neurons of the paragigantocellular, parabronchial nuclei of the medulla and the locus coeruleus (LC), the arginine-vasopressin (AVP) neurons of the paraventricular nuclei (PVN) of the hypothalamus and other majorly noradrenergic (NE) cell groups in the medulla and pons (Vegiopoulos and Herzig, 2007). The peripheral components of the stress system include the efferent sympathetic adrenomedullary system, the peripheral limbs of the HPA axis; the efferent sympathetic-adrenomedullary system; and components of the para-sympathetic system (Belda *et al.*, 2015).

Chronic stress and intensified levels of HPA axis hormones can have damaging effects in captive animals including negative feedback control of reproductive functions (Smith and Dobson, 2002), tissue atrophy and suppression of the immune system (Steward, 2003). Chronic stress leads to high levels of glucocorticoids which is also known to cause gastrointestinal ulceration, depression, bone mass reduction, imbalance of electrolyte, hypertension, loss of calcium, and growth inhibition (Francesca, 2018). Thus, refining the health and general welfare of captive animals requires recognising what stressful environmental situations and developing control strategies.

2.3. Stressors of Wild Animals in Captivity

Wide variety of stressors are experienced by wild animals in captivity. These include habitat loss, different kinds of strange noises, artificial natural environment, odours which they are not accustomed to, aberrant lighting and fluctuations in temperatures (Madliger and Love, 2014; Eikenaar *et al.*, 2019). In addition, the animals are susceptible to stressors specific to captivity such as decreased space for movement, decreased flight space, increased human activities, reduced human-animal interface, less feeding options, upkeep in social groups that are not identical and other restrictions towards expressing their normal behaviour (Batson *et al.*, 2017).

However, through stress, animals gain different experiences especially by discovering of new environmental components and enhanced breeding. These are typically seen to have a positive effect while in captivity (Hawkey, 2017). According to Cauchoix *et al*., (2017), the natural body stress response may not be harmful intrinsically but entails a compound feedback mechanism involving the activation of the HPA and release of glucocorticoids that maintain the body in an allostatic state. Wielebnowski (2003) further adds that it is important to monitor/control the type of stress a captive animal is experiencing in order to know whether it is beneficial or harmful to the animal. Chronic damaging stress can become injurious to captive animals if they are not able to cope (Brosschot and Verkuil, 2018). Any form of stress that is persistent may bring about negative effects on the body such as neurodegeneration coupled with impaired cognition and cardiovascular detriment (Eikenaar *et al.*, 2019).

Insufficient acclimatization can cause stress, which could lead to the clamping down state of the mind, amplified exhibition of platitudinal behaviour and decreased propagative conduct henceforward dictating the need to survey it (Bernabucci *et al.*, 2010). Another clue pointing to stress in mammals in confinement can be determined by measuring and quantifying the level of cortisol and corticosterone in faeces and blood (Wheeler *et al.*, 2013).

The different wild animals in captivity have slight or no self- restriction over the period, kind of illumination, temperatures, sounds, odours or guests to which they are exposed. Wheeler *et al*., (2013) notes that behavioural alteration is an animal's "primary line of defence" as a rejoinder to stressors. If animals are continuously exposed to the various stressors, acute or chronic often con-

tributes to a sudden change in an animals' physiological and behavioural response followed by cross sensitization, which greatly affect the HPA axis (Belda *et al.*, 2015; Ozella *et al.*, 2017).

2.3.1. Visitor Categories

Zoos have been in existence since early 18th century and have provided avenues for public enjoyment, education and research (Mason, 2010; Landa *et al.*, 2017). Zoo visitors since the beginning of time have been the main source of revenue for operational costs hence are an essential component in running of the zoos. Their importance to the zoos has in turn resulted into research devoted to understanding the relationship between people, the zoo and zoo animals (Davey, 2006). Through various evolutionary stages, research now includes studying visitors' population characteristics, animals' psychological and behaviour responses including other aspects all aimed at humanising the significances of zoos (Strandin *et al*., 2018).

Among other roles, zoos are important in animal protection as well as conservation while at the same time educating the public on the existence of diverse animal species and the importance of conserving and protecting them. Zoos also play a crucial role in creating awareness and changing local communities attitudes towards wildlife (Ozella *et al.*, 2017).

Visitor effects on zoos are not always unfavourable to the animals especially in cases where there is continued positive animal welfare and interaction between the animals and human caretakers (Hosey, 2000; Cole and Fraser, 2018). Several studies have shown that some animal species are not negatively affected by human onlookers and will exhibit natural conduct in their presence (Hosey, 2000) while in other species such as captive bonobos (*Pan paniscus*), human audiences have an enriching effect (Eikenaar *et al.*, 2019). According to Sekar *et al.*, (2008), a positive reac-

tion from some animals often instigate human interactions, which researchers believe might be enriching to the animals. However, majority of the studies have also shown that captive animals are negatively impacted by visitor interactions. Stressors in captive animals often have a detrimental effect on an individual, are usually exhibited by decreased movement in their enclosures, and increased hostility towards caretakers. Hosey, (2005), MacLeod *et al.,* (2018) and many other studies have measured glucocorticoids; cortisol and corticosterone to understand the effect of these stressors on the health and well-being of these animals (Vera *et al*., 2019). During stress, the HPA axis is activated which causes the release of glucocorticoid from the adrenal cortex in mammals (Bhimte *et al.*, 2018). Glucocorticoid metabolites assessment are often performed when assessing stress as they are the main body hormones produced in response to a stressful situation. These metabolites can be measured from the faeces of various animal species (Morrow *et al.*, 2002). To assess the physiological impact of stress on wild animals in captivity, faecal glucocorticoid assay accords a non-invading approach (Franceschini *et al.,* 2008). Glucocorticoids are produced as stress hormones and play a key role in research as they are often used to assess stress in vertebrate animals including birds, fish, mammals and reptiles (Rajagopal *et al*., 2011). In captive wild animals, using blood glucocorticoid variables is deterred as restraint and handling induce stress hence compromising the accuracy of the assays (Franceschini *et al.*, 2008).

The presence of humans in zoos may be inevitably unfair and unfavourable to wild animals because they lack a chance to express their natural behaviour without worrying about who is watching (Hosey, 2000). According to Love *et al.,* (2017), human visitors may have a negative influence on animals in captivity which may cause and/or exacerbate health conditions. Numerous research papers have illustrated how human interaction with these animals have negatively af-

fected them leading to abnormal behavioural changes like increased aggression and reduced exploration (Hosey, 2005; Rajagopa *et al*., 2011; Landa *et al.*, 2017). Most of the wild animals are social and over time have been observed isolating themselves while others develop behavioural complications in the presence of humans (Courtney *et al*., 2018).

Contact with protracted stressful environments has implications to the long-standing health of zoo animals such as causing suppression of immune system, reduced fertility rate and promotion of anomalous behaviours such as self-inflicted injuries (Gaskill and Garner, 2017). Nevertheless, an animal's reaction to zoo guests is dependent on the individual species or on how its kind respond (Hosey, 2005). Other studies contrary to this state that continuous exposure of animals to zoo visitors plays an important role in enriching some wild species by decreasing stress in some animals (Morgan and Tromborg, 2007). A few researchers have however reported minimal to zero effects of human visitors on animals in captivity (Synder, 1975; Melfi and Thomas, 2016).

Studies carried out on caged lion-tailed macaques (*Mzacaca silenus*) highlighted an increase in abnormal behaviour following an increase in the numbers of human viewers (Mallapur *et al*., 2005). Changes in the behaviour of the target species were observed when visitor numbers were either high or low. Adult orang-utans (*Pongo pygmaeus*) in Chester Zoo, United Kingdom have also been observed to take accessible sacks made up of papers to cover themselves regularly when visitor number is high during the daytime while new-borns always stay with their mothers. In India, high stress levels were recorded on bison (*Bos gaurus*) at the Arignar Anna Zoological Park when visitor numbers were high (Serrats *et al.*, 2017). Richardson (2015) puts weight on the importance of visitor regulation in zoos for the welfare of the animals in captivity. More studies

emphasize on this by showing linkages of stress of wild animals in captivity as a result of human interaction (Hosey, 2005; Touma and Palme, 2005; Davey, 2006). The adverse effect of stress on feeding habits has also been documented though the specific pathways responsible are still contested (Strandin *et al*., 2018). These studies are yet to explain the exact effect on appetite resulting from stress coming from an interaction within glucocorticoid and leptin (Ozella *et al.*, 2017).

2.3.2. Temperature Humidity Index (THI)

A number of parameter have been developed to help recognize some of the impacts on animals as influenced by environmental factors (Cauchoix *et al.*, 2017). However, few ecological parameters have been comprehensively studied and include temperature and moisture (RH). The utmost familiar comfort index is the Temperature-Humidity Index (THI) whose initial establishment was done by Thorn (1958) before its subsequent acceptance by the U.S. Weather Bureau (1959) as the ideal index for humans.

The Indian Metrological Department claimed that India has a varied diversity of physiographic structures that have created varying climatic conditions. There are highlands where snow is near everlasting, while in some areas, temperature go above 50°C. Daily temperature ranges in the coastal belts fall between 7-8°C though this is dependent on location. In India, there are three major seasons; summer, monsoon and winter. Maximum temperature in summer is 50°C. A temperature of up to 4-5°C is noted in winter while during monsoon season, it rains very heavily about 800 mm. All these factors can affect stress levels of an individual animal (Rajagopal *et al*., 2011)

A rise in temperature has the ability to affect unfavourably the morphology and physiology of cells, which will in turn alter their membrane structure and function (Debata, 2017). This will in turn interfere with RNA processing due to oxidative metabolism, causing impaired transcription (Mashaly *et al.*, 2004). However, only a certain number of stressors from the surroundings are able to activate the hypothalamus pituitary-adrenal cortical and sympathetic-adrenal medullary axes (Saitou, 2013).

