Assessing the Welfare of Semi-captive African Elephants (*Loxodonta africana*) Using Self-Directed Behaviours and Levels of Faecal Glucocorticoid Metabolite Concentrations

by

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Picture by Jackson, T., 2017

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I. Introduction

The report of the Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services (IPBES) in 2019 found that around 1 million animal and plant species are threatened with extinction (IPBES, 2019); in 2021, the International Union for Conservation of Nature (IUCN) reported that at least 85 % of the species on the Red List are threatened with extinction due to habitat loss (IUCN, 2021); two northern white rhinos remaining in the world due to rampant poaching; every 26 minutes, an African elephant is killed by poachers for its tusks, meaning that 20,000 elephants are killed every year (IFAW, 2021). These are only a few facts showing the rapid decline of biodiversity worldwide directly caused by human activities. This so called `Sixth Extinction’, has led to increased conservation efforts, and zoos and aquariums have become powerful tools for animal conservation and education in order to recover species at the brink of extinction and their habitats (Kendall and Bergl, 2018).

At the moment, more than 800,000 animals are living in accredited zoological institutions in the United States alone (Maple and Perdue, 2013; AZA, 2021) and it is estimated that 1 out of 7 species of endangered terrestrial vertebrates are kept in zoos worldwide (Escobar-Ibarra *et al.*, 2021). Each of these individuals are key for the conservation of its species, therefore, ensuring their welfare is not only crucial for their survival, but is also our ethical responsibility (Maple and Perdue, 2013). As a result, animal welfare science has become an established and growing research study field in order to improve the welfare of animals living, not only in zoological institutions but also in laboratories, production and domestic settings. Nevertheless, assessing animal welfare is challenging. There is no single measurement and different tools and approaches need to be integrated in order to have an objective view (Dawkins, 2006; Scott *et al.*, 2017). Results may be difficult to interpret or contradictory due to specific properties of the variables that are being measured (Mason and Mendl, 1993).

Among different welfare assessment tools, non-invasive methods, such as observing and quantifying changes in behaviour (e.g., stereotypies, displacement behaviours), and measuring concentration levels of faecal glucocorticoid metabolites (FGM), have become increasingly popular and there is evidence supporting them as valid indicators of welfare when using correctly (Palme, 2019).

Displacement behaviours have been observed in a wide variety of taxa, yawning in domestic dogs, chewing behaviours in mice, preening in birds, self-scratching in primates, elephants and humans (Zeigler, 1964; Whitehouse, 2018). Among different types of displacement behaviours that have been described (Zeigler, 1964), self-directed behaviours (SDBs) are the most observed displacement behaviours and there is evidence showing that under situations of psychological stress their frequency increases, suggesting that they may be used as indicators of emotional states in non-human primates (Maestripieri *et al.*, 1992; Troisi, 2002).

In this literature review, I will focus on outlining current research on self-directed behaviours (SDBs) as behavioural indicators of stress and their possible use as a welfare assessment tool. Also, I will discuss the studies being made to understand the possible link between SDBs and levels of faecal glucocorticoid metabolites (FGM) as a non-invasive approach to assess animal welfare.

II. Displacement Behaviours

Animal behaviour is integrated by individual movement patterns that occur as part of a series of functionally related behaviours, characterized by their directedness (Zeigler, 1964). However, in 1940, Tinbergen and Kortlandt, through independent animal observations, reported patterns of behaviours that seemed to happen out-of-context, apparently irrelevant to the situation, in which they appear (Kortmulder, 1998; Press, 2011). These behaviours received the name of “displacement activities”. They are part of the natural behaviour repertoire of the animal and tend to occur when an individual is facing a situation of conflict, frustration or uncertainty (Maestripieri et al., 1992; McFarland, 1966).

Displacement behaviours are a group of movement patterns that have been reportedly to be expressed when an individual is in a situation of uncertainty, stress, conflict or frustration (McFarland, 1966; Delius, 1967; Maestripieri *et al.*, 1992; Troisi, 2002). They have been identified in different social species and taxonomic groups, from primates and humans (Maestripieri *et al.*, 1992; Castles, Whiten and Aureli, 1999; Leavens *et al.*, 2001; Troisi, 2002) to different species of birds and fishes (Press, 2011). Even though there is no specific definition of what a displacement behaviour is, they have been observed to occur when the individual encounters motivational conflict, frustration to carry out or fulfil behavioural needs and uncertainty (Delius, 1967; Maestripieri *et al.*, 1992).

The underlying function of displacement activities remains unclear. Despite of the evidence suggesting that they may be serve as a coping mechanism to regulate stress and anxiety, it has also been suggested that they may serve also as a mitigator of social uncertainty in macaques (Castles, Whiten and Aureli, 1999; Whitehouse, 2018). In order to understand better why these behaviours occur and their possible application to assess welfare, not only in animals but also humans, further research is crucial.

Among different types of displacement behaviours (e.g., displacement feeding, displacement drinking), self-directed behaviours (SDBs) are the ones that have been the most observed in humans and non-human primates. Researchers have found a link between their expression and negative emotional states such as, anxiety, internal conflict and frustration (Maestripieri *et al.*, 1992; Schino *et al.*, 1996; Castles, Whiten and Aureli, 1999; Mohiyeddini and Semple, 2013).

III. Stress, Welfare and Self-Directed Behaviours

There are different definitions and concepts of stress; it can be referred to how an individual responds to different stimulus, or to a physiological or psychological state, and it can vary from low, mild or severe (Whitehouse, 2018). In a physiological approach, stress is defined as the disruption of an organism's homeostasis and the ability that the individual has to adapt to that challenge (Carlstead and Brown, 2005). Throughout a stressful experience, there are physiological changes in the organism, such as the release of glucocorticoid (GC) occurring in the hypothalamic–pituitary–adrenocortical (HPA), which is a key element to successfully cope with a stressful event (Lane, 2006). After the GC is released by the HPA axis, metabolites are excreted through urine and faeces, which can be extracted and measured in order to try to evaluate the welfare state of an individual (Lane, 2006; Palme, 2019). These physiological changes can be measured and quantified by assessing level changes in FGM for example, in order to determine stress levels in an individual (Whitehouse, 2018). However, it is important to understand that positive arousal can also cause stress hormone levels to increase, such as mating, new enrichment or new food (Harris, M., Sherwin, C. and Harris, 2008).

The stress response of an organism, such as glucocorticoid release, is useful and adaptative in the short-term, however, high levels of glucocorticoids for long periods of time can develop into chronic stress, leading to diseases, self-injury behaviours and even death (Mostl Palme, R. and Mostl, E., Palme, 2002; Carlstead and Brown, 2005). Chronic stress in captive animals can be common, especially in animals that live in complex societies, animals who have extensive home ranges and where mimicking their natural environment and species-specific needs is not possible (Veasey, 2006). Taking into account the number of animals living under human care, it is key to develop tools that allow us to determine if an animal may be suffering from chronic stress before irreparable damages or injuries originate. It is important to remark that the levels of FGM can increase not only due to stressful events, but also due to diet, age, sex, oestrus cycle and other individual characteristics (Lane, 2006), therefore it is crucial to include the study and assessment of behavioural changes along with the physiological response.

In humans and animals, the psychological challenge of a stressful experience can manifest in stress associated behaviours, such as the display of stereotypic or displacement behaviours, such as self-directed behaviours (SDBs) (Whitehouse, 2018). In the following sections I will be focusing on the discussion of research of SDBs as stress associated behaviours and their use as a non-invasive welfare assessment tool.

IV. Self-Directed Behaviours and Physiological Measurements

Self-directed behaviours (SDBs) such as self-scratching, self-grooming and self-touching, are the most observed displacement behaviours in primates and in humans. Pharmaceutical experiments have provided evidence of a link between the frequency of SDBs and negative affective states, such as anxiety stress and uncertainty (Maestripieri *et al.*, 1992; Schino *et al.*, 1996; Castles, Whiten and Aureli, 1999; Mohiyeddini and Semple, 2013), showing that SDBs may be indicative of mild and general level of stress (Higham *et al.*, 2009). Research studies done in humans, have also provided evidence suggesting that a possible function of displacement behaviours could be to mediate the levels of anxiety and the experience of stress (Mohiyeddini and Semple, 2013). Therefore, the study of SDBs as welfare indicators has become a promising approach to understand the correlation between behaviour and stress.

