UNIVERSIDADE DE LISBOA

FACULDADE DE MEDICINA VETERINÁRIA

A PILOT WELFARE ASSESSMENT STUDY IN THREE CAPTIVE AFRICAN ELEPHANTS (*Loxodonta africana*) AT BARCELONA ZOO

MIGUEL TRINDADE DE MELO

ORIENTADOR:
Doutor Hugo Fernández-Bellon

COORIENTADOR:
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JÚRI
PRESIDENTE:
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VOGAIS:
Doutora Maria Teresa da Costa Mendes Vítor Villa de Brito
Doutor Hugo Fernández-Bellon

ORIENTADOR:
Doutor Hugo Fernández-Bellon

COORIENTADOR:
Doutor Virgílio da Silva Almeida

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Nome: Miguel Trindade de Melo

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"In the end we will conserve only what we love, we will love only what we understand, and we will understand only what we are taught."

Baba Dioum, 1968

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ABSTRACT

The welfare of wild animals in captivity is an increasingly discussed topic these days. The African elephant (*Loxodonta africana*) is an intelligent, charismatic animal, with specific social and environmental needs. The species *L. africana* is currently considered endangered by the International Union for Conservation of Nature. The decreasing number of individuals in the wild is mainly due to poaching for ivory. African elephants housed in captivity act as ambassadors for their wild counterparts, enabling the research on factors that may impact health, longevity, or reproduction, that may be extrapolated to wild populations. Hence, the importance of studying and promoting the welfare of these animals in man-made environments is highlighted.

This study sought to develop a tool to assess the welfare of three adult female African elephants (E1, E2, and E3) housed at Barcelona Zoo, Spain. For this purpose, saliva samples were collected on a daily basis to measure cortisol, as well as behavioral observations, between October and December 2018.

Salivary cortisol is increasingly used when studying changes in animal welfare. It is considered an effective method for quantifying cortisol levels, capable of detecting acute stress fluctuations, in addition to being a less invasive method than measuring cortisol in the blood. The objectives of this study were to establish baseline cortisol values for the three individuals of the species *L. africana*, and to explore the relationship between salivary cortisol and stereotypic behavior, specifically head bobbing.

Saliva cortisol values ranged from 0.123 to 3.307 ng/ml. The results showed that the animal with the highest mean cortisol, E3, also corresponded to the animal that had a higher rate of stereotypic behaviors. However, the positive correlation between cortisol and head bobbing was not statistically significant (p-value = 0.71). During the study period, a change in the hierarchy was observed among the three elephants based on the analysis of social behaviors. In terms of agonistic behaviors, E2 faced E3 a total of 50 times. In addition, these animals allocate a large part of their time foraging.

This pilot study contributes to increasing the psychological well-being of elephants in captivity by elucidating which factors influence well-being and stereotypic behavior. Projects of this nature are essential for the proper management of these magnificent animals in captivity.

Keywords: African elephant, Cortisol, Saliva, Behavior, Well-being.

RESUMO

O bem-estar de animais selvagens em cativeiro é um tema muito debatido atualmente. O elefante Africano (*Loxodonta africana*) é um animal inteligente e carismático, com necessidades sociais e ambientais específicas. Presentemente, a espécie *L. africana* encontra-se em perigo, segundo a União Internacional para a Conservação da Natureza, sendo a caça furtiva para venda de marfim considerada a principal razão para a diminuição do número de indivíduos em estado selvagem. Os elefantes africanos em cativeiro atuam como embaixadores dos seus congéneres selvagens, possibilitando o estudo de fatores que podem afetar a sua saúde, longevidade ou reprodução, podendo ser extrapolados para populações selvagens, o que reforça a importância de estudar e promover o bem-estar destes animais em ambientes artificiais.

Este estudo focou-se no desenvolvimento de uma ferramenta de avaliação do bem-estar em três elefantes Africanos adultos (E1, E2 e E3), fêmeas, alojados no Jardim Zoológico de Barcelona, Espanha. Foram realizadas diariamente colheitas de saliva para medição de cortisol, assim como observações de comportamento, entre outubro e dezembro de 2018.

O cortisol salivar é muito utilizado no estudo das alterações do bem-estar animal. É um método eficaz para quantificar os níveis de cortisol, capaz de detetar flutuações agudas de *stress*, além de ser menos invasivo do que a medição de cortisol no sangue. Os objetivos deste estudo foram estabelecer valores basais de cortisol dos três indivíduos da espécie *L. africana*, e investigar a relação entre os valores de cortisol salivar e comportamentos estereotipados, nomeadamente *head bobbing*.

Os valores de cortisol na saliva variaram entre 0.123 to 3.307 ng/ml. O animal com a média de cortisol mais elevada, E3, correspondia também ao que apresentava uma maior taxa de comportamentos estereotipados. No entanto, a correlação positiva entre o cortisol e *head bobbing* não foi estatisticamente significativa (p-value = 0.71). Neste período, observou-se uma alteração na hierarquia entre os três elefantes, com base na análise dos comportamentos sociais. Em termos de comportamentos agonísticos, E2 enfrentou E3 num total de 50 vezes. Além disso, foi possível observar que estes animais alocam grande parte do seu tempo ao consumo de alimentos.

Este estudo piloto contribui para o aumento da avaliação e conhecimento do bem-estar psicológico dos elefantes em cativeiro, ao elucidar que fatores influenciam o bem-estar e o comportamento estereotipado. Projetos desta natureza são essenciais para um maneio adequado destes magníficos animais em cativeiro.

Palavras-chave: Elefante Africano, Cortisol, Saliva, Comportamento, Bem-estar.

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LIST OF ABBREVIATIONS AND SYMBOLS

ACTH – Adrenocorticotropic hormone

CITES – Convention on International Trade in Endangered Species of Wild Fauna and Flora

CRH – Corticotropin-releasing hormone

EIA – Enzyme immunoassay

FAWC – Farm Animal Welfare Council

GC – Glucocorticoid

HPA – Hypothalamic-pituitary-adrenal

IUCN – International Union for Conservation Nature

kg – kilogram

km^2 – square kilometer

m – meter

ng/ml – nanogram per milliliter

PVN – Paraventricular nuclei of the hypothalamus

REM – Rapid eye movement

RIA – Radioimmunoassay

UK – United Kingdom

1. ACTIVITIES DEVELOPED DURING THE CURRICULAR TRAINEESHIP

1.1. Barcelona Zoo

The 6th-year curricular traineeship was accomplished at the Barcelona Zoo, in Barcelona, Spain. The program period started on the 1st of October 2018 and finished on the 4th of January 2019, adding 560 hours of practical training.

The veterinary team was composed of three veterinarians, Doctor Hugo Fernández, the head veterinarian and co-supervisor of the trainee, Doctor Vanessa Almagro, and Doctor Mariángeles Huzman, and by two veterinary assistants, Aurora Pazos and Begoña Teruel.

During the traineeship, the trainee integrated the veterinary team and participated in the daily management of the animals housed at Barcelona Zoo. This task included partaking in the morning clinical and welfare rounds with the zookeepers throughout the zoo.

Specifically, these are some of the medical procedures mostly performed during the trainee period:

- Handling and restraining mammals, birds, and reptiles.
- Performing and interpreting diagnostic imaging procedures, with or without animal handling, of several body parts.
- Performing necropsies.
- Discussing clinical cases, diagnoses, therapies, and medical decisions.
- Managing and administrating distinct drugs.
- Inducing and monitoring, fixed or volatile anesthesia, inside the surgery room or in a zoo enclosure.
- Performing medical training sessions.
- Performing coprology diagnoses.
- Helping in different surgeries and complex procedures such as monitoring ovariohysterectomy, abdominal ultrasound of a female Komodo Dragon, sedation and nodulectomy of a Cayman, sedation and surgery of a Leopard.

In order to assess the physical well-being of wild animals in captive settings, carry out ongoing treatments, or safely perform diagnostic techniques, there is a need to resort to sedation and immobilization, often resorting to tele-anesthesia. At Barcelona Zoo, the trainee was responsible for monitoring during anesthesia, including heart and respiratory rate, oxygen saturation, and body temperature. He was also in charge of data recording of such procedures: timestamps, drugs administration, animal behavior reaction throughout the operation, as well as checking all the material necessary for the procedures.

The trainee also participated in a Journal Club, where he presented to the entire veterinary team and discussed two major topics: “Stereotypic behavior in African Elephants”, and “Vaccination in birds against West Nile Virus”.

1.2. Clinic for Birds, Reptiles, Amphibians and Fish, Justus-Liebig University

A second traineeship was performed by the author lasting 300 hours. From 1st of April 2019 until 31st of May 2019, the trainee worked at an exotic animal clinic, which belongs to the Justus-Liebig University, in Giessen, Germany. This facility also functions as a wildlife rehabilitation center, mostly taking in injured wild birds with the intention of returning them to their natural habitat.

Under the supervision of Doctor Dominik Fischer, the clinical director, the following routine activities were carried out: handling and performing clinical examinations in many different species of birds and reptiles; assisting in the execution and the interpretation of diagnostic imaging procedures, such as radiography, ultrasonography, and endoscopy; discussing clinical cases, from diagnostic to therapy; monitoring anesthesia of surgical procedures; performing necropsies of wild or pet animals.

Additionally, we had the opportunity to visit the Nuremberg Zoo with the objective of collecting semen by electroejaculation from a male Harpy eagle (*Harpia harpyja*) specimen, the largest and most powerful raptor found throughout its range.

Finally, the author contributed to the European Association of Avian Veterinarians’ (EAAV) newsletter, issue of July 2019, with a revision article on the distribution of West Nile Virus in Europe, entitled “Update on West Nile Virus in European Countries”.

2. INTRODUCTION

The *Loxodonta africana* is classified as an endangered species since 2021. Poaching for ivory is considered the main reason for the decreasing number of individuals in the wild. African elephants housed in captivity act as ambassadors for their wild counterparts, enabling the research on factors that may impact health, longevity, or reproduction, data that may be extrapolated to wild populations.

The welfare of captive animals in zoo settings is a topic being increasingly discussed and studied. The well-being of these animals kept in human care is carefully monitored by the public, who demands better housing and management conditions.

A growing number of zoological parks are searching for a welfare assessment tool to provide continuous monitoring of individual well-being. However, most welfare studies in captive contexts are costly, time-consuming, and challenging. This is in part due to the low number of individuals of the same species, a wide variety of husbandry and housing conditions between institutions, and little opportunity to perform experimental rather than observational studies. Hence, there is a need for a rapid, affordable, non-invasive, and straightforward welfare assessment method that can easily be integrated into zoo management practices.

Barcelona Zoo is home to a group of three adult female African elephants. At the time of this project, these elephants were showing possible signs of stress, namely stereotypic behavior and social disputes. Thus, there was a need to create a practical management protocol to frequently monitor the welfare of the elephants. For that purpose, a combination of behavioral and physiological indicators of welfare started to be measured on the zoo's daily management routine.

3. LITERATURE REVIEW

3.1. The African Elephant (*Loxodonta africana)*

Elephants are the only survivors of the order Proboscidea, belonging to a single family named Elephantidae, and classified into two different genera – *Loxodonta* and *Elephas*. The general opinion of taxonomists is that three species should be recognized: the African savanna, or bush, elephant (*Loxodonta africana*), the African forest elephant (*Loxodonta cyclotis*), and the Asian elephant (*Elephas maximus*) (Fowler and Mikota 2006). However, there has been much discussion as to whether or not the savanna and the forest African elephant should be considered one single species. The lack of exact geographical distributions for these two and the existence of areas with hybrid populations contribute to this controversial debate (Mondol et al. 2015). Nonetheless, very few African forest elephant individuals are held in captivity (Clubb and Mason 2002), and therefore, for the purpose of this thesis, the term "African elephant" will be used to refer to the savanna species.

