Aus dem Leibniz-Institut für Zoo- und Wildtierforschung

eingereicht über den Fachbereichs Veterinärmedizin

der Freien Universität Berlin

Non-invasive detection methods of steroids and its application in cheetahs (*Acinonyx jubatus*) in European zoological gardens

Inaugural dissertation to obtain the academic degree

doctor medicinae veterinariae (Dr. med. vet.)

submitted to the Department of Veterinary Medicine

of the Freie Universität Berlin

by

CARSTEN MARIO LUDWIG

Veterinarian from Coblenz

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CONTENT

CHAPTER 1: GENERAL INTRODUCTION

1.1 Importance of zoological gardens in conservation

The role and function of zoological gardens changed radically over the past centuries (Nogge 1993, Hosey 2013). Simple exhibition of exotic animals to the public is not accepted any more by visitors in developed countries. This is reflected in an ongoing development from the classic menagerie of the 19th century to a zoological garden as a powerful conservation instrument of the modern time (Conde et al. 2011, 2013). It is estimated that 700 million visitors are passing through the gates of zoos and aquaria worldwide per year (Gusset and Dick 2011). The latest conservation strategy of the World Association of Zoos and Aquariums (WAZA) aims to use this enormous interest of the public to develop and establish conservation strategies for species and ecosystems (Barongi et al. 2015). The modern zoological garden links ex-situ breeding programs with in-situ conservation projects to build bridges from cities to natural ecosystems (Conde et al. 2011, 2013). One aim is the education of people, who often have their first contact with wildlife by visiting the zoological gardens in their cities (Ballantyne et al. 2014, Barongi et al. 2015). In an entertaining way, zoological gardens can raise interests and inform people on ongoing challenges in the environment (Barongi et al. 2015). The amount of research supported by and carried out in and by zoos is impressive and contributes to a better understanding of the biology of species as well as the interaction of species with their habitats (Hosey et al. 2013). It is therefore an essential contribution to the protection and conservation of endangered species (Conde et al. 2011). No other group of institutions has the practical experience and scientific knowledge to keep and breed thousands of animal species, thereby creating an enormous potential of contributing to wildlife conservation (Barongi et al. 2015). At least 400 breeding programs are supervised in Europe by the European Association of Zoos and Aquaria (EAZA) (EAZA 2017). Zoological gardens also use

flagship species such as the golden lion tamarin (*Leontopithecus rosalia*) as an example to inform and protect an entire ecosystem, e.g., the Atlantic rain forest in Brazil (Kierulff and Oliveira 1996, Beck 2001).

The cheetah (*Acinonyx jubatus*) is another flagship species, although one that does not breed well in zoological gardens (Marker and O'Brien 1985, Wildt et al. 1993) even though it breeds well in the wild (Laurenson et al. 1992, Wachter et al. 2011). It was the aim of this study to investigate the underlying reasons for the difference of reproductive performance in zoological gardens and free-ranging populations. Although husbandry conditions have been much improved in the last decades (Marker et al. 2018), breeding success of cheetahs in captivity remains poor (Versteege 2013). A frequently cited potential cause for low reproductive performance in captivity is allostatic load or "stress" (Jurke et al. 1997, Terio et al. 2004). Therefore, the first hypothesis of this study was to investigate whether female cheetahs with a good reproductive performance have a lower allostatic load than non-reproducing females.

1.2 Allostatic load ("stress")

The term "stress" designates a stimulus triggered reaction of an organism, either an external - such as an environmental condition - or internal stimulus (Cannon 1932). A stress response generally involves regulatory mechanisms of the body to ensure the survival of the organism in dangerous situations (Chrousos and Gold 1992). These take place on a physiological level and are designed to restore homeostasis, i.e. the balance of bodily functions (Cannon 1932). The main components of a stress response are the stimulation of the sympathetic-adrenal medullary system and the hypothalamic-pituitary-adrenal (HPA) axis (Chrousos and Gold 1992, Minton 1994). The concept of allostatic load (Romero et al. 2009) has recently been

proposed as a model to replace ambiguous aspects in the original concept of “stress” and specify more precisely which rules govern bodily, physiological and mental homeostasis.

1.2.1 Acute stress response

By definition, an acute stress situation is a stimulus or “stressor-induced”, suddenly occurring, time-limited activation of the stress system for coping with an acute challenge (Sapolsky 1987, Moberg 2000, Charmandari et al. 2005). This can for example be an attack by a predator or conspecific competitor (Romero and Butler 2007). The famous "fight-or-flight" response is mainly triggered by the sympathetic-adrenal medullary system. The term "fight-or-flight" refers to the immediate delivery of energy in the form of glucose to the cells through gluconeogenesis and lipolysis (Cannon 1932, Matteri et al. 2000). Furthermore, the cardiovascular system is activated and general alertness and responsiveness of the organism increases (McEwen and Sapolsky 1995). The two most important hormones in the stress response are the catecholamines epinephrine and norepinephrine, previously known as adrenaline and noradrenaline (Romero and Butler 2007). Upon detection of a stressor, both catecholamines are released by the adrenal medulla and the nerve terminals of the sympathetic nervous system. These hormones are produced continuously and stored in secretory vesicles. Consequently, a release of catecholamines can occur rapidly, i.e., within seconds after detection of a stressor. The animal has the energy and capacity to “fight-or-flight” (Romero and Butler 2007) by activating a number of responses such as increasing visual acuity, heart rate, brain blood flow, arousal and gas exchange efficiency in the lungs (Romero and Butler 2007).

The activation of the hypothalamic-pituitary-adrenal (HPA) axis is slower than the one of the sympathetic-adrenal medullary system. This is because it first needs to release the

corticotropine releasing hormon (CRH) from the hypothalamus to release the adrenocorticotropic hormone (ACTH) via the pituitary gland (Goymann and Wingfield 2004). ACTH stimulates the synthesis and release of glucocorticoids such as cortisol from the adrenal cortex (Fig. 1). Both systems are controlled by negative feedback mechanisms of the hormones on the hypothalamus and the pituitary gland, respectively. Similar to catecholamines, glucocorticoids can help coping the organism with the challenge, particularly if the stressor is short and acute. In such situations the short-term release of glucocorticoids helps vertebrates to overcome potentially dangerous situations through the fast mobilization of glucose (Romero et al. 2009).

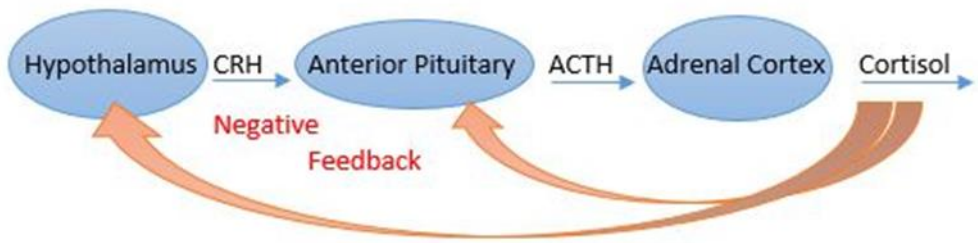

Fig. 1: Basic hypothalamic–pituitary–adrenal axis summary (corticotropin-releasing hormone=CRH, adrenocorticotropic hormone=ACTH).

1.2.2 Chronic stress response

The described physiological changes of stress normally do not last long, but sometimes organisms are in a nearly constant state of high alertness, i.e., are in chronic stress. The state of chronic stress is normally reflected by a prolonged high secretion of glucocorticoids (Wright et al. 2007). Chronic stress responses can be deleterious for the organism because continuously elevated blood cortisol levels can lead to an inhibition of the production of interferon gamma (IFN-γ) and thus an increase in disease susceptibility (Munck et al. 1984, Wiepkema and Koolhaas 1993, Sapolsky 2005, Cabana et al. 2018). It can also lead to a higher susceptibility of upper respiratory infections (Cohen et al. 1997) or gastrointestinal disfunction

such as the marmoset wasting syndrome in callitrichids (Cabana et al. 2018). Chronic stress can also affect the cardiovascular system by leading to hypertension (Sapolsky 2005) or elevated heart rate (Sapolsky and Share 1994). Furthermore, reduced reproductive performance and survival have been observed, thus chronic stess can also reduce Darwinian fitness (Moberg 1987, Liprap 1993, Ferin 1999, Bonier et al. 2009, Angelier et al. 2010). The "captive stress hypothesis" is therefore discussed as one possibility for the low reproductive performance in captive cheetahs (Jurke et al. 1997, Terio et al. 2004). Terio et al. (1999) validated a radio immunoassay (RIA) for measuring fecal glucocorticoid metabolites (fGCM) in captive cheetahs with the help of ACTH challenges and stressful handling. They described an elevation of blood serum cortisol minutes after ACTH injection in immobilized cheetahs and detected a raise of fGCM in feces with a delay of 48 to 96 hours. Using this RIA, study animals showed an increase in fGCM 24 to 72 hours after exposure to exogenous stressors such as transfer of the animal to another enclosure within the institution, immobilization or, for females, introduction to a male (Terio et al. 1999).

Several factors have been previously assessed which might function as a frequent or repeated stimulus causing chronic stress. Unsuitable housing conditions such as predators nearby or crowded social living conditions were shown to increase allostatic load in some species (McEwen and Wingfield 2010) and suppress ovarian activity (Jurke et al. 1997). Jurke et al. (1997) found a difference in fGCM levels between reproducing and non-reproducing female cheetahs measured with a RIA. Females with high cortisol levels (> 200 ng/g) seemed compromised in their ovarian cycling as measured by fecal estrogen concentrations whereas the reproducing females had lower cortisol levels and were not compromised in this respect (Jurke et al. 1997). In another study, fGCM measurements with a cortisol EIA were compared with measurements of a corticosterone RIA in eight carnivore species, including the cheetah

(Young et al. 2004). The results were similar, suggesting that EIAs are a good alternative to RIAs when the use of radioactive substances is unwanted or restricted, such as in zoos (Young et al. 2004). Female cheetahs in North American zoos had significantly higher stress responses than their free-ranging or captive conspecifics on Namibian farmland, with the latter two groups indistinguishable from each other (Terio et al. 2003, 2004). The stress responses were determined in terms of the adrenal corticomedullary ratios, i.e., the ratio of the length of the cortex and medulla of the adrenal glads, of dead cheetahs and the baseline concentrations of fGCM in alive cheetahs (Terio et al. 2003, 2004). On Namibian farmland, captive cheetahs are fenced in large enclosures in their natural habitat, thus exposed to the same natural weather conditions as their free-ranging conspecifics, which explains this result (Wachter et al. 2011). This result was also consistent with ultrasonographic measurements of the adrenal glands in a separate study, which were similar in size for free-ranging and captive Namibian cheetahs (Wachter et al. 2011). Repeated and regular ACTH release by the adrenal cortex (zona fasciculata), typical for chronic or persistent exposure to stressors, first results in hypertrophy and then hyperplasia of the adrenal cortex. As a consequence of these changes, an increase in the size of adrenal glands can be used as a marker of chronic stress (Estivariz et al. 1992). Because adrenal mass, width, corticomedullary ratio and corticomedullary hyperplasia can also change with age and not only with allostatic load (Gillis-Germitsch et al. 2016, Kirberger and Tordiffe 2016), it is useful to measure allostatic load with fGCM when this is possible, such as in zoological gardens.