One might expect that elevated levels of anxiety would ultimately manifest as physiological stress, and that SDB and FGM measures would therefore be positively correlated, nevertheless, this remains unclear. In 2009, Higham, et. al., studied wild populations of olive baboons *Papio hamadryas Anubis*) in order to understand the possible correlation between rates of SDBs and FGM. Their results showed no association between these two variables. Results such as this one may support the hypothesis of SDBs as a coping mechanism, helping to reduce stress levels (Higham *et al.*, 2009).

There is limited data and research on the relationship between SDBs and glucocorticoid output in wild and captive animals. There have been a few studies investigating the specific link between these two non-invasive measures in captivity conducted primarily with non-human primates, and have produced contradictory results (Castles, Whiten and Aureli, 1999; Leavens *et al.*, 2001; Troisi, 2002). In addition, there are also studies investigating the use of SDBs as indicators of emotional states in humans and recent studies have begun to investigate the relationship between physiological and behavioural indices of stress and anxiety in humans (Troisi, 2002).

V. Self-Directed Behaviours as Animal Welfare Assessment Tool

The study of animal welfare has been challenging to define. It encompasses a wide range of disciplines such as veterinary medicine, behavioural ecology, evolution, neuroscience, animal behaviour, genetics, cognitive science and the study of consciousness (Dawkins, 2006). Even though physical health is a critical part of the welfare of humans and animals, it does not represent the entire picture of how the animal is responding and perceiving its environment (Dawkins, 2006).

The main objective of animal welfare science is to study the welfare state of animals by trying to make inferences on how an animal is feeling by using quantifiable and objective data. It also studies possible factors that may contribute to a state of poor or good welfare in order to improve also our understanding of human relationships with other animals (Ward, Sherwen and Clark, 2018). Nevertheless, assessing welfare can be highly challenging, there is no single measurement of good animal welfare. Therefore, different tools, approaches and indicators need to be integrated (Dawkins, 2006). Some of the welfare indicators can be physical condition and health, physiological measurements and behavioural observations (Ward, Sherwen and Clark, 2018).

An effective animal welfare assessment must be non-invasive, rapid, low-cost, reliable and accurate. This is why behavioural observations are one of the most useful approach. In zoo animals, behavioural changes and expression of abnormal behaviours can be indicators of poor welfare and through behavioural observations they can be identified and take action to improve the well-being of those individuals (Escobar-Ibarra *et al.*, 2021).

In both non-human primates and human subjects, displacement behaviours appear in situations characterized by social tension and are likely to reflect increased autonomic arousal (Troisi, 2002). However, whether increased rates of SDBs are likely to be associated with increased faecal glucocorticoid output is still unknown and despite studies being done, the results are contradictory and inconclusive (Troisi, 2002; Higham *et al.*, 2009; Mohiyeddini and Semple, 2013).

Non-invasive techniques have been applied most frequently in studies of captive, free-ranging, production or wild mammals, for example to explore the stress related costs of group living (Sands and Creel 2004), to investigate the anxiety reducing benefits of conflict management (Schino 1998) or to assess the fitness impacts of elevated stress (Pride 2005). In addition, given the clear link between animals' well-being and the stress levels they experience, these approaches have also been used to address key applied questions relating to captive animal welfare (Honess and Marin 2006; Lane 2006).

VI. Conclusion

Since the first reports of displacement behaviours, done by Tinbergen and Kortlandt in 1940, it was suggested that these behaviours were linked to animals experiencing stress, frustration and uncertainty (Zeigler, 1964; Press, 2011). Currently, the link between animals experiencing stress and high rates of self-directed behaviours (a type of displacement behaviours), it is well supported and recognized in humans and non-human primates (Maestripieri *et al.*, 1992; Castles, Whiten and Aureli, 1999; Mohiyeddini and Semple, 2013; Whitehouse, 2018), however, their evolutionary background and function remains unclear.

The measurement of the frequency of SDBs has been proven to be a promising tool as non-invasive indicators of stress, anxiety and social uncertainty in humans and non-human primates (Castles, Whiten and Aureli, 1999; Mohiyeddini and Semple, 2013; Whitehouse, 2018), yet, research on other social species continue to be limited or non-existent.

Despite the use of SDBs as a possible indicator of negative emotional state, it is essential to understand the physiological changes that are linked to the expression of those behaviours, in order to use them as a reliable measurement to assess welfare. Nonetheless, studies that have been investigated this, are limited and their results have been contradictive and inconclusive (Castles, Whiten and Aureli, 1999; Higham *et al.*, 2009).

The use of non-invasive welfare assessment methods is key to improve and maintain healthy captive animal populations, and the use of SDBs and FGM as indicators are a promising approach to identify stress related behaviours before the development of chronic stress, stress-related diseases and in some cases, death of individuals (Veasey, 2006).

The suggestion that SDBs may function as an indicator to evaluate relationship security among individuals, can be particularly useful in animals with complex societies living in limited space facilities such as zoos. Further studies focusing on the possible correlation between SDBs and physiological measurements, such as FGM, are essential to keep developing non-invasive but reliable and accurate tools to assess animal welfare.

VII. References

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1. Abstract

Elephants are one of the most popular species, attracting visitors around the world, but their social complexity, high cognitive abilities and behavioural repertoire makes them one of the most challenging species to maintain in captivity. Concerns regarding captive elephant welfare has been increasing and non-invasive methods are key to assess their well-being without causing further stress on the animals, improving their management in captivity and therefore, their survival.

New approaches to assess animal welfare are based on non-invasive behavioural and physiological indicators. Measuring levels of faecal glucocorticoids metabolites (FGM) has become a reliable and well-known tool to measure stress in different species, including African elephants. Self-directed behaviours (SDBs) are a group of stress related behaviours that have been linked to emotional states of anxiety, stress and social uncertainty, but their correlation with FGM levels remains unclear.

The fact that in the present study a positive correlation between FGM levels and SDBs (p-value=0.024) was found, it is important to take these results with caution and further research on the correlation of both variables is recommended. Furthermore, we found no effect of visitors on FGM levels and SDBs did not find a correlation between number of visitors and levels of FGM, but there was a significant effect on the rates of SDBs. The rank of the elephants did not show to have a significant effect on the FGM levels, on the other hand, the rank did show a significant effect on rates of SDBs (p-value=0.028). Finally, we found a positive correlation between the levels of FGM and rates of SDBs in

the herd of African elephant. Further research on SDBs expressed by elephants should be investigated

Key words: Self-directed behaviours, FGM, African elephants, stress, animal welfare, non-invasive welfare assessment.

2. Introduction

Zoos and aquariums have become powerful tools to counteract the rapid decline of biodiversity worldwide, helping to recover critically endangered species and maintaining other populations from their continuous decline (Kendall and Bergl, 2018). Each individual plays a key role in the conservation of their own species, by either being part of breeding and reintroduction programs, or as ambassador animals to raise conservation awareness among visitors. As a result, zoos face the ethical obligation to provide and maintain the highest standard of welfare for all animals under their care.

Captive animals may experience stress due to limited space, negative social interactions and inadequate enclosures, leading to the decrease of their welfare (Escobar-Ibarra *et al.*, 2021), which can result in low reproduction rates, poor health, immunosuppression and premature death, compromising also the survival of species (Veasey, 2006). Whereas there are clear indicators of poor welfare (e.g., physical injuries or low body mass), assessing the overall welfare is challenging since there is no single measurement of good animal welfare (Dawkins, 2006). Different tools, approaches and indicators need to be integrated, such as evaluation of physical condition and health, physiological measurements and behavioural observations (Ward, Sherwen and Clark, 2018).

Elephants are one of the most popular species, attracting visitors to zoos and wildlife parks around the world, nonetheless, they are one of the species with more welfare concerns when living in captivity, and one of the most challenging species to maintain due to their social complexity, high cognitive abilities, behavioural repertoire and specific environmental needs (Szott *et al.*, 2020). An increasingly used approach to assess welfare in elephants is the measurement of glucocorticoid metabolites in faeces (FGM). It has been validated as non-invasive technique to determine levels of stress hormones (Stead, Meltzer and Palme, 2000). Nonetheless, behavioural observations are crucial to reach an objective and reliable conclusion of the welfare state of an individual.

