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The use of Non-Invasive Stress Biomarkers for Assessing Welfare of Non-domestic Felids in Captivity

Bachelorprojekt i Husdyrvidenskab

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Division of labour

Division of labour has been equally divided and we both vouch for the full context of this project.

Abstract

With the continuous decline in many species of Felidae, the necessity of ex situ conservation is more prominent than ever, yet many species suffer from reproductive difficulties in captivity. This is hypothesised as being a consequence of chronic stress, which in turn also compromises welfare. Thus, it is essential to establish what factors are inducing continuous or chronically elevated cortisol levels and address these.
When measuring cortisol in plasma, the invasiveness and immediate stress response will potentially cause biased data. The relatively new approach of measuring cortisol non-invasively, reduces the need for handling the animals. This makes it a useful approach for free-living as well as captive animals and offers the advantage of not biasing the results, as a consequence of initial stress responses.
Numerous biotic and abiotic factors might cause variations in cortisol concentrations, which encumbers interpretation of data. Thus, to conduct accurate stress and welfare assessments, knowledge of these needs to be implemented.
When examining stress factors in both captive and free-living felids, it becomes evident that these are so distinct that it encumbers comparison and thus, the use of GC levels of the animals themselves as a baseline, has been widely applied.
The few studies comparing the two groups found conflicting results, which encourages further studies, applying this approach and taking the various stress factors into account.
We have found several indicators that captive felids suffer from chronically elevated cortisol levels due to captivity-related stressors, which lead us to conclude that captive felids experience distress to such an extent, that it causes deleterious effects on their physiology and welfare.
It is evident that more knowledge is needed on stress assessment in felids, and we recommend further studies to implement the various factors and to attain more knowledge of the coherence between captivity, welfare and reproductive difficulties, in order to help the ex situ conservation of non-domestic felids, as well as increase welfare of captive felids.

Resumé

Grundet et kontinuerligt fald i antallet af flere arter af kattedyr, er nødvendigheden for artsbevarelse vigtigere end nogensinde. Dog lider mange arter, i fangenskab, under reproduktionsbesvær, hvilket tyder på at være en konsekvens af kronisk stress og kompromitteret velfærd. Det er derfor essentielt, at danne et overblik over hvilke faktorer der inducerer gentagende eller kronisk forhøjede cortisolniveauer og tage højde for disse.
Når cortisol måles i plasma kan det invasive indgreb og øjeblikkelige stressrespons, potentielt være årsag til bias i data. Ved at måle cortisol non-invasivt, hvilket er en relativ ny metode, reduceres behovet for håndtering af dyret ved prøvetagning. Derfor er dette en anvendelig metode til både vildtlevende dyr og dyr i fangenskab og har den fordel ikke at påvirke resultaterne som konsekvens af det øjeblikkelige stressrespons.
Adskillige biotiske og abiotiske faktorer kan forårsage variationer i cortisolkoncentrationerne, hvilket besværliggør fortolkning af data og viden om disse faktorer må derfor implementeres, for at kunne udføre præcise stress- og velfærdsvurderinger.
Ved at undersøge stressfaktorerne i hhv. vildtlevende kattedyr og kattedyr i fangenskab, ses det tydeligt at forskellene mellem disse besværliggør en eventuel sammenligning. Derfor er anvendelsen af dyrets egen GC-baseline en udbredt tilgang i stedet.
Kun få undersøgelser på området har forsøgt at sammenligne kattedyr i fangenskab og i det fri, og disse fandt tilmed modstridende resultater. Dette opfordrer derfor til flere undersøgelser, der anvender denne metode og samtidig tager højde for de forskellige stressfaktorer.
I dette projekt har vi fundet adskillige indikationer på, at kattedyr i fangenskab lider under kronisk forhøjede cortisolniveauer, som resultat af stressorer fra et liv i fangenskab. Dette leder os til den konklusion, at kattedyr i fangenskab er udsat for stress i så høj grad, at det er fysiologisk ødelæggende og reducerer velfærd.
Det er tydeligt, at der er behov for mere viden om stressvurdering hos kattedyr og vi anbefaler at fremtidige studier implementerer de forskellige faktorer, for at tilstræbe mere viden omkring sammenhængen mellem fangenskab, velfærd og reproduktionsproblemer, for dermed at støtte artsbevarelsen af ikke-domesticerede kattedyr, samt at øge velfærden for disse.