1.3 Assessing hormone concentrations

The traditional measurement of stress hormones such as cortisol in the blood is not reliable, because the handling of animals itself induces a stress response, and this response is visible

within minutes in the blood (Sapolsky 1982, Wingfield et al. 1994). Therefore, a non-invasive approach is needed to measure concentrations of glucocorticoids and other hormones. Cortisol is a glucocorticoid and one of the most important "stress" hormones (Elenkov and Chrousos 2002, Matousek et al. 2010). Glucocorticoids are a class of steroid hormones consisting of a 4-ring carbon backbone with different hydroxyl groups and carbon side chains attached at various places around the rings (Fig. 2). The side chains and their locations on the carbon rings determine which steroid it is (Romero and Butler 2007). Thus, cortisol is very similar to other steroid hormones such as testosterone or estradiol (Fig. 2). Because of this similarity, cross-reactions with other steroid hormones can occur when measuring metabolites in the feces with a competitive EIA. Circulating steroid hormones are usually metabolized by the liver and excreted as conjugates via the kidneys into the urine or via the bile into the gut (Palme et al. 1996). Steroid metabolism studies in domestic cats demonstrated that 85 % of the hormones were excreted via the feces and the rest via the urine (Shille et al. 1990, Brown et al. 1994, Graham and Brown 1996). Cat species usually defecate once a day (gut passage 12 – 24 hours), thus hormones excreted with a circadian rhythm will be pooled in the feces over the period of a day. Measuring fGCM in felids, including the cheetah, is therefore likely to be a good approach as it focuses on the signal caused by key stressors rather than instantaneous or rhythmic changes in hormone concentrations. After defecation several factors such as temperature, bacterial enzymes, the presence of UV-light and humidity can affect concentrations of fGCM (Khan et al. 2002). Thus, feces have to be collected fairly rapidly, within several hours, and properly stored (Khan et al. 2002, Terio et al. 2002, Blekhman et al. 2016, Pribbenow 2016). Also, assays aiming to measure fGCM or other steroid hormone metabolites in feces must be carefully validated for each species and both sexes (Touma and Palme 2005, Benhaiem et al. 2012). Pharmacological stimulation of the HPA

axis is necessary to evaluate whether stimulation of glucocorticoid production leads to a measurable increase of fGCM. Typically, an injection of ACTH is used to stimulate adrenocortical activity, thereby increasing the secretion of cortisol from the adrenal glands (Fig. 1; see also Goymann et al. 1999, Touma and Palme 2005). In addition, validation requires an injection of radioactively labelled hormones (e.g. ^{3}H labelled cortisol) to determine by high-performance liquid chromatography (HPLC) whether the targeted conjugates or metabolites are measured in the feces (Benhaiem et al. 2012). Chapter 2 presents a comparison of four EIAs and investigates the most reliable one (the corticosterone-3-CMO EIA) further with ^{3}H labelled cortisol and HPLC (see section 1.5). The chapter also presents the stability of the fGCM over time and indicates the time period in which the samples should be collected and stored.

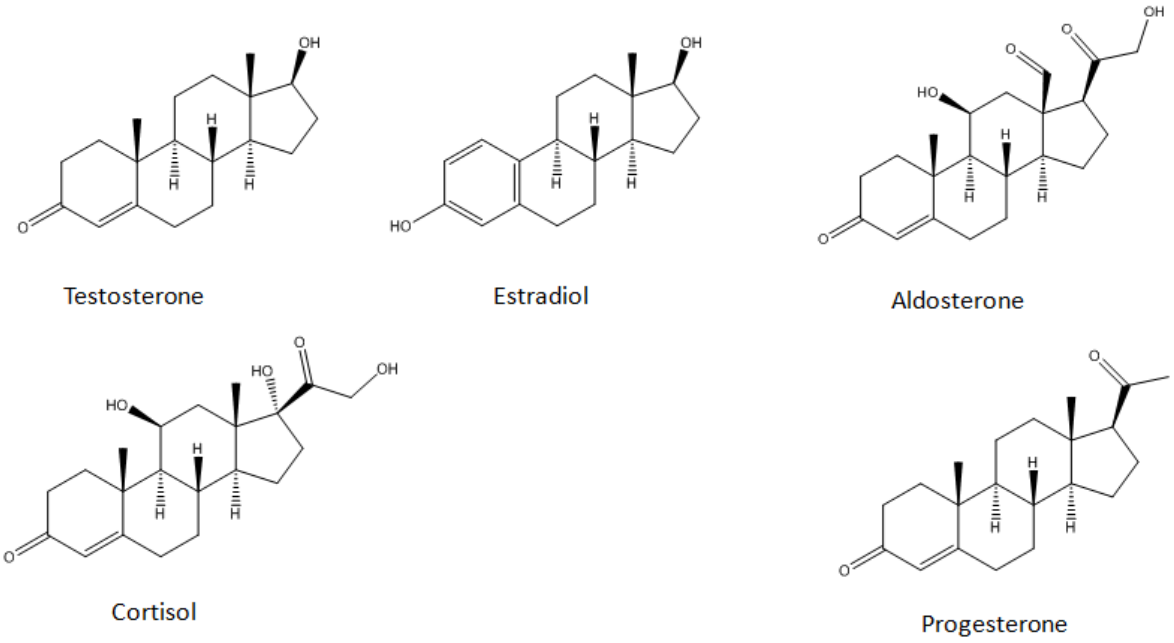

Fig. 2: Biochemical structure of steroid-hormones.

Testosterone is produced and secreted at varying quantities during the life history of a male, starting at sexual maturity. It plays a key role in fertility, reprodcutive function and sexual behavior of males (Hiller-Sturmhoefel and Bartke 1998, Tarttelin et al. 1998, Shahidi 2001, Romeo et al. 2003, Duke et al. 2014). Similarly to glucocorticoids, it is regulated by a

complex series of feedback mechanisms orchestrated by the hypothalamic-pituitary-gonadal axis (Ray and Choudhuri 2011, Koehrle et al. 2014,). Testosterone secretion underlies a diurnal rhythm with 4 - 5 peaks during the day (Bertschinger et al. 2008). Thus, serum concentration can vary decisively several times within one day. Testosterone concentration in feces (fTM) also is pooled over approximately 24 hours. A reliable EIA measuring fTM would therefore be a useful tool to monitor endocrine status and reproductive development of male cheetahs. When applied in tandem with an EIA that measures fGCM, cross-reactions with glucocorticoids can be avoided. Chapter 3 presents the validation and identification of an EIA for fTM in cheetahs by conducting a physiological challenge with gonadotropin-releasing hormone (GnRH) and HPLC analyses (see section 1.5).

1.4 Hypotheses suggested for low reproduction in female cheetahs

Besides the "captive stress hypothesis" mentioned in section 1.2.2, several other hypotheses were suggested as a cause for the low reproductive performance of female cheetahs. These are the "genetic monomorphism hypothesis", the "captive reproductive suppression hypothesis", the "innate rhythm hypothesis" and the "asymmetric reproductive aging hypothesis", which are presented in detail below. Chapter 4 investigates these hypotheses in cheetahs in European zoos (see section 1.5).

1.4.1. "Genetic monomorphism hypothesis"

Numerous studies discussed lack of genetic variation as a cause for the low reproductive performance in captive cheetahs (O'Brien et al. 1983, O'Brien et al. 1985, Wayne et al. 1986, Kieser and Groeneveld 1991, Menotti-Raymond and O'Brien 1993, Freeman et al. 2002, Drake et al. 2004). The ancestreal cheetah population was assumed to have passed through a

demographic bottleneck at the end of the last ice age (Menotti-Raymond and O'Brien 1993). A couple of more recent declines were proposed as resulting from direct and indirect anthropogenic impact, and all these declines were suggested to have led to inbreeding and thus a low genetic variability (O'Brien et al. 1983, 1985, 1987). This low genetic variability was often termed "genetic monomorphism" and included a low biochemical genetic variation based on a low number of polymorphic allozyme loci and a low variability at the major histocompatibility complex (MHC) based on an apparent lack of skin graft rejection (O´Brien et al. 1983, 1985). Wayne et al. (1986) compared skull measurements of the cheetah with other felid species and suggested that there were hints of inbreeding in the population history of the cheetah. Macroscopic anatomy of dental dimensions (odontometric asymmetry) of cheetahs was compared with that of other felid species. The observed asymmetry in cheetahs was discussed as a consequence of low genetic variability (Kieser and Groeneveld 1991). Thus, the cheetah became a model of conservation genetics in textbooks and publications. Subsequent studies concentrated on the comparison of mitochondrial DNA and detected a lower intraspecific population variability in cheetahs than in other cat species such as ocelots (*Leopardus pardalis*) or margays (*Leopardus wiedii*) (Freeman et al. 2002). This did not prevent the researchers to identify different mitochondrial DNA clusters (Freeman et al. 2002). A study of the MHC class II loci revealed limited genetic variability (Drake et al. 2004). More recent studies found that cheetahs might be not as genetically uniform as assumed (Castro-Prieto et al. 2010). The investigation of MHC class I and II diversity in free-ranging cheetahs in a large sample size and with modern methods revealed a higher diversity of MHC than previously reported, but still lower than those of other felid species (Castro-Prieto et al. 2010).

Irrespective of the exact genetic variability, the crucial question is whether the genetic makeup of the cheetah hampers its reproductive performance. Cheetahs in zoological gardens

often do not breed well, but free-ranging cheetahs are fertile and reproduce well (Laurenson et al. 1992, Wachter et al. 2011). This has been demonstrated in free-ranging cheetahs on commercial farmland in Namibia, where females were always either cycling, pregnant, lactating or accompanied by their offspring (Wachter et al. 2011) and in the Serengeti National Park in Tanzania, where females quickly became pregnant again after their offspring were killed by lions or spotted hyenas (Laurenson et al. 1992). Thus, it is likely that other reason(s) underlie the low reproductive performance in captivity.