Croze, Lee and Moss, (2011) identified self-directed behaviours in elephants, such as, when an individual seems listening or observing, while twisting the tip of its trunk back and forth, or trunk twisting, foot-swing, raising and tentatively swinging its foreleg intermittently, specifically in situations when they were experiencing ambivalence, apprehension, social uncertainty or insecurity. However, little we know about the underlying function and identification of SDBs in elephants and the research that has been done is mainly focused in human and non-human primates (Maestripieri *et al.*, 1992; Castles, Whiten and Aureli, 1999).

Research on elephant welfare using FGM measurements has been growing along with our understanding on stress physiology in elephants, however, due to their complex social life and large behavioural repertoire, stress related behaviours are challenging to understand, as well as the link between both aspects.

The aim of the study is to assess the welfare of a herd of semi-captive African elephants formed by 10 individuals, living at the Knysna Elephant Park in South Africa using observation of self-directed behaviours (SDBs) and measuring levels of faecal glucocorticoid metabolites concentration (FGM), by asking three questions:

1) Do the visitors have a significant effect on the levels of FGM and rates of SDBs?
2) Do the different ranks that the individuals occupied in the herd have an effect in their FGM levels and SDBs frequencies?
3) Is there a correlation between the levels of FGM and rates of SDBs in African elephants?

3. Materials and Methods

3.1 Ethics Statement

This study was completed under the approval of the Ethical Review Committee at the Royal (Dick) School of Veterinary Studies, with the VERC reference number 47.21. The physiological and behavioural data collected for this study was non-invasive and did not involve direct contact or interaction with the animals.

3.2 Study Area and Elephant Population

The focused group for the study was a herd of 10 African elephants living at the Knysna Elephant Park in South Africa, from May to July 2021. The herd's composition (Table 1) is similar to a wild African elephant herd, with a greater number of females. The oldest female being the matriarch, seven other females and two young males. The major distinction from the study herd to a wild group is the absence of close genetic kin, with only two elephants being related (mother and daughter). The elephants are able to roam, graze and forage the 50-hectare park freely under the supervision of the elephant guides (from 7:30 – 17:00) and they also have access to indoor and outdoor shelters.

Table 1. Composition of the African herd at the Knysna Elephant Park.

Name	Sex	Year of birth	Related to (whom)
Sally	Female	1989	NA
Nandi	Female	1993	Thandi (daughter)
Thandi	Female	2003	Nandi (mother)
Keisha	Female	2003	NA
Thato	Female	2008	NA
Amari	Female	2000	NA
Shanti	Female	2002	NA
Madiwa	Female	2005	NA
Mashudu	Male	2007	NA
Shungu	Male	2007	NA

3. 3 Faecal Sample Collection and Freeze-Drying Process

The faecal samples were taken twice a day for each elephant, in three different periods of time. The first sample was the baseline for the study and it was taken before the behavioural observation (T0), the second sample was collected 24 hours after the observations (T24) and the third one, 36 hours after (T36). The specific periods of time, were selected based on previous studies showing that the peak concentrations of stress hormones metabolites in elephants is between 20-25 hours after the stressful event (Stead, Meltzer and Palme, 2000). Between 13-14 grams were taken from the centre of the dung and put inside of sealed cooling bag, between two ice packs to avoid and slow down the process of hormone decay. The bags were labelled with the name of the individual, date and time of collection. The sample collection was repeated three times (cycle 1=C1, cycle 2=C2 and cycle 3=C3) for each elephant, in different weeks for the length of the study.

For the freeze-drying process, the dung had to be prepared by loading them on metal trays and loaded into a freezer. Afterwards, the samples on the metal trays were taken from the freezer and put into a pre-cooling freeze dryer for at least 10 Minutes, which was run until the temperature reached -45 ºC. For drying the samples, the temperature was lower than -40 ºC and the vacuum pump was activated. After 24 hours the samples were removed. Lastly, the samples were naturally defrosted, ensuring the water was drained from a tube. The defrosting process took around 36 hours.

3.4 Measuring faecal steroid metabolites with EIA

The freeze-dried faecal samples from the elephant herd were sent from the Knysna Elephant Park to the Scotland’s Rural Collage (SRUC) in Edinburgh for the measurement of faecal steroid metabolites with enzyme immunoassays (EIA) following the protocol of by Möstl 2009. However, some of the reagents had to be substituted from the original protocol due to COVID-19 restrictions and logistic problems (see appendix 1 for further specifications).

For the extraction process, between 0.050 – 0.055 g of dried faeces were weighed, 3 ml of 80 % methanol was added, then they were vortexed (VWR VX-2500 Multi-Tube vortexer) for 15 minutes at 2500 rpm and then centrifuged (HERMLE Z 200 A) for 10 minutes at 2500 rpm. The extracts were labelled and stored in the freezer at -20 ºC before the measurements with EIA.

3.5 Measurement with EIA

To measure glucocorticoids and metabolites using an enzyme immunoassay (EIA), the preparation of two coating buffers was needed, an EIA assay buffer, a washing solution and a stop solution. For the first coating procedure, 1 mg of anti-rabbit IgG was diluted in 750 ml of coating buffer (see appendix 3) and 0.25 ml per well of diluted IgG was dispensed into three Maxisorp 96 well flat-bottomed plates (MTP), then sealed and incubated at room temperature overnight. Afterwards, the coating solution was discarded and 250 μl/well of second coating buffer was added to the plates. The plates were then left to incubate at room temperature (RT) for minimum 3 hours before use.

3.4.3 Assay procedure, MTP loading and Absorbance measurement

For the assay procedure, the plates were washed three times using washing solution with the “EIA” program in the BioPlex Pro Washed Station, the remaining liquid was removed by blotting the MTP on paper towels, without touching the plates. Before loading the plates, an aliquot of antibody (11-oxoaetiocholanolone-17-CMO:BSA), biotin enzyme label and standard (11- oxoaetiocholanolone (5ß-androstane-3α-ol-11,17-dione)) had to be defrosted at room temperature for 1 hour. To prepare the standards, 230 µl of assay buffer was added into the tube and vortexed, then it had to be settle for 5 minutes before loading the plate.

For the loading plate process, the volume of controls and standards were dispensed (Figure 1), adding 100 µl of biotin-steroid and 100 µl of antibody solution and put on a MTP shaker overnight at 4 ºC.

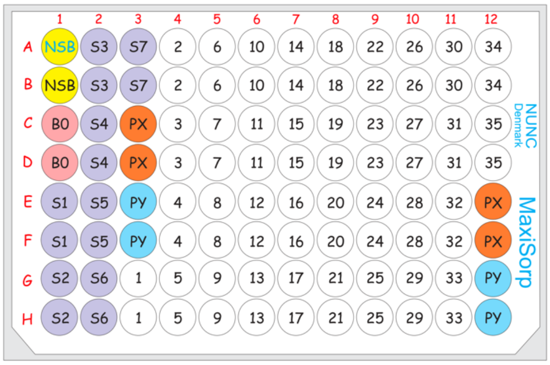

Figure 1. Arrangement of standards, pools and samples on the MTP, showing the nonspecific binding (NSB) and zero binding (B0), standards and sample into the MTP. For this study only one QC was used.

After incubation, the plates were washed with washing buffer 4 times using the BioPlex Pro Washed Station and blotted dry, then 250 µl of streptavidin-HRP (R&D Systems, Inc. 314509) from R&D ELISA duo set kit horseradish peroxidase conjugate was added to each well and incubated for 45 minutes at room temperature in a shaking plate. For the substrate, the plates were washed again four times with washing buffer, blotted dry and

250 µl of KPL SureBlue TMB Microwell Peroxidase Substrate (SERA CARE CAT No. 5120-0083) was added, incubating in the dark at room temperature and shaking for 45 minutes, checking every 10 minutes to ensure colour saturation was not reached and to stop the reaction, 50 µl of 1M HCl was added after

To measure the absorbance, the MultiSkan plate reader was used with reference filter: 620 nm & Measuring filter: 450 nm (Figure 2). After reading, the standard curve was visualized, as well as the R^2 value. In addition, values for unknowns and controls with their standard deviation (SD) and coefficient of variance (CV %) were calculated and exported to Microsoft Excel for further analysis.