A prolonged exposure to either extremes of temperature can adversely raise the levels of catecholamines and cortisol in the body (Asres and Amha, 2014). In males, ecological factors for instance temperature and humidity may cause stress that can contribute to low sperm count which is unswervingly relational to a reduction in reproductive levels in females that results from reduced rates of conception and high death of embryos (Gaskill and Garner, 2017).

In India, twenty-four, two (2) months old New Zealand white rabbits (*Oryctolagus cuniculus*) were arbitrarily selected from sections of rabbits at Krishi Vigyan Kendra, Thrissur, Kerala and were raised throughout the hot season from March to May. Weekly collected cortisol showed that the rabbits were stressed during afternoon hours (Smitha *et al.*, 2011). Current global warming and other environmental factors are also playing important role in stress of the animals. In addition, heat stress can exist for nearly the whole year especially in semi-arid and tropical areas. There are four (4) important ecological factors that influence heat stress on animals. They include humidity, air movement, radiation and dry bulb temperature. However, little research has been done to explain how temperature in combination with the other factors make an animal suffer from heat stress (Buffington *et al.*, 1981; García-Ispierto *et al.*, 2007)

2.4 Assessment of Stress

Glucocorticoids have been described as the effective indices to measure and quantify stress levels in many species (Turner *et al*., 2003). As a result of their prospective influence on physiological status, measuring glucocorticoids function is important in studies related to animal welfare, evolutionary ecology and conservation biology (Muehlenbein *et al.*, 2012; Hing, 2016).

Stress levels of individual animals can be reported from glucocorticoid concentrations in various samples from animals for instance blood, saliva, hair, urine or faeces (Mastromonaco *et al.*, 2014). Collection of most of these samples require invasive methods and or restraint of animal species leading to acute stress responses making them less ideal in studying stress in wild animals. Methods that do not require handling of animals for measurements of glucocorticoid (GC) concentrations serve as dependable indicators of adrenocortical activities and physiological stress piles in a variety of species (Kersey and Dehnhard, 2014; Nemeth *et al.*, 2016; Wolfensohn *et al.*, 2018).

As a result, non-invasive monitoring by use of faecal steroid metabolite assays is now increasingly popular. Measurements in faeces are not fundamentally affected by short-term variations of circulating GC levels, since GCs are processed by the liver and excreted via the digestive system (Goymann, 2005). Accordingly, faecal GC metabolites (FGMs) mirror overall stress loads and stress reactions in a long time frame and are preferred validation of stress load especially in wild animals (Morrow *et al.*, 2002; Touma & Palme, 2005 Franceschini *et al.*, 2008; Rajagopal *et al*., 2011; Bhimte *et al.*, 2018).

2.4.1 Assessment of Faecal Cortisol

A number of approaches are available for the approximation of cortisol from biological specimen (Ward *et al*., 2018). Plasma assessment of glucocorticoid levels has been indispensable in the evaluation of cortisol (Goymann, 2005). Faecal samples are currently being used as biological specimens to measure cortisol levels in animals (Sopinka *et al.*, 2015). There is a constant practice of faecal glucocorticoid metabolites related studies by many researchers to evaluate glucocorticoids which are known as a stress hormones released by wild as well as domestic animals (Touma and Palme, 2005; Fanson *et al.*, 2017). Various techniques are used in the extraction of metabolites from faecal samples. The metabolites have different polarities hence necessitating appropriate procedures for the extraction process (Crespi *et al.*, 2013; Morrison *et al*., 2016).

The unpredictability in adrenocortical activity also known as stress response, is a new area of research for conservation scientists. Through stress the immunity can decrease, show adverse effects on reproduction or even change animal behaviour (Millspaugh and Washburn, 2004). This technique of glucocorticoid assessment is non-invasive and very attractive allowing sample collection for research that not stressing animals, particularly the endangered ones and/or those that are highly responsive to external stimulus (Beehner and Bergman, 2017).

Presence of steroid metabolites in the faeces is a representation of pooled endocrine activity, resulting from the intestinal passage of steroid metabolites in the faeces over the previous several hours (Keay *et al.*, 2009; Forristal *et al.*, 2016; Fanson *et al.*, 2017). The liver metabolizes as steroids and the products excreted through bile or urine into the faeces. As they pass through the intestines, the metabolites of the steroids have a chance of being absorbed back into the circulation between the stomach and the liver (Schwarzenberger, 2007). However, the intestinal passage

results in a delay between steroids circulation in the plasma and appearance in the faeces; which may correlate with the time taken by the bile to go through the intestinal passage to the rectum. Thus, faecal steroid analysis is preferred because over time, it presents a hormone profile which is more dampened with little interference from acute stress and daily rhythm.

There are many advantages of using faecal steroid analysis as opposed to the more traditional analysis which has been used for years (Touma and Palme, 2005). The most obvious is that the technique does not introduce variables because it is non-invasive to the subjects involved. Faecal glucocorticoids are not sensitive to researchers' induced errors, which can be caused by normal changes in glucocorticoid fluctuations. They are also not affected by minor environmental changes and restraining of animals. Depending on the species, the glucocorticoid delay time in faeces takes up to six to twenty-four hours from primary glucocorticoids once released in the body (Fanson *et al.*, 2017). In small-size animals or stress-prone species, faecal samples have become a suitable option which could be used to analyse hormones in the plasma or serum (Moulder *et al.*, 2018). Faecal steroid analysis has also been applied on species with different sizes, ranging from animals as big as elephants to those that are as small as mice (Touma and Palme, 2005). However, there is need for proper validation of faecal steroid assay which relates closely to the metabolism of steroids (Schwarzenberger, 2007). Other than estimating glucocorticoid from a faecal sample, the effectiveness and non-invasive attribute of this technique should also be tested.

The limitation of faecal steroid analysis is the existence of a large number of several faecal metabolites which are also present in species that are closely related. Experiments on the metabolism

of radioactively labelled steroids have given remarkable insight into the excretion and metabolism of hormone metabolites through urine and faeces which has helped in developing techniques for faecal steroid analysis (Kersey and Dehnhard, 2014). The pathway of excretion differs significantly between steroids within the same species and among species.

Because of this, it may alter the results. However, a number of studies, in which there was either difficulty in blood sampling regularly or circumstances which rendered the activity impossible in species such as wildlife which are often prone to stressors in captivity, opted for substitute hormonal analysis using faecal samples (Moulder *et al.,* 2017). Faecal cortisol analysis has previously been applied in many animal species including whales, elephants and even mice (Touma & Palme, 2005). There is however a need for befitting faecal cortisol assays which relates closely to the metabolism of steroids (Touma & Palme, 2005; Schwarzenberger *et al*., 2007).

2.4.2 Radioimmunoassay (RIA) Technique

Enzyme-linked Immune Assays (EIA) and Radio immuno Assays (RIA) are applied on an equivalent occurrence to measure FGC and their metabolites in numerous animals (van Bodegom *et al*., 2017). However, EIA are now replacing RIA (Zheng *et al*., 2019). EIA is considered to be less superior to RIA based on the fact that it has kits that contains antibodies developed to conglomerate with a particular un-metabolized Glucocorticoid hormone in blood plasma as either cortisol or corticosterone and other metabolites from FGM (Geerlings and Gerritsen, 2017). This therefore indicates that faecal cortisol assessment in wild animals gives more definitive results than mean measuring plasma cortisol.

The technique in carrying out RIA involves competitively binding or displacement reaction where two antigens able to bind to one antigen together, the higher concentrated antigen binds more extensively displacing the other antigen. The antigen that is allowed to bind is the radio-labelled one (Zheng et al., 2019). It is a sensitive invitro assay that measures consolidation of matter in the body using concentrations of antigens (Bowie, 2018).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

The study was carried out in Rajiv Gandhi Zoological Park and Wildlife Research Centre (RGZP; 18° 27′ 10.46″ N and 73° 51′ 40.23″ E at an altitude of 560m) Pune, Maharashtra, India (Figure 3.1). The area of the facility is about 130-acre which is divided into a snake park, animal rescue center and a zoo. Animals in the zoo includes bear, sambar, barking deer, nilgai, chinkara, elephant, lion, fox, jackal, wolf, Indian gaur, tiger, leopard, spotted deer and blackbuck (About zoo, 2014).