1.4.2. "Captive reproductive suppression hypothesis"

In captivity, husbandry conditions try to simulate the natural environments of the animals as good as possible. Nevertheless, some factors such as climate, vegetation, social structure and distance to conspecifics might differ from their natural environment. Free-ranging cheetahs have larger home range sizes (Caro 1994, Broomhall et al. 2003, Marker et al. 2018, Melzheimer et al. 2018) than zoological gardens can offer. Recommendations for zoos vary between a few m² in husbandry guidelines for facilities in North America (Ziegler-Meeks 2009) to at least 100 m² per cheetah in Germany (BMEL 2014). The important issue is to try to meet all the essential needs of cheetahs under husbandry conditions such as food, health and safety. Concerning reproduction, mate selection cannot be performed as in the wild, but the partners are brought together under husbandry management decisions. Free-ranging males are either solitary or live in groups of two to four males, mainly brothers, whereas females are solitary except when accompanied by her offspring (Caro 1994, Marker 2002). In zoological gardens, cheetahs are frequently housed in pairs or groups (Wielebnowski et al. 2002). Research on females permanently housed together indicated that there is a mechanism of reproductive suppression between the animals (Wielebnowski et al. 2002, Kinoshita et al.

2011). When female cheetahs were housed in pairs for 6-month periods, most pairs showed prolonged anoestrus (Wielebnowski et al. 2002). Shortly after they were separated and kept by themselves, females rapidly resumed estrus cyclicity (Wielebnowski et al. 2002). Another study illustrated that a female permanently housed together with a male did not reproduce (Kinoshita et al. 2011). The only female which bred successful in the study was separated from other cheetahs for more than a year (Kinoshita et al. 2011). It is currently the perceived conventional wisdom that housing in individual compartments and long-term separation areas are beneficial for breeding cheetahs in captivity (Meltzer 1999, Kinoshita et al. 2011).

1.4.3. "Innate rhythm hypothesis"

It can be beneficial for free-ranging animals to have their offspring during a time of the year when environmental circumstances maximise the chance of rearing offspring successfully. For example, many free-ranging ruminant species show reproductive seasonality and give birth during a particular period of the year (Zerbe et al. 2012). This can depend on a particular vegetation period or the availability of other seasonal resources, anti-predator strategies or photoperiodism (Zerbe et al. 2012). In cheetahs, such an innate rhythm was also discussed (Terio et al. 2003). It was suggested that reproductive cycling is triggered by an endogenous circannual rhythm, because female cheetahs kept in semi-captivity on Namibian farmland showed seasonality in anestrus periods (Terio et al. 2003). This differed from hormone profiles of cheetahs kept in North American zoos (Brown et al. 1996). It was argued that this circannual rhythm might therefore require factors that are only present in the natural environment such as rainfall during the wet season or high prey availability (Terio et al. 2003). Wachter et al. (2011) rejected predictions derived from the innate rhythm hypothesis by investigating with ultrasound the activity of the inner reproductive organs of free-ranging and captive female

cheetahs in Namibia and by demonstrating that there is no breeding season in free-ranging Namibian cheetahs.

1.4.4. “Asymmetric reproductive aging hypothesis”

Female reproductive organs such as the uterus and the ovaries are exposed to varying concentrations of hormones during reproductive cycles. For example, during estrus high estrogen peaks are measured. In the wild, females normally become pregnant early in life, because they find a suitable mating partner shortly after they turn sexually mature and become sexually interested (Caro 1994, Kelly et al. 1998, Mills et al. 2017). During pregnancy and lactation there are no estrogen peaks and the dominant hormones are progesterone and prolactin, respectively. Frequent fluctuations of estrogen concentrations during periods without reproductive activity cause faster aging of the inner reproductive organs, which has the consequence of reduced cycling capacities and induce pathological lesions of these organs (Hermes et al. 2004, Wachter et al. 2011). This phenomenon, termed “asymmetric reproductive aging” (ARA), was first described in white rhinoceros (*Ceratotherium simum)*, Asian elephants (*Elephans maximus*) and African savanna elephants (*Loxodonta africana*) in zoological gardens (Hildebrandt et al. 2000, Hermes et al. 2004, 2006). In zoological gardens, females are often prevented from reproducing early, often due to a lack of a suitable mating partner in the institution. Time consuming transfers between institutions, planned with the help of a centralized studbook, must be organized and conducted before reproduction can take place (Versteege 2013). The phenomenon of ARA is often observed in older, nulliparous females which have been exposed to frequently repeated fluctuations of estrogen for the longest time. These females are at risk to enter into an early and irreversible reproductive quiescence and lose their fertility earlier than females which successfully reproduced earlier

in their life (Hildebrandt et al. 2000, Hermes et al. 2004, 2006). A link between nulliparity and infertility because of pathological changes in reproductive organs has also been described for canine and feline species in captivity, including cheetahs (Munson et al. 2002, Crosier et al. 2011, Asa et al. 2014, Penfold et al. 2014, Saunders et al. 2014). These studies suggest similar underlying mechanisms as described for ARA and termed the phenomenon "use-it-or-loose-it" (Penfold et al. 2014). Early pregnancy and lactation are protective mechanism against ARA because they reduce estrogen fluctuations, which are thought to be responsible for the damaging effects on the inner reproductive organs demonstrated in several species such as white rhinoceros, Asian elephants and African savanna elephants and tigers (*Panthera tigris*) (Hildebrandt et al. 2000, Hermes et al. 2004, 2006, Penfold et al. 2014, Saunders et al. 2014). Wachter et al. (2011) performed a study on free-ranging and captive Namibian cheetahs and showed that the reproductive characteristics of females were consistent with the ARA hypothesis.

1.5 Objectives of this thesis

The first objective of this thesis was to characterize and validate an EIA to measure fGCM non-invasively as a representative proxy for allostatic load in cheetahs. This assay was used in captive female cheetahs in European zoological gardens to detect whether reproducing and non-reproducing females differ in their allostatic load ("captive stress hypothesis"). Another objective was to characterize and validate an EIA to measure fTM in cheetahs to exclude cross-reactions between glucocorticoid and testosterone metabolites in feces. Two other hypotheses which were suggested to explain the low reproductive performance of female cheetahs in captivity were investigated in this thesis: "the captive reproductive suppression hypothesis" and "the asymmetric reproductive aging hypothesis".

The results of my studies are presented in three manuscripts in chapters 2 to 4:

Chapter 2 describes the development and validation of an EIA to measure stress hormones in feces of cheetahs. The specific aims were to:

- compare four EIAs to identify the most sensible and reliable assay for cheetahs,
- test whether the assays measure significant increases in fGCM after ACTH challenges in a male and a female captive cheetah in Germany,
- conduct a radio-metabolism study with ^{3}H labelled cortisol to characterize the fGCM detected by the assay,
- run a HPLC analysis to determine whether the targeted metabolites are measured,
- test how long fGCM remain stable in the feces after defecation,
- assess whether the assay performs also well in captive cheetahs in South Africa.

Chapter 3 describes the development of an EIA to measure testosterone metabolites in feces of cheetahs. The main aims of this study were to:

- test whether the assay measures significant increases in fecal testosterone metabolites (fTM) after a physiological challenge with GnRH and compare this with the values after a placebo injection,
- conduct a radio-metabolism study with ^{3}H labelled testosterone to characterize the fTM detected by the assay,
- run an HPLC analysis of radiolabeled and immunoreactive testosterone metabolites to determine whether the targeted metabolites are measured,
- develop a reliable tool to get information about the reproductive function of male cheetahs and conduct a biological validation by testing the EIA on free-ranging cheetahs in Namibia,
- test how long fTM remain stable in the feces after defecation,

- conduct cross-reaction tests with EIAs for fTM and fGCM to verify the specificity for both EIAs.

Chapter 4 investigates several hypotheses concerning the low reproductive performance of female cheetahs in five European zoological gardens:

- the “captive stress hypothesis” was investigated by comparing fGCM of female cheetahs with a good reproductive performance with females that did not breed,
- the “captive reproductive suppression hypothesis” was investigated by comparing the reproductive performance of animals under different social and housing conditions,
- the “asymmetric reproductive aging (ARA) hypothesis” was investigated by comparing the reproductive history of the study animals and associated pathological or clinical findings of the reproductive organs.

The “genetic monomorphism hypothesis” and the “innate rhythm hypothesis” have been rejected already for cheetahs in a previous study using free-ranging and captive cheetahs in Namibia, whereas the predictions of the “ARA hypothesis” were consistent with the data from cheetahs in free-ranging and captive populations in Namibia (Wachter et al. 2011). In this Chapter the “ARA hypothesis” was also investigated for cheetahs in European zoos. The results were consistent with the predictions, together with the results of the “captive reproductive suppression hypothesis”.

Chapter 5 discusses and summarizes the key findings of this thesis in a broad context and highlights their importance for the management of cheetahs in zoological gardens.

CHAPTER 2: MANUSCRIPT 1

Characterisation and validation of an enzyme-immunoassay for the non-invasive assessment of faecal glucocorticoid metabolites in cheetahs (*Acinonyx jubatus*)

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In: General and Comparative Endocrinology 2013, 180: 15-23.

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CHAPTER 3: MANUSCRIPT 2

Validation of an enzyme-immunoassay for the non-invasive monitoring of faecal testosterone metabolites in male cheetahs (*Acinonyx jubatus*)

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CHAPTER 4: MANUSCRIPT 3

Asymmetric reproductive aging in cheetah (*Acinonyx jubatus)* females in European zoos

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Research article

Asymmetric reproductive aging in cheetah (*Acinonyx jubatus*) females in European zoos

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Abstract

For conservation breeding and management, such as re-introduction programmes, reproductively healthy animals are essential. Low reproductive performance in captive animals is therefore of great concern in species which are judged to be vulnerable or endangered in the wild. Hence, it is important to understand the mechanisms that lead to low reproductive performance in captivity. Here, a long-term study on 12 captive cheetah females is presented as an example to test predictions derived from three hypotheses, the captive 'stress', captive reproductive suppression and asymmetric reproductive aging hypotheses. Parous and nulliparous cheetah females did not differ in their faecal glucocorticoid concentrations, suggesting that allostatic load ('stress') might not affect reproductive performance. Consistent with the captive reproduction suppression hypothesis, females permanently kept together with other adult females (or males) did not reproduce until they were individually introduced to a single male when in oestrus. In addition, reproductive performance of these females was determined by their age and reproductive history, as predicted by the asymmetric reproductive aging hypothesis. Asymmetric reproductive aging arises when first breeding attempts and first pregnancies of females are substantially delayed, thereby ensuring frequent cycle fluctuations of oestrogen concentrations which subsequently result in reproductive tract pathologies. Our results suggest that conservation breeding facilities should breed cheetah females as early as possible, keep them separate from adult males or other females and introduce them to single males for breeding purposes when in oestrus only.