Figure 2. Automated plate reader Thermo scientific Multiskan FC.

3.5 Behavioural Observations of Self-Directed Behaviour's (SDB's)

The behavioural observations were recorded from the 10th of May to the 22th of June, in two different times of the day, the first in the morning and the second in the afternoon. AERU personnel trained to identified 16 different SDBs (Table 2) recorded the behaviours into an Excel sheet. The observers followed a focal elephant for 30 minutes and all the SDB's displayed were recorded continuously, as well as other behaviours or activities that the elephant was doing using an ethogram (Appendix 2). In addition, every five minutes during the observation period, it was recorded which elephant was the closest to the focal individual, the closest person (tourist, guide or volunteer), and the distance between the focal individual (Table 3).

Table 2. SDBs identified and recorded by the AERU staff for the present study.

Behaviour	Description	
Trunk curl	t-curl	
Trunk out curl	t-out-curl	
Trunk to own body	t-own-body	
Trunk to own ear	t-own-ear	

Trunk to own eye	t-own-eye	
Trunk to own foot	t-own-foot	
Trunk to own head	t-own-head	
Trunk to own leg	t-own-leg	
Trunk to own mouth	t-own-mouth	
Trunk to own temporal gland	t-own-temporal-gland	
Trunk to own trunk	t-own-trunk	
Trunk to own tusk	t-own-tusk	
Trunk spring	t-spring	
Trunk suck	t-suck	
Trunk swing	t-swing	
Trunk twist	t-twist	

Table 3. Classification of the nearest neighbour of the focal elephant, measured in meters.

Nearest neighbour	A: Touching	C: 6-10m	E:16-25 m	G: 50 m
	B:1-5 m	D:11-15	F:26-50 m	OOS: out of sight

3.6 Data Analysis

3.6.1 FGM and Behavioural analysis

The results showed by the MultiSkan plate reader were exported to Microsoft Excel for further analysis and calculations. In Microsoft Excel, the ng/g of faeces were calculated using the equation:

$$\frac{\boldsymbol{ng}\ (\text{steroid})}{\boldsymbol{g}\ (\text{faeces})} = \frac{\boldsymbol{pg(per\ well)} \cdot \boldsymbol{V_e} \cdot \boldsymbol{d}}{\boldsymbol{w_f} \cdot \boldsymbol{V_s} \cdot \mathbf{1000}},$$

where the mass per well calculated by the EIA-reader is given in pg, V_e is the extract volume, d is the dilution factor, w_f is the faecal weight in g, V_s the sample volume, which will be multiplied by 1000 to have the results expressed in ng. The percentage of recovery, the Intra-CV% and Inter-CV%, the average of the coefficient of variance CV% of the unknown samples were calculated. For the Intra-CV%, it was only possible to calculate it for plate 3, since for plate 1 and plate 2, one of the controls did not show values. Both, the Intra-CV% and Inter CV% were calculated using the formula:

$$C_v = \frac{\sigma}{\mu} * 100,$$

where α is the standard deviation of the concentration values and μ is their mean value. For the analysis of the behavioural data, the frequencies of SDBs and the recorded information during the observations were arranged in Microsoft Excel for the descriptive statistics and then exported to Minitab v10 2020 for the statistical analysis.

Both, the behavioural and the physiological data were analysed for normality using the Anderson-Darling test. The total count of SDBs and the ng FGM/g faeces were tested for normality using the Anderson-Darling test, showing both a p-value <0.005, not meeting the assumptions for normality. Therefore, they were transformed into a log_{10} scale (logTSDBs and log FGM) for further analysis.

The total of the frequencies of SDBs and the ng FGM / g faeces were transformed to a log_{10} scale, and afterwards, a Mixed Effect Model was run using Minitab. For the mixed effect model, the estimation method was maximum likelihood (ML) and the test method for fixed effects was Kenward-Roger in order to control the small sample size of the study. The statistical significance was defined as $p < 0.05$. The elephants were treated as a random effect throughout the analysis, the time (T0, T24 and T36) and cycle (C1, C2, and C3) as fixed factor. The visitor variable was analysed by the number of visitors at the time of the observation, by their presence (1) or absence (0), treating it as a covariant variable in the model; also, the variable was categorized by few numbers of visitors = 1 (less than 9 visitors), or high number of visitors = 2 (more than 9 visitors). The social ranking of the elephants was determined by the AERU staff, in a descending order, starting with Sally, the matriarch as number 1 (Table 4), and it was treated as a covariant for the statistical analysis.

The mixed effect model was run to investigate significant differences between the total count of SDBs over the three cycles, the rank of the elephants, the visitor number, treated as categorical variable and time. The response variable was the total number of SDBs transformed in a $\log_{10}$ scale, the elephants as a random effect, the cycles, category of visitors and time as fixed effects and the rank as a covariant variable.

The mixed effect model was also adjusted to analyse the interaction between the Log FGM and the interaction of log total SDBs and time. Time was treated as a fixed factor, log total SDBs as a covariant variable, and in addition, the interaction between log total SDBs and time was added to the model.

Table 4. Table of the ranks of the elephants classified by the AERU staff

Elephant	Sex	Rank
Sally	F	1
Nandi	F	2
Thandi	F	3
Thato	F	4
Keisha	F	5
Amari	F	6
Shanti	F	7
Madiwa	F	8
Mashudu	M	9
Shungu	M	10

4. Results

4.1 Results of the Faecal Steroid Metabolites

The three EIA plates passed quality control checks with acceptable R^2 values. The average percentage recovery of controls was 97.97 %, inter-plate CV% was 9.37 %. However, one control of plate 1 and one control of plate 2 showed no results. Therefore, the calculation of Intra-CV% it was only possible for plate 3 and intra-plate CV%, which was 5.68 %. The average coefficient of variance of the samples was 34.09 %. For plate 1 and plate 2 it was not possible to calculate the Intra-CV% since we only got results for one of the two controls. For plate 3, Intra-CV% was 5.68 %, and the Inter-CV% was 0.37 and the percentage of recovery was 97.9 % and samples which had a CV% of over 15 %, were included in the analysis.

4.2 Influence of Rank, Visitors and time on FGM levels

The results of ng FGM / g of faeces were tested for normality using the Anderson-Darling test, showing a p-value <0.005, not meeting the assumptions for normality. Therefore, it was transformed into a $\log_{10}$ scale and analysed with the Anderson-Darling test, showing a p-value of 0.222 (mean=1.6803, SD= 0.3222). Figure 3 shows the levels of log FGM seemed to be higher in T0 (baseline) than 24 hours (T24) and 36 hours (T36) after the behavioural observations, and higher in cycle 2 (Figure 3). A mixed effect was used to analyse if there were significant statistical differences between log FGM and the social rank, number of visitors and time (Table 6). The model showed no significant differences of log FGM and the social rank (p-value=0.579), no significant differences in regards to the number of visitors (p-value=0.641) and no significant differences in regards of time (p-value for T0 = 0.594 and p-value for T24 = 0.353).

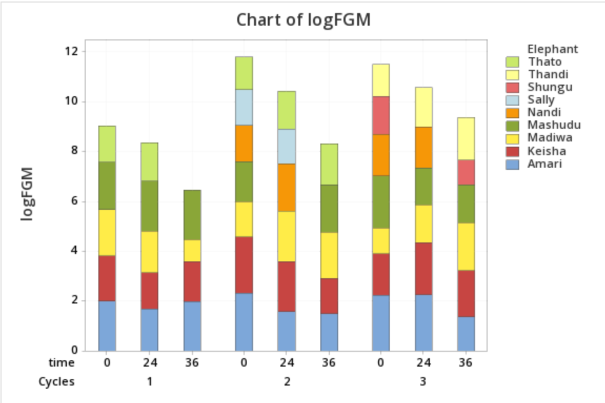

Figure 3. Graph showing the changes in the levels of FGM in the elephants labelled by rank 24 and 26 hours after the behavioural observation and in the three different cycles.