Introduction

Since the first modern zoo opened in France in 1793 (national geographic w.y.), the development of living conditions of captive animals has changed from museum-like environments, where animals were kept in small cages with no environmental enrichment, to large enclosures designed to mimic the natural habitats of the animals more closely, with the possibility of activation and space for the animals to roam in, as it is seen today. With the continuous decline in populations of non-domestic felids, as a consequence of habitat loss and poaching (Brown 2011), zoos today play an important part in the ex situ conservation of endangered species. Thus, it is important for the captive felids to be able to reproduce efficiently as they are able to in the wild, provided their natural habitats are not endangered (Graham & Brown 1996; Ludwig *et al.* 2013).

In spite of the improvements of the environment of captive animals, there are still indications of the levels of stress being higher than in free-living animals. These indicators can be found among the non-domestic felids kept in captivity, where observations are pointing towards stereotypic behaviour, such as stereotypic pacing, excessive grooming, self-plucking of hair and tail and paw sucking (Carlstead *et al.* 1993). Reproductive difficulties (Ludwig *et al.* 2013) and diseases (Terio *et al.* 2004), which can compromise welfare as well as breeding programmes for conservation purposes, are also observed. Thus, it is of importance that methods are available for assessing stress levels in non-domestic felids.

Uncertainties can arise when assessing welfare of animals based on ethology alone. The results can be biased by subjective analysis and problematic to quantify, as it is usually based on subjective observations, albeit still adopting a scientific approach. Thus, by supplementing ethology-based observations with physiology, more accurate data can be obtained. In this project, we are examining the validity of using non-invasive sampling for measuring cortisol and transferring the results to assessment of stress and welfare, to see if this is applicable for assessing these parameters.

By measuring cortisol levels in blood, saliva, urine, faeces and hair, it is possible to quantify stress. The use of invasive sampling, for instance blood sampling, can alter cortisol levels by exposing the animal to stress and thus can be less suitable in non-domestic felids, as it causes a rapid elevation of cortisol levels in plasma. Also, it does not provide an extended time frame of cortisol levels, as some of the non-invasive sampling techniques do.

Measuring cortisol levels using non-invasive sampling, is a fairly new technique and it is interesting to examine whether this is a valid approach (i.e. if it contributes to less stress and offers clearer results). With non-invasive sampling, cortisol levels can be obtained without altering the levels in the samples by stressing the animal. There are several non-invasive approaches for measuring cortisol levels in felids (faeces, urine, hair and saliva) and these sampling media offer a variety of time frames of cortisol fluctuations. Saliva samples offer a more immediate display of stress levels (Sheriff *et al.* 2011) while faecal and urine samples offer an insight of the stress level over several hours (Touma & Palme

2005), whereas the presence of cortisol in hair is an accumulation over a duration of weeks or months (Sheriff *et al.* 2011).
The sampling of cortisol in faeces is of special advantage in assessing stress levels in free-living animals, as it is not necessary to be in direct contact with the animal. Due to faeces being fairly accessible in the wild, it can easily be used when measuring cortisol levels in both free-living and captive animals and this is reflected in the majority of the literature used in this project.
During this project we mainly use literature based on non-domestic felids, where the captive species used, are suffering from reproductive difficulties or stereotypic behaviour, yet we draw parallels to other mammals at times, which will be stressed in the text. The literature used has not been consistent when applying the term 'cortisol' and 'glucocorticoids' (GCs), and instead the less specific term 'corticoids' is occasionally used. As this has been used in a stress-assessment context, we have interpreted this use as concerning only stress hormones and implemented it so in our project.
We cover a variety of consequences of high cortisol levels throughout this project. Because high levels of cortisol, stress, compromised welfare, and reproductive challenges are highly connected, we use these terms somewhat interchangeably, as we assume that chronically elevated levels of cortisol equal high levels of stress and thus reduced welfare, which in turn is causing reproductive difficulties.
Previous knowledge of non-invasive sampling is limited and exists mainly from studies on other species than felids, why this project has great relevance, as it assembles the essential factors needed in order to conduct a satisfying study on stress assessment of felids.