Introduction

Several mammalian species exhibit low reproductive performance in captivity (Carlstead 1996; Hermes et al. 2004, 2006; Hildebrandt et al. 2000; Steiner et al. 2015; Wielebnowski 1998). Successful reproduction in captivity is desirable because it can improve general and reproductive health and supplies the individuals required for conservation management activities, such as re-introduction programmes (Carlstead 1996; Ebenhard 1995; Hermes et al. 2004; Wielebnowski 1998). The cheetah (*Acinonyx jubatus*) is known to be difficult to breed in captivity (Lindburg et al. 1993; Marker and O'Brien 1989; Marker-Kraus and Grisham 1993; Versteege 2013; Wildt et al. 1993), as few breeding centres are consistently successful in their breeding attempts over longer periods of time (Bertschinger et al. 2008; Versteege 2013). Breeding in captivity will become more important in future as the global population of cheetah declines progressively (Durant et al. 2017; Weise et al. 2017). Cheetah females kept in zoological gardens have been observed to exhibit irregular cycling, anoestrous periods, reproductive suppression or lack of sexual arousal (Brown et al. 1996; Marker-Kraus and Grisham 1993; Wielebnowksi et al. 2002a). In contrast, free-ranging cheetah females have a higher reproductive performance in terms of conception rate than cheetahs in zoological gardens, resuming cycling shortly after the loss of a litter and readily becoming pregnant again (Caro 1994; Laurenson et al. 1992; Marker-Kraus et al. 1996; Wachter et al. 2011).

The possible reasons for this difference in performance between captive and free-ranging cheetahs have been previously discussed in the context of five hypotheses: (1) the "genetic monomorphism hypothesis", which suggests that the low genetic variability of cheetahs (Drake et al. 2004; Freeman et al. 2002; Menotti-Raymond and O'Brien 1993; O'Brien et al. 1983, 1985) is linked to low fertility and high cub mortality (Brown et al. 1996; Wildt et al. 1993); (2) the "captive 'stress' hypothesis", which suggests that unfavorable husbandry conditions increase allostatic load, for example, from crowded social living conditions (McEwen and Winfield 2010; Romero et al. 2009), which in turn suppresses ovarian activity (Jurke et al. 1997); (3) the "captive reproductive suppression hypothesis", which suggests that pheromones of jointly-housed females directly suppress ovarian activity of females living within the same enclosure (Kinoshita et al. 2011; Wielebnowski et al. 2002a); (4) the "innate rhythm hypothesis", which suggests that reproductive cycling is triggered by an endogenous circannual rhythm (Terio et al. 2003); and (5) the "asymmetric reproductive aging (ARA) hypothesis", which suggests that frequent fluctuations of oestrogen concentrations cause faster aging of and diminished functionality of reproductive organs and pathological lesions of the reproductive tract (Hermes et al. 2004; Wachter et al. 2011). This phenomenon has been described in African and Asian elephants (*Loxodonta africana* and *Elephans maximus*) and white rhinoceros (*Ceratotherium simum*) kept in zoological gardens (Hermes et al. 2004, 2006; Hildebrandt et al. 2000). It is observed in nulliparous females, particularly in older ones, because these individuals are exposed for the longest period to regular and frequent fluctuations of oestrogen concentrations. Such females enter into a non-reversible early reproductive quiescence in terms of hampered conception and lose a substantial part of their reproductive life compared to females which have successfully reproduced, are reproductively healthy and enter a natural senescence process (Hermes et al. 2004, 2006; Hildebrandt et al. 2000).

A link between nulliparity and endometrial hyperplasia, which can lead to reduced fertility, as well as between reproductive history and likelihood of future reproduction has also been described for several captive canine and feline species, including cheetahs (Asa et al. 2014; Crosier et al. 2011; Munson et al. 2002; Penfold et al. 2014; Saunders et al. 2014). These functional relationships are also encompassed by the ARA hypothesis, although the studies did not label them as such. Penfold et al. (2014) suggested for such relationships the term "use-it-or-lose-it hypothesis" as a more apt description. Contrary to the mechanism suggested by the ARA hypothesis, the captive reproductive suppression hypothesis assumes a mechanism that is a reversible process, because apparently reproductively suppressed captive cheetah females resumed cyclicity when they were separated from conspecifics (Wielebnowski et al. 2002a).

A study on free-ranging and captive cheetahs in Namibia, which simultaneously investigated all hypotheses except the captive reproductive suppression hypothesis found that the data were only consistent with the ARA hypothesis (Wachter et al. 2011) and rejected predictions from the genetic monomorphism, captive 'stress' and innate rhythm hypotheses. This study compared cheetahs held locally in captivity in very large enclosures of at least 10,000 m^2 per animal in natural habitat with free-ranging cheetahs in Namibia. Cheetahs living in such conditions of captivity therefore did not necessarily experience the same conditions as captive cheetahs kept in zoological gardens elsewhere. In particular, these conditions were unsuitable to test the captive reproductive suppression hypothesis which requires females to be jointly housed and in close proximity of each other. In the Namibian study, captive and free-ranging cheetahs did not differ in their size of adrenal glands, a proxy for chronic elevation of allostatic load ('stress') as measured by ultrasonography during immobilisation (Wachter et al. 2011). Cheetahs kept in zoological gardens, however, have larger adrenal glands and higher concentrations of faecal glucocorticoid metabolites (fGCM), the latter being a non-invasive hormonal measure and proxy for short-term increases in allostatic load, than free-ranging Namibian cheetahs (Terio et al. 2004). Increased allostatic load can suppress ovarian activity in many mammals (Breen et al. 2005; Dobson and Smith 1995; Ferris and McCue 2010; Rivier and Rivest 1991), although not necessarily in all of them (Hofer and East 1998). Thus, it is possible that in zoological gardens ARA and the effect of increased allostatic load both negatively affect reproductive health in female cheetahs.

In this study, parous and initially nulliparous female cheetahs kept in zoological gardens were investigated, with the latter being females that were brought into breeding situation but failed to breed. Predictions on reproductive age, reproductive history and allostatic load derived from the captive 'stress', captive reproductive suppression and ARA hypotheses were simultaneously tested. Here, the "genetic monomorphism hypothesis" and the "innate rhythm hypothesis" were not investigated because it was already demonstrated that the reproductive performance of cheetahs is not linked to their genetic makeup and that there is no endogenous circannual rhythm triggering reproductive activity (Wachter et al. 2011). The captive 'stress' hypothesis predicts that nulliparous females should show higher fGCM concentrations than parous ones. The captive reproductive suppression hypothesis predicts that cheetah females kept together with other females are unlikely to breed. Nulliparous captive Namibian cheetah females developed pathologies on their reproductive tract, such as hydrosalpinx, hydrometra and connective tissues on ovaries, all known to potentially hamper reproduction (Munson 1993; Munson et al. 2002; Wachter et al. 2011), at a mean age of 5.6±1.2 years. The ARA hypothesis therefore predicts that females kept in zoological gardens conceive their first litter when they are younger than 5.6 years of age. Females older than that and brought into a breeding situation for the first time would be likely to remain nulliparous. Pathologies on reproductive tracts may also occur in canine and feline females treated with contraceptive products (Asa et al. 2014; Munson et al. 2002; Penfold et al. 2014). It was therefore verified that none of the cheetah females in this study was ever treated with a contraceptive.

Methods

Study animals and facilities

In this long-term study, the reproductive history of eight adult cheetah females was monitored throughout their lifetimes (mean age at death: 9.9±2.0 years, range: 7.1–12.9), while four females were monitored most of their lifetime (their first 14.6±1.5 years of life) until the 31st of December 2018. According to the studbook (Versteege 2013), six females were nulliparous, five females had given birth previously to one (n=4) or two (n=1) litters and one female was pregnant (X012) during the period when faecal samples were collected for this study (see below). Thus, six females were classified as nulliparous and six females as parous and this data set was used to test the prediction derived from the captive 'stress' hypothesis (Table 1).

All animals were kept and monitored throughout their lives, in European zoological gardens. During the period of faecal sample collection, one animal was kept in Austria, three in Denmark, six in Germany, one in Portugal and one in Switzerland. Five females were kept alone in their enclosure, the others were kept together with another female (X011), a male (X008 and X013) or their offspring (n=4) aged 2.9, 3.4, 8.5 and 16.1 months at the start of faecal sample collection (Table 2). Prior to faecal sample collection, one animal was kept in Austria, two in the Czech Republic, one

Table 1. Identity of cheetah females (♀), social group in the enclosure before first potential sexual encounter (between a single female and a single male without other cheetahs in the enclosure), age of first potential sexual encounter, age at first conception, pathologies in reproductive tract detected by necropsy, and number of litters produced. Never refers to the date of death (X008: 10.2 years, X009: 12.9 years and X011: 12.2 years) or the end of the monitoring period on the 31st of December 2018 (X013: 15.7 years).

♀ ID	Conspecifics in same enclosure prior to first potential sexual encounter		Age at first potential sexual encounter (years)	Age when first litter was conceived (years)	Pathologies in reproductive tract	Reproduced	Number of litters
	♀	Male					
X002	-	-	2.9	2.9	Not investigated	Yes	1
X004	-	-	2.8	3.5	None found[a]	Yes	1
X005	-	-	1.9	4.4	Not investigated	Yes	2
X006	-	-	3.9	4.3	Not investigated	Yes	2
X007	-	-	3.8	5.0	Not investigated	Yes[b]	1
X008	1	-	8.9	Never	None found[a]	No	0
X009	Various	Various	8.6	Never	Not investigated	No	0
X010	Various	-	5.0	5.9	Paraovarian cysts	Yes[c]	1
X011	1	-	Unknown, most likely never	Never	Paraovarian and uterine cysts	No	0
X012	-	-	5.2	5.4	Not investigated	Yes[d]	1
X013	-	-	Never	Never	Not investigated	No	0
X014	-	-	4.1	4.3	Not investigated	Yes	1

a: X004 and X008 were diagnosed with gastritis; b: X007 was nulliparous during the faecal sampling period of this study, but gave birth 5.7 months after the sampling period terminated; c: X010 was nulliparous during the faecal sampling period of this study, but gave birth 7.3 months after the sampling period terminated; d: X012 was pregnant during the faecal sampling period of this study.

in Denmark, two in Germany, two in Ireland and four in the Netherlands. Back then, seven females were kept alone in their enclosure, X008 was kept together with her mother, X009 with other females and males, X010 with a variable number of other females, X011 with one other female and X013 with a male (Table 1). It is unknown whether—although it is suspected to be unlikely that—X011 was brought together with a male. Thus, to test the predictions derived from the captive reproductive suppression hypothesis, seven females were classified as being kept alone and three females (X008, X009, X010) as being kept with other females. X011 and X013 were excluded from this analysis.

After the collection of faecal samples from these females was completed, two of the nulliparous females (X010 and X007) and one female that had previously given birth to one litter (X006) gave birth to a litter 5.7, 7.3 and 20.3 months later, respectively (Table 1). Thus, to test the predictions derived from the ARA hypothesis, four females were classified as nulliparous and eight females as parous.