Table 6. Table of results of the coefficients using the mixed effect model to measure differences between log FGM of elephants, their rank and number of visitors.

Coefficients					
Term	Coef	SE Coef	DF	T-Value	P-Value
Constant	1.607993	0.170892	20.66	9.409406	0.000
Rank	0.013096	0.022984	11.82	0.569793	0.579
VisitorsOBS	-0.004092	0.008720	49.62	-0.469217	0.641
time					
0	0.030450	0.056685	42.55	0.537191	0.594
24	0.055507	0.059138	42.54	0.938603	0.353

Afterwards, the effect of the visitors on log FGM was analysed by their presence or absence. The results showed a p-value of 0.764, therefore, the presence or absence of visitors did not show to have a significant effect with respect of the log FGM of the elephants or their rank (Table 7).

Table 7. Table of results of the coefficients using the mixed effect model to measure differences between log FGM of elephants, their rank and presence (1) or absence (0) of visitors.

Coefficients					
Term	Coef	SE Coef	DF	T-Value	P-Value
Constant	1.543780	0.156826	12.85	9.843905	0.000
Rank	0.015994	0.022388	8.71	0.714412	0.494
time					
0	0.030502	0.057531	39.17	0.530187	0.599
24	0.058530	0.060027	38.98	0.975057	0.336
PresenceV					
0	-0.023087	0.076332	28.95	-0.302453	0.764

In the three different models, the age and sex of the elephants was also analysed, however, there was no significant difference between both variables and the log FGM levels.

4.3 Influence of Rank, Visitors and time on frequencies of SDBs

During the length of the study, Sally, the matriarch, showed higher number of SDBs (156 SDBs) than any other elephant, and Shungu (rank 9) showed the smallest number of SDBs (10 SDBs) (Figure 4). The most frequent SDBs observed among the 10 elephants were trunk out curl (t-out-curl) with a total of 316 repetitions and the less frequent were trunk to temporal gland and trunk swing, which were not observed at all (Figure 5).

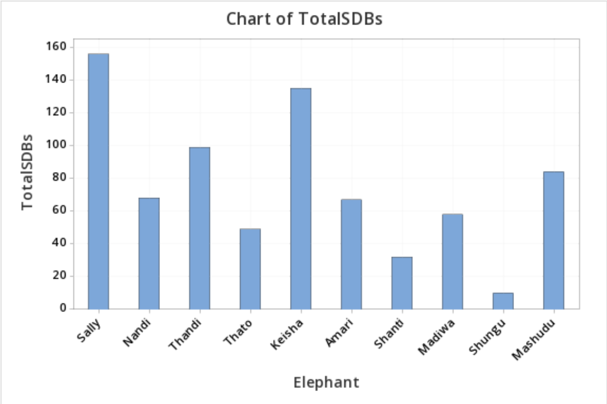

Figure 4. Graph showing the total counts of SDBs showed by all elephant during the study.

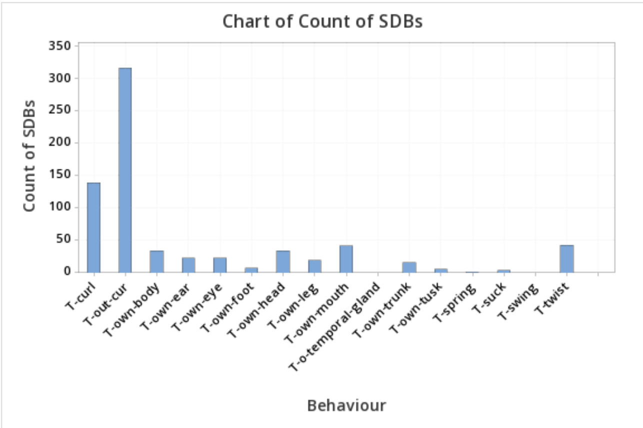

Figure 5. Total count of each SDBs by all elephants throughout the observational study.

The total number of SDBs of each elephant was compared with the number of visitors, (less than 9 visitors =1, 9 or more= 2) in each cycle (1, 2 or 3). Showing the highest number of SDBs expressed (267 of SDBs) were during cycle 1, with higher number of visitors

(Figure 6). The comparison shows that the highest number of SDBs was expressed during cycle 1, with the presence of high number of visitors.

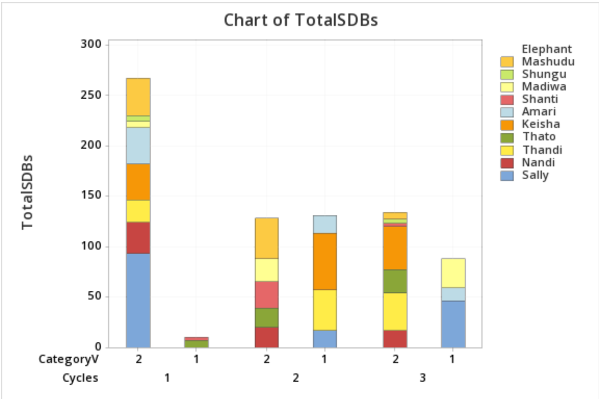

Figure 6. Bar chart showing the total of SDBs expressed by the elephants in the three different cycles, when visitor number was less than 9 (1) or more than 9 (2) during the behavioural observations.

The model showed no significant difference in respect with the rank of the individuals (p-value=0.231), however, there were significant differences when comparing log total SDBs and cycle 2 (p-value=0.028) and with higher number of visitors (p-value=0.014) (Table 8).

Table 8. Table of results of the coefficients using the mixed effect model to measure differences between log total of SDBs of elephants in respect of their rank, cycles time and visitors.

Coefficients					
Term	Coef	SE Coef	DF	T-Value	P-Value
Constant	1.488835	0.206863	9.08	7.197211	0.000
Rank	-0.043524	0.033880	9.01	-1.284672	0.231
Cycles					
1	-0.027706	0.044660	41.73	-0.620379	0.538
2	0.098984	0.043544	42.13	2.273203	0.028
CategpryV					
1	0.096932	0.037902	46.21	2.557422	0.014
time					
0	-0.005912	0.039306	40.72	-0.150401	0.881
24	-0.017179	0.041188	40.81	-0.417098	0.679

4.3 Interaction between FGM levels and SDBs frequencies.

To analyse the correlation between the levels of FGM and the SDBs, a linear regression was done, which showed a positive correlation (S=0.308, R^2=10 %, R^2(adj)=8.2 %) (Figure 7). Afterwards, a Pearson correlation showed a significant correlation between the variables (p-value=0.024).

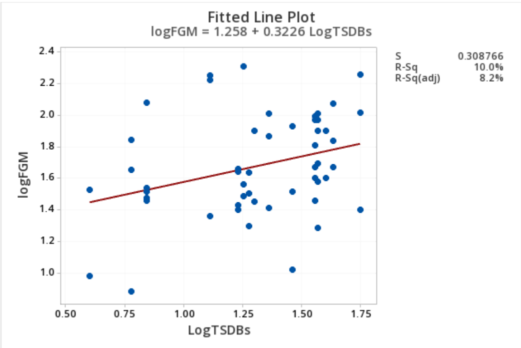

Figure 7. Graph of the linear regression between log FGM and log total SDBs.

Figure 8 shows an increase in the slope of the regression line over the three different times. Therefore, we expect a stronger growth of variable log FGM when variable log Total SDBs increases at later times.

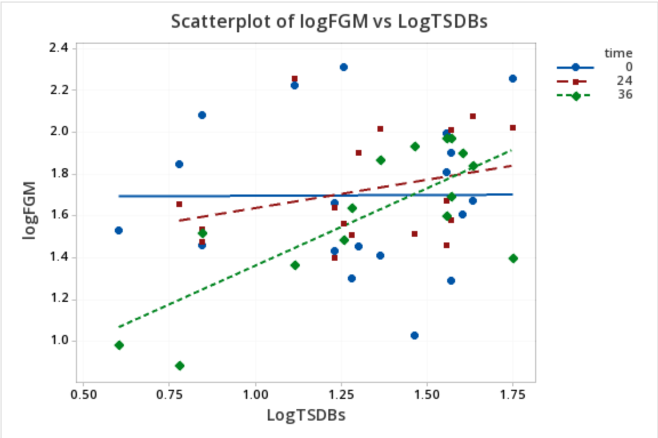

Figure 8. Scatterplot showing the interaction between log FGM and log total SDBs in the three different times of measurements.