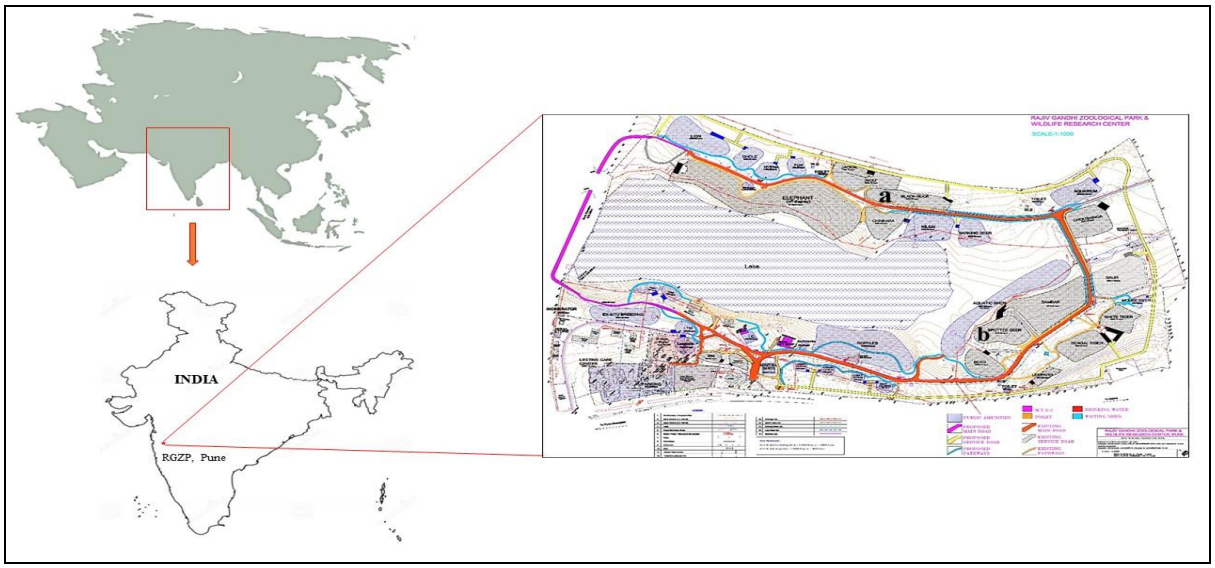

Figure 3.1: Map showing layout of Rajiv Gandhi Zoological Park, Pune location in India, Asia (Inset map), Blackbuck enclosure (**a**) top centre and Spotted deer enclosure (**b**) right centre. (Source: Director's office RGZP, Pune).

The city of Pune is rated among the most rapidly developing cities in the region of Asia-Pacific. The last official census in 2011 recorded over 9 million citizens living in the Pune district with a land mass of 15,642 km^2 (About Pune., 2019). The Pune urban mass constituted more than half of the people living in the district making up about 75% of the total population. Hinduism postulates the importance of all animals and encourages respect towards animal in similar accord to humans. Therefore, the people frequently visit animal institutions like zoo in order to accord respect to these animals.

3.2 India's Climatic Conditions

The meteorological department in India records a total of four (4) different climatic seasons in India. The coldest period is during the winter season where the temperature ranges between 10°C and 25°C having a humidity of 56%. The summer season mostly begins in April up until July in North western India but ends in June in other areas. Monsoon season starts in July and ends in November whereas the post monsoon season begins in October up until November. Post monsoon season is generally known as the 'October Heat'. The average temperature during this period gets up to 35°C with an average humidity of up to 68%. However, the Pune city goes through a hot semi-arid climate having temperatures between 19°C and 33°C.

3.3 Study Animals

Two (2) species were selected from the zoo for the purpose of the study. One of the species was selected from the Bovidae family and Antilopinae sub-family. It is referred to as the Indian Antelope (*Antelope cervicapra)* also called by the name 'blackbuck'. The blackbuck consisted of six

(6) adult males and six (6) adult females with an average age of 5.4 ± 0.55 years. Similarly, the spotted deer also consisted of six (6) adult males and six (6) adult females with an average age of 5.5 ± 0.45 years according to the zoo records. All the animals were born and brought up in RGZP and lived in an open enclosure in the same premises. The animals were given the right quantity of water and food according to their nutritional requirements. The feeding time was between 11am and 12noon each day. The animals for each species were selected based on good health and adequate physical condition. These animals were also identified through the use of ear tags and specific characteristics and markings on the body of the animals.

3.4 Study Design

This study was conducted during two (2) seasons which included the October heat from 18th October 2017 to 5th November 2017 and the winter season from 20th December 2017 to 7th January 2018. The temperature and humidity were measured every day during the course of the study with the help of an automatic hygrometer (GOOSEBERRY Digital Hygrometer Thermometer Humidity Meter with Clock LCD Display HTC-1). The average maximum temperature during the October heat day was 37.17°C whereas the average minimum temperature was 25.47°C. The average humidity for the October heat was 44.44%. while, the average humidity for the winter season was 29.27%. Also, the average maximum temperature for the winter season was 29.77°C. The average minimum temperature was 14.27°C. The Temperature Humidity Index (THI) was calculated with the formula specified in (1) (Kelly *et al*., 1971).

$$THI = (1.8\times T+32) - (0.55 - \frac{0.55RH}{100})\times [(1.8\times T +32) - 58] \quad (1)$$

Where THI is Temperature Humidity Index, T is Temperature and RH is Relative Humidity

Therefore, the mean THI observed during the October heat was 80.97 and 69.50 for the winter season. Three (3) weeks out of each season were selected for the sample collection. However, the samples were collected on only three (3) days in each week which included Sunday, Wednesday and Friday. Twelve samples from each species was collected on each of those days in the week (six (6) from the male and six (6) from the female) making a total of twenty-four samples collected per day.

3.5 Zoo Visitor Category

The visitor category in this study was described as the total number of visitors to the RGZP on selected days. Specific data was not available on the number of people visiting the spotted deer and blackbuck display area. The study was conducted in three (3) different visitor category conditions; high, medium and low/zero visitor categories as shown below in (Table 3.1).

Table 3.1: Visitor Category According to Days Selected for Sample Collection.

Seasons	Days of the week	Visitor Category	Mean Visitors
October heat	Wednesday	Low/Zero	0
	Friday	Medium	7887
	Sunday	High	13326
Winter	Wednesday	Low/Zero	0
	Friday	Medium	6369
	Sunday	High	14066

The mean zoo visitor numbers varied during the three (3) sampling days of the week (Wednesday, Friday and Sunday) in both seasons October Heat and Winter (Table 3.1). The electronic ticket sale recorded an average of 13696 visitors on Sunday and an average of 7128 visitors on

Friday during the study period. However, there were no visitors on Wednesday because the zoo is closed on that day.

3.6 Faecal Sample Collection

A pilot study was carried out for a week in order to understand the defecation pattern and identification of study animals. The actual sample collection was done between 12noon to 6pm on Sunday, Wednesday and Friday per week. The selected animals were strictly observed with the help of CCTV cameras (D3D D8862) and personal observation of the individual study animals defecating during sample collection. The samples were collected immediately after defecation and aseptically stored within two (2) hours. A total of twenty-four faecal samples were collected daily into plastic bottles that were closed tightly using a screw cap and packed into cool boxes containing ice packs. Samples were adequately labelled according to the species, tag number, sex, and date they were collected. Thereafter, the samples were transported from the enclosures to the zoo laboratory and lyophilised for about 24 hours (Model: Freeze dry system/freezone '2.5 Labconco) then stored at under -20°C (Hunt and Wasser, 2003) awaiting further transportation to the study laboratory.

3.7 Determination of Cortisol Levels

The samples were transferred from RGZP to the Department of Veterinary Physiology Bombay Veterinary College, Parel, Mumbai 400012, Maharashtra, India for the extraction procedure.

3.7.1 Extraction of Steroids from Faeces

Samples were transported to Department of Veterinary Physiology, Bombay Veterinary College after completion of field work in the lab sample was crushed and thawed in a polythene pouch before the onset of the extraction procedure. The extraction procedure was carried out with some modifications based on the method explained by Wasser *et al.*, (2004). To begin, 2gm of the crushed faecal sample was weighed and mixed with up to 15ml distilled water in 50ml polypropylene tubes. Then the sample was passed through a process of vertexing for about five (5) minutes and centrifuged at 4200rpm for twenty minutes. The filtrate was collected into a clean tube and 10000 microlitre of dichloromethane was pipetted and vortexed for one minute. The sample was then allowed to stand for phase separation. The aqueous phase was thrown away, while the rest was shifted into clean RIA tubes and the organic contents were dehydrated using a nitrogen evaporator (Model: Caterpillar, Speedovap, Takahe analytical instruments). 500µl of buffer assay (20 mMol. Trishydroxyaminomethane, 0.3 Mol. NaCl 0.1% Bovine serum albumin and 0.1% tween 80; Ph 7.5) (Rajagopal *et al*., 2011) was pipetted into the RIA tubes and permitted to stand still for 15 minutes. The content was then mixed with a vortex for one (1) minute and the resulting sample was entirely shifted into an eppendorf tube and stowed at -80°C for RIA analysis.

3.7.2 Radioimmunoassay (RIA)

The samples were transferred from the Department of Veterinary Physiology Bombay Veterinary College to the Radio Immunoassay Laboratory, Department of Veterinary Physiology and Biochemistry, College of Veterinary Science and Animal Husbandry, Anjora Durg, Chattisgarh

491001, India approved by BARC (Bhabha Atomic Research Centre, Mumbai) for the Radioimmunoassay analysis.

Cortisol estimation was done by RIA using a commercial kit (Immunotech, Czech Republic) as per (Smitha *et al.*, 2011). Cortisol concentration were measured in all the 432 extracted faecal samples using a modified RIA as explained by Huber *et al*., (2003). Intraassay and Interassay coefficient of variation in cortisol concentration were calculated at less than 8.6% and 11.6% respectively.

Material used

- RIA kit for cortisol (IM 1841 cortisol C. T. RIA kit; Immunotech, Czech Republic) and the kit comprised of the following;
    - Anti-cortisol monoclonal antibody-coated tubes
    - ^{125}I-labelled cortisol tracer
    - Reference calibrator set. Calibrators containing from 0-2000Nm of cortisol
- Pipettes and disposable tips
- Electronics Corporation of India Limited (IC4702A, I-125 Gamma counter)

Faecal cortisol concentrations were determined by [^{125}I] radio immunoassay procedure using a commercial kit mentioned above. Before beginning the test, all samples and reagents were brought to room temperature. Disposable tips were used for pipetting and dispensing of reagents. The desired numbers of antibody-coated tubes were labelled and secured in a holder. 50µl of calibrator or control or SEF sample was dispensed into the antibody-coated tubes and labelled accordingly. 500 microliters of tracer were then pipetted into tubes coated with antibody. These

tubes were vortexed and placed into an incubator at 25°C on orbital shaker for 60 minutes. After incubation the contents of the antibody-coated tubes was decanted from the tubes (except for two (2) tubes meant for total count) and, to determine the amount of radioactivity present, each tube was counted for one (1) minute on an automatic gamma counter. Standards (0, 10.5, 21, 63, 215, 770, 2200Nm/ml) and controls were included with each set of unknowns. The concentrations of unknown samples were interpolated from the logit-log representation of the calculated percent bound versus the standards.