All cheetahs were zoo-born animals, fed with whole prey or beef meat and had water available ad libitum. Enclosure sizes and substrate in the facilities differed, but all animals had outdoor exhibits with natural fluctuations of daylight and huts or smaller facilities as indoor enclosures.

All procedures undertaken for this study were approved by the Ethics Committee on Animal Welfare of the Leibniz Institute for Zoo and Wildlife Research (IZW).

Collection of faecal samples

Faecal samples were collected between February 2010 and May 2011 with sample periods ranging from 11 days to 23 days for each female. This sampling period is longer than commonly used

Table 2. Identity and origin of cheetah females (♀), their age, social group composition in the enclosure, including number and age of offspring, at the time when the collection of faecal samples commenced (between February 2010 and May 2011, depending on the zoo [zoological garden]).

♀ ID	Studbook number	Zoo	Age (years)	Conspecifics in same enclosure at start of faecal sample collection				
				♀	♂	Offspring		
						n	Age (months)	
X002	2147	A	3.9	-	-	4	8.5	
X004	2144	B	4.0	-	-	4	3.4	
X005	1853	C	7.9	-	-	2	16.1	
X006	2088	C	4.8	-	-	4	2.9	
X007	2083	C	4.8	-	-	-		
X008	1769	D	9.4	-	1	-		
X009	1881	E	7.8	-	-	-		
X010	2060	F	5.5	-	-	-		
X011	1636	G	11.9	1	-	-		
X012	2137	H	5.6	-	-	-		
X013	1883	I	8.0	-	1	-		
X014	1979	F	5.8	-	-	-		

to determine levels of fGCM concentrations in feline species (Terio et al. 1999, 2004; Wells et al. 2004; Wielebnowski et al. 2002b). Faecal sampling was conducted at a random time period in relation to the age of the females and their potential breeding activities. The collection of faecal samples commenced when these females were between 3.9 and 11.9 years of age, with a mean of 6.6±2.4 years (Table 2).

Enclosures were checked for fresh faeces daily in the morning or in the morning and afternoon during husbandry routines. Eight to 24 faecal samples were collected per female for the measurement of fGCM concentrations. When more than one animal was kept in an enclosure (Table 2), food for the female in focus was prepared with blue food colorant (brilliant blue, FCF, Sensient Food Colors Europe, Geesthacht, Germany) to permit the allocation of faeces to the correct individual. All faecal samples were homogenised and frozen at -20 °C directly after collection and stored at -80 °C at the IZW until analyses were conducted.

Extraction of faeces and enzyme immunoassay (EIA)

First, 0.5 g of wet faeces were dried for 22 h in a freeze drier (EPSILON1-4, LSC plus, Martin Christ GmbH, Germany). After powdering the dried faeces, 0.1 g of well-mixed powder were extracted with 0.9 ml of 90% methanol with gentle shaking on a horizontal shaker for 30 min. After centrifugation at 3000 rpm for 15 min, the supernatant was transferred into a new tube and diluted 1:1 with water, and aliquots of 20 µl were subjected to the EIA to measure concentrations of fGCM.

The methanol extracts were analysed with a corticosterone-3-CMO immunoassay that was proven highly sensitive for measuring fGCM in cheetahs (Ludwig et al. 2013). The antibody of the EIA was polyclonal and raised in rabbit against corticosterone-3-CMO-steroid coupled with bovine serum albumin (BSA). The corresponding 3-CMO-peroxidase was used as label for the EIA (Ludwig et al. 2013).

Data analysis

Due to the small sample sizes non-parametric tests were conducted, that is, Mann-Whitney U and Fisher exact tests. Power analyses were performed for some of the non-significant results to ask what statistical power there was to discover a difference if there really was one. The calculations of the statistical power took into account how large the observed effect sizes were and considered the sample size or the degrees of freedom for each statistical test. For this purpose a general model was applied for power analysis developed and presented by Murphy et al. (2014), using their One-Stop-Calculator (Murphy et al. 2014, tab 'eResources' at the book's website, https://www.routledge.com/products/9781848725881). All other statistical analyses were performed with Systat 13 (Systat Software, Inc., San Jose, California, USA). The level of significance was set at 5% and all tests were two-tailed. All mean values are given with standard deviations (SD).

Results

Captive 'stress' hypothesis

The mean fGCM concentration of all 12 females was 1049.3±575.4 ng/g. There was no difference of fGCM concentrations between parous and nulliparous females (Mann-Whitney U test, n_{parous} 6, $n_{nulliparous}$=6, U=14, P=0.52, Table 1, Table 3). The result did not change when the two females that gave birth for the first time 5.6 and 7.3 months after collection of faecal samples were categorised as parous (n_{parous}=8, $n_{nulliparous}$=4, U=11, P=0.40). The power of finding a difference if there really was one was modest to moderate (0.121 for the equivalent two-sample t-test for the first comparison, 0.197 for the second), which was not surprising given the modest differences (effect size) observed between the two groups.

The pregnant female X012 had a mean fGCM concentration that was 4.0 times higher than the mean fGCM concentrations of the other 11 females (mean±SD: 840.3±435.0 ng/g). Exclusion of X012 from the analyses did not change the result (n_{parous}=5, $n_{nulliparous}$=6, U=14, P=0.86; n_{parous}=7, $n_{nulliparous}$=4, U=11, P=0.57). The power of finding a difference if there really was one was again modest to moderate (0.051 for the equivalent two-sample t-test for the first comparison, 0.103 for the second), which again was not surprising given the modest differences (effect size) observed between the two groups.

Captive reproductive suppression hypothesis

Four females were housed together with one or more other female(s) before collection of faecal samples commenced (X008, X009, X010, X011, Table 1). The managers of the facilities tried to breed X008, X009 and X010 with males when the females were thought to be in estrus, based on behavioral signs such as rolling, in these housing conditions. X011 is unlikely to have been brought together with a male. None of them produced litters while being housed together with other females. X008 was kept together with her mother until the latter died and from then on, at the age of 8.9 years, was permanently kept with a male. She never reproduced until she died at the age of 10.2 years. Female X009 was kept together with various numbers of females and males and was introduced alone to a single male for the first time at the age of 8.6 years, but did not reproduce until she died at the age of 12.9 years. Female X010 was kept together with several different females between her age of 2.6 years and 5.0 years, with several breeding attempts starting at the age of 2.6

Table 3. Number of faecal samples collected per cheetah female (♀), mean, SD and peak of faecal glucocorticoid metabolite (fGCM) concentrations in dry matter.

♀ ID	n	FGCM (ng/g)		
		Mean	SD	Peak
X002	8	924.3	297.0	1235.9
X004	11	1068.2	489.5	2108.2
X005	12	1034.5	406.9	1972.3
X006	18	711.2	330.5	1167.3
X007	16	1414.7	698.5	2813.0
X008	11	744.6	637.9	1971.5
X009	18	937.9	816.1	2777.1
X010	24	623.3	393.7	1661.7
X011	11	801.7	364.0	1289.9
X012	14	3348.3	2120.4	6491.7
X013	13	590.8	148.6	846.0
X014	20	392.5	201.9	778.1

years, but never reproduced. From the age of 5.0 years onwards, X010 was kept alone and at the age of 5.2 years was introduced alone to a single male for the first time, which was three months before the period of faecal sampling began. She was regularly introduced alone to the male after he advertised by vocalisations that she was in oestrus and she showed normal signs of oestrus in this constellation. She conceived her first litter at the age of 5.9 years. Another female (X013) was permanently kept together with a male between her own ages of 3.7 and 15.7 years (when the monitoring period ended) and never reproduced. Females housed on their own with no other adults present (n=7) had a much, and significantly, higher chance of successful reproduction (100%) than females jointly housed together with other females (n=3, 0%; Fisher exact test, P=0.0083).

Asymmetric reproductive aging hypothesis

Seven of eight parous females (87.5%) conceived their first litter when they were younger than 5.6 years of age, that is, the mean age when captive females in Namibia developed pathologies on their reproductive tracts (Wachter et al. 2011). The eighth female (individual X010) conceived her first litter at the age of 5.9 years (Table 1). Two females had also conceived at the age of 6.3 years (individuals X005 and X006) and were both, as predicted, pluriparous.

Two of the four nulliparous females were brought into a breeding situation, that is, an encounter between a single female and a single male without other cheetahs in the enclosure, for the first time at the age of 8.9 years (individual X008) and 8.6 years (individual X009), respectively (Table 1). Both females did not conceive until they died at 10.2 years and 12.9 years, respectively. Female X013 was 8.0 years old when faecal samples were collected for this study. She had been permanently kept together with a male since she was 3.7 years old and had never reproduced until the end of the monitoring period when she was 15.7 years old. From the studbook, it is not known whether female X011 was ever brought into a proper breeding situation, but she was 11.9 years old when faecal samples were collected for this study and had never reproduced until she died at the age of 12.2 years (Table 1). The association between presence (or absence) of successful reproductive activity if the first potential sexual encounter was initiated before (or after) the age of 5.6 years was highly unlikely to result from chance alone (Fisher exact test, P=0.01).

Necropsy reports were available from four females that died (X004, X008, X010, X011, Table 1), with information on pathologies of reproductive organs from three females (X008, X010, X011). The two nulliparous females X008 and X011 died at the age of 10.2 years and 12.2 years. X008 showed no pathologies on the reproductive tract, whereas X011 showed paraovarian and uterine cysts. The parous female X010 died at the age of 8.2 years and had paraovarian cysts. The parous female X004 died at the age of 7.1 years and was diagnosed with chronic gastritis and enteritis, whereas the nulliparous X008 was diagnosed with gastritis and nephritis.

Discussion

Low reproductive performance in zoological gardens can be problematic in species that are vulnerable or endangered in the wild (Arnold 1995; Brown et al. 2004; Marker and O'Brien, 1989; Marker-Kraus and Grisham 1993). Breeding and re-introduction programmes rely on reproductively healthy females (and males). It is therefore crucial to understand the mechanisms of low reproductive performance in captivity (Brown et al. 2004, Crosier et al. 2011; Saunders et al. 2014; Walker et al. 2004). Asymmetric reproductive aging (ARA) has been previously described in females of African and Asian elephants, as well as for white rhinoceros kept in zoological gardens (Hermes et al. 2004, 2006; Hildebrandt et al. 2000), in female domestic horses (*Equus caballus*) (Hinrichs 1997), female laboratory rats (*Rattus norvegicus*) (Sopelak and Butcher 1982), captive cheetah females in Namibia (Wachter et al. 2011) and free-ranging Sumatran rhinos (*Dicerorhinus sumatrensis harrissoni)* (Kretzschmar et al. 2016).

Our findings are consistent with the ARA hypothesis and our previous study in that the main factors for reproductive health in cheetah females are age and reproductive history. In one of the parous females, paraovarian cysts were detected during necropsy. Such cysts have been demonstrated not to hamper reproduction (Munson 1993; Wachter et al. 2011). In the nulliparous female, paraovarian and uterine cysts were found during necropsy, with the latter known to hinder reproduction in severe cases (Munson 1993), but this case was a mild one. This female was 12.2 years old when she died and it was not known whether she was ever put into a breeding encounter as a single female with a single male.