The mixed effect model was adjusted to analyse the interaction between the Log FGM and the interaction of log total SDBs and time. Time was treated as a fixed factor, log total SDBs as a covariant variable, and in addition, the interaction between log total SDBs and time was added to the model. Results showed a significant difference between log FGM and the log Total SDBs and time interaction (p-value = 0.040) (Table 9).

Table 9. Table of results of the coefficients using the mixed effect model to the interaction between log FGM and log total SDBs in respect of time

Coefficients					
Term	**Coef**	**SE Coef**	**DF**	**T-Value**	**P-Value**
Constant	1.241751	0.189715	42.59	6.545342	0.000
LogTSDBs	0.310591	0.141559	44.31	2.194078	0.034
time					
0	0.484937	0.219298	43.09	2.211313	0.032
24	0.139698	0.245395	46.41	0.569276	0.572
LogTSDBs*time					
0	-0.345908	0.163551	43.01	-2.114995	0.040
24	-0.063149	0.181886	45.90	-0.347188	0.730

5.Discussion

The aim of the study was to assess the welfare of African elephants using SDBs and concentration levels of FGM, which can be indicators of stress. The study investigated whether visitor presence had an effect on both indicators and if the social rank of the individuals may also have an effect on SDBs rates and levels of FGM. Finally, it was also investigated the correlation between both, in order to provide evidence for further studies to use them as tools for assessing animal welfare.

5.1 FGM levels

Before analysing the data to answer the questions for the study, it was necessary that the EIA measurements were reliable enough to analyse them, and even though there are missing control values of two of the EIA plates, the results for quality control showed that the measurements were reliable enough to further analyse the data (Möstl, 2009). However, it is important to take into account some of the limitations of the study. For the faecal sample collection, it was challenging to collect them at exact points in time (24 hours and 36 hours after the behavioural observations), so it is suggested instead of having specific times, to have a time range in which the faecal samples can be collected. The time points of when to collect the faecal samples were established based on previous studies showing that the time between the release of FGM due to a stressful stimuli and the excretion of their metabolites was between 20-33 hours, with a peak within 20-25 hours, this study was done in Asian and African elephants (Stead, Meltzer and Palme, 2000).

The results for the present study indicated that the number of visitors did not showed a significant effect on the FGM levels of the elephants (p-value=0.679), nor with the presence (1) or absence (0) of visitors (p-value= 0.328). This is a different result of what has been shown previously, where they found that levels of FGM in African elephants increased as the number of tourists increased and with their presence (Szott *et al.*, 2020). However, it is important to notice that in this particular study, we were not able to analyse the specific interactions that the visitors have with the elephants in the park. Some of the activities involved a closer interaction between the visitors and the elephants (feeding activity) or more distant (walking activity), which may establish limitations in the study and in the results. Therefore, it is suggested that further studies may focus on analysing the FGM levels of the elephants with respect of the different activities that the park has available for the visitors to have a broader view of what may cause (or not) changes in the stress hormones of the animals.

Among the 10 elephants, Amari, with the number rank number 6, showed the highest mean of FGM levels throughout the study (mean =1.873 ng FGM/g faeces), along with Keisha (mean=1.7937 ng FGM/ g faeces). It is known that in elephant societies, females may use aggression to establish and maintain their social rank, showing higher concentrations of FGM (Burks *et al.*, 2004). With this in mind, we would have expected Sally (rank 1), to have higher concentrations of FGM, not Amari or Keisha. The social subgroup of Amari is formed by Keisha on top (rank 5), then Amari and then Shanti (rank 7), so their high FGM levels may be a result of maintaining their social rank. On the other hand, Shungu (rank number 9) had the lowest concentration level (mean= 1.260 ng FGM/g faeces). However, when analysing the rank on both mixed effect models, there were no significant differences between FGM concentration levels and the rank of the individuals.

Commented [SH1]: Unit?

Commented [SH2]: (rank number?)

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In both statistical models that were used, we did not find significant differences between sexes. It is well known that male elephants may show increasing levels of FGM when they reach sexual maturity and when they experience the phenomenon of musth which has been linked to increasing stress hormone levels (Ganswindt *et al.*, 2003). However, the rest of the year, it has been reported that male elephants show lower concentrations of FGM than females (Hunninck *et al.*, 2017). The male elephants in the study are still considered to be young (Mashudu and Shungu, both 14 years old) and that may be why they were not showing any significant differences in comparison to the females. However, it would be of interest to investigate the changing FGM levels of these males as they growth to adulthood.

There are different factors that may have contribute to the results shown in this study. First, it is important to clarify that the values with high coefficient of variance (more than 15 %) were also included in the statistical analysis, which may decrease the reliability of the results. We had also missing values of some of the individuals in different time points and in different cycles, so the data that was analysed small. Additionally, it is also necessary to take into account the skills and experience of the pipetting during the extraction and measurement of the faecal metabolites, since the technique may have an important influence on the results. For future studies, it is also suggested to do the EIA measurements with more faecal samples, with more time point and over a longer period of time. In the present study we run three plates, and in duplicates, however, more broader studies analysing FGM, analyse five plates and the samples, controls and standards are done in triplicates or quadruplicates in order to have a broader range of data for the analysis (Mesa-Cruz, Brown and Kelly, 2014).

For future studies it is also suggested to consider factors that may influence the FGM levels in African elephants, such as the study design, protocols, methods for collecting the faecal samples, methods for storage, validation of EIA and statistical analysis.

5.2 Frequencies of SDBs

During the length of the study, Sally, the matriarch showed the highest frequency of SDBs (156 SDBs) among the ten elephants, and Shanti, the number 7 in the rank, the lowest one (10 SDBs). Matriarchal family units of African elephants are usually composed by smaller groups, within each social group, it is known that high ranking females may use agnostic behaviours to establish and maintain their rank, as a consequence, it is possible

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to see an increase in the glucocorticoid excretion (Burks *et al.*, 2004). Other studies also have provided evidence that weak social bonds, or groups without older matriarchs may have an increase in intra-social competition (Szott *et al.*, 2020). The herd of the present study is formed by non-related individuals which may be influencing FGM levels in certain individuals, also increasing conflicts and aggression within the herd.

When comparing the number of SDBs in the presence or absence of visitors, Amari (rank 6), showed the highest number of SDBs displayed when visitors where present. The statistical analysis showed that there was a significant difference (p-value= 0.010) between the total of SDBs and the visitors. Providing evidence that the presence of visitors may have an influence on the frequencies of SDBs displayed by the elephants.

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After analysing the data and running the statistical analysis, the present study provides evidence that the presence of visitors may be influencing increasing rates of SDBs (p-value= 0.014) in the herd of elephants. Even though it was not possible to find previous studies investigating the visitor pressure and the rates of SDBs in African elephants, there are studies that have provided evidence that African elephants showed higher levels of FGM, when the number of visitors was higher (Hunninck *et al.*, 2017). This may indicate that visitor number may increase overall stress levels in this animals. There has been reports of negative impacts on animal welfare due to tourism, increasing levels of stress hormones in wildlife, increasing risk disease, lowering reproduction success and their overall fitness. However, the lack of research on this matter does not allow us to fully understand the impact that tourist may have on wildlife, especially in animals such as elephants, which attract visitors from all over the world (Szott, Pretorius and Koyama, 2019). Previous studies have reported that elephants are more likely to move away from large numbers of tourists, and FGM levels showed increasing levels when the number of tourist were also higher (Szott, Pretorius and Koyama, 2019).

Further studies investigating in more detailed the relation between SDBs and the possible effect of the number of visitors are key to unravel the function of these behaviours and use it as an indicator to improve elephant management and welfare.

It was not possible to support the hypothesis that visitors may be the cause of the increasing rates of SDBs in the elephants. Even though in the presence of visitors there were more SDBs, after running the statistical model, there was no significant difference.