In agreement with Kingdon (1979), the African elephant is the largest terrestrial animal in the world. Female elephants can range from 2400 to 3500 kg in mass and stand 2.4 to 3.4 m at shoulder height. Male elephants are even larger, weighing from 4000 to 6300 kg and standing 3 to 4 m tall.

Figure 1. African elephant (*Loxodonta africana*) at Barcelona Zoo (original).

3.1.1. Ecology and social structure

African elephants (*Loxodonta africana*) are generalist herbivores, mainly feeding on grasses, leaves, twigs, bark, herbs, roots, flowers, and fruit. The digestive system of this species most closely resembles the one of horses. As a non-ruminant, due to the low nutritional quality of their diet and low digestive efficiency, wild elephants spend a large portion of their day, between 60 and 80%, searching for food to fulfill their nutritional needs (Owen-Smith 1988; Clubb and Mason 2002). Similarly, captive elephants allocate a significant proportion of their time budget foraging (Horback et al. 2014).

Burt (1943, p. 351) defined home range as “the area traversed by the individual in its normal activities of food gathering, mating and caring for young”. The average home range size for an African elephant herd is about 1975.7 km^2 (range 100 – 5527.0 km^2) (Clubb and Mason 2002). This area is largely dependent on the availability of food and water. Since the abundance of these resources varies seasonally, the extent of elephant movements also changes throughout the year. During the rainy season, home ranges tend to be smaller because of food and water being readily available. Yet, during the dry season elephants have to move large distances to find food and water, which become scarcer due to the lack of rain (Osborn 2004). It has even been reported by Nowak (1995) annual migrations covering several hundred kilometers in search of food and water. In zoo settings, nutritional needs are ensured through good husbandry practices, and so the functional need for walking is reduced (Holdgate et al. 2016).

As mentioned above, rainfall strongly influences range size, but perhaps, today, human pressure plays an even bigger role in influencing the spatial distribution of African elephants in the wild. Osborn (2004) showed that home range size is more closely related to the amount of land in which elephants can roam unperturbed, than to rainfall patterns. A reason for this is the difference in land use for agriculture between dry and wet areas. In the latter, agriculture tends to be more intensive and so the elephant home range is more limited when compared to dry areas.

Concerning social structure, the *L. africana* is a very gregarious species, said to have “one of the most advanced of all mammalian social systems” (Dublin 1983, p. 291). Social bonds are particularly strong between females, living in close-knit family groups of 8-12 related individuals (Schulte 2000; Charif et al. 2005). A typical family consists of an old, experienced female (the matriarch), being responsible for leading the herd, her dependent offspring, and adult females with their respective calves, including pre-pubescent males. However, these males abandon their natal group once they reach adolescence (10 to 15 years of age) (Clubb and Mason 2002). Wittemyer et al. (2005) identified four distinct tiers of social interaction in African elephants: mother-calf units, families, bond groups, and clans. The most basic tier is the mother-calf unit (tier 1), followed by family units (tier 2). Bond groups (tier 3) occur when different family units, typically up to 5, interact with each other. Finally, bond groups can sometimes aggregate and establish a clan with as many as hundreds of individuals (tier 4) (Clubb and Mason 2002). Furthermore, Wittemyer et al. (2005) also described the fission-fusion nature of elephant society. It appeared that the third and fourth tier were season dependent, in response to ecological changes like resource scarcity, while family units were stable over time. Nonetheless, fusion of unrelated individuals into a social unit has been observed in the wild, proving that bonding is not exclusively based on kinship (Moss 1988).

The human-created social structure in captivity for African elephants is an attempt to simulate wild groups. Females are housed in single-sex groups while males are set on a separate facility, most of the time alone (Schulte 2000). Nevertheless, Schulte (2000) also emphasized the differences between the adult captive population and its wild counterpart: groups tend to be smaller in captive settings, the number of calves per group is generally very low or null, group members are generally unrelated, males are removed at a much earlier age, and there are some mixed-species facilities with African and Asian elephants housed together. These variations in group size and composition can have strong implications on elephant welfare since species-specific social complexity is of paramount importance for many species in captivity (Carlstead and Shepherdson 2000).

3.1.2. Conservation

Once assumed to be widely distributed across sub-Saharan Africa (Thouless et al. 2016), the range of African elephants (*L. africana*) is now patchily distributed across 24 countries, according to Gobush et al. (2021) (Figure 2). Although large range continuous tracts persist in Central and Southern Africa, elephant distribution is becoming increasingly fragmented across the continent, resulting in smaller populations particularly in West Africa (CITES Secretariat et al. 2013; Thouless et al. 2016).

According to the International Union for Conservation Nature (IUCN), the *L. africana* is listed as an “Endangered” species since 2021 (Gobush et al. 2021), preceded by “Vulnerable” in 2004, and “Endangered” back in 1996 (Blanc 2008).

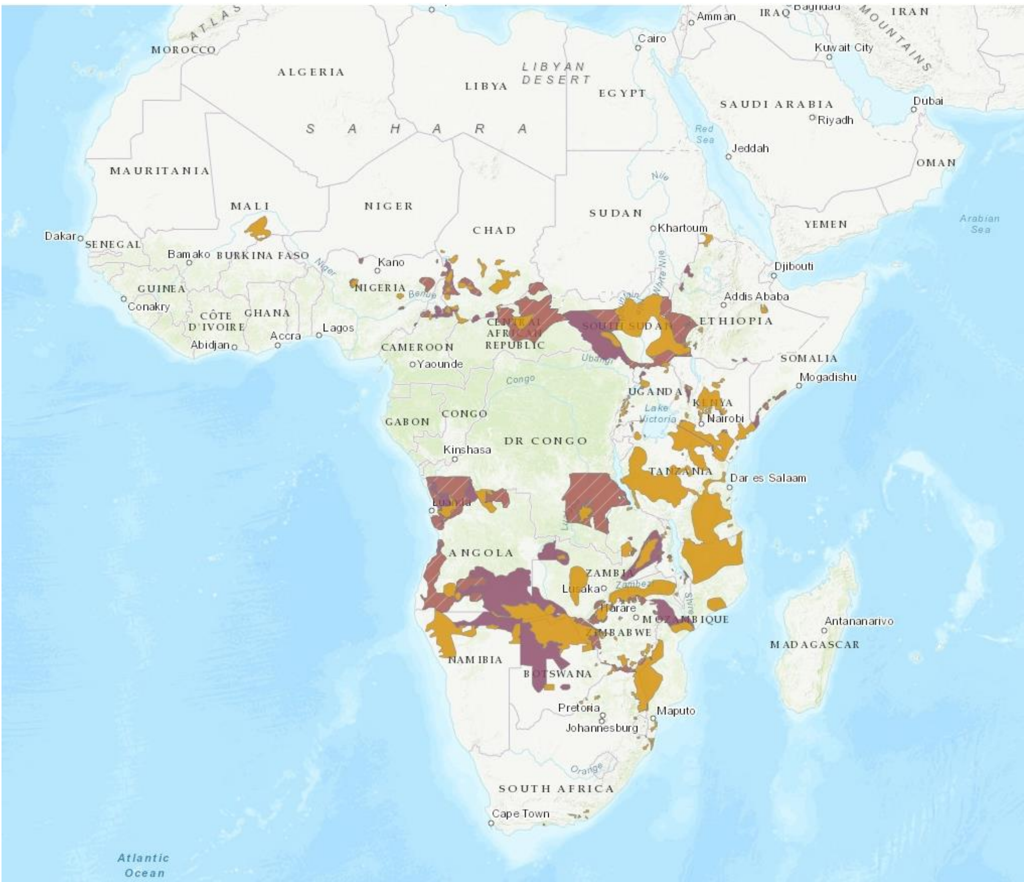


Figure 2. African elephant (*L. africana*) geographic range (source: Gobush et al. 2021).

From the 24 range states, all agreed to be bound by the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), except for South Sudan. Elephant populations of Botswana, Namibia, South Africa, and Zimbabwe are in Appendix II of CITES, meaning international trade in these specimens from these four countries may be authorized under strict regulation. Populations from the other range states are included in Appendix I of CITES, and so international trade for commercial purposes is prohibited (CITES 2017).

As mentioned in the latest African Elephant Status Report (Thouless et al. 2016), there is an estimated number of 415,428 ± 20,111 elephants in areas surveyed, less 118,000 individuals in comparison with 2007. This is the first continental decline in elephant populations reported in 25 years and is believed to be driven by a combination of three major factors: habitat loss, increasing human-elephant conflict, and, in particular, poaching for ivory. Despite the recent growing awareness of the conservation crisis, there has been a dramatic rise in illegal hunting since 2006, only comparable with the surges of 70s and 80s, more than doubling the weight of illegal ivory traded globally (CITES Secretariat et al. 2013; UNEP et al. 2013; Thouless et al. 2016).

The current hunting rate of elephants exceeds the intrinsic growth capacity of the species, leading to a conservation crisis of global significance, with an imminent threat to their existence (Wittemyer et al. 2014). Consequently, every single population of elephants, either *in-situ* or *ex-situ*, can play a vital role in the survival of the species (Cameron and Ryan 2016). Furthermore, through the study of zoo elephant collections, valuable research conclusions can be drawn, for instance factors that impact health, longevity, and reproduction, that may be extrapolated to wild populations (Cameron and Ryan 2016).

3.2. Welfare

For several decades now, animal welfare has been a highly debated topic concerning farm animals. However, animal welfare focusing on exotic animals such as those kept in zoos and aquariums collections is a fairly recent development (Kagan et al. 2015). Before discussing this subject, it is imperative to clarify the meaning of some concepts commonly used in this field of study. The most relevant terms used in this thesis are welfare, stress, and stressor. As of today, there is still a lack of consensus on the definition of animal welfare. Welfare is defined by Williams et al. (2018) as a "concept which encompasses both mental and physical health, engagement with the physical or social environment and the opportunity to exhibit control or choice". The second term, stress, is described as a biological response elicited when an individual experiences a threat to its homeostasis (Moberg 2000), and the environmental stimuli that lead to this threat are known as "stressors" (Möstl and Palme 2002).

In 1979, the Farm Animal Welfare Council (FAWC) announced recommendations to improve the welfare of all livestock animals, which became known as the "Five Freedoms": freedom from hunger and thirst; freedom from discomfort; freedom from pain, injury, and disease; freedom from fear and distress; freedom to express normal behavior while providing proper facilities and social companions (FAWC 1979). These principles are also valid for zoo animals, even though welfare in zoos is becoming a new area of expertise since modern ones are expected to provide long, high-quality life to each individual (Meehan et al. 2016).

In recent years, there has been an increasing public interest in the care and management of captive animals. Alongside this growth of public interest, questions like whether or not zoos can satisfy animals' social and environmental needs, and thus provide good welfare standards, rose as well. This is particularly evident for a charismatic species like the African elephant, which easily draws the attention of animal protection organizations. Moreover, due to their social nature and large home ranges in the wild, elephants represent a management challenge for zoo institutions. Hence, it is understandable why zoo elephant welfare is a timely and relevant subject (Carlstead et al. 2013; Meehan et al. 2016).

A growing number of zoological parks are demanding a welfare assessment tool able to provide continuous monitoring of individual well-being. However, most welfare studies in captive contexts are costly, time-consuming, and challenging. This is in part due to the low number of individuals of the same species, a wide variety of husbandry and housing conditions among institutions, and little opportunity to perform experimental rather than observational studies. Hence, routine welfare assessment should be designed to be fast, affordable, non-invasive, and straightforward, in order to become part of daily management and allow each institution to establish a customized welfare assessment system (Whitham and Wielebnowski 2009; Williams et al. 2018)

For instance, in the United Kingdom (UK), zoos must show evidence of elephant welfare improvement to continue to house elephants, underlining the need for an objective system for tracking welfare progress (Zoos Forum Elephant Working Group 2010). Furthermore, Hill and Broom (2009) proposed that an animal's previous experiences and background may impact its ability to cope with challenges. African elephants are a species with a lifespan up to 70 years in the wild (Wiese and Willis 2004). In captivity they can have a wide range of housing and care practices (e.g. zoological institutions, circuses, sanctuary) and origins (wild or captive-born) (Williams et al. 2018).