The percent bound was calculated for each standard using the equation:

$$\text{Percent bound} = \frac{Calibrator\ or\ control\ or\ unknown\ sample\ counts}{Net\ maximum\ total\ binding\ counts} \times 100$$

3.7.3 Calculations of Cortisol Concentrations

After getting concentration of cortisol values for each sample, they were expressed in ng/gm of faecal material by using the following equation;

Cortisol concentration in ng/lit × 0.362 = cortisol concentration in ng/ml

(To convert nM/lit into ng/ml, multiply results by 0.362 as per kit instructions)

$$\textbf{Cortisol concentration in ng/0.5 ml} = \frac{\textbf{Cortisol concentration in ng/ml}}{\textbf{2}}$$

(The concentration was calibrated in 500µl of extracted sample)

$$\textbf{Cortisol concentration in ng/gm} = \frac{\textbf{Cortisol concentration in ng/0.5ml}}{\textbf{2}}$$

(2gm of faecal sample was used to get 0.5ml of extracted sample).

3.8 Statistical Analysis

R- software was used to perform all statistical analyses (Fiske & Chandler, 2011). ANOVA (Kruskal-Wallis test) was used to determine if there were significance differences in the mean faecal cortisol concentration as affected by visitor numbers during winter and October heat separately. Linear regression was used to determine if there was relationship (positive or negative) between temperature humidity index and faecal cortisol concentration during winter and October heat. Wilcoxon rank sum (Mann Whitney) was to compare the difference in faecal cortisol concentration between male and female. The level of significance was determined at $\alpha \leq 0.05$.

3.8.1 R Scripts

Kruskal Wallis Anova: > kruskal.test(variable 1 ~ variable 2, data = data)

Simple Linear Regression: > data.lm = lm(variable 1 ~ variable 2, data=data)

Multiple Linear Regression: > data.lm = lm(variable 1 ~ variable 2 + variable 3, data=data)

Wilcoxon Rank Sum Z Test: > wilcox.test (variable 1 ~ variable 2, data=data)

CHAPTER FOUR

4.0 RESULTS

4.1 Effect of Visitors numbers on Blackbuck and Spotted Deer

Visitor numbers had a significant effect ($p < 0.05$) on faecal cortisol metabolites of blackbuck during Winter but not on spotted deer. However, visitor numbers did not have any significant effect ($p > 0.05$) on both species during October Heat (Table 2). During Winter, mean faecal cortisol metabolites of blackbuck were significantly affected by zoo visitors' categories (Kruskal-Wallis test, $\chi 2 = 7.62$, df = 2, $p = 0.02$) (Table 2). The Kruskal- Wallis test with post hoc comparison Tukey test showed that the mean faecal cortisol concentration was significantly ($p < 0.05$) higher during the high levels of zoo visitor category [Sunday (1.42 ± 0.46 ng/gm)] than during medium visitor category [Friday (1.18 ± 0.28 ng/gm)] and low/zero visitor category [Wednesday (1.18 ± 0.24 ng/gm)] (Figure 7 and Figure 8). Mean faecal cortisol metabolites of Blackbucks during medium and zero visitor category were not statistically different from each other ($P>0.05$: Table 4.1).

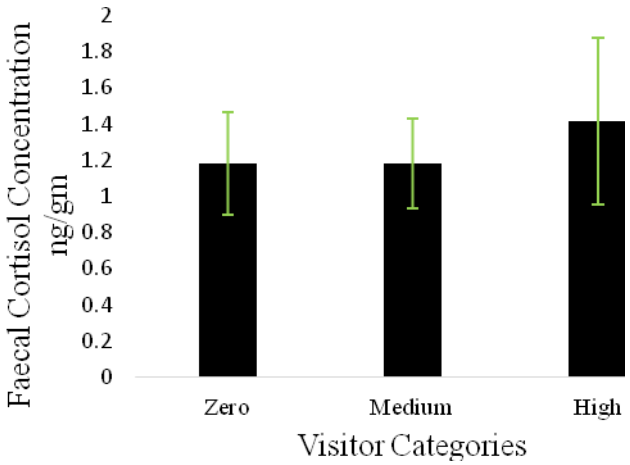

Figure 4.1: Effect of visitors' categories on faecal cortisol concentration (ng/gm) of Blackbuck during Winter (November-December). Faecal cortisol concentrations ng/gm mean for zero (1.18 ± 0.28ng/gm) and medium (1.18 ± 0.24ng/gm) visitors' categories was the same and significantly different from high visitors' category ($\alpha = 0.05$)

Table 4.1: Effect of Visitor Category on Blackbuck and Spotted Deer During October Heat and Winter.

Mean faecal cortisol concentration (ng/gm) during the three (3) levels of zoo visitor category. Low/Zero visitor category (Wednesday) is when visitors were absent, medium visitor category (Friday) is when the number visitor present were between 3000 to 13000 and high visitor category (Sunday) is when the visitors present were above 16000. Means with the same letters in the same column are not significantly different at $p<0.05$. Values in parentheses are Faecal Cortisol (FC), Standard Deviation (SD), Degrees of Freedom (DF), Chi-square value ($\chi 2$) and statistical significance level (P-value).

Signifiance codes : 0.001 '***' 0.01 '**' 0.05 '*' 0.1 '.' 1

Species	Seasons	Visitor Category	Mean FC ± SD	DF	$\chi 2$	P-value
Blackbuck	October Heat	Low/Zero	1.065 ± 0.19a			
		Medium	1.130 ± 0.22a	2	0.0832	0.9592
		High	1.111 ± 0.27a			
	Winter	Low/Zero	1.180 ± 0.29a			
		Medium	1.180 ± 0.25a	2	7.616	0.02219*
		High	1.417 ± 0.46b			
Spotted Deer	October Heat	Low/Zero	1.274 ± 0.21a			
		Medium	1.414 ± 0.24a	2	3.1987	0.2
		High	1.433 ± 0.60a			
	Winter	Low/Zero	1.318 ± 0.21a			
		Medium	1.343 ± 0.27a	2	0.5399	0.76
		High	1.429 ± 0.39a			

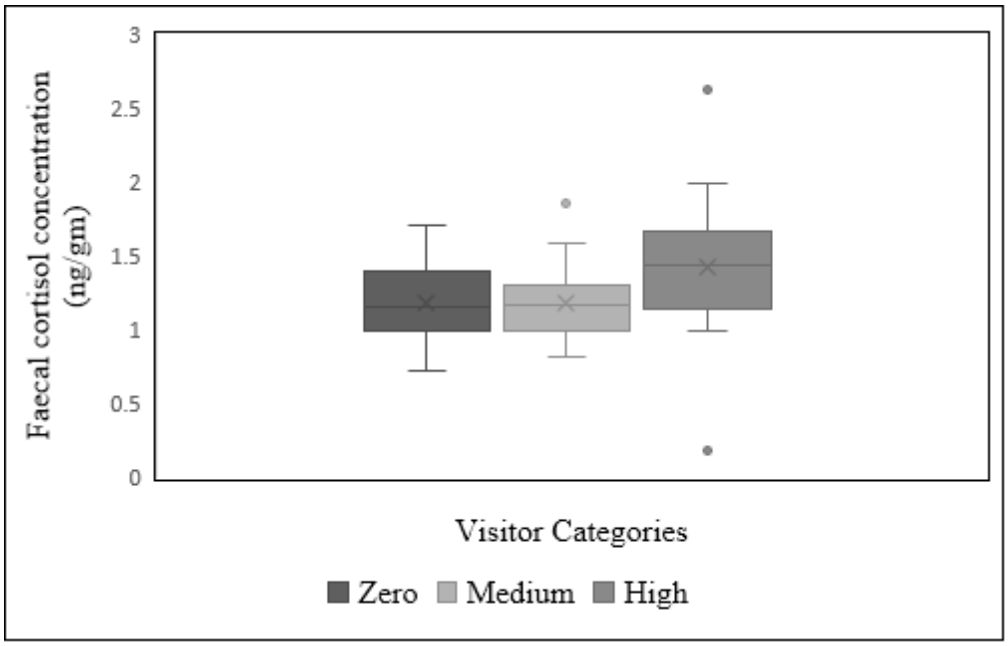

Figure 4.2: Faecal cortisol concentrations of blackbuck at Rajiv Gandhi Zoological Park and Wildlife Research Centre (RGZP), Pune, Maharashtra, India. Box Plot showing comparison between mean faecal cortisol concentration at zero, medium and high visitor categories. Mean faecal cortisol concentration of Zero and Medium visitor categories were same ($p<0.05$) but statistically different from High visitor category ($p>0.05$) Points situated on the plot are outliers which are not included in the final analysis.

4.2 Effect of Temperature Humidity Index (THI) on Blackbuck and Spotted Deer

There was a significant negative correlation between Temperature Humidity Index (THI) and faecal cortisol level in Blackbuck during Winter (Figure 4.3.a: Simple linear regression: $r^2 = 0.15$, $F = 12.14$, $p = 0.001$). There was no significant effect of THI on mean cortisol levels of Black-buck during October heat (Figure 4.3.b: simple linear regression: $r^2 = 0.02$, $F = 1.344$, $p = 0.2502$).