In regards to the effect that the social ranking may have on the levels of FGM and expression of SDBs, the elephants that are below the first level of their group, showed high rates of SDBs, suggesting that they may encounter social conflicts between them and other elephants due to availability for resources for example. Nonetheless, after running the statistical analysis, there were no significant differences between the different rankings.

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In order to be able to use SDBs as indicators of the emotional inner state of animals, it is essential to understand the evolutionary function that these behaviours may have. Among different hypothesis developed over time in regards to the function of SDBs (Zeigler, 1964; McFarland, 1966), there is one proposal suggesting that they can also have a communication function among groups and between individuals (Whitehouse, 2018). Previous studies have provided circumstantial evidence suggesting that SDBs may be a coping mechanism to deal with uncertainty, anxiety and stress (Higham *et al.*, 2009). However, recent studies are providing evidence that stress related behaviours, and specifically SDBs, may be also a type of communication among gregarious animals, specifically in macaques (Whitehouse, 2018). Although there is no sufficient evidence acknowledging that SDBs serve as a form of communication, it has been observed that when an individual is expressing a stress related behaviour, others that near, start to showing it too (Whitehouse, 2018). Yawning has been classified as a displacement behaviour (Maestripieri *et al.*, 1992), and it has been reported to be contagious in at least four different species of mammals (Gallup *et al.*, 2015). There are reports of contagious yawning in dogs, (Maestripieri *et al.*, 1992; Harr, Gilbert and Phillips, 2009), in budgerigars (Melopsittacus undulatus) (Gallup *et al.*, 2015), in chimpanzees (Yamanashi and Matsuzawa, 2010), and in gelada baboons (Mancini, G. and Palagi, E., 2009). There are have been reports observing SDBs to be contagious in rhesus macaque, Japanese macaque and *Macaca fuscata* (Whitehouse, 2018).

Even though, in the present study we recorded data of which elephant was near the focal individual during the behavioural observations, were not able to investigate if there were any correlation between the frequencies of SDBs and the nearest neighbour. We encourage that future studies also investigate the possible communication function of SDBs in African elephants.

The statistical model in this study, we did not find a significant difference in the expression of SDBs between sexes. This is likely to be because of the age of the males investigated in this study, in addition to the fact that the herd is composed only by to males.

5.3 FGM levels and frequencies of SDBs

The study found a significant positive correlation between changes in Log FGM and changes in the SDBs frequencies through time, and a Pearson correlation showed a moderate positive correlation between both variables. However, the lack of research studies investigating the possible correlation of the two indicators is limited. In 2009, a study researching the correlation between rates of SDBs and levels of FGM in female wild olive baboons (*Papio hamadryas anubis*) concluded that they are not correlated and they suggest that SDBs may have a coping mechanism to regulate stress in this primates (Higham *et al.*, 2009). On the other hand, independent studies done in macaques, provided circumstantial evidence of the possible relation between SDBs and physiological changes by showing that the frequency of SDBs increased along with their heart rates (Maestripieri *et al.*, 1992). However, the direct relation between both responses remains unsolved.

The correlation between levels in FGM and SDBs have been studied mainly in human and non-human primates (refs), however, the results have been contradictory, inconclusive and focused on human and non-human primates. There are very limited studies focusing on how these two indicators of stress may be related in other species. It is important that future studies have broader data and throughout a longer period of time, since the FGM

Commented [SH9]: Of elephant studies?

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levels in African elephants changed during dry or wet season it is recommended that future studies may be cover both seasons (Ganswindt *et al.*, 2008).

Commented [SH11]: Why is this? refs

After analysing the results of the present study, it is crucial to take into account also the individual differences between the elephants. Each elephant in the herd have experience different traumatic events throughout their lives (such as poaching, separation from their birth family, abandonment, physical injuries etc.). Many of them came from unknown backgrounds and stressful experiences that may have an influence in how they respond to different stimuli, including company of other elephants, staff of the same park and visitors. So, it is suggested that in future studies, the individual differences are taken into account when analysing changes in their physiology and in their behaviour.

6. Conclusion

The present study was able to demonstrate that the number of visitors may have a significant effect on the FGM levels or in the increasing rates of SDBs of the herd. This result is supported by previous studies investigating also the effect of visitors on FGM levels in African elephants. Furthermore, the research did not find significant differences in the levels of FGM in regards to the rank of the individuals. Nevertheless, we did find a significant effect of the rank in regards to the rates of SDBs, which is supported by other studies done in social primates. And finally, we were able to show a positive and significant correlation between the FGM levels and the rates of SDBs in the herd of African elephants living at the Knysna Elephant Park. Since some of the values of the FGM levels had high CV%, additionally, it is necessary to take into account the limited sample size and missing values occurring during the study. This research provides a promising pathway to follow for future studies focusing on the understanding of elephant physiology, behaviour and welfare.

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General Discussion of Results and Criticisms of Methodology

I. Sample Collection and Sample Size

The original plan for my project was to travel to South Africa to carry out the behavioural observations of self-directed behaviours on the herd of African elephants living at the Knysna Elephant Park. I was going to learn how to collect the faecal samples and I was going to undergo a training period to learn how to identify SDBs in African elephants. However, due to travel restrictions caused by the COVID pandemic, I was not able to travel and I could not be in charge of the faecal samples, nor of the behavioural observations. The AERU team, located at the Knysna Elephant Park, carried out the collection of the faecal samples and the behavioural observations for my project. Even though the AERU staff are well trained on how to collect faecal samples and are trained to identify SDBs in the elephants, it is important to take into account that I had no control over the collection sample process and it was challenging to organize the logistics, understand the challenges to collect the faecal samples and to record the behaviours. The time of the year when the study was carried out, is also a factor that is necessary to take into account since there is evidence suggesting that the levels of FGM of elephants changes and is influence by the dry or wet season, increasing levels of FGM during dry season due to lack of resources (Ganswindt *et al.*, 2008).

II. FGM analysis with EIA

Different reagents that are used in the original protocol (Möstl, 2009), were not available because of the COVID pandemic, therefore, for the present study it was necessary to substitute with other reagents. The inter-CV% and intra-CV% of our plates, were reliable enough to continuing to analyse the data, however, the CV% of many of our samples were higher than 15 %, which is too high to validate some of our results (Mesa-Cruz, Brown and Kelly, 2014). Additionally, it is very important to take into account the experience that is needed in the laboratory when measuring FGM. When loading the plates with the controls and samples, having good pipetting experience can be key in order to have reliable measurements since some of the reagents are light and temperature sensitive, having the necessity to be accurate but fast, and for me, it was my first practical experience measuring FGM with EIA.

Furthermore, the standards and samples to analyse FGM levels, are usually done in triplicates or quadruplets in order to have more reliable results of the samples (Yarnell, Purcell and Walker, 2016). However, due to limitations of time for the study, the controls, standards and samples were done in duplicates. This may have caused having missing values and high CV% of our samples (more than 15 %).

In addition, it is important to take into account the small sample size was small, having only 10 elephants for the study. Even though the AERU staff and myself tried to organize the collection samples as often as possible to have a broader data, limitations of time and personnel, restricted the data collection of the study.

III. Statistical analysis

There were different factors that limited the exploration of the research study such as missing values in the data, leading to have few data points to analyse. In addition, the small sample size of ten individuals, limited time to collect the samples and limited personnel trained to record the behavioural observation also represented limitations for the analysis and the have to take into account when interpreting the results of the study. Nonetheless, the model used for the statistical analysis proved to be reliable and even though we are confident about our results, further investigation is recommendable.

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Appendices

Appendix 1:

Full description of the protocol developed by Möstl E., 2009, indicating with * the reagents that were substituted for the present study.