To effectively measure welfare, the present alternatives are animal-based indicators, and resource-based or environmental parameters (Whitham and Wielebnowski 2009). Williams et al. (2018) assessed the reliability and validity of animal-based indicators applied to captive elephants published in peer-reviewed literature. The animal-based indicators were first sorted into three categories: behavioral, physiological, and physical. Statistical evidence of improved welfare state was associated with reduced cortisol levels and reduced stereotypies. Additionally, increased lying rest time and exhibition of positive social interactions were strongly associated with reduced cortisol levels and reduced stereotypies, although not fully validated. It is important to highlight that most of these indicators express a cumulative welfare state, rather than a current state. Therefore, a variety of behavioral, physical, or physiological indicators should be employed to reliably assess the welfare of captive elephants (Hill and Broom 2009).

Another possible way to measure welfare is by comparing baseline activity budgets of captive elephants with wild elephant time budgets, or in new social or environmental conditions, as suggested by Veasey (2006).

3.3. Behavioral measures

3.3.1. Stereotypies

Several behavioral welfare indicators can be used to assess the zoo elephants' welfare (Williams et al. 2018). Among these behavioral welfare indicators, stereotypies are the most frequently used (Mason and Veasey 2010). Stereotypies are "repetitive, invariant behavior patterns with no obvious goal or function", as defined by Mason (1991). They are normally associated with normal motor patterns, such as those found in routine activities like foraging, grooming, or eating. At first, there may be some variation in the behaviors, but over time they become less diverse. Stereotypic behaviors may differ substantially among species and individuals within a species (Tynes 2010).

In elephants, these behaviors typically consist of rhythmic head movements, known as head bobbing, body swaying, or repetitive pacing, and often emerge in situations of frustration, unavoidable stress or fear, restraint, and lack of stimulation (Rees 2009). It is believed that stereotypies are a way of coping with stress and, according to some researchers, there is a link between stereotypic behaviors and animals unable to express their full range of species-typical behaviors at some point in their lives (Mason 1991; Swaisgood and Shepherdson 2005).

3.3.1.1. Causes of Stereotypic behavior

Captive animals living in confined environments usually develop stereotypies (Kelling 2008). Gruber et al. (2000) demonstrated that stereotypic rates increased when the elephants were chained or picketed *versus* penned. However, space is not always the critical variable. For instance, Forsyth et al. (2007) showed that spatial complexity, a variable measuring the level of enrichment, strongly influenced stereotypic levels in female mice housed in cages. Hence, both the quantity and quality of the environment should be considered when discussing ways to improve the welfare of a captive species.

Besides spatial considerations, the social experiences of captive elephants are thought to influence the manifestation of stereotypic behavior. Due to the highly social nature of elephants and their ability to form strong bonds with conspecifics and with humans (e.g. caretakers), there is an interesting relationship between stereotypy performance and sociality (Greco et al. 2016).

However, the common denominator among animals exhibiting stereotypic behavior is the presence of frustration. Environments that are insufficient in terms of variability and complexity are prone to induce a state of frustration in housed animals because they cannot engage in highly motivated behaviors, leading to prolonged negative subjective states, which may alter processing in the brain's cortical-basal ganglia system, ultimately promoting stereotypic behavior (Rushen and Mason 2006).

Motivated behaviors are characterized by an appetitive phase, the first phase of a behavioral series, followed by a consummatory phase. In some cases, motivation is regulated by negative feedback, for instance, when a leopard kills a prey (consummation is successful), the motivation to perform the appetitive behavior is reduced, and the leopard stops hunting. The term "appetitive" does not exclusively applies to hunt, and may refer to many other behaviors, such as foraging, exploring, or searching for a mate. What motivational theories propose is that, often in captive settings, the appetitive phase of a behavior does not culminate in appropriate consummatory behavior, or the consummatory behavior does not follow a normal period of appetitive behavior. The failure of the negative feedback loops frequently leaves animals in a high state of motivation, resulting in frustration-related stress (Rushen and Mason 2006; Würbel 2006).

A range of development and genetic factors are believed to predispose individuals to develop stereotypic behaviors. These factors are not necessarily the same as those environmental factors that elicit them (Tynes 2010). The lack of environmental complexity can be damaging, especially to young, developing animals. Multiple studies have shown that these animals have fewer neurons in the brain, decreased dendritic branching and spine density, reduced synaptic connectivity, and a higher incidence of stereotypies than those kept in enriched environments (Cabib 2006; Lewis et al. 2006).

Management practices may also affect stereotypic behavior. Multiple studies have shown that environmental enrichment, unpredicting feeding schedules, and positive reinforcement-based training, contribute to lower stereotypic behavior in many non-elephant species (Greco et al. 2016).

Some life history events like experiencing inter-zoo transfers or the death of a herd mate can disrupt social relationships, which may promote stereotypic behavior (Greco et al. 2016). Age is another factor to consider, with older elephants presenting higher rates of stereotypic behavior than their younger conspecifics (Haspeslagh et al. 2013). Moreover, there seems to be a difference between captive-born and wild-captured animals. Based on several studies of primates, rodents, and carnivores, captive-born animals are more susceptible to developing stereotypic behavior than their wild-captured animals (Mason 2006).

3.3.1.2. Stereotypies and Welfare

The relationship between well-being and the showing of stereotypies is complex as these may persist after the removal of the stressor that triggered its development, and thus may represent a previous welfare state rather than the current (Mason and Latham 2004). Moreover, whilst stereotypies are believed to be elicited by suboptimal environments, they have been associated with good or neutral welfare states almost as many times as with poor welfare ones (Williams et al. 2018). Therefore, while the development of a stereotypic behavior may be indicative of a suboptimal environment, the performance of established stereotypies does not necessarily represent compromised welfare.

It has been suggested that if a stereotypy takes more than 10% of an animal's activity budget or causes bodily injury, it should be considered detrimental to welfare. However, there are difficulties in creating an arbitrary cutoff to be used to assess welfare (Broom 1983). Nonetheless, changes in the frequency or intensity of a stereotypic behavior may be useful when paired with other measures and if the causes that triggered the expression of the stereotypy are known. Any increase in the level of expression of stereotypies may indicate a reduction in welfare state. By contrast, a decrease in stereotypies not caused by direct prevention may be indicative of improved welfare (Mason and Latham 2004). In adult animals, it may not even be possible to eliminate stereotypic behaviors, only decrease their dependence on such behaviors (Kiley-Worthington 1990). In the review made by Williams et al. (2018), stereotypies are referred to as "an important and well-supported indicator of welfare" when used alongside other measures and in cases where it is possible to monitor its variation over time.

Finally, another reason to address stereotypic behavior when discussing welfare in zoo animals is the public perception of this behavior. As it is seen as abnormal behavior, ways to reduce its frequency may not only improve the elephants' welfare but also the public perception of elephants exhibits (Rees 2009).

3.3.2. Resting

Achieving adequate rest is crucial for animals' good health and welfare (Botreau et al. 2007). Research has suggested that night-time and rest behavior can be used as a reliable assessment of welfare (Abou-Ismail et al. 2007). Wild and captive elephants are known to be active during much of the night (Wyatt and Eltringham 1974; Wilson et al. 2006). Often captive elephants are not allowed to roam their outdoor enclosures at night and are limited to their smaller indoor enclosures (Yon et al. 2019). The major time of resting in elephants is in the early hours of the morning (00:00h-06:00h) (Schiffmann et al. 2018).

These same authors underlined the importance of sleep for the elephant, and the performance of this natural behavior should be even more essential in stressed, physically injured, or geriatric elephants. It is believed that rapid eye movement (REM) sleep, a state defined by a general loss of voluntary muscle tone, is only achieved when the animal does not have to actively support its body against gravity (Gonfalone and Jha 2015). Therefore, presuming elephants need a certain but unknown amount of REM sleep, they might have to lie down or lean to relax the musculature. Instead, an elephant in a free-standing position might only achieve slow wave sleep, since there is a continuous tension of anti-gravity musculature (Schiffmann et al. 2018). In horses, Williams et al. (2008) revealed the occurrence of REM sleep during lying rest, while a standing position was found to be incompatible with the state of REM sleep.

Three types of causes for insufficient lying rest were identified by Bertone (2006) in horses: pain-associated; environmental insecurity; and monotony-induced. Typically in stressed, ill, or geriatric elephants, there is a decrease in time spent in lying rest, which is compensated with an increase in time spent in standing rest (Laws et al. 2007; Holdgate, Meehan, Hogan, Miller, Rushen, et al. 2016). This shift in sleep behavior is usually seen as the result of the animal's anticipation that it may struggle to return to a standing position if it lies down (Schiffmann et al. 2018). Furthermore, it is well documented that elephants in captivity are prone to degenerative joint disorders in old age and, consequently, often avoid lying rest (Miller et al. 2016), while younger, healthy, and comfortable-feeling elephants prefer recumbent rest (Laws et al. 2007; Holdgate, Meehan, Hogan, Miller, Rushen, et al. 2016). Hence, there seems to exist a close relationship between lying rest and age in elephants (Schiffmann et al. 2018).

Asher et al. (2015) considered lying rest an indicator of welfare since it represents the optimal opportunity for elephants to enter REM sleep. An optimal level of lying rest for elephants remains to be identified. Nonetheless, short periods spent asleep may be indicative of poor welfare in some species, as well as excessive sleep may suggest underlying illness or even boredom. Additionally, in the review done by Williams et al. (2018), sleep and rest behavior were associated with other welfare indicators, despite not being a validated indicator. A reduction in the frequency of sleep was correlated with increased stereotypies and associated with stressful events, such as travel (Laws et al. 2007; Williams et al. 2018). Furthermore, evidence suggests that increased lying rest, combined with other validated measures, could be used as a behavioral measure of welfare in zoo-housed elephants (Williams et al. 2018).

3.4. Physiological measures

3.4.1. Cortisol

Glucocorticoids (GCs) are steroids produced by the adrenal glands and are generally associated with stress (Ralph and Tilbrook 2016). The environmental stimuli that threaten homeostasis are called “stressors”, while the corresponding defense mechanisms set to re-establish homeostasis are known as “stress responses” (Möstl and Palme 2002). These responses include behavioral changes, adjustments in the immune system, and activation of the autonomous nervous system (resulting in the release of catecholamines) and the neuroendocrine system. The latter often induce an increase in GC synthesis through the activation of the hypothalamic-pituitary-adrenal (HPA) axis, so it is common to measure these hormones when evaluating stress and well-being not only in livestock animals but also in wild animals (Sheriff et al. 2011; Palme 2012).

3.4.1.1. Anatomophysiology of the HPA axis

The hypothalamus, the anterior pituitary gland, and the cortex of the adrenal glands form the HPA axis. A wide range of different stimuli can activate it. This is supported by the myriad of signals transmitted to the paraventricular nuclei of the hypothalamus (PVN) from various organs and systems (brain stem, subfornical organ, limbic system, and other hypothalamic nuclei) which can carry both internal and external information (Manteuffel 2002). The PVN consists of specialized neurons with axons that reach the median eminence, being responsible for the production and release of corticotropin-releasing hormone (CRH), as well as arginine vasopressin, into the hypophyseal portal blood system (Ralph and Tilbrook 2016). In turn, this hypophysiotropic neuropeptide stimulates the corticotrope cells in the anterior pituitary to synthesize and cleave the precursor molecule pro-opiomelanocortin into adrenocorticotropic hormone (ACTH) (Delitala et al. 1994; De Kloet et al. 2005). Ultimately, once released to the bloodstream, ACTH acts on the fascicular zone of the adrenal cortex culminating in GC production. In all mammals except rodents, the main active GC is cortisol (Mormède et al. 2007; Ralph and Tilbrook 2016).