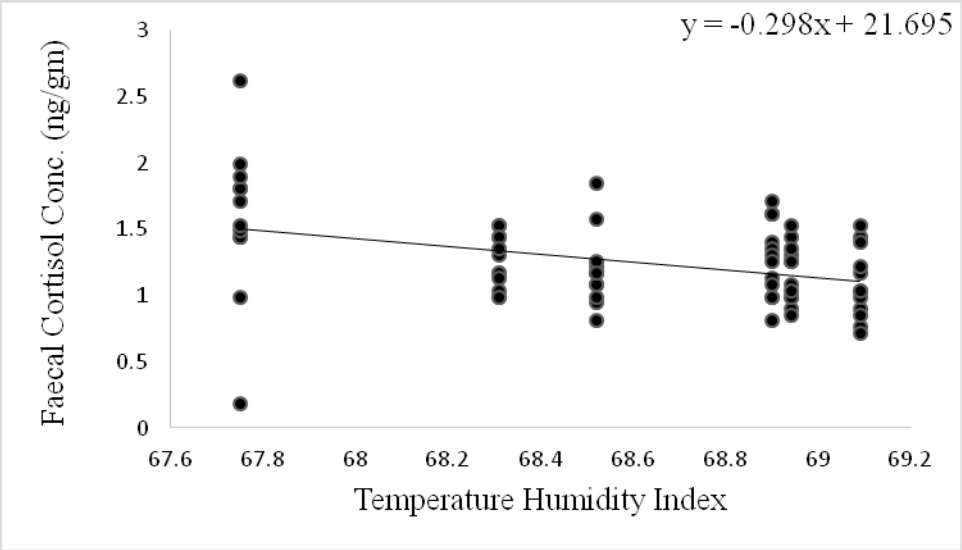

Figure 4.3.a: Effect of Temperature humidity index (THI) on mean faecal cortisol concentration (ng/gm) of Blackbuck at Rajiv Gandhi Zoological Park and Wildlife Research Centre (RGZP), Pune, Maharashtra, India during Winter. The mean faecal cortisol concentration showed negative significant relationship with Temperature Humidity Index. Linear regression equation y=-0.298x + 21.695.

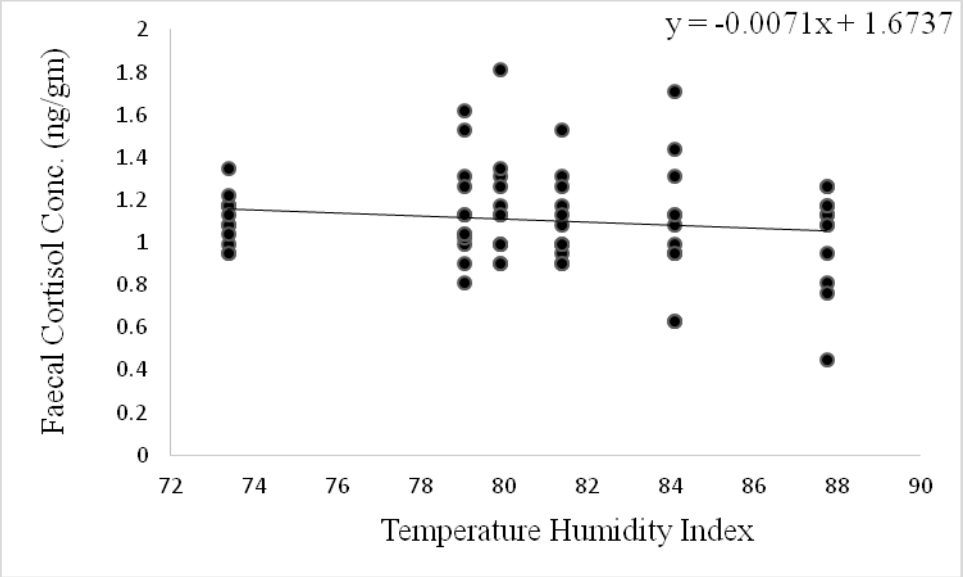

Figure 4.3.b: Effect of Temperature humidity index (THI) on mean faecal cortisol concentration (ng/gm) of Blackbuck at Rajiv Gandhi Zoological Park and Wildlife Research Centre (RGZP), Pune, Maharashtra, India during October Heat. The mean faecal cortisol concentration showed a negative relationship with Temperature Humidity Index which was not significant at 5% significance level. Linear regression equation y = -0.0071x + 1.6737

There was a significant negative correlation between Temperature Humidity Index (THI) and faecal cortisol level in Spotted deer during Winter (Figure 4.4.a: Simple linear regression: r^2 = 0.08, F = 5.707, p = 0.0196). There was no significant effect of THI on mean cortisol levels of Spotted deer during October heat (Figure 4.4.b: Simple linear regression: r^2=0.28, F = 2.025, p = 0.1592).

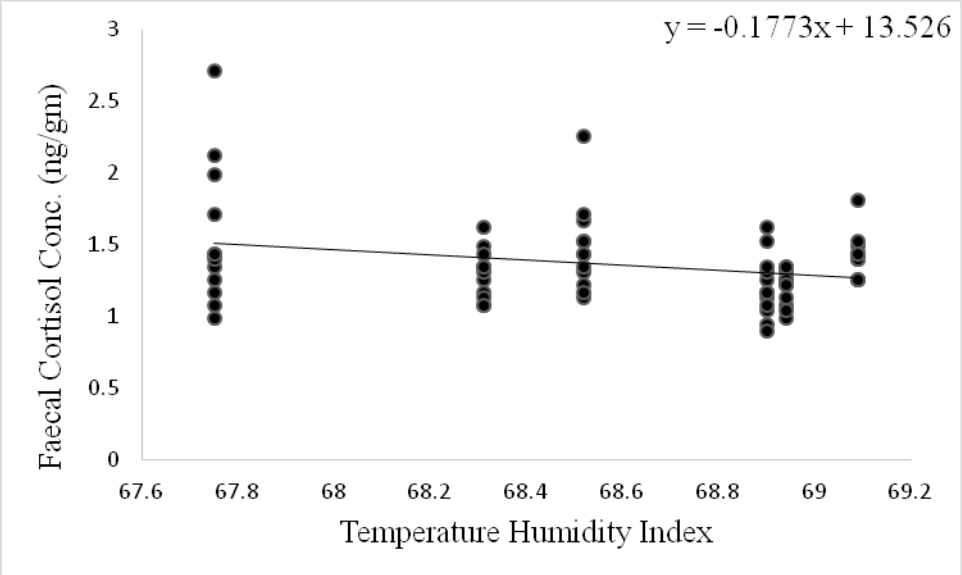

Figure 4.4.a: Effect of Temperature humidity index (THI) on mean faecal cortisol concentration (ng/gm) of Spotted deer at Rajiv Gandhi Zoological Park and Wildlife Research Centre (RGZP), Pune, Maharashtra, India during Winter. The mean faecal cortisol concentration showed negative significant relationship with Temperature Humidity Index. Linear regression equation y=-0.1773x + 13.526.

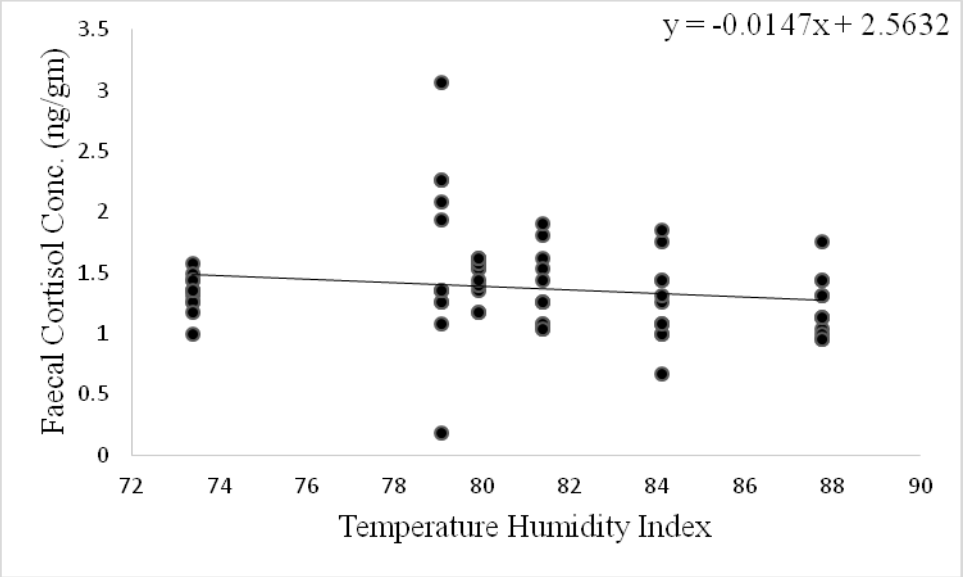

Figure 4.4.b: Effect of Temperature humidity index (THI) on mean faecal cortisol concentration (ng/gm) of Spotted deer at Rajiv Gandhi Zoological Park and Wildlife Research Centre (RGZP), Pune, Maharashtra, India during October Heat. The mean faecal cortisol concentration showed a negative relationship with Temperature Humidity Index which was not significant at 5% significance level. Linear regression equation y = -0.0147x + 2.5632.

4.2.1 Effect of Temperature Humidity Index and Visitor Number on Faecal Cortisol Metabolites of Spotted deer and Blackbuck

There was no significant interaction effect between temperature humidity index and visitor numbers for Spotted Deer on mean cortisol metabolites concentrations during October heat (r^2 = 0.02, DF = 69, F = 2.09, p = 0.13) as well as winter (r^2 = -0.0003, DF = 69, F = 0.98, p = 0.37).