Hypothesis

We hypothesise that the cortisol levels of captive, non-domestic felids are increased compared to non-domestic, free-living felids. We believe that high levels of cortisol equals reduced welfare. However, it is plausible that free-living felids will have high levels of cortisol on occasions, due to naturally occurring environmental factors, which cause elevated stress levels, but not necessarily reduced welfare.

Objective

During this project, we wish to establish the relationship between stress and reduced welfare, and if chronic stress equals bad welfare. We will review what factors affect stress in each group respectively, in order to evaluate if there is a basis for comparison and if so; if captive felids have higher cortisol levels, for which we strive to use GC levels of free-living felids as baselines.
Finally, we wish to examine whether measuring cortisol levels non-invasively, is a valid method for assessing stress and welfare in non-domestic felids in captivity, along with what factors are important to consider when sampling and analysing.

1. Basic endocrinology of cortisol

1.1 The physiology of stress responses

Stressor-induced activation of the hypothalamus-pituitary-adrenal (HPA) axis results in a series of neural and endocrine adaptations, known as 'the stress response'. This response is responsible for allowing the body to make the necessary physiological and metabolic alterations that are needed to cope with the demands of threats to homeostasis (Miller & O'Callaghan 2002).

According to Moberg (2000), the stress response can be divided into three stages: the recognition of a stressor, the biological defence and the consequence of the stress response, where the last stage determines whether the animal is suffering from distress or simply is experiencing a passing response, that will have no significant impact of the fitness and welfare of the animal.

The stress response begins with the nervous system perceiving a stressor (i.e. a potential threat to homeostasis). Whether or not the stressor is genuine is not physiologically important, as it is the perception itself that jump-starts the cascade of responses. When the central nervous system (CNS) perceives a threat, it starts a biological defence consisting of combinations of the four general biological defence responses: the behavioural response, the autonomic nervous system (ANS) response, the neuroendocrine response or the immune response (Moberg 2000).

The neuroendocrine response activates the HPA axis (see Figure 1). The pathway begins with the anterior hypothalamus releasing corticotropin-releasing hormone (CRH), a hormone that activates the pituitary gland to release adrenocorticotropic hormone (ACTH) into the general circulation. ACTH travels to the adrenal cortex through the bloodstream and activates it to secrete the species-specific GCs; cortisol or corticosterone (with cortisol being the primary GC excreted in felids) from the adrenal gland. The corticosteroids increases metabolism and alter body fluids, and thereby blood pressure. The response of hormones released from the adrenal cortex is prolonged, as their effects on stress response ranges from minutes to hours.

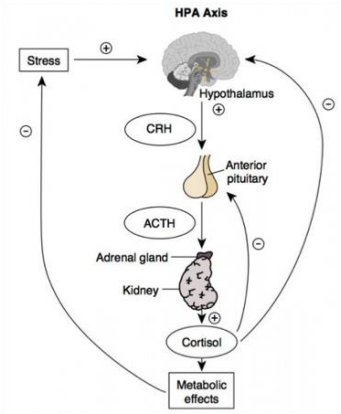

Figure 1. The HPA axis. UNSW embryology (2012)

Secretion of cortisol through the HPA axis is regulated by a negative feedback system including the hypothalamus, the anterior pituitary and the adrenal glands. Under a normal stress response, cortisol functions as the negative feedback signal and inhibits both CRH and thus ACTH secretion (Sjaastad *et al.* 2010). The body strives to maintain GC levels within margins and interference at any level of the axis will influence the other components via feedback loops (Miller & O'Callaghan 2002). During prolonged stress, the plasma concentrations of ACTH are high and the mass of the adrenal cortex increases resulting in continually high-level secretion of GCs.

The adrenal cortex (see Figure 2) produces and releases corticosteroids (GCs and mineralocorticoids). In the following, most emphasis will be put on GCs, as these are the primary hormones involved in the stress response regarding the fight or flight response. The half-life of cortisol varies from hours to weeks, which makes its effects on stress prolonged. This also makes cortisol a suitable hormone for measuring stress non-invasively, as it can be tracked within the body for a longer period, compared to stress hormones with immediate and intermediate effects on the stress response.