Measurement with EIA	
1.Buffers and Solutions	**1.1 Coating Buffer:** - $1.59g\ Na_2CO_3$ (Merck 106392 or Sigma S-7795) - $2.93g\ NaHCO_3$ (Merck 106329 or Sigma S-6014), dissolve and fill up to 1L with DDW, adjust to pH 9.6 with HCl (1mol/l) **1.2 HCl (1mol/l):** - 920ml DDW +80ml 37% HCl (Merck 100317 or Sigma H-1758) **1.3 Assay Buffer:** - 2.42 g Trishydroxyaminomethane (Merck 108382 or Sigma T-1503), 20 mmol/l - 17.9 g NaCl (Merck 106404 or Sigma S-9625), 0.3 mol/l) - 1ml Fetal Bovine Serum albumin (FBS)*

	- 1 ml Tween 80 (Merck 822187 or Sigma P-8074) dissolve and fill up to 1 l with DDW and adjust to pH 7.5 with (about 17 ml) HCl (1 mol/l) filter through Sep-Pak C18. **1.4 Second Coating Buffer:** - 3.146 g Trishydroxyaminomethane - 23.3 g NaCl (Merck 106404 or Sigma S-9625) - 13 ml FSA (Sigma A-4503)* - 1.3 g Sodium azide (Merck 106688) dissolve and fill up to 1.3 l with DDW and adjust to pH 7.5 with (about 40 ml) HCl (1 mol/l). **1.5 Washing Solution:** - 0.5ml Tween 20 (Merck 822184); add 2.5 l DDW **1.6 KPL SureBlue Reserve TMB Microwell Peroxidase Substrate*** - 1.36g Sodium acetate (Merck 6267) = 10mmol/l, dissolve and fill up to 1 l with DDW and adjust to pH 0.5 with (~8ml) 5% citric acid (Merck 100244) **1.7 Enzyme Solution for Stretavidin-reaction:** - 30ml assay buffer - +0.001ml streptavidin-HRP from ELISA duo set kit horseradish, the working solution has to be prepared immediately before use. **1.8 KPL SureBlue Reserve TMB Microwell Peroxidase Substrate*** **1.9 Stop reagent: 1 mol/l HCl**

2.Coating of microtitre plates with (MTP)	- For 30 MPT, prepare a solution of 1 mg IgG (Sigma R2004-5x1MG add 1 ml DDW to a portion of mg) dissolved in 750 m coating buffer. Dispense 0.25 ml/well of diluted to the MTP (F96 MaxiSorp, No. 442404, Co. Nunc, Denmark). - Incubate the plate at room temperature overnight. - Discard the solution and refill each well with 0.3 ml "second" coating buffer (see 1.4.) Cover the filled MTP with parafilm and keep it at room temperature until use.
3. Reagents (Stock solutions)	Keep all stock solutions frozen at -20°C until use. Dilute 0.01 ml of a stock solution (1 mg steroid per ml methanol) with 20 ml of assay buffer.
4.Working dilutions	4.1. Standard Dispense 0.15 ml (0.2 ml when lyophilized) of assay buffer to one portion of standard vial, shake and wait 20 min. Dilute this solution 1 : 2.5 seven times. Mix well after each step (500 pg till 2 pg per).10 µl - In our lab, this is done with a Hamilton Microlab dispenser 1000 (0.09 ml standard + 0.135 ml assay buffer). - Alternatively, you may take the whole portion of the standard vial (0.05 ml) with the 0.15 ml assay buffer, and add another 2.3 ml assay buffer (you have to transfer everything to a larger vial). This results in a concentration

	of 500 pg per 50 µl assay buffer, which needs to be further diluted (1:2.5; 1 ml + 1.5 ml buffer). Standard, antibody and biotin label solution have to incubate 20 minutes before you can work with them.
5.Assay procedure	**Plate washing:** Before use, wash coated MTP three times with washing solution. Remove the rest of liquid by blotting the MTP on paper towels. Do not touch the underside of the plate **Pipetting of standards, pool and samples:** Dispense assay buffer for nonspecific binding (NSB) and zero binding (0), standards (4.1.), pool X and Y (PX, PY), and sample into the MTP (see Fig. below) prepared earlier (5.1.).
6.Absorbance measuring	The absorbance was measured at 450 nm (reference filter: 620 nm) with an automated plate reader (Thermo scientific Multiskan FC)

Appendix 2:

Ethogram for the behavioural observations of the African elephants at the Knysna Elephant Park

Code	Behaviour	Description
FG	Feeding - Grazing	Eating grass or shrubs
FB	Feeding - Branches	Eating tree branches, leaves or bark. Does **not** include suikerbossie

FS	Feeding - Straw/Lucerne	Eating straw or lucerne
FP	Feeding - Pellets	Eating pellets
FF	Feeding - Fruit	Eating fruit and/or vegetables
FO	Feeding - Oats	Eating oat hay meal mix
FM	Feeding - Miscellaneous	Eating something else, e.g. sweet potato, shrubs from suikerbossie. Does not include flowers
W	Walking	Walking with all four legs moving in a steady pace
WF	Walking - Fast	Walking with all four legs moving at a fast pace - this is markedly swifter than usual pace (often when approaching the barrier for fruit)
WG	Walking - Grazing	Walking and grazing simultaneously
WB	Walking - Branches	Walking and eating branches simultaneously
WGU	Walking - Guide	Walking due to commands given or action taken by a guide
WW	Walking - Walk	Walking with guests and guided along pre-determined path for the elephant walk experience
S	Standing	Standing stationary for at least 2 seconds, not performing any other general behaviour
SB	Standing - Barrier	Standing at the feeding barrier for tourists, not eating fruit
SLB	Standing – Leaning Barrier	Standing and leaning on the feeding barrier when there are no tourists or no feeding occurring
ST	Standing - Tourist	Standing in response to guest, due to commands given by a guide
SR	Standing - Walk	Standing due to elephant walk activities (waiting for guests)

SS	Sleeping - Standing	Standing motionless with trunk relaxed, tip flopped on ground or draped over an object
SL	Sleeping - Lying down	Lying down on either side sleeping, eyes closed
TW	Training - Walk	Walking for training purposes
TO	Training - Other	Training activities not covered in other categories- e.g. lying down on command, lifting leg etc
CG	Command from Guide	A guide is giving the elephant a command other than walking e.g. being held back from barrier
I	Interacting	Deliberately interacting, sparring or playing with another elephant
IG	Interacting - Guide	Deliberately interacting with a guide, not for training purposes or in response to commands
IV	Interacting - Volunteer	Deliberately interacting with a volunteer or other member of staff
IT	Interacting - Tourist	Deliberately interacting with a tourist
PE	Playing enrichment	Using any part of the body to play or interact with an enrichment device
FE	Feeding enrichment	Eating food rewards from an enrichment device
WEN	Walking enrichment	Walking while carrying an enrichment device
TE	Trunk to enrichment	Investigating an enrichment device: trunk extended within 10cm or touching the enrichment
OD	Other - Dust throw	Picking up dust with the trunk and throwing it over own body
OM	Other - Mud throw	Picking up wet mud with the trunk and throwing it over own body

OP	Other - Play	Playing with an object, or chasing animals or birds
OWP	Other - Water Play	Playing in large quantities of water, swimming, splashing, spraying water etc
ODr	Other - Drink	Sucking water into the trunk, then ejecting the water into own mouth
OSc	Other - Scratch	Scratching a part of their body on a surface such as a barrier or tree
O	Other	General behaviour not defined in this list
Stereotypic behaviours: must be performed repetitively for at least 10 seconds or for 3 repetitions		
SW	Sway	Standing in one place and moving the front of the body from side to side; the back end stays still
WE	Weave	Standing in one place and moving the body in a snakelike motion; the back end follows the front
RO	Rock	Rocking the body forward and backwards; weight is shifted from back feet to front feet
PA	Pace	Walking forwards around or along the inside of a containing fence, repetitively
RT	Route trace	Walking forwards, backwards and/or side to side in a particular pattern of steps
Hbb / Hnod	Head bob/nod	The head moves repetitively up and down while the elephant is standing still
TUR	Tusk rub	Rubbing a tusk repetitively against bars or objects
BAR	Bar bite	Closing the mouth around enclosure bars and biting (does not include biting on enrichments)

SHS	Shuffle	Feet make constant small movements on the ground; elephant does not leave its position
Codes indicating why behaviour was not recorded at this time		
OOS	Out of sight	The elephant is out of sight or the observer can't see its behaviour clearly
NIS	Not in sight	The elephant is outside the boma and the observer is watching the inside, or vice versa
NR	Not recorded	The observer was not able to record at this time because they were busy with something else