There was a statistically significant interaction effect between temperature humidity index and visitor numbers for Blackbuck in winter (r^2 = 0.06, DF = 69, F = 3.53, p = 0.03), but not in October heat (r^2 = 0.002, DF = 69, F = 1.10, p = 0.33).

4.3 Faecal cortisol metabolites between Adult Male and Adult Female Blackbucks and Spotted deer's

Mean faecal cortisol concentrations for Blackbuck male (1.12 ± 0.19 ng/gm) and females (1.09 ± 0.25 ng/gm) during October heat were not significantly different (Figure 4.5.a; Wilcoxon rank sum (Mann Whitney) test: z = -0.95 and Prob |z| = 0.3422) from each other. Similarly, mean faecal cortisol values for blackbuck male (1.24 ± 0.39 ng/gm) and female (1.27 ± 0.32 ng/gm) during Winter were not significantly different (Figure 4.5.b; Wilcoxon rank sum (Mann Whitney) test: z = 0.525 and Prob |z| = 0.5997).

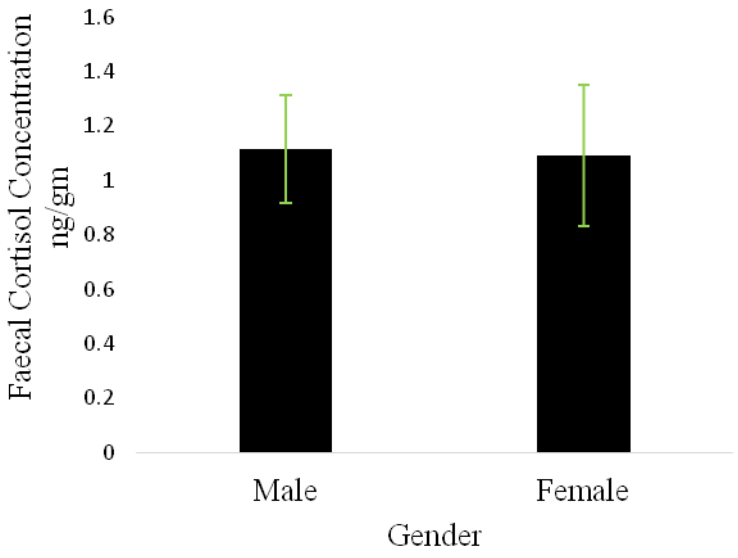

Figure 4.5.a: The faecal cortisol concentration in male and female blackbuck at Rajiv Gandhi Zoological Park and Wildlife Research Centre (RGZP), Pune, Maharashtra, India during October heat. There was no significance difference in faecal cortisol concentrations between male *(1.12 ± 0.19 ng/gm)* and female *(1.09 ± 0.25 ng/gm)* blackbuck with z-value of -0.95 and Prob |z| = 0.3422.

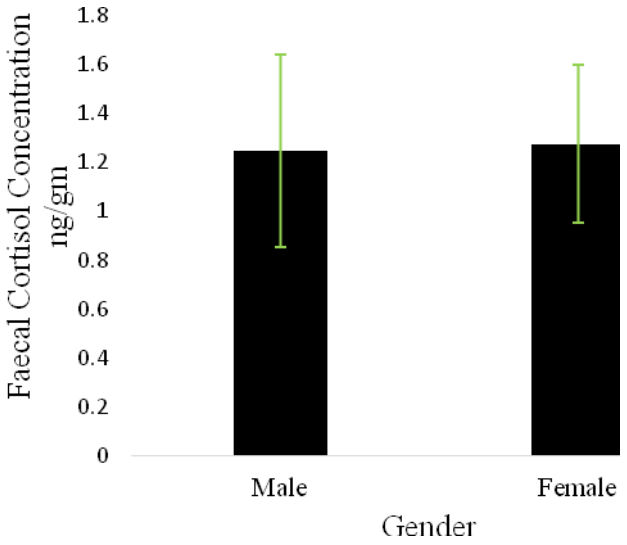

Figure 4.5.b: The faecal cortisol concentration in male and female blackbuck at Rajiv Gandhi Zoological Park and Wildlife Research Centre (RGZP), Pune, Maharashtra, India during Winter. There was no significance difference in faecal cortisol concentrations between male *(1.24 ± 0.39 ng/gm)* and female *(1.27 ± 0.32 ng/gm)* blackbuck with z-value of 0.525 and Prob |z| = 0.5997.

Mean faecal cortisol values did not significantly vary between male (1.40 ± 0.46 ng/gm) and female (1.33 ± 0.30 ng/gm) Spotted Deer during October heat (Figure 4.6.a; Wilcoxon rank sum (Mann Whitney) test: z = -0.615 and Prob |z| = 0.5383). Likewise, mean faecal cortisol concentration for male (1.36 ± 0.25 ng/gm) and female (1.36 ± 0.34 ng/gm) Spotted Deer during Winter were not significantly different (Figure 4.6.b; Wilcoxon rank sum (Mann Whitney) test: z = -0.57 and Prob |z| = 0.5684).

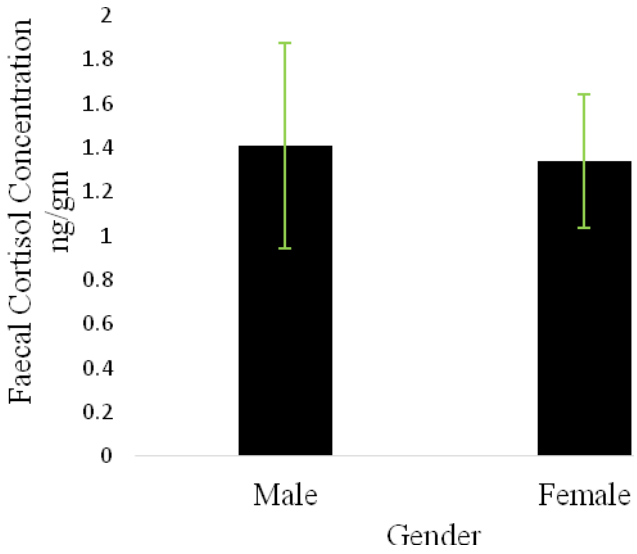

Figure 4.6.a: The faecal cortisol concentration in male and female Spotted Deer at Rajiv Gandhi Zoological Park and Wildlife Research Centre (RGZP), Pune, Maharashtra, India during October heat. There was no significance difference in faecal cortisol concentrations between male *(1.40 ± 0.46 ng/gm)* and female *(1.33 ± 0.30 ng/gm)* Spotted Deer with z-value of -0.615 and Prob |z| = 0.5383.

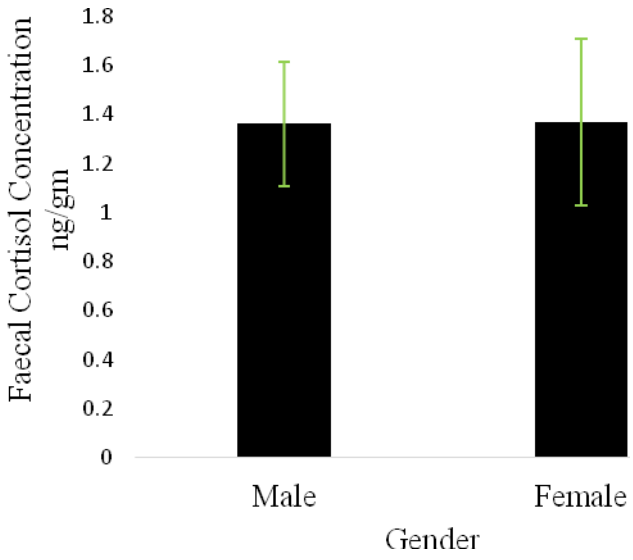

Figure 4.6.b: The faecal cortisol concentration in male and female Spotted Deer at Rajiv Gandhi Zoological Park and Wildlife Research Centre (RGZP), Pune, Maharashtra, India during Winter. There was no significance difference in faecal cortisol concentrations between male *(1.35 ± 0.25 ng/gm)* and female *(1.36 ± 0.34 ng/gm)* Spotted Deer with z-value of 0.525 and Prob |z| = 0.5997.

CHAPTER FIVE

5.0 DISCUSSION

5.1 Effect of Visitor Categories on Blackbuck and Spotted Deer

From this study, visitor numbers had an effect on faecal cortisol concentration of blackbuck but not spotted deer. The results showed that the faecal cortisol concentrations of blackbuck were significantly affected by visitor numbers during winter. The highest faecal cortisol concentrations occurred when the blackbucks were exposed to high number of zoo visitors, whereas the lowest faecal cortisol concentrations were exhibited when there were no visitors in the zoo. These results indicate that exposure to high number of visitors would be most stressful to blackbuck while similar experience may not affect the spotted deer. These findings agree with that of Hosey, (2005) who reported that although interactions between visitors and animals in captivity may negatively impact some animals, some species may be tolerant. The pattern observed on the blackbuck agrees with Hosey, (2000) that interactions between visitors and animals in captivity negatively impacts animals in captive facilities.

Our results could also be explained through the behaviour of visitor practices while in the zoo as explained by Rajagopal *et al*., (2011) who studied blackbuck in India. He observed that visitor practices while in the zoo such as teasing, shouting and trying to cause physical harm to the animals caused increased stress in the blackbucks. Indeed, blackbucks in our study were observed constantly jumping up and down and exhibiting stereotypic pacing and repeated walk back and forth in presence of visitors *(personal observation).* In addition, the presence of the visitors and their activities around the enclosures which are not spacious enough could cause chronic stress.