This pathway is controlled by the GCs themselves. The secreted cortisol interacts with glucocorticoid receptors in the pituitary, the PVN, and central nervous system, ceasing the initial steps of the HPA axis through a negative feedback loop, which leads to the regularization of the axis activity levels (Manteuffel 2002). Around 15-30 minutes after a stressor, plasma corticosteroid concentrations typically hit peak levels, and return to basal levels within 60-90 minutes (De Kloet et al. 2005). However, this timeline should not be seen as a rule since the severity of the stressor determines the level at which GCs are elevated. Under the influence of an acute stressor, the feedback mechanism promptly decreases the amount of GC to pre-stressor levels. On the other hand, when the stressor is chronic, the feedback signals exerted in the HPA axis are weak and the system takes longer to return to normality (Sheriff et al. 2011).

Regarding transportation, most circulating cortisol (around 90%) is bound to proteins, more specifically corticosteroid-binding protein and albumin (Gayrard et al. 1996). The corticosteroid-binding protein is a specialized glycoprotein that binds cortisol with high affinity and low capacity, whereas albumin binds with low affinity and high capacity (Breuner and Orchinik 2002). It is important to note that only the free or unbound fraction of GCs is able to cross cell membranes, and so bound cortisol is considered biologically inactive (Bright 1995).

3.4.1.2. Collection and storage

One of the most traditional ways of determining GC output is via collection and analysis of blood samples (Palme 2012). This method is becoming less popular due to its invasive nature, since the handling and venipuncture of the animal can be stressors themselves, ultimately leading to a biased elevation in plasma cortisol levels (Menargues et al. 2008). Alternatively, minimally invasive collection of samples such as saliva, urine, faeces, milk, hair/feathers, or eggs are increasingly being used in animals (Cook 2012; Palme 2012).

Non-invasive methods to assess adrenocortical activity must be validated for each species, including physiological and biological validation (Touma and Palme 2005).

Physiological validation is performed by chemically inducing physiological changes in circulating GC levels, the so-called ACTH challenge test. After the injection of ACTH, there is a significant increase in plasma GC concentrations, soon followed by a decrease. This pattern should be reflected in the concentration of salivary GCs after a certain delay time (Touma and Palme 2005). A second method may be used to physiologically validate the measurement of cortisol in saliva, the dexamethasone suppression test. Dexamethasone is an artificial steroid that acts like endogenous GCs, activating the negative-feedback mechanism of the HPA axis and ultimately reducing circulating GC levels (Sapolsky et al. 2000). Therefore, a decrease in salivary cortisol levels should occur after the injection of dexamethasone. Finally, a strong positive correlation between plasma and saliva GCs concentrations may also be indicative that the applied assay is valid (Touma and Palme 2005).

To the author's knowledge, there is not a single study validating the measurement of GCs in saliva with hormonal (ACTH or dexamethasone) challenges, in African elephants. These procedures normally require a large sample size and more conditions in terms of animal management, both of which may not be feasible or desirable, notably in wild or zoo-kept animals. Nevertheless, salivary cortisol concentrations reliably reflect circulating cortisol in the blood (Grand et al. 2012). Hernandez et al. (2014) identified a 10-minute time lag between peak cortisol concentrations in plasma and saliva, in cattle.

Biological validation is also important, serial samples are collected before and after a known stressful event such as capture, immobilization, transportation, anesthesia, novelty, social agonistic encounters, different housing conditions, or human disturbance (Touma and Palme 2005). In African elephants, Kelling (2008) showed a significant increase in salivary cortisol levels after using a novel training program, as well as a spike in cortisol levels when each elephant was placed in an isolation stall with its dominant conspecific.

Similar to other terrestrial mammals, cortisol production follows a circadian rhythm in African elephants, with plasma and salivary cortisol concentrations peaking during the morning and decreasing throughout the day (Menargues et al. 2012; Casares et al. 2016). Furthermore, the results obtained by Casares et al. (2016) suggest that there is also a seasonal variation in the concentrations of salivary cortisol. In Asian elephants (*Elephas maximus*), the maximum cortisol levels were registered in October and then decreased until reaching the minimum concentration in April (Menargues et al. 2012). Another advantage of using a matrix like saliva is that only the free hormone is able to pass to the saliva, which occurs within a few minutes after the initial stimuli, whereas blood assays measure total GC concentrations rather than only reflecting free GC concentrations (Sheriff et al. 2011; Casares et al. 2016).

Since the collection and storage of saliva samples are relatively easy, salivary cortisol may be analyzed frequently, which is of particular importance when monitoring acute stress (Queyras and Carosi 2004). Elephant saliva has been proven to be useful to study cortisol levels and also to detect rapid and brief increases in its concentrations (Mason and Veasey 2010).

After sampling, cortisol is very stable in saliva and samples can be stored at room temperature for up to 4 weeks without significant reduction of its levels (Kirschbaum and Hellhammer 1989). Garde and Hansen (2005), upon researching the long-term stability of salivary cortisol, showed that saliva samples may be stored at 5ºC for 3 months and at -20ºC for 1 year without deterioration of cortisol concentrations. Repeated thawing and freezing of saliva samples up to four times was also found to not influence cortisol measurements (Garde and Hansen 2005).

Two highly sensitive competitive binding immunoassays are commonly used to accurately measure GC levels: radioimmunoassay (RIA) and enzyme immunoassay (EIA) (Sheriff et al. 2011).

3.4.1.3. Cortisol and Stereotypic behavior

According to Dantzer and Mormède (1983), the two main explanations for the development of stereotypies are: i) compensatory function to deal with a lack of stimulation; ii) mechanism to cope with excess stimulation. Stereotypies should either increase or decrease arousal levels. Therefore, it is possible to test between these alternatives by examining the effects of performing stereotypies on the pituitary-adrenal activity. If stereotypies decrease arousal, pituitary-adrenal activity should decrease, but if they are stimulating and enhance arousal, pituitary-adrenal activity should increase, resulting in increased cortisol levels.

A study made by Kelling (2008) with African elephants investigated the link between swaying, a type of stereotypic behavior, and salivary cortisol concentrations. For this purpose, multiple saliva samples were collected after the observation of stereotypic behavior. They found a negative correlation between the two indicators, strongly suggesting that stereotypic behavior decreases arousal levels.

3.4.1.4. Cortisol and Welfare

Stress responses followed by GC production are essential for an individual to cope with their environment and challenging situations, and so these mechanisms should not be considered intrinsically bad. While short-term elevated GC concentrations contribute to a positive outcome in life-threatening situations, when the HPA is stimulated too much or for long periods (chronic activation) there can be deleterious effects to the organism, ultimately leading to biological functions impairment (Wingfield et al. 1998; Palme 2012).

When evaluating stress responses, one must be careful in the interpretation of data since the activation of the HPA axis is very context-dependent. Since either beneficial or detrimental events can trigger adrenal activity, there is a general agreement that a single measure (e.g. cortisol concentration) is insufficient, and instead, a series of different indicators (e.g. physiological and behavioral) should be considered (Rushen et al. 2011; Palme 2012). Besides, individual characteristics such as sex, age, physiological stage, and life history can influence the functioning of the HPA axis (Mormède et al. 2007).

4. MATERIAL AND METHODS

4.1. Study objectives

The main objective of this pilot study was to develop a relatively simple but useful management protocol to assess the welfare of three African elephants housed at the Barcelona Zoo. A secondary objective was to establish a baseline cortisol value for each individual. Additionally, behavioral data was also collected to determine rates of stereotypic behavior, current social relationships and dominance hierarchy, and how the elephants spend the majority of their time (i.e., activity budgets). Finally, we searched for a link between GCs levels and some behaviors, specifically stereotypies and sleep.

H0: Behavior does not correlate with cortisol levels; H1: one or more of these measures of behavior correlate with cortisol levels.

4.2. Material and methods

4.2.1. Type of study

Observational case study.

4.2.2. Study period

Three months, from October 2018 to December 2018.

4.2.3. Study population

The study population of the present study was three adult female African elephants (*Loxodonta africana*), residents of the Barcelona Zoo (Barcelona, Spain, 41º23'16.0'' N 2º11'28.0'' E).

All three animals were born in the wild.

The youngest, codename E1, was born in approximately 1985 (35 years old, at the time of this study). E1 was first captured and transferred to a circus, where she was housed until 2007. Then, she was moved to another zoological garden, Bioparc Valencia, where she stayed for 5 years. Finally, in 2012, E1 arrived at Barcelona Zoo.

Codename E2, thought to have been born in 1973 (47 years old) was initially housed at Terra Natura theme park, Benidorm. She has been living at Barcelona Zoo, since 2002.

Lastly, the eldest female, codename E3, was born in 1968 (52 years old). She was captured in 1993. Before arriving at Barcelona Zoo, in 2009, E3 was housed at Aqualeon Zoo, Tarragona.

Standing out in the clinical history of the elephants was that E2 suffered episodes of colic, often presenting a distended abdomen, and E3 sometimes showed signs of pain in one limb, namely lameness, caused by a sole abscess on the right forelimb.

The information regarding the three animals is summarized in Table 1.

Table 1. Identification of the African elephants housed at Barcelona Zoo at the time of this study.

Codename	Sex	Birthdate	Age	Origin	Observations
E1	F	1985	35	Wild born. Transferred from Bioparc Valencia.	Previously housed in a circus company.
E2	F	1973	47	Wild born. Transferred from Terra Natura.	Chronic colic episodes.
E3	F	1968	52	Wild born. Transferred from Aqualeon Zoo.	Sole abscess on right forelimb.

4.3. Elephant’s facilities

The elephants are housed in an exhibit that totals 2839 m^2. The enclosure is made of an outdoor area and an indoor facility.

They spend most of their day in the outdoor area, approximately from 0800h to 1730h. This area attempts to recreate the natural habitat of the elephant. It consists of two pools, a mud wallow, logs for scratching and tusking, and a crane system that allows the food to be above the ground, allowing the elephants to reach for the food, simulating feeding from a tree.

For the purpose of this study, this outdoor area was subdivided into three different paddocks (P1, P2, P3), as shown in Figure 3.

The indoor facility or barn (B) was where the animals rested during the night.

Zoo visitors can watch the elephants in their naturalistic outdoor area, as well as in their interior facility.

Figure 3. Aerial view of the African elephants' enclosure at Barcelona Zoo, 2018. Adapted from: http://www.icc.ca

P1- Paddock 1;

P2- Paddock 2;

P3- Paddock 3;

B- Barn.

4.4. Diet

Regarding nutrition, the main diet is based on hay and pellets provided twice a day, one in the morning (at around 0800h) and other in the afternoon (at around 1700h). Additionally, fruits and vegetables, such as apples, cabbages, and carrots are given to the elephants throughout the day.

4.5. Training

Each elephant is trained once a day, at around 1300h. The keepers used ingredients of their diet besides hay and browse for reinforcement. The training area is located on P2, kitted out with a metal fence acting as an interface between the animal and the keeper, providing a safer handling of the elephants (Figure 4). This management system employed at Barcelona Zoo is named "protected contact".

Figure 4. Blood sampling of an African elephant (*L. africana*) with protected contact interface at Barcelona Zoo, 2018 (original).

4.6. Behavioral data collection

The author observed each elephant between one to two hours per day, for 33 days, between November 20, 2018, and January 4, 2019.

The behavioral data were collected in two different time periods: 10:00h-11:00h, and 15:00h-16:00h.

In total, the elephants' behavior was observed for 16 hours in the morning and 32 hours in the afternoon, adding up to 48 hours of elephant behavior recorded. The initial objective was to do the same amount of observation time in the morning and in the afternoon, to compare possible differences in behavior between the two day times. However, since the majority of medical and surgical procedures at Barcelona Zoo are scheduled for the early hours of the day, the author could not get as many morning observations as in the afternoon. Additionally, a week before the beginning of the study, we observed the elephant group daily to make adjustments on the ethogram and to get the elephants used to our presence.