Most captive cheetahs in zoological gardens are managed by a breeding programme (Versteege 2013) providing recommendations to exchange animals between different institutions for breeding and conservation purposes to preserve genetic variability and prevent inbreeding. Due to logistical reasons, including official regulations on epizootic diseases, the transport of an animal from one facility to another can sometimes be delayed for months or even years. As a result, captive cheetah females often do not breed early in their life. This is unfortunate since early pregnancy and lactation are protective mechanisms against ARA, as demonstrated in several species such as African and Asian elephants, white rhinoceroses, cheetahs and tigers (*Panthera tigris*) (Hermes et al. 2004, 2006; Hildebrandt et al. 2000; Penfold et al. 2014; Sounders et al. 2014; Wachter et al. 2011).

The present findings are also consistent with the captive reproductive suppression hypothesis. Similar to other studies, females kept together with one or more other females did not reproduce (Kinoshita et al. 2011; Wielebnowski et al. 2002a). One of these females (X010) after being kept together with several other females until the age of 5.0 years, was introduced for the first time by herself to a single male during estrus and then produced a litter, demonstrating that the non-reproducing state can be reversible (Wielebnowski et al. 2002a).

The mechanism of reproductive suppression remains unclear. In mammalian species for which reproductive suppression is not a standard life history stage (Hofer and East 1998), suppression might be linked to agonistic behavior of behaviorally incompatible females (Wielebnowski et al. 2002a). In the wild, female cheetahs are solitary and only accompanied by their dependent offspring (Caro 1994; Marker 2002), thus they might not cope well when permanently housed with other females. Although two or more cheetah females kept in one enclosure do not show open aggression towards each other, such unnatural social grouping can sometimes result in agonistic interactions (Wielebnowski et al. 2002a).

One female (X013) was permanently kept together with a male and never produced a litter. With free-ranging cheetah males and females living separately except when mating (Caro 1994; Marker 2002), it might be likely that not enough sexual interest and arousal can be generated when both sexes are permanently kept together. The complex neuro-chemical system that controls the mechanisms necessary for mating behavior (Holstegge and Huynh 2011) is likely to be adversely affected by such housing situations.

Allostatic load in captivity may have many sources, although zoological gardens attempt to imitate the natural environment and many improvements in husbandry were implemented during the last decades (Hoage and Deiss 1996; Kuehn 2002; Morgan and Tromborg 2007; Shepherdson and Mellen 1998). Possible stressors

might be nearby enclosures with natural predator species (Mellen 1991; Rawlins 1972; Wielebnowski 1998), artificial light, exposure to unnatural sounds or odors, uncomfortable temperatures or disturbance by visitors (Morgan and Tromborg 2007; Wielebnowski et al. 2002b). Yet the observed differences of dry fGCM concentrations between parous and nulliparous females in our population were not significant, although the power of finding a difference if there really was one was modest. One female had very high fGCM concentrations. Her pregnancy might have been responsible for inducing a high glucocorticoid level, as is well known for other species (Doerr et al. 1989; Raeside and Ronald 1981). A longer time period of faecal sample collection might have revealed a clearer result, and also additional measures to quantify allostatic load (Edes et al. 2018). Gastritis, a disease that develops in cheetahs under stressful conditions (Munson et al. 2005), was also found in parous and nulliparous females, suggesting no negative effect on reproduction, but also here, additional data would be valuable to further investigate this connection. Thus, with a caution note, the captive 'stress' hypothesis is unsuitable to explain the lack of reproduction in these cheetah females.

Conclusions

1. The present study suggests that females should be bred by the age of 5.6 years to maintain fertility for many years and to avoid the onset of asymmetric reproductive aging.
2. The study also suggests that females are more likely to come into oestrus when kept alone and only then should they be introduced to single males for breeding purposes.
3. This approach is likely to increase the reproductive performance and health of cheetah females in captivity and therefore increases the chances of success for breeding and re-introduction programmes.

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CHAPTER 5: GENERAL DISCUSSION

The main outcome of this thesis was that the regulalrly detected poor reproductive performance of female cheetahs in European zoos was strongly and directly linked to the breeding management of this species. Females which were bred early in life showed better reproductive performance and less pathologies on their reproductive organs than females which were brought into their first breeding situation late in life (Ludwig et al. 2019). This phenomenon is known as “asymmetric reproductive aging” (ARA) (Hildebrandt et al 2000, Hermes et al 2004, Hermes et al 2006). It was also important for the breeding success to keep females alone in the enclosure before they were introduced to a male for mating (Ludwig et al. 2019). This was because females in the same enclosure can induce reproductive suppression in other females and males in the same enclosre might induce a lack of sexual arousal in females (Ludwig et al. 2019). Allostatic load was no factor that affected the reproductive performance of females. This was measured with a newly validated enzyme immunoassay (EIA) to measure glucocorticoid metabolites in cheetah feces.

5.1 Measuring allostatic load (“stress”) in cheetah feces

In our study we verified a new EIA to measure fecal glucocorticoid metabolites (fGCM) in cheetahs. We demonstrated with the injection of radioactive ^{3}H labelled cortisol the high affinity to immunoreactive fGCM in a male and a female cheetah in two German zoos. Furthermore, we validated the EIA with ACTH challenges in two and five captive cheetahs in Germany and South Africa, respectively. The antibody we used (corticosterone-3-CMO) was similar to an antibody used in a previously validated RIA often used in studies including cheetahs (Wasser et al. 2000, Wielebnowski et al. 2002b, Terio et al. 2004, Young et al. 2004). We demonstrated that this corticosterone EIA is a very suitable alternative to the RIA which

works with radioactive substances and thus requires specialized facilities and cannot be universally applied. An EIA can be used anywhere, particularly when facilities prefer not to work with radioactive substances. We measured a 2.7 to 11.9 fold increase of fGCM compared to the baseline fGCM after ACTH challenges (Ludwig et al. 2013). This is similar to the increase detected with the RIA in cheetahs (11.5 fold increase) (Young et al. 2004) and in other species (Wielebnowski et al. 2002b, Young et al. 2004). This indicates that this EIA is as reliable as the RIA in measuring fGCM in cheetahs. We also tested three other EIA with antibodies against other conjugates of corticosterone and cortisol (cortisol-3-CMO EIA, corticosterone-21-HS EIA, cortisol-21-HS EIA). These assays showed no increase in fGCM after ACTH challenges (Ludwig et al. 2013), although in a previous study an increase of 11.5 fold was demonstrated with a cortisol-3-CMO EIA (Young et al. 2004). This demonstrates that EIAs measuring with the same antibody might lead to different results in different laboratories.

Species differences need to be taken into account before an EIA is applied that measures fGCM and was developed for another species. The EIA must be validated for every species because there might be species-specific differences in metabolites. For instance, different species might digest or process native hormones in different pathways, which may make a particular EIA suitable for application in one species but unsuitable for another species. For example, the cortisol-3-CMO EIA successfully used in cheetahs in Young et al. (2004) did not detect an increase of fGCM in clouded leopards (*Neofelis nebulosa*) in the same study and the cortisol-3-CMO EIA that did not work for cheetahs in our study (Ludwig et al. 2013) was identified as a working well in spotted hyenas (Benhaiem et al. 2012).

The increase of fGCM in feces was detected with a delay of 24 to 48 hours in our study (Ludwig et al. 2013) which matches the normal gut passage time in this species and is consistent with previous studies (Young et al. 2004). We stored the fecal samples collected

within one hour after defecation directly at -20°C. Under field conditions, however, this is often not practicable. We therefore investigated how quickly the fGCM concentrations changed over time at temperatures between 0°C and 4°C, simulating the temperature in a cool box taken to the field. Although the variation of the measured fGCM concentrations increased with storage time, the fGCM did not degrade within 22 hours, the maximum time of this stability experiment. Several factors such as UV-light, sunshine, rain, bacterial activity and insect digestion of feces can influence fCGM concentration before collection and might lead to unreliable results (Touma and Palme 2005). In case of missing freezing capacities, fecal samples can also be stored in ethanol at ambient temperatures (Terio et al. 2002). Additional variation might be caused by factors affecting the production of fGCM in the digestion system of cheetahs such as the microbiome in the gut or individual differences in liver metabolism or steroid hormone metabolism. Such factors might be a reason for slightly different fGCM excretion patterns between our study animals. One male exhibited two small fGCM peaks whereas all other study animals exhibited one high peak (Ludwig et al. 2013). Another indication that there might be individual differences in digestion and/or metabolisation of hormones was the observation that four cheetahs with similar native cortisol concentrations in the blood after ACTH challenges (29.1 ± 1.8ng/ml) had substantial differences in their fGCM concentrations (Terio et al. 1999).

Our EIA showed some cross-reactivity (2%) with progesterone (Ludwig et al. 2013). This might affect fGCM values in pregnant females with high levels of progesterone in blood and its metabolites in feces (Adachi et al. 2011). Other steroids did not cross-react with our EIA. This was particularly encouraging, as cross-reactions with testosterone are a frequent occurrence in enzyme validation studies of other EIAs, and might affect fGCM measurements

in male cheetahs. This topic was part of chapter 3 (Pribbenow et al. 2016) and is discussed in the next section.

5.2 Measuring testosterone metabolites in cheetah feces

The validation of an EIA to measure fecal testosterone metabolites (fTM) in cheetahs was an important step in our study to demonstrate that there was no cross-reaction with our EIA for fGCM (Ludwig et al. 2013). In other species, for example in spotted hyenas or in African savanna elephants, cross-reactions between fGCM and fTM have been demonstrated (Ganswindt et al. 2013, Pribbenow et al. 2015). We validated an epiandrosterone (epi-A) EIA with a GnRH challenge, a testosterone challenge and a ^{3}H labelled testosterone injection, and detected the expected peaks in fTM 24 hours after injection (Pribbenow et al. 2016). Epi-A is the major fecal testosterone metabolite in cheetahs as demonstrated in HPLC analyses of radiolabelled fTM (Pribbenow et al. 2016). Using fecal samples from the ACTH challenge experiment (Ludwig et al. 2013) we demonstrated that there was no cross-reaction with fGCM.

The fTM were stable under different conditions of sample storage for up to 72 hours, the maximum time of the stability experiment (Pribbenow et al. 2016). This is important because it is often not possible to obtain fresh fecal samples in the field or from animals in zoological gardens. Fecal samples are often exposed to various environmental factors before they are frozen and analyzed in the laboratory, which may influence fecal metabolite measurements (Wasser et al. 1988, Terio et al. 2002, Abaigar et al. 2010, Hodges and Heistermann 2011). We therefore measured fTM concentration over time in samples being exposed to low ambient temperatures in Germany and high ambient temperatures in Namibia and demonstrated that fTM did not degrade during the first 3 days (Pribbenow et al. 2016).