The increased levels of abnormal behaviours per observation observed presumably associated with higher levels of faecal cortisol is only evidenced when large number of visitors are present around the animal enclosures. This could explain why blackbuck was most affected in the presence of visitors especially when the number of visitors was high. In general, the increased level of faecal cortisol in blackbuck as indicated by the results of this study and stereotypic pacing can be best described as a response towards visitor presence and category. Visitors feeding animals, shouting and teasing adversely affected animals in captivity as reported by Birke, (2002). The observed positive relationship between physiological stress in blackbucks and visitor number in this study matches the findings of Li C. *et al*., (2007) working on the influence of enclosure size and animal category on faecal cortisol concentration of wild animals. Hosey, (2005) working on effect of visitors on zoo animals found that visitor numbers increased aggression in Pere David's deer *(Elaphurus davidianus)* stags. Aggression has been found to be an indicator of stress (Hosey, 2005). Rajagopal *et al*., (2011) also reported that Zoo visitor numbers affected faecal cortisol metabolites and behaviour of the endangered Indian blackbuck *(Antilope cervicapra).* Other studies including that of Keay *et al*., (2009) found similar results that captive animals physiological stress is negatively affected by high visitor category. Taken together, the results of this study and those of these other authors support the idea that presence of visitors may be detrimental to some wild animals in zoos. In the long run, this may negatively affect zoo animal welfare (Davey, 2006). These results are however on contrary to the results found by Todd, (2007) working on effect of visitor numbers on Diana Monkeys *(Cercopithecus diana)*. In this study, noise level was positively related to visitor number which in turn affected foraging and play by

Diana monkeys. Such active behaviours may provide short term stress relief, thus reducing faecal cortisol concentrations (Todd *et al*., 2007).

Findings from this study indicate that October heat did not have effect on blackbuck and spotted deer. These results suggest that both spotted deer and blackbucks are tolerant to variation in visitor numbers during October heat. Findings of this study are similar to that of Tilbrook et al., (2017), who also observed that spotted deer is not significantly affected by high human audiences due to their social organization which consists of gregarious units of herds. Further, the spotted deer could have not experienced any effect as a result of habituation from daily and routine human audiences (Cole and Fraser, 2019). In addition, the lack of effect of visitor categories during October heat for both blackbuck and spotted deer could be attributable to high adrenal activity variations in both species (Dancer and Burn, 2019) which is further emphasized by physiological status and perception of the environment. Similarly, these results agree with the finding of Davey, (2006) that some zoo animals are not affected by human audiences due to habituation as a result of continuous human-animal interaction.

5.2 The Role of Environmental Factors (Temperature Humidity Index) in the Level of Faecal Cortisol Metabolites on Blackbuck and Spotted Deer

The results of this study showed that temperature humidity index (THI) significantly affected both blackbuck and spotted deer during winter but not during October heat. As the THI decreases, the faecal cortisol in blackbuck and spotted deer increases. This study indicated a significant negative relationship in blackbuck and spotted deer faecal cortisol concentrations and the Temperature Humidity Index (THI) during the winter season. The negative relationship reported

in this study could be due to seasonal variation with response to parameters such as minimum ambient temperature and cold stress (Dikmen and Hansen, 2009). In this study, cold stress and ambient temperature could be some of the factors leading to significant effects on the faecal cortisol concentration in the blackbuck and spotted deer during winter. The observed results could also be due to drastic change of weather conditions within a short period of time which can interfere with adrenocortical activity (Dikmen and Hansen, 2009, Mason *et al*., 2010). The adrenocortical hormones are known to help in daily regulation of physiological status of the body (Dikmen and Hansen, 2009). The change in adrenocortical activity as the cause of increased production of faecal cortisol could be explained by the concept reported by Huber *et al*., (2003) and Allwin *et al*., (2016) that environmental factors are stressors to zoo animals by altering their adrenocortical hormones.

The circadian rhythm absence during winter in spotted deer and blackbuck could be due to limited amount of daylight accompanied with the constant need to produce metabolic heat (Dikmen and Hansen, 2009) which in turn may result in changing level of faecal cortisol production. Besides, the elevated concentration of faecal cortisol as observed in our study may be attributed to the process of adapting to the harsh environmental conditions especially the cold weather during winter (Bubenik *et al*., 1998). The increased levels of faecal cortisol in blackbuck during cold seasons could also be due to the shift in body metabolism (Dikmen and Hansen, 2009). Alteration of body metabolism ensure that enough energy is produced to keep the body warm in the absence of surrounding balminess (Saltz and White, 1991).

Several vertebrate species modulate faecal cortisol concentrations seasonally (Huber *et al*., 2003) with higher levels during adverse weather conditions (Millspaugh and Washburn, 2004). The results of our study agree with that of Saltz and White, (1991), Bubenik *et al*., (1998), Huber *et al*., (2003) who reported that the drastic change in weather condition affects the production of faecal cortisol. Other studies including that of Allwin *et al*., (2016) who correlated the faecal cortisol metabolites in free ranging Nigiri Tahrs (*Nilgiritragus hylocrius*) with meteorological parameters also supports the finding of this study. Taken together, these results support the idea that environmental factors such as temperature and humidity affect some wild animals in captive facilities and may in long run impact on the welfare of these animals (Huber *et al*., 2003).

This is the first study of faecal cortisol concentration on spotted deer as affected by Temperature Humidity Index (THI). Similar variation in faecal cortisol concentration being significant during winter season have been reported on mule deer (*Odocoileus hemionus*) by Saltz and White, (1991), white-tailed deer (*Odocoileus virginianus*) by Bubenik *et al.,* (1983) and reindeer *(Rangifer tarandus)* by Bubenik *et al.,* (1998). Other studies however have reported contrary results, including a study conducted by Millspaugh and Washburn, (2004) on North American elk (*Cervus elaphus)* who reported a significant change in faecal cortisol concentration in summer and not in winter. Correspondingly, Ringberg *et al*., (1978) also reported no significant difference in faecal cortisol concentration between winter and summer in Reindeer. These divergent findings may be due the differences in climate or species.

5.3 Faecal cortisol metabolites between Adult Males and Adult Females

The sex of blackbuck and spotted deer did not have significant effect on faecal cortisol as shown by the result of this study. Adult males and females of both species showed relatively similar faecal cortisol metabolites irrespective of visitor categories or season (winter or October heat). This might be attributed by exposure of both males and females to similar environmental conditions and activities throughout the study period. It can then be inferred that both species received similar treatments and stress levels which did not lead to variation of faecal cortisol. Additionally, coexistence between captive male and female blackbucks in the same enclosure during the non-breeding season might have led to lack of variation of faecal cortisol concentration in both sexes during the study period. In addition, both male and female spotted deer and blackbuck experienced same captive stress level since they are compelled to live conspecifics in the enclosure. The findings of this study are supported by that of Bubenik *et al.,* (1998) and Huber *et al.,* (2003) who found that adult male and female reindeer and red deer did not have significant difference in faecal cortisol in the studies conducted in Alaska and Austria. Similarly, findings of Bubenik *et al.,* (1998) who reported that male and female reindeer have similar cortisol concentration during non-breeding seasons also supports the results of this study. A study on red deer *(Cervus elaphus),* did not also find significant difference between sexes (Huber et al., 2003).

CHAPTER SIX

6.1 Conclusions

1. It can be deduced that visitor numbers predict faecal cortisol concentration in captive blackbuck in winter but not during October heat.
2. Higher levels of faecal cortisol concentration in blackbuck are associated with high visitor category in the zoo.
3. The mean visitor numbers at the zoo was positively correlated with faecal cortisol concentration on blackbuck during Sundays when high number of visitors were recorded.
4. Spotted deer was not significantly affected by variation in visitor number in the zoo during winter and October heat.
5. Spotted deer was tolerant to large number of visitors in the zoo.
6. Spotted deer may have been habituated to the presence of visitors in the zoo.
7. The effect of visitors on stress response of wildlife are variable and may depend on different factors and may vary across species.
8. The temperature humidity index affected both spotted deer and blackbuck during winter during which the adrenocortical activity of these animals have been interfered with and the daily regulation of body physiological status altered.
9. Temperature humidity index negatively correlated with faecal cortisol concentration in blackbuck and spotted deer.
10. THI did not affect blackbuck and spotted deer during October heat.
11. Sex did not predict stress levels in blackbuck and spotted deer in the zoo.

6.2 Recommendations

It is recommended that the existing enclosures to be modified for blackbuck by addition of hidden observation sites that ensure that these animals do not have visual contact with visitors. Measures to reduce negative visitors' effects such as noise, movement, teasing or even causing physical harms should be enhanced in the zoo. Animal welfare conservation programs with good and appropriate husbandry practices should be done to ensure the welfare of these animals is protected in an effort to the process of conserving and managing these wild animals. Moreover, problems related to physiology and animal health should be continuously assessed and appropriate prevention measures taken to enhance the well-being and reproductive efficiency of the zoo animals.