The behaviors were recorded into a recording sheet. Behaviors may be regarded as states or as events. States are characterized to have appreciable durations, while events are instantaneous (Altmann 1974). Therefore, two distinct behavior sampling methods were used in this study. For recording behavior states, such as forage and locomotion, instantaneous scan samplings (Altmann 1974) were performed every 5 minutes in 1-hour sessions. This quantifies the proportion of time each elephant spent in a specific activity, also known as time budget. Behavior events like physical contact between two individuals were recorded by continuous focal group sampling (Altmann 1974). Hence, the elephants were followed around in a way that allowed us to maintain eye contact with at least two individuals, and thus record every social interaction for the duration of the session.

Besides diurnal behaviors, the location within the enclosure was also recorded, making it possible to measure the time each elephant spent on each area of the environment.

The ethogram (Table 2) used was adapted from (Horback et al. 2014). It covered solitary, stereotypic, and social behaviors – both affiliative and agonistic.

Table 2. Elephant behavioral ethogram.

Category	Behavior	Definition
Behavior state	Forage	Collecting, consuming and/or searching with trunk close to the ground for food or water, while standing still or walking.
	Locomote	Walking or running (except while feeding or stereotyping).
	Rest	Standing or lying without engaging in another listed behavior.
	Self-Directed	Engaging in bathing, or sustained dusting and rubbing of body with the environment.
	Stereotypy	Repetitive behavior with no obvious purpose (head bobbing).
	Enrichment Use	Individual moves, pushes, tosses or picks up objects within its environment or keeper-provided items.
Behavior event	Trunk touch	Contact of the trunk with a conspecific in a nonaggressive manner.
	Strike	The aggressor hits another elephant with its head, trunk or limb.
	Push	One elephant contacts another and gradually forces/ pushes against the other, often causing it to move.
	Threat	Aggression between conspecifics that do not involve contact. Includes charge or mock charge and threat display with ears erect and held outward.

In order to observe the nocturnal behavior of the elephants, more specifically lying rest, installed video surveillance cameras at the barn were used. Video recordings of the previous night were analyzed daily by the keepers, to quantify time that each elephant spent lying rest.

4.7. Saliva sampling

Saliva sampling took place at the start of the training session, between 13:00h and 14:00h. The elephants were trained to open their mouths on cue. Saliva sampling was made as swiftly as possible, normally within 1 to 2 minutes, to prevent increased cortisol by activation of the HPA axis. In total, 144 saliva samples were obtained, 48 per elephant.

Saliva sampling was performed with sterile, disposable swabs Sugi ® eye spear pointed tip (Figure 5).

Figure 5. Saliva sampling of an African elephant (*L. africana*) at Barcelona Zoo, 2018 (original).

Afterward, the tip of the swab was placed inside an Eppendorf tube labeled with the elephant's name and date. The samples were temporarily stored in a domestic freezer at -18ºC and, at the end of the day, moved to another freezer and stored at -80ºC. Finally, the samples were shipped to the Department de Sanitat i d'Anatomia Animals, Facultat de Veterinària, Universitat Autònoma de Barcelona.

4.8. Salivary cortisol

For salivary cortisol analysis, a commercial enzyme immunoassay (EIA) kit (Cortisol ELISA KIT; Neogen® Corporation, Ayr, U.K.) was used. This is an ELISA (Enzyme-Linked ImmunoSorbent Assay) for the quantitative analysis of cortisol levels in biological fluid. This test kit operates based on direct competition between the enzyme conjugate and the cortisol in the sample for a limited number of binding sites on the antibody-coated plate.

The testing was carried out at the Laboratory of the Department de Sanitat i d’Anatomia Animals, Facultat de Veterinària, Universitat Autònoma de Barcelona, in accordance with the instructions provided by the manufacturer.

The sample or standard solution is first added to the microplate. Next, the diluted enzyme conjugate is added and the mixture is shaken and incubated at room temperature for one hour. During the incubation, competition for binding sites is taking place. The plate is then washed removing all the unbound material. The bound enzyme conjugate is detected by the addition of substrate which generates an optimal color after 30 minutes. Quantitative test results may be obtained by measuring and comparing the absorbance reading of the wells of the samples against the standards with a microplate reader at 650 nm. The extent of color development is inversely proportional to the amount of cortisol in the sample or standard. For example, the absence of cortisol in the sample will result in a bright blue color, whereas its presence will result in decreased or no color development.

This EIA kit presents cross-reactivity with prednisolone (47.4%), cortisone (15.7%), 11-deoxycortisol (15.0%), prednisone (7.83%), corticosterone (4.81%), 6β-hydroxycortisol (1.37%), 17-hydroxyprogesterone (1.36%), deoxycorticosterone (0.94%), progesterone (0.06%), and all other steroids (<0.06%).

As previously mentioned, there is an individual variation in stress responsivity. To reduce the influence of this factor, each elephant acted as its own control (Palme et al. 2000).

4.9. Statistical analysis

For descriptive statistics, we used Microsoft Excel 365® and R® (version 3.6.1) along with the extension Rcmdr (R commander). The statistically significant level was settled at a p-value of <0.05 in all tests performed. The normality of the distribution was assessed by carrying out a Shapiro–Wilk test.

Baseline cortisol concentrations were calculated by iteratively excluding all points greater than the mean plus 2.5 times the standard deviation (SD) until no values were higher than the threshold (mean + 2.5 SD) (Fanson et al. 2013). All samples that exceeded the aforementioned threshold were considered peaks of cortical activity.

Although only three elephants were enrolled in the study, since each one served as its own control, the power of the statistics is dependent upon the number of samples taken.

5. RESULTS

5.1. Salivary Cortisol

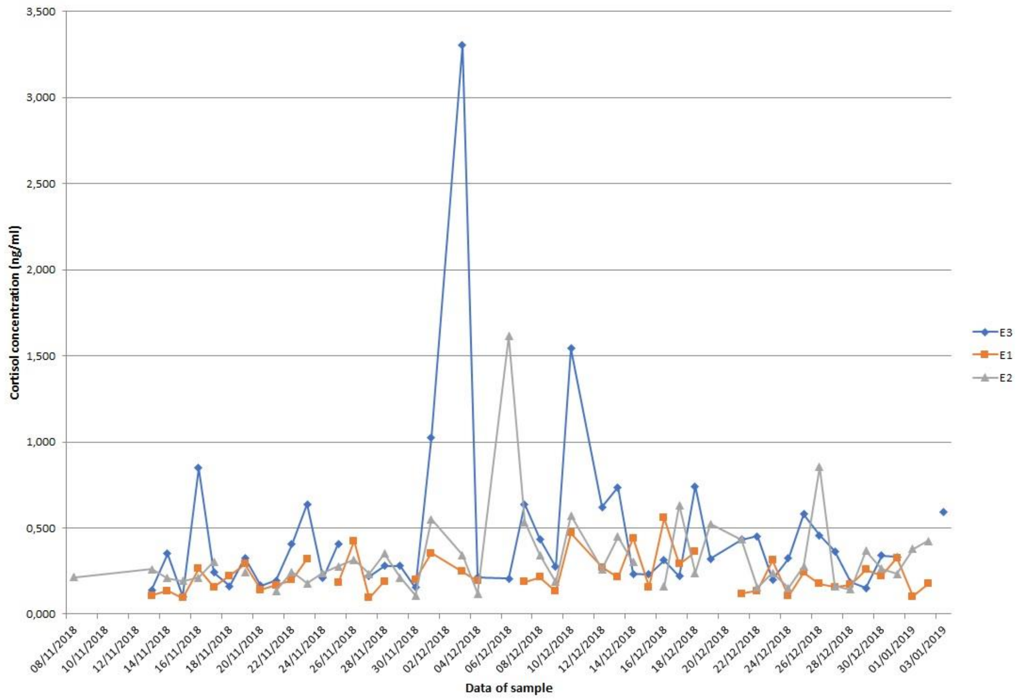
Average salivary cortisol levels was highest for E3 (mean ($\bar{x}$) = 0.458 ng/ml, standard deviation (SD) = 0.512). Intermediate levels were found for E2 ($\bar{x}$ = 0.330 ng/ml, SD = 0.249), while E1 had the lowest cortisol levels ($\bar{x}$ = 0.227 ng/ml, SD = 0.108) (Table 3). Additionally, E1 showed a minimum of 0.123 ng/ml and a maximum of 0.560 ng/ml. For E2, the minimum value was 0.158 ng/ml, and the maximum value was 1.613 ng/ml. Finally, E3 presented a minimum of 0.242 ng/ml and a maximum of 3.307 ng/ml.

Table 3. Means and standard deviations of cortisol (ng/ml) for each elephant (2018).

Elephant codename	Mean Cortisol (ng/ml)	Standard Deviation	n
E1	0.227	0.108	43
E2	0.330	0.249	45
E3	0.458	0.512	45

By looking at the daily salivary cortisol values of the three elephants, assembled in Graph 1, E1 was the more inconspicuous since she showed the least variation cortisol levels, only presenting a small elevation above 0.500 ng/ml. In contrast, E2 and E3 cortisol concentrations varied more. On the 6th of December 2018, E2 had a notable spike in cortisol levels. Even more remarkable was E3's cortisol levels on the 3rd of December 2018, exceeding the 3.000 ng/ml mark. This was the highest cortisol value throughout the whole study.

Graph 1. Daily salivary cortisol values (ng/ml) of the three African elephants.



In 2014, saliva samples of the African elephants in Barcelona Zoo had also been collected and subsequently analyzed to measure cortisol levels. The results are summarized in Table 4. E2 was the elephant with higher mean cortisol in 2014 ($\bar{x} = 0.411$ ng/ml, SD = 0.535).

Table 4. Means and standard deviations of cortisol (ng/ml) for each elephant (2014).

Elephant codename	Mean Cortisol (ng/ml)	Standard Deviation	n
E1	0.222	0.280	21
E2	0.411	0.535	26
E3	0.263	0.324	29

Table 5 gathers the results of baseline cortisol concentrations obtained for the three elephants according to the methodology described by Fanson et al. (2013). It also describes the cutoff values, and the proportion of peaks (calculated by dividing the number of cortisol spikes by the total number of samples). The baseline mean (±SD) salivary cortisol concentrations ranged from 0.202 ± 0.074 ng/ml to 0.338 ± 0.171 ng/ml. E3 was the elephant with the highest baseline cortisol (0.338) and baseline cutoff

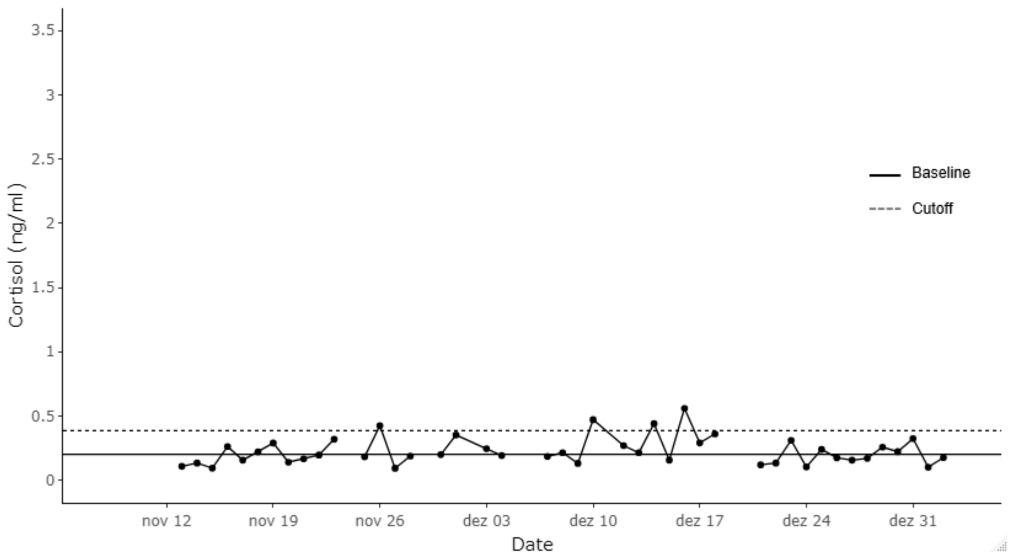
(0.766). Although E1 showed the highest proportion of peaks (9.3%), the amplitude of her cortisol spikes was much lower when compared with the other two elephants.