The epi-A EIA can also be a useful tool to determine the reproductive status of captive male cheetahs and the influence of different husbandry conditions and social organisation on testicular activity. Although some cheetah males live in groups of two to four males in the wild (Marker 2002, Caro 2014, Melzheimer et al. 2018), it could be that within these groups males establish a dominance hierarchy and subordinate males in these groups may become reproductively suppressed by the more dominant males. If so, some males kept in groups in captivity might not become fertile and would become unsuitable for reproduction in the facility. In a group of three cheetah males in a German zoo, only one male was fully sexually active, whereas the others had smaller testes sizes and lower sperm quality (I Lueders, unpublished data). This is interesting but also inconsistent with previous studies where androgen concentrations were higher and ejaculate quality was better in group-housed cheetah males (Koester et al. 2017).

Our study did not reveal differences in fTM between one adult and one juvenile male cheetah in a German zoo (Pribbenow et al. 2016). This might be due to the low sample size in our study, although previous studies showed that testosterone concentrations of captive adult males were significantly lower than those of their free-ranging conspecifics in Namibia (Terio et al. 2004). We therefore tested our epi-A EIA with faeces from 76 free-ranging adult male cheetahs, juvenile male cheetahs and adult female cheetahs and measured high, low and very low fTM concentrations for adult males, juvenile males and adult females, respectively (Pribbenow et al. 2016). Thus, our EIA is suitable to measure fTM in male cheetahs.

5.3 Cause(s) of low reproductive performance in captive female cheetahs

We found no significant differences in fGCM concentration between reproducing and non-reproducing females, or between parous and nulliparous females (Ludwig et al. 2019). This

demonstrates that the “captive stress hypothesis” does not explain the low reproductive performance of female cheetahs in European zoological gardens. As it is known that North American captive cheetahs might have higher allostatic loads than free-ranging ones (Terio et al. 2004), our result implies that female cheetahs can reproduce successfully even when stressed. Zoological gardens invested much research-based knowledge and money to improve husbandry conditions for cheetahs and other animal species in exhibits (Hoage and Deiss 1996, Kuehn 2002, Morgan and Tromborg 2007, Hosey et al. 2013). There are still several possible stressors which might cause chronic high allostatic loads, such as exposure to unnatural light and sound, particularly in urban zoological gardens, temperatures and humidity that differ from those in natural habitats or repeated disturbance by visitors (Wielebnowski et al. 2002b, Morgan and Tromborg 2007). Furthermore, in zoological gardens, natural predators such as lions, spotted hyenas or leopards are often kept close to cheetah enclosures, or can be heard or smelled by cheetahs, which may be stressful to cheetahs (Rawlins 1972, Mellen 1991, Wielebnowski 1998).

One female in our study was pregnant during the time fecal samples were collected and had much higher values of fGCM than other females (Ludwig et al. 2019). Pregnancy is known to be a cause of high allostatic load also in other species such as harbour seals (*Phoca vitulina*) and humans (Raeside and Ronald 1981, Doerr et al. 1998). During pregnancy, blood concentrations of progesterone, a steroid hormone very similar to glucocorticoids, are constantly high (Adachi et al. 2011). Therefore, cross-reactions of our EIA with progesterone metabolites are a possible reason for the high fGCM concentrations. We think this is unlikely because our EIA was validated against other steroid hormones and showed a minimal cross-reaction with progesterone (Ludwig et al. 2013). Also, in the HPLC analysis, no immunoreactive

peaks at the locations of typical fecal progesterone and other gestagen metabolites in cheetah were detected (Ludwig et al. 2013).

Predictions derived from the “innate rhythm hypothesis” and “genetic monomorphism hypothesis” were already rejected in previous studies (Kieser and Groeneveld 1991, Castro-Prieto et al. 2010, Wachter et al. 2011). They were not tested again in this study as all study animals came from very similar husbandry conditions.

The “asymmetric reproductive aging (ARA) hypothesis”, which is linked to the breeding management, and the “captive reproductive suppression hypothesis”, which is linked to social husbandry conditions, were investigated in this thesis to understand the mechanisms of low reproductive performance of captive female cheetahs. ARA is recognised to be a serious problem in other species managed in zoological gardens such as Asian or African savanna elephants (Hildebrandt et al. 2000), white rhinoceros (Hermes et al. 2004, 2006) or domestic horses (Hinrichs 1997). It is an irreversible process once it has started, so that the individuals concerned are lost as potential breeders for the institutions and breeding programs (Hermes et al. 2004, 2006). Breeding programs are in charge to make suggestions for animal transfers between institutions to prevent late breeding in females (Schwammer and Fruehwirth 2015, van Wees and Damen 2015). The organization and coordination of such transfers can be time consuming and take years until all the paperwork and pre-shipment health testing is done. Such breeding programs are also in charge of the captive cheetah populations in zoological gardens and face similar time constraints (Marker and O´Brien 1989, Versteege 2013). As a consequence, it is likely that animals are offered an opportunity to breed later in life than under natural conditions in the wild (Laurenson et al. 1992). This can become a problem for females, since lactation and pregnancy can protect against ARA as shown in other species (Hildebrandt et al. 2000, Saunders et al. 2014). Our findings on female cheetahs in European

zoos were consistent with the predictions from the ARA hypothesis and in line with previous studies conducted in captive female cheetahs on Namibian farmland (Wachter et al. 2011). During necropsies we also identified more pathological lesions in the reproductive organs of nulliparous than parous females. In one female with a positive breeding history we found paraovarian cysts which, however, do not hamper reproduction (Munson 1993, Wachter et al. 2011). In study species experiencing very low population densities such as the Sumatran rhinoceros (*Dicerorhinus sumatrensis harrissoni*), ARA has also been described for free-ranging females (Kretzschmar et al. 2016). In this situation, home ranges of males and females do not overlap anymore due to anthropogenic factors such as habitat loss or fragmentation or poaching. This resulted in females meeting mating partners only rarely, preventing them to come into a breeding situation early in their life and thus developing irreversible pathologies of their inner reproductive organs from regular estrogen impact due to uninterrupted cycling (Kretzschmar et al. 2016).

Reproductive suppression was described in captive female cheetahs in previous studies (Wielebnowski et al. 2002a, Kinoshita et al. 2011). In contrast to ARA, this phenomenon is reversible and females will start to cycle again once the females are separated from other cheetahs (Wielebnowski et al. 2002a). We confirmed this phenomenon for females permanently or mainly housed together with other females. They did not reproduce when introduced to males to mate. One female was brought into a breeding situation alone with a male later in life after being kept alone for a while and this female successfully reproduced (Ludwig et al. 2019). Deviation from natural social structures such as being permanently housed together with adult female conspecifics leads to agonistic interactions which possibly suppress cycling but the actual mechanism is not yet fully understood (Wielebnowski et al. 2002a). When females are permanently kept together with males, they did not conceive in

our study or in a previous study (Wielebnowski et al. 2002a, Ludwig et al. 2019). In these cases, no agonistic interactions were observed, but perhaps not enough sexual arousal was produced to establish a successful breeding situation. In such housing situations the complex neuro-chemical system important for mating behavior (Holstegge and Huynh 2011) might be disturbed. The breeding and re-introduction programs of European zoos can only be successful with healthy and reproducing animals to support the vulnerable populations in the wild. It is therefore crucial to optimize the management of breeding programs following the latest scientific results.

5.4 Conclusion and perspectives

Free-ranging animals and their conspecifics under human care live in an environment under continuous changes through human influence. Hormones regulate many important body functions and are major players in helping animals to adapt to these changing circumstances. With the EIA we characterized and validated a powerful assay to non-invasively measure allostatic load in cheetahs. We found no significant differences between breeding and non-reproductive females. This is promising because much has already been done in the past to improve housing conditions of zoo animals in modern zoological gardens. Our study results were consistent with the predictions of the “asymmetric aging hypothesis“ and the “captive reproductive suppression hypothesis”. Thus, studbook coordinators and curators should try to transfer cheetahs early in life and bring them into a breeding situation as soon as they are sexually mature. Furthermore, a social management compatible with social structures in the wild and a breeding situation in which one cat and one tomcat have the chance to mate in a separate enclosure might optimize the chances of breeding in this rare and sensitive species. These recommendations might increase the breeding success of cheetahs of the European

breeding program (EEP). It is essential to record as much information as possible to allow retrospective studies of the reproductive history of as many cheetahs as possible.

We showed that the epi-A EIA provides a powerful and non-invasive tool to evaluate testicular activity in male cheetahs. This assay permits future studies of the impact of life history parameters and environmental conditions on reproductive activity in male cheetahs. It remains unclear which role the management of males play for a successful breeding. More research on testosterone secretion levels of males kept singly or in groups is recommended to understand the mechanisms of possible sexual suppression in males.

Both EIA that we characterized and validated measured reliable results and we demonstrated the stability of fGCM and fTM in feces over many hours. It is not yet known how stable samples really are when collected several hours or even days after defecation in other, more tropical ambient temperatures. In zoological gardens it is possible to determine the approximate time of defecation relatively reliably because of management routines, thus various situations could be simulated to test the stability of fecal hormone metabolites.

This study demonstrates that research in zoological gardens can contribute to understand mechanisms affecting reproductive health and contribute to conservation efforts of modern zoos. Some environmental factors will always differ between captive and free-ranging animals. Therefore, it is crucial to study both captive and free-ranging animals and to use all available knowledge to improve the conditions for animals kept in our care. Non-invasive hormone measurements give us the opportunity to improve our understanding of physiological and pathological responses to stress and will thus be beneficial for the conservation of this species.

ZUSAMMENFASSUNG

Nicht invasive Bestimmungsmethoden von Steroidhormonen und deren Anwendung bei Geparden (*Acinonyx jubatus*) in europäischen Zoos

Die bisherigen Erfolge in der Züchtung von Geparden (*Acinonyx jubatus*) in menschlicher Obhut sind bis heute nicht zufriedenstellend. Seit Jahrzehnten wird an den Ursachen dieser Problematik geforscht. Bis vor kurzem ging man noch davon aus, dass der genetische Flaschenhals, durch den die Population entstehungsgeschichtlich gegangen ist, maßgeblichen Anteil an dem Phänomen hat. Untersuchungen im Freiland zeigten jedoch, dass freilebende Geparde regelmäßig und problemlos reproduzieren, wenn sie in Ökosystemen leben, in denen keine Löwen (*Panthera leo*) und Tüpfelhyänen (*Crocuta crocuta*) vorkommen, die ihre Jungtiere töten. Davon unterscheiden sich die Populationen in den Zoos weltweit entscheidend. Hier kommt es selten zu erfolgreichen Nachzuchten, obgleich Löwen und Tüpfelhyänen als Mortalitätsursache der Jungtiere ausscheiden. Verschiedene Gründe wurden hierfür untersucht. Unter anderem wurde vermutet, dass die unterschiedlichen Lebenssituationen der Tiere im Freiland und in Zoos zu einer unterschiedlichen Belastung führen, und somit die erhöhte Allostase („Stress“) in begrenzter und gemanagter Umgebung zu einer schlechteren Reproduktion führt. Allerdings gelingt es einigen Einrichtungen immer wieder verlässlich, mit Geparden Nachzuchterfolge zu erzielen. Auch wenn die Stressbelastung in zoologischen Gärten höher als im Freiland sein sollte, ist zu erwarten dass sich die erfolgreich züchtenden Haltungen in einem oder mehreren Faktoren von den nicht erfolgreich züchtenden Haltungen unterscheiden.

Diese Dissertation wurde durchgeführt, um diese Faktoren zu ergründen und gegebenenfalls Empfehlungen zur besseren Erhaltungszucht dieser bedrohten Tiere in Zoos zu ermöglichen. Hauptaugenmerk lag auch in dieser Arbeit zuerst auf der Untersuchung der

allostatischen Belastung der Zootiere. Die Konzentration von Glukokortikoiden im Körper wird gewöhnlich als ein Maß für den Stress bei Wirbeltieren verwendet. Blutabnahmen unter Narkose führen allerdings binnen kürzester Zeit zu einer akuten Erhöhung dieser Hormone im Blut, die keinen Rückschluss auf vorausgehende Werte mehr zulassen. Daher sollte die Probennahme berührungsfrei erfolgen, um Handling-Effekte bei der Messung der Glukokortikoid-Messwerte zu vermeiden. Hierzu eignet sich bei Katzen besonders der Kot, um die Metabolite der Glukokortikoide zu messen. Bisher gab es keinen für Geparde validierten Enzyme-Immunoassay (EIA), der die fäkalen Glukokortikoid-Metabolite (fGCM) messen konnte und sie auch charakterisierte, sowie die Affinität des EIA bestimmte. Die vorhandenen Radio-Immunoassays (RIA) werden auf Grund der Radioaktivität oft nur ungern eingesetzt. Daher wurde in **Kapitel 2** ein neuer EIA für Geparde entwickelt und technisch wie biologisch validiert und charakterisiert. Der EIA dokumentierte erfolgreich eine erhöhte Konzentration von fGCM nach der experimentellen Zuführung von adrenocorticotropen Hormon (ACTH) bei einem männlichen und einem weiblichen Gepard. Diese experimentelle Zuführung von Hormonen mit dem Nachweis eines erwarteten Ergebnisses wird als „Hormonchallenge“ bezeichnet und ist die hierfür allgemein akzeptierte Form einer biologischen Validierung. ACTH führt über eine Aktivierung der Nebennierenrinde zu einem schnellen Anstieg von Glukokortikoiden im Blut. Dieser Anstieg ist mit Verzögerung von etwa einem Tag dann auch im Kot nachweisbar.

Glukokortikoide (wie z.B. Cortisol) gehören zu den Steroidhormonen. Da sich Cortisol-Metabolite nur unwesentlich von anderen Steroidhormon-Metaboliten (wie z.B von Testosteron) unterscheiden, war eine eindeutige Abgrenzung zu den fGCM im Kot wichtig. **Kapitel 3** beschreibt einen zweiten entwickelten EIA, der Testosteron-Metabolite im Kot detektiert. Dieser unterstreicht nicht nur die gut trennbare Messung von Testosteron-

Metaboliten zu anderen Steroidhormon-Metaboliten, sondern könnte in Zukunft auch Aussagen über die reproduktive Aktivität männlicher Tiere berührungsfrei erlauben. Auch der Testosteronassay wurde mit Hormonchallenges biologisch validiert. So wurde ein erhöhter Testosteronanstieg nach der Injektion von Gonadotropinreleasinghormon (GnRH) nachgewiesen. GnRH bewirkt in der Hypophyse eine Freisetzung von Gonadotropinen (Follikel stimulierendes Hormon (FSH), Luteinisierndes Hormon (LH)), die wiederum auf die Geschlechtsorgane wirken. LH bewirkt beim männlichen Säuger durch Aktivierung der Leydig-Zellen der Hoden u.a. einem Anstieg von Testosteron im Blut. Dieser war im Kot nachweisbar.

Kapitel 4 verglich fGCM von reproduzierenden und nicht reproduzierenden weiblichen Geparden, um zu untersuchen, ob die beiden Gruppen unterschiedliche Stresswerte aufwiesen, was nicht der Fall war. Es war zudem bereits bekannt, dass Schwierigkeiten in der Reproduktion in menschlicher Obhut auch nicht durch die geringe genetische Variabilität der Geparde verursacht wurde und es keine(n) endogene(n) jahreszeitlichen Umweltsignale (Zeitgeber) gibt, der/die die Reproduktion auslöst/en. Daher wurden die Vorhersagen zweier weiterer Hypothesen untersucht, um zu verstehen, welche Faktoren einen Einfluss auf den Fortpflanzungserfolg von Gepardenweibchen haben könnten. Dies waren die Hypothesen (1) fehlende Fortpflanzung aufgrund der reproduktiven Unterdrückung durch Artgenossen, 2) fehlende Fortpflanzung aufgrund der beschleunigten Alterung der inneren Geschlechtsorgane (ARA = *asymmetric reproductive aging*) bei Weibchen, die sich nicht in jungen Jahren fortpflanzen. Die lebensgeschichtlichen Daten bezüglich der reproduzierenden und nicht reproduzierenden weiblichen Geparde in fünf zoologischen Gärten in Europa sind mit den Vorhersagen beider Hypothesen kompatibel.

Die Diskussion der gesamten Doktorarbeit und der neuen Erkenntnisse behandelt **Kapitel 5.** Darin werden auch Empfehlungen für die zoologischen Gärten und das europäische

Erhaltungszuchtprogramm (EEP) vorgestellt, damit in Zukunft die unbefriedigenden Nachzuchterfolge dieser Spezies der Vergangenheit angehören.

SUMMARY

Non-invasive detection methods of steroids and its application in cheetahs (Acinonyx jubatus) in European zoological gardens

In captivity, cheetahs (*Acinonyx jubatus*) continue to be a challenge to breed. Scientists have worked for decades to identify the underlying reasons for this problem. Until recently, it was assumed that the genetic bottleneck the cheetah population is assumed to have gone through during its history contributed to its low reproductive performance. However, studies on free-ranging populations showed that cheetahs reproduce regularly and successfully when they live in ecosystems without their main competitors, the lions (*Panthera leo*) and spotted hyenas (*Crocuta crocuta*) which kill their cubs. This is different in zoological gardens where there is no impact of competitors but where successful breeding is still a rare event. Various potential causes for this lack of apparent fertility have been suggested and investigated. One suggestion was that housing and environmental conditions in captive cheetahs may be responsible for a higher allostatic load ("stress") in the zoo animals, which in turn might result in a low reproductive performance. However, some facilities have successfully and regularly bred cheetahs. Even if allostatic load in cheetahs under human care may be higher than in the wild, zoological gardens with high breeding success should differ in one or several factors from those with low breeding success.

This dissertation was conducted to identify these factors and develop recommendations on how to improve the success of cheetah breeding programs in zoological gardens. The first step was the investigation of the allostatic load of study animals in European zoological gardens. The concentration of glucocorticoids in the body is commonly used as a biological marker of the allostatic load of vertebrates. However, the conventional procedure of collecting blood under anesthesia causes itself an immediate increase of glucocorticoids in

the blood which makes an assessment of the true value impossible. In order to avoid any distortions of glucocorticoid concentrations through handling of animals, samples must be obtained in a minimally invasive manner. In feline species, feces are particularly well suited to measure glucocorticoids. Previously, there was no validated enzyme-immunoassay (EIA) available that measured and had characterised fecal glucocorticoid metabolites (fCGM) in cheetahs and that determined the affinity of the EIA. The existing radio-immunoassays (RIA) are often avoided because of the radioactivity involved in the measurements. **Chapter 2** therefore presents the characterization and biological as well as technical validation of an EIA for cheetahs. The EIA demonstrated a significant increase in fGCM after experimental injection of adrenocorticotropic hormone (ACTH) in a male and a female cheetah. This experimental injection of hormones and the proof of the predicted result is called a “hormone challenge” and is equivalent to a biological validation. ACTH activates the adrenal cortex and results in a fast increase of glucocorticoids in the blood. This increase can be detected with a delay of approximately one day in the feces.

Glucocorticoids such as cortisol belong to the steroid hormones. Cortisol metabolites slightly differ in their biochemical structure from other steroid hormone metabolites such as testosterone. Therefore, a clear differentiation of fGCM from other steroid hormone metabolites was essential. In **chapter 3**, another EIA was characterized and validated to detect testosterone metabolites in feces. It also demonstrates the differentiation between fecal testosterone metabolites (fTM) and other steroid hormone metabolites. Further, this EIA allows to conduct minimally invasive measurements on the reproductive activity of male cheetahs. The testosterone assay was also biologically validated with hormone challenges. After injection of gonadotropin releasing hormone (GnRH), an increase of fTM was detected. GnRH causes a release of gonadotropins (follicle stimulating hormone (FSH), luteinizing

hormone (LH)) in the pituitary gland. In male vertebrates, LH activates the Leydig cells in the testis and leads to an increase of testosterone in the blood. This increase was detected in the feces of cheetah males.

Chapter 4 compared fGCM of reproducing and non-reproducing cheetah females in zoological gardens to examine whether they differ in their allostatic load. This was not the case. It was already known that low reproductive performance is not linked to the low genetic variability ("genetic monomorphism hypothesis") in cheetahs nor to an endogenous seasonal trigger ("innate rhythm hypothesis"). Therefore, in this dissertation, the predictions of two other hypotheses were investigated to understand which factors might influence the reproductive success of female cheetahs. The hypotheses investigated were (1) a lack of reproduction because of reproductive suppression by conspecifics, (2) a lack of reproduction because of the faster ageing of inner reproductive organs (ARA = asymmetric reproductive aging) in those females which did not reproduce early in their life. The life history data on reproducing and non-reproducing female cheetahs in five European zoological gardens were consistent with the predictions by both hypotheses.

The discussion of the entire thesis and its scientific results is compiled in **chapter 5**. It also introduces recommendations for zoological gardens and the European breeding program (EEP) to improve the reproductive success of this species in the future and make reproductive failure of cheetah part of history.

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Selbständigkeitserklärung

Hiermit erkläre ich, dass ich die vorliegende Doktorarbeit selbständig und ohne unzulässige Hilfe oder Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe.

Berlin, den 18.09.2019

Carsten Ludwig