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APPENDICES

Appendix 1 Visitor number

Phase I								
Day	**Date**	**Adult**	**Child**	**Foreigner**	**Govt.Edu Trip**	**Pvt .Edu Trip**	**Handicap**	**Total Visitor**
Wednesday	18-Oct-17	0	0	0	0	0	0	0
Friday	20-Oct-17	11731	1827	3	387	0	8	13956
Sunday	22-Oct-17	11614	2725	28	459	0	12	14838
Wednesday	25-Oct-17	0	0	0	0	0	0	0
Friday	27-Oct-17	4828	1576	9	136	88	12	6649
Sunday	29-Oct-17	10770	2690	25	171	72	8	13736
Wednesday	1-Nov-17	0	0	0	0	0	0	0
Friday	3-Nov-17	2544	484	2	9	15	1	3055
Sunday	5-Nov-17	9078	1934	7	146	232	8	11405
Total		**50565**	**11236**	**74**	**1308**	**407**	**49**	**63639**
Phase II								
Day	**Date**	**Adult**	**Child**	**Foreigner**	**Govt.Edu Trip**	**Pvt .Edu Trip**	**Handicap**	**Total Visitor**
Wednesday	20-Dec-17	0	0	0	0	0	0	0
Friday	22-Dec-17	2753	1105	5	783	665	64	5375
Sunday	24-Dec-17	12774	2835	3	515	575	8	16710
Wednesday	27-Dec-17	0	0	0	0	0	0	0
Friday	29-Dec-17	6174	1977	16	706	1434	1	10308

Sunday	31-Dec-17	11265	2097	8	80	556	0	14006
Wednesday	3-Jan-18	0	0	0	0	0	0	0
Friday	5-Jan-18	2225	327	2	411	457	1	3423
Sunday	7-Jan-18	8968	1622	23	554	310	4	11481
Total		**44159**	**9963**	**57**	**3049**	**3997**	**78**	**61303**

Appendix 2 Environmental data of October Heat 2017

Number	1	2	3	4	5	6	7	8	9
Date	18-Oct	20-Oct	22-Oct	25-Oct	27-Oct	29-Oct	1-Nov	3-Nov	5-Nov
Day	Wed	Friday	Sunday	Wed	Friday	Sunday	Wed	Friday	Sunday
Temperature									
Max	39	40	38.5	37.8	36.4	35.8	36	35.1	36
Min	20	32	23.1	31.5	27.8	29	17	25	23.9
Humidity									
Max	51	76	95	40	70	46	82	60	62
Min	22	29	27	26	26	24	17	20	27

Average									
Temperature	29.5	36	30.8	34.65	32.1	32.4	26.5	30.05	29.95
Humidity	36.5	52.5	61	33	48	35	49.5	40	44.5

THI	79.65	85.59	77.56	87.76	81.39	84.09	73.79	79.91	79.07

Appendix 3 Faecal Cortisol data of October heat 2017

Number	1	2	3	4	5	6	7	8	9
Date	18-Oct	20-Oct	22-Oct	25-Oct	27-Oct	29-Oct	1-Nov	3-Nov	5-Nov
Day	Wednesday	Friday	Sunday	Wed	Friday	Sunday	Wed	Friday	Sunday
Animals	**Cortisol Con-centration ng/gm**								
Spotted dear male									
1	1.99	1.71	0.81	1.31	1.26	1.76	1.35	1.62	1.08
2	2.17	1.53	1.35	0.95	1.81	1.44	1.35	1.35	1.94
3	1.71	1.71	1.53	1.13	1.62	1.85	1.26	1.53	2.26
4	1.62	1.71	2.08	1.13	1.53	1.44	1.31	1.58	3.07
5	2.08	1.35	1.85	1.04	1.9	0.99	1.58	1.17	0.18
6	2.17	1.62	2.35	0.99	1.08	1.08	1.26	1.17	1.35
Spotted dear fe-male									
1	1.62	1.26	0.67	0.95	1.26	1.08	1.17	1.53	2.08
2	1.35	1.53	1.26	1.31	1.44	1.26	1.49	1.58	2.26
3	1.35	1.17	1.71	1.76	1.04	1.31	1.44	1.4	1.26
4	1.53	1.26	0.99	1.44	1.08	0.99	1.44	1.62	1.26
5	1.62	1.26	3.16	1.13	1.26	1.08	1.35	1.62	1.35
6	1.44	1.71	1.31	1.44	1.04	0.67	0.99	1.44	1.35
Blackbuck male									
1	2.26	1.22	0.45	0.81	1.08	1.13	1.17	1.13	1.53
2	1.99	1.35	2.71	0.76	1.17	0.95	0.95	1.31	1.31
3	1.81	1.62	1.71	1.13	1.13	1.08	1.08	1.17	0.99
4	1.99	1.53	1.35	1.08	1.31	1.31	1.17	1.26	1.62
5	1.62	1.44	2.62	0.95	1.26	0.95	0.99	0.99	1.26
6	1.44	1.62	1.71	1.13	0.99	0.63	1.35	0.9	1.13
Blackbuck female									
1	1.35	1.08	1.99	1.26	0.95	1.44	0.95	0.9	1.13
2	1.17	1.17	2.35	1.08	0.9	0.99	1.08	0.99	1.02
3	1.31	1.35	2.26	1.26	0.99	1.71	1.17	1.13	1.04
4	1.71	1.04	1.71	1.53	0.99	0.95	1.04	1.81	0.9

5	1.44	1.26	1.62	1.17	0.9	1.13	1.13	0.99	0.81
6	1.81	1.49	2.62	1.17	0.45	0.63	1.22	1.35	1.04

Average Cortisol ng/gm									
Days	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Spotted Deer Male	1.95	1.6	1.66	1.09	1.53	1.42	1.35	1.4	1.64
Spotted Deer Female	1.48	1.36	1.51	1.33	1.18	1.06	1.31	1.53	1.59
Blackbuck Male	1.85	1.46	1.75	0.97	1.15	1	1.11	1.12	1.3
Blackbuck Female	1.46	1.23	2.09	1.24	0.86	1.14	1.09	1.19	0.99

Appendix 4 Environmental data of Winter 2017-18

Number	1	2	3	4	5	6	7	8	9
Date	20-Dec	22-Dec	24-Dec	27-Dec	29-Dec	31-Dec	3-Jan 18	5-Jan 18	7-Jan 18
Day	Wed	Friday	Sunday	Wed	Friday	Sunday	Wed	Friday	Sunday
Temperature									
Max	30.2	30.7	29	30.8	29.4	30.2	30	29.2	28.5
Min	21	13.8	13.1	13	13.5	12.6	13.9	14.6	13
Humidity									
Max	21	49	52	53	50	55	60	59	44
Min	10	10	10	10	10	9	10	8	7

Average									
Temperature	25.6	22.25	21.05	21.9	21.45	21.4	21.95	21.9	20.75
Humidity	15.5	29.5	31	31.5	30	32	35	33.5	25.5

THI	76.36	69.77	67.86	69.09	68.52	68.31	68.9	68.94	67.75

Appendix 5 Faecal Cortisol data of Winter 2017-18

Number	10	11	12	13	14	15	16	17	18
Date	20-Dec	22-Dec	24-Dec	27-Dec	29-Dec	31-Dec	3-Jan 18	5-Jan 18	7-Jan 18
Day	Wed	Friday	Sunday	Wed	Friday	Sunday	Wed	Friday	Sunday
Animals	**Cortisol Con-centration ng/gm**								
Spotted dear male									
1	2.98	1.49	0.9	1.81	1.53	1.08	1.26	1.08	1.35
2	2.26	1.17	1.67	1.49	2.26	1.35	1.04	1.26	1.26
3	2.53	0.9	1.17	1.4	1.44	1.49	0.95	0.99	1.44
4	1.26	1.17	1.08	1.53	1.31	1.17	1.31	1.26	1.4
5	2.71	0.85	1.08	1.4	1.67	1.35	1.35	1.31	1.44
6	1.99	0.9	1.26	1.26	1.71	1.62	1.13	1.26	0.99
Spotted dear fe-male									
1	2.71	1.26	0.95	1.44	1.22	1.44	1.62	1.22	1.08
2	2.35	1.13	1.17	1.44	1.13	1.13	1.17	1.13	1.71
3	2.8	0.9	0.95	1.26	1.53	1.26	0.9	1.35	1.17
4	1.71	0.95	1.04	1.4	1.35	1.08	1.53	1.35	2.71
5	1.94	0.99	1.44	1.44	1.17	1.31	1.17	1.04	1.99
6	2.62	1.08	1.26	1.26	1.44	1.35	1.08	1.22	2.12
Blackbuck male									
1	2.03	0.67	1.4	1.17	1.17	1.53	0.99	1.31	1.44
2	1.94	0.9	0.95	0.76	1.08	1.17	1.4	1.26	1.71
3	2.35	1.35	1.08	0.9	1.58	1.31	1.35	0.99	1.81
4	1.71	1.17	1.08	1.04	1.08	1.17	1.31	0.9	0.18
5	0.99	0.72	1.76	1.22	1.17	0.99	1.71	1.44	1.81
6	2.62	0.9	0.81	0.99	1.22	1.04	0.99	0.99	2.62
Blackbuck female									
1	1.99	0.99	0.85	1.44	0.81	0.99	1.62	1.08	1.44
2	2.44	1.35	0.76	1.85	0.72	1.53	1.13	1.26	1.49
3	2.08	0.9	1.17	1.04	0.95	1.13	1.62	1.04	1.99

4	2.44	0.63	0.9	1.53	1.26	1.44	1.26	0.85	0.99
5	1.71	0.95	0.85	0.85	1.17	1.44	1.08	1.35	1.9
6	2.62	0.58	1.22	1.4	0.99	1.35	0.81	1.53	1.53

Average ng/gm									
Days	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**
Spotted Deer Male	2.28	1.08	1.19	1.48	1.65	1.34	1.17	1.19	1.31
Spotted Deer Fe-male	2.35	1.05	1.13	1.37	1.3	1.26	1.24	1.21	1.79
Blackbuck Male	1.94	0.95	1.18	1.01	1.21	1.2	1.29	1.14	1.59
Blackbuck Female	2.21	0.9	0.95	1.35	0.98	1.31	1.25	1.18	1.55