Table 5. Baseline cortisol and baseline cutoff values for each elephant (2018).

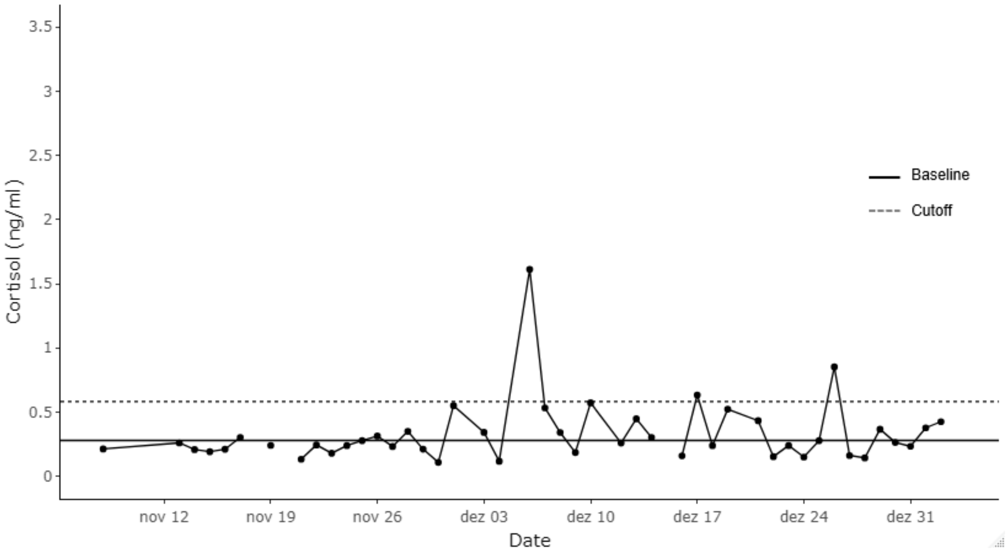
Elephant codename	Baseline Cortisol (ng/ml)	Baseline cutoff (ng/ml)	Proportion of peaks (%)
E1	0.202 ± 0.074	0.387	9.3
E2	0.280 ± 0.121	0.583	6.7
E3	0.338 ± 0.171	0.766	8.9

Graphs 2, 3, and 4 display the variation in cortisol levels throughout the study. Regarding E1, there are four cortisol spikes, represented by the dots above the cutoff: 0.425 ng/ml on November 26; 0.472 ng/ml on December 10; 0.441 ng/ml on December 14; and 0.560 ng/ml on December 16. E2 had three values over the cutoff: 1.613 ng/ml on December 6; 0.634 ng/ml on December 17; and 0.853 ng/ml on December 26. Finally, E3 showed four GC elevations: 0.849 ng/ml on November 16; 1.026 ng/ml on December 1; 3.307 ng/ml on December 3; and 1.543 ng/ml on December 10.

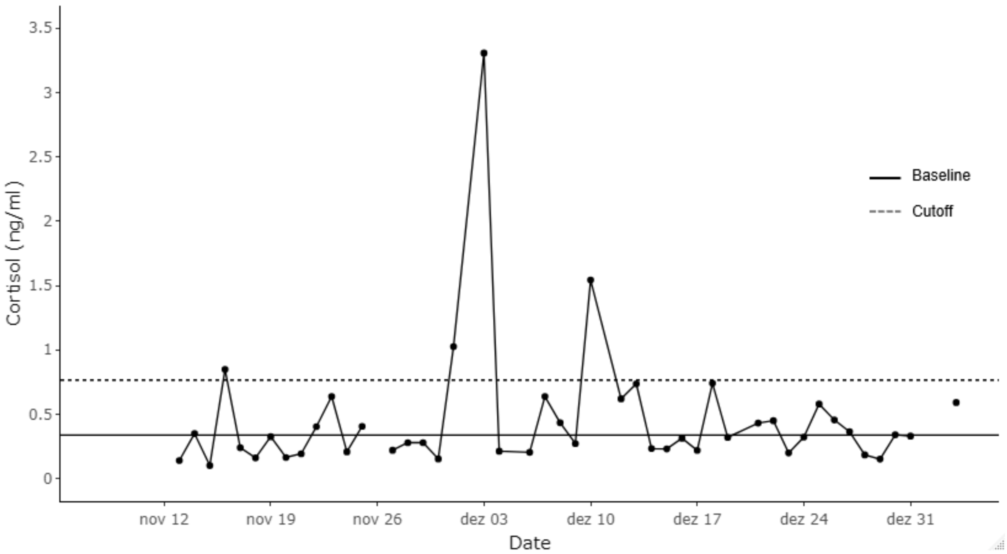
Graph 2. Longitudinal representation of salivary cortisol levels (ng/ml), E1 (2018).



Graph 3. Longitudinal representation of salivary cortisol levels (ng/ml), E2 (2018).

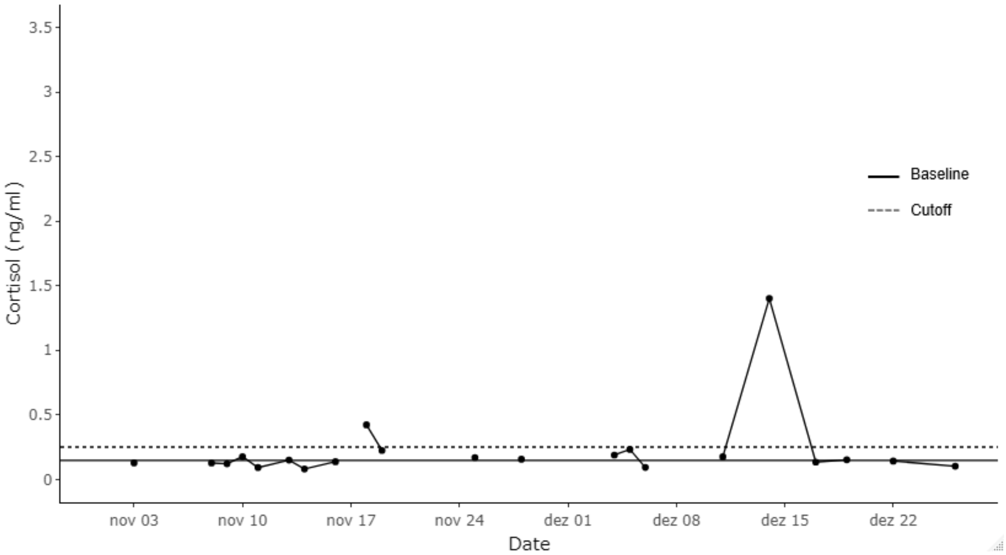


Graph 4. Longitudinal representation of salivary cortisol levels (ng/ml), E3 (2018).

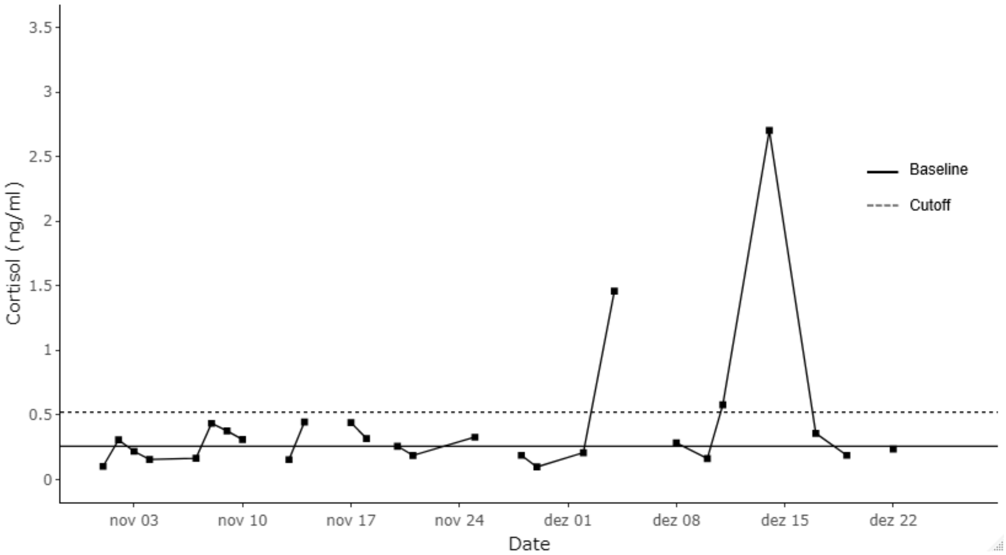


Baseline cortisol concentrations were also obtained from the saliva samples collected in 2014 (Graphs 5, 6 and 7).

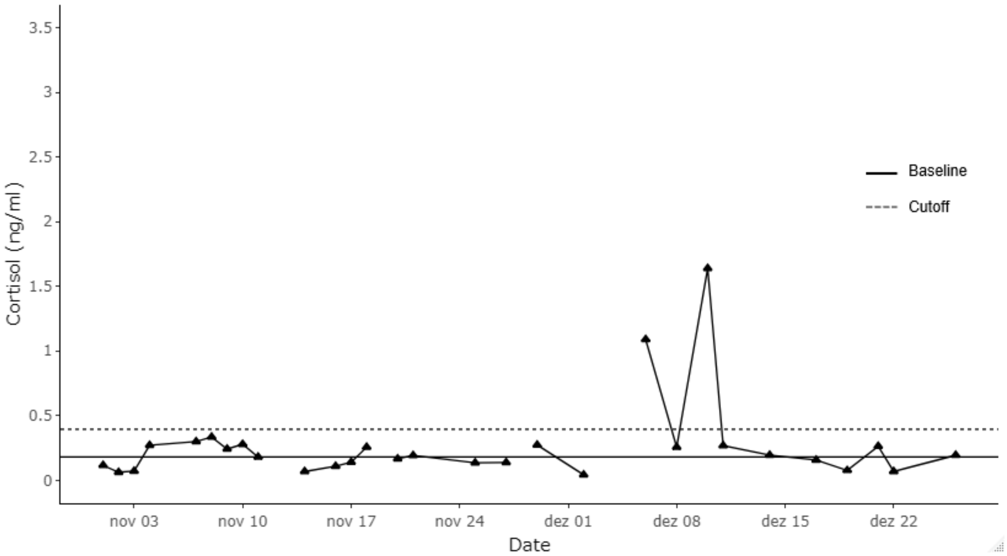
Graph 5. Longitudinal representation of salivary cortisol levels (ng/ml), E1 (2014).



Graph 6. Longitudinal representation of salivary cortisol levels (ng/ml), E2 (2014).



Graph 7. Longitudinal representation of salivary cortisol levels (ng/ml), E3 (2014).



In 2014, the baseline mean (±SD) salivary cortisol concentrations ranged from 0.149 ± 0.042 ng/ml to 0.259 ± 0.106 ng/ml. The elephant with highest baseline cortisol and baseline cutoff was E2 instead of E3. E2 was also the elephant with the highest proportion of peaks (11.5%) (Table 6).

Table 6. Baseline cortisol and baseline cutoff values for each elephant (2014).

Elephant codename	Baseline Cortisol (ng/ml)	Baseline cutoff (ng/ml)	Proportion of peaks (%)
E1	0.149 ± 0.042	0.253	9.5
E2	0.259 ± 0.106	0.523	11.5
E3	0.182 ± 0.086	0.396	6.9

5.2. Lying Rest

Mean duration of lying rest was highest for E1 ($\overline{x}$ = 2.80 h, σ = 1.27), followed by E3 ($\overline{x}$ = 2.58 h, σ = 1.50) (Table 7). From the trio of elephants, E2 showed the lowest mean time spent lying rest during nighttime ($\overline{x}$ = 2.14 h, σ = 1.13).

Table 7. Means and standard deviations of time spent in lying rest, for each elephant.

Elephant codename	Mean Sleep (h)	Standard Deviation	n
E1	2.802	1.271	48
E2	2.142	1.127	48
E3	2.580	1.502	48

5.3. Activity budget

The overall activity budget of the elephants is described in Table 8.

Table 8. Mean proportion of activity budget spent on selected behaviors for each elephant.

Elephant codename	Behavior	Mean (%)	Standard Deviation (%)
E1	Forage	81.08	18.90
	Locomotion	3.85	5.27
	Rest	6.25	12.56
	Grooming	1.28	4.00
	Enrichment	1.93	4.35
	Head bobbing	1.22	3.93
E2	Forage	77.39	18.67
	Locomotion	5.45	6.54
	Rest	12.67	16.30
	Grooming	0.96	2.57
	Enrichment	0.32	1.55
	Head bobbing	0.00	0.00
E3	Forage	67.14	23.99
	Locomotion	2.41	4.25
	Rest	7.38	9.79
	Grooming	1.44	3.78
	Enrichment	0.48	1.88
	Head bobbing	13.17	18.86

For all elephants, the largest proportion of scans was spent foraging. E1 led the foraging time (81.08%), followed by E2 (77.39%), while E3 had only 67.14% of the scans.

Regarding locomotion, E2 spent the most time walking (5.45%), followed by E1 (3.85%) and E3 (2.41%).

E2 was the only elephant that did not express any stereotypic behavior. E1 spent a relatively low time performing head bobbing (1.22%), but E3 spent a substantial amount of time (13.17%) displaying this abnormal behavior. Even more surprising, on December 1, 2018, E3 spent 51.9% of the observations head bobbing, corresponding to one of the days in which cortisol levels (1.026 ng/ml) were way above the cutoff.

5.4. Incidents of aggressive behavior

E2 struck E1 once in the morning. E3 also struck E1 once during a morning session. E1 did not hit another herd mate (Table 9).

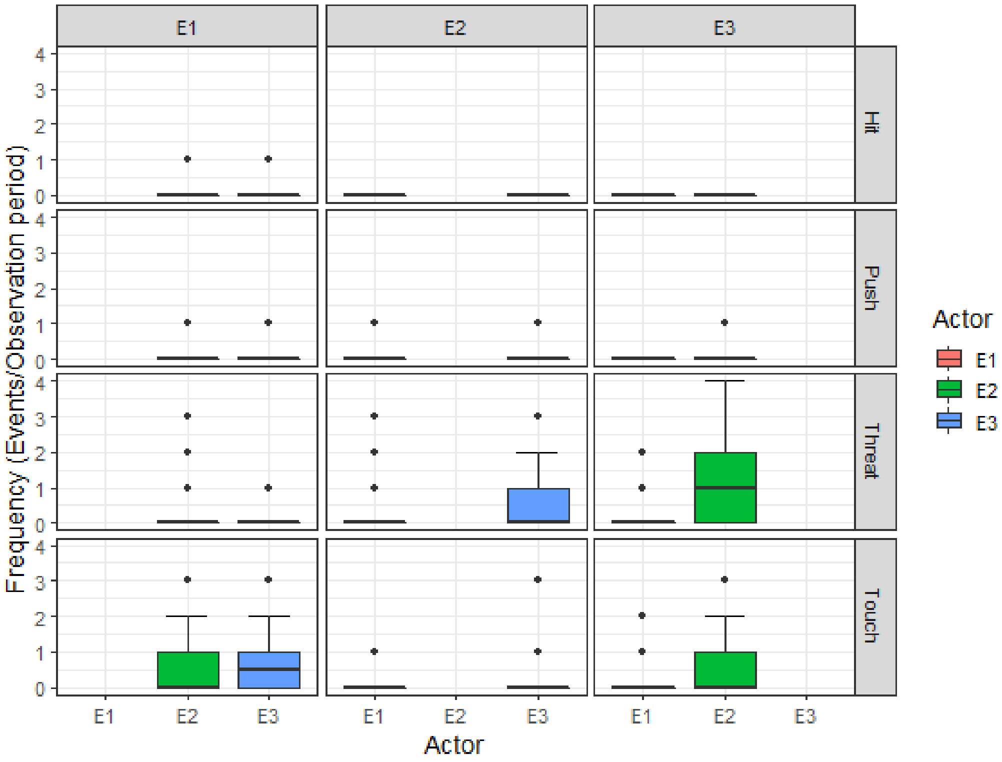
Table 9. Incidents of aggressive behavior recorded per elephant.

Actor \ Recipient	E1	E2	E3
E1		Strike = 0 Push = 2 Threat = 11	Strike = 0 Push = 0 Threat = 11
E2	Strike = 1 Push = 3 Threat = 14		Strike = 0 Push = 2 Threat = 48
E3	Strike = 1 Push = 2 Threat = 2	Strike = 0 Push = 1 Threat = 30	

However, E1 was observed to push E2 twice. E2 pushed E1 three times, once during the morning, and twice during the afternoon. Furthermore, E1 never pushed E3, but E1 was pushed by E3 twice. E2 pushed E3 twice, both in the afternoon, while E3 pushed E2 once. Threats were observed more frequently. E1 threatened both E2 and E3 11 times. E2 threatened E1 14 times, and the latter was also threatened by E3 twice. Finally, E2 threatened E3 14 times during morning sessions and 34 times during afternoon sessions (N = 48 times). E3 also threatened E2 9 times during the morning and 21 times during the afternoon (N = 30 times).

E2 threatened E3 with a median of 1 and a maximum value of 4 per observation period. Whereas the median of E3 threatening E2 was 0 and a maximum of 2 per observation period (Graph 8).

Graph 8. Frequency of social behaviors per session, per elephant.



5.5. Incidents of affiliative behavior

Different frequencies of affiliative behavior between elephants were also observed. E1 performed the lowest number of trunk touching of the trio (N = 14). E2 touched E1’s mouth 19 times and was the elephant that showed more affiliative behavior towards E3 (N = 22 times) (Table 10).

E3 had the highest number of trunk touching behavior towards E1 recorded (N = 35 times), but she only touched E2 10 times.

Table 10. Incidents of affiliative behavior (trunk touch) recorded per elephant.

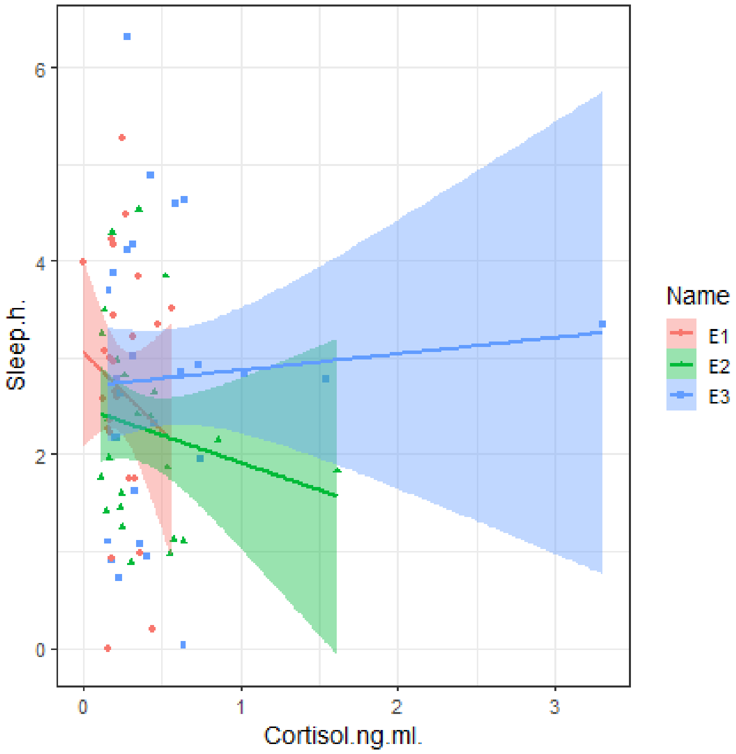
Recipient **Actor**	E1	E2	E3
E1		4	10
E2	19		22
E3	35	10	

5.6. Relationship between cortisol levels and specific behaviors

5.6.1. Relationship between cortisol levels and sleep hours

An objective of this study was to search for possible relationships between cortisol levels and specific behaviors, namely lying rest and head bobbing. For the former, we compared the levels of cortisol with the amount of lying rest each day, testing the hypothesis if higher levels of cortisol measured during daytime were associated with less amount spent on lying rest position during nighttime (Graph 9).

Graph 9. Relationship between cortisol (ng/ml) and sleep (hours) for each elephant.



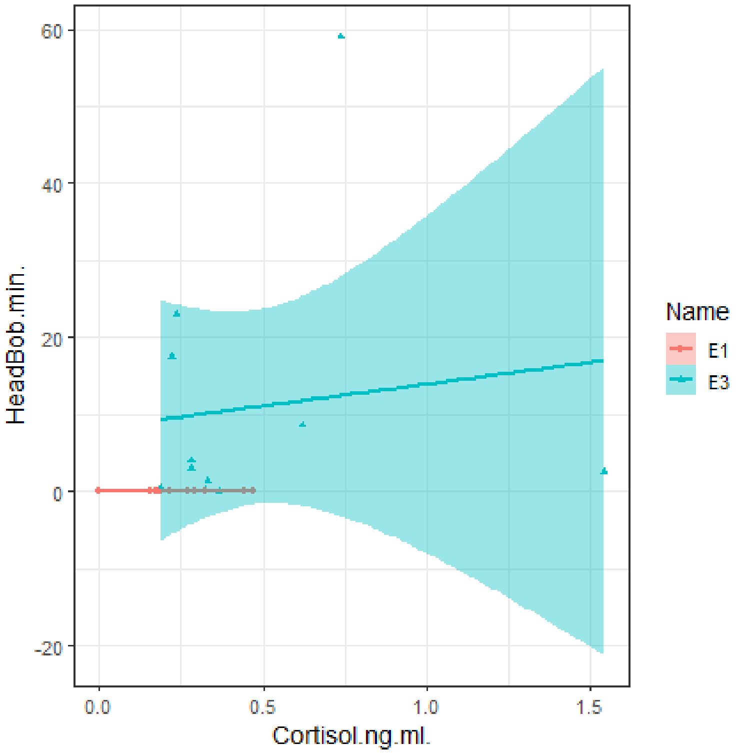
Results were not statistically significant (E1 p-value = 0.36; E2 p-value = 0.38; E3 p-value= 0.70). Thus, we did not identify any association between levels of cortisol and the amount of lying rest each day.

5.6.2. Relationship between cortisol levels and head bobbing

E2 was not included in this analysis since she did not perform any stereotypic behaviors during the study period.

We compared the levels of salivary cortisol with the amount of time spent head bobbing during morning observations (Graph 10).

Graph 10. Relationship between cortisol (ng/ml) and head bobbing (min) for each elephant.



For E3, there was a possible positive correlation between cortisol levels and head bobbing, however, it was not statistically significant (Pearson Test, p-value = 0.71). For E1, we did not find an association between the two variables. Hence, we cannot claim that there is a correlation between cortisol levels and head bobbing.

6. DISCUSSION

Overall, this pilot study provides helpful insights into the aspects of psychological well-being of captive elephants, including stereotypic behavior. Additionally, it discusses the pros and cons of using a combination of behavior ethogram with salivary cortisol measurements to evaluate elephant welfare. We will first discuss the results obtained from observing the elephants' behavior, followed by the analysis of the salivary cortisol. Finally, we will be making a general comment on the welfare of the African elephants at Barcelona Zoo.

6.1. Behavior

The behavior results will be discussed considering the following dimensions: activity budget distribution (including stereotypic behavior), social behaviors, and lying rest.

Activity budget distribution

Behavioral data provided an estimation of how these elephants spent time. In this part of the discussion, we focused on behaviors like foraging and stereotypies since they are the most relevant for our study.

E2 and E1 spent their time mostly eating, 81.08% and 77.39%, respectively, while E3's time spent eating was much lower (67.14%). Nevertheless, the three elephants showed similar feeding time budgets to those reported in wild ones, between 60% and 80% (Clubb and Mason 2002), and thus were considered normal.

E1 and E2 showed low rates of stereotypic behavior (1.22% and 0%, respectively), while E3 spent 13.17% of her time performing head bobbing. E3's higher rate of stereotypic behavior might be related to the lesser time spent foraging. A study by Rees (2009) showed that increased stereotypic behavior in captive Asian elephants was related to a decrease in feeding and the thwarting of appetitive behavior. Throughout the day, as the food supply in the outdoor enclosure decreases, the need to forage (appetitive behavior) increases. As this behavior is limited in captive settings due to the size of the enclosure, a state of frustration is created, promoting stereotypic behavior.

The negative correlation proposed by Rees (2009) between feeding and stereotypic behavior aligns with E3's behavior observations. He also suggested that the inability to exhibit feeding behavior may elicit stereotypic behavior, and that stereotypic behavior may be reduced by encouraging feeding activity.

Another factor that may influence E3's higher rate of stereotypic behavior is age. In elephants, multiple studies have shown that stereotypic behavior increases in frequency with age (Kurtr and Garai 2001; Haspeslagh et al. 2013).

E1 also engaged in head bobbing. Previous life experiences such as inter-zoo transfers or the fact she was previously housed in a circus could have contributed to this. In this type of environment, the available space is usually very limited, and the animals are penned or, even worst, chained, which are proven to elicit stereotypic behavior (Gruber et al. 2000).

Social behaviors

Dominance and direction of aggression appear to be shifting in the investigated elephant trio. E3, being the eldest female, was the dominant elephant, the matriarch of the group. However, during the period of observation, the keepers anecdotally reported a change in the hierarchy, which was later confirmed based on the observed agonistic interactions between the elephants. E2 started being dominant to E3, who was still dominant to E1. E2 and E1 also appeared to have a closer relationship than either had with E3. This difference was confirmed through observations of affiliative behavior. Overall, levels of aggression were high, with 116 incidents of one elephant threatening another in 48 hours of observation. Affiliative behaviors, namely trunk touching, occurred less frequently than aggression. Trunk touching was observed 100 times, mostly between E3 and E1, around 45 times (45%).

All these interactions observed among the elephants could be interpreted as the presence of an unstable hierarchy, given that when a hierarchy evolves from instability to a stable situation, aggressive encounters should progressively decrease. Although better known for their cooperative behavior and being closely related, female elephants in the wild also demonstrate aggressive interactions (Dublin 1983). These aggressive acts tend to be related to dominance or competition for resources and are thought to establish social roles in the artificial family groups housed in captivity (Douglas-Hamilton 1973). In fact, at Barcelona Zoo, we noticed that these agonistic behaviors often happened after the provision of food.

These changes in the hierarchy of the group might also help explain E3's higher rate of stereotypic behavior. Some studies found that socially isolated animals are more prone to engage in stereotypic behavior (Kurtr and Garai 2001).

Lying rest

Regarding time spent in lying rest, E2 was the elephant that presented the lowest mean amount of sleep, around 2.14 hours per day. On the other hand, E1 showed the highest average daily time in recumbent sleep (2.80 h). In African elephants, the mean time spent sleeping ranges between 3.10 to 6.90 h/day, although the times recorded in the wild showed lower results (3.17 to 3.36 h/day) (Gravett et al. 2017).

Schiffmann et al. (2018) revealed that time spent in lying rest usually decreases with age, and, in this study, the youngest elephant of the group (E1) was the one with the most time spent in recumbent rest position. In captivity, older elephants are known to be prone to degenerative joint disorders and, consequently, avoid lying down to sleep (Miller et al. 2016). Additionally, the social conflicts within the group may contribute to some reluctance to lie down in zoo elephants (Koyama et al. 2012).

Elephants that present a low amount of time of recumbent sleep often compensate with standing rest (Schiffmann et al. 2018). This was confirmed with E2, as the animal with the lowest mean amount spent in lying rest during the night, was also the elephant that rested more, in standing position, during the day (12.67%).

6.2. Salivary Cortisol

Cortisol physiology is complex because the HPA axis is highly sensitive to a variety of environmental, physiological, and emotional stimuli. Additionally, differences based on species, sex, age, early life experiences, social status, and diurnal, seasonal, and life history variations may all affect GC concentrations (Touma and Palme 2005).

The saliva cortisol values obtained in this study (2018) ranged from 0.123 to 3.307 ng/ml. These results are not very far from those found in 2014, which ranged from 0.045 to 2.702 ng/ml. Furthermore, the means found in the current study of 0.227, 0.330, and 0.458 ng/ml are lower than the salivary cortisol means reported by Kelling (2008), ranging from 0.30 to 0.54 ng/ml.

However, E3 showed an increase in baseline salivary cortisol levels along with a higher baseline cutoff amplitude and proportion of cortisol peaks, when comparing the results from 2018 with 2014. A possible reason for this increase was the occurrence of social disputes, particularly between E3 and E2. For instance, Casares et al. (2016) registered a twofold increase in salivary cortisol concentrations of both individuals after a fight between two zoo African elephants. The incident took place at 14:30h and the saliva samples were collected at 16:00h (1 hour and 30 minutes later).

Factors related to social structure, such as the presence of conspecifics and aggressive interactions, can elicit physiological responses. According to Serres-Corral et al. (2021), stress and, therefore, elevated GC levels, can occur either in high or low-ranking individuals depending on the social organization of the species or the population.

Age may also be a factor in adrenocortical activity. The youngest elephant, E1, had the lowest average cortisol concentrations, while the eldest, E3, was the one with the highest cortisol values. Age is a significant predictor of serum and urinary mean cortisol concentrations in Asian elephants, with concentrations being lowest in the 0-10 age group, higher in the age group spanning 11–60 years, with 41–60 years being highest (Glaeser et al. 2020). Both E3 and E2 fall within the category between 41-60 years.

Regarding the relationship between cortisol concentrations and behavior, Kelling (2008) found that stereotypic behavior decreased cortisol levels, suggesting that it was a way to deal with excess stimulation. In our study, although the elephant with the highest mean cortisol, E3, was also the one that performed head bobbing the most, there was not a statistically significant association between these two variables. One possible explanation for this result was the time lag between the performance of stereotypy and saliva sampling since cortisol values in the saliva change rather quickly.

Similarly, the relationship between cortisol concentrations and lying rest did not show statistically significant results. This relationship should be further investigated with a different cortisol medium other than saliva or blood so that the cortisol concentration can be representative of a few days instead of minutes to hours (for example, feces).

Overall, neither chronically elevated nor suppressed concentrations of GCs were observed on this group of elephants.

6.2.1. Welfare

The African elephants at Barcelona Zoo are experiencing some changes in the dynamics of the group. This social stressor may elicit an increase in rates of stereotypic behavior as a coping mechanism. Specifically, E3 is showing a stereotypic rate above the 10% threshold defined by Broom (1983). This, in combination with the increase in cortisol concentrations when compared with 2014, may suggest the start of a decreased welfare state.

However, it is not possible to truly qualify the elephants' welfare since most of the selected indicators had no reference values before the start of the study. In fact, the major contribution of this pilot study is the acquisition of reference values for the different indicators of welfare. It is required to continue monitoring these indicators to allow the detection of changes in the welfare state of each elephant. Furthermore, in Barcelona Zoo there is already a software in place, named ZIMS Care and Welfare, that turns the input of data a straightforward process. Thus, these indicators will work as a foundation for a future welfare monitoring of the three African elephants housed in Barcelona Zoo. As mentioned in the beginning, a rapid, affordable, non-invasive, and straightforward routine that could easily become part of the daily management protocol.

7. CONCLUSIONS

Despite being carried out in a very small group of elephants, this pilot study made a modest contribution in the validation of salivary cortisol, in conjunction with behavior, to investigate elephant welfare.

First, it provided an important collection of objective behavioral and physiological data in captive African elephants, such as activity budgets and baseline cortisol values that can be used as a future reference, and the quantification of dominance and social relationships.

Second, we demonstrated that salivary cortisol is a useful minimally invasive measure of physiological stress if the animals are willing to cooperate, and samples can be collected during or immediate (ideally within 30 min) after the stressor. Furthermore, we showed that stereotypic behavior varied both qualitatively and quantitatively among elephants. Although the elephant with the highest mean cortisol, E3, was also the one that engaged in stereotypic behavior the most, in the present study no statistically significant correlation was found between these two variables.

Finally, this study contributed to a wider perspective on elephant welfare. Instead of attempting to classify welfare in a binary way (i.e., good versus bad), it focused on acquiring the tools to evaluate its progression over time. Hence, we identified and selected a set of indicators, namely salivary cortisol, stereotypic behavior, activity budgets, social behaviors, and lying rest, that will allow the welfare assessment of the group of African elephants housed in Barcelona Zoo, when used simultaneously on a regular basis. These indicators are the basis of a welfare assessment tool that will enable the recognition in the event of welfare deterioration, and, in turn, facilitate the adoption of new and better management protocols that, utterly, promote better welfare.

8. LIMITATIONS

Although the study has reached its aims, there were some unavoidable limitations. One of the most obvious limitations of this study is the small sample size. This was an exploratory study, limited by the availability of subjects. Due to this restriction, any findings are unavoidably ineligible for generalizations. Nevertheless, trends found across all three subjects should be repeated with future studies with elephants, allowing a more focused and effective approach. Furthermore, the data collected are still informative and the small sample size is fairly compensated by calculating statistics on individual animals separately. Additionally, the exploratory nature of this work generates a needed framework for further research utilizing salivary cortisol.

Additionally, the time lag between the recording of behaviors and the sampling of saliva should have been shorter since cortisol levels in the saliva change rapidly. This may have limited some associations between the two different types of indicators.

Finally, during the study period, construction works were taking place in an enclosure near the elephants' facilities. It was not possible to measure the impact of possible disturbances caused by an increased level of noise, but if it did occur, this environmental variable may have affected the three elephants.

9. RECOMMENDATIONS

As already mentioned, this study contributed to a wider perspective on elephant welfare. Furthermore, the current work has provided a great framework to further build upon. As such, a great deal of work is suggested by the results of this study.

For future research, it would be useful to repeat some aspects of this study with additional elephants. Of course, baseline values would have to be established for any additional subject. Additional baseline cortisol analyses would allow further exploration between cortisol and behavior connection. For example, additional trends between cortisol and stereotypic rate, percent of time spent consuming food, and any dominance or aggression. Other studies should also examine the cases in which increased cortisol occurred after positive events, such as novel training and enrichment to examine the effects of these husbandry events.

Additionally, an interesting future project would be studying the potential impact of the undergoing construction works at the zoo, not only in terms of sound but also regarding ground vibrations.

To effectively study the relationship between cortisol and head bobbing, saliva samples should be collected soon after, within 30 minutes, the occurrence of the behavior. Another option would be to use a different type of non-invasive cortisol sample, such as feces.

All in all, the goal for keeping elephants in captivity should not be to eliminate all sources of stress, just those that may be detrimental to welfare, either physiologically or psychologically.

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11. APPENDICES

Appendice I – Behavior recording sheet.

Date: ___/___/___

Time (min)	E1	E2	E3
0			
5			
10			
15			
20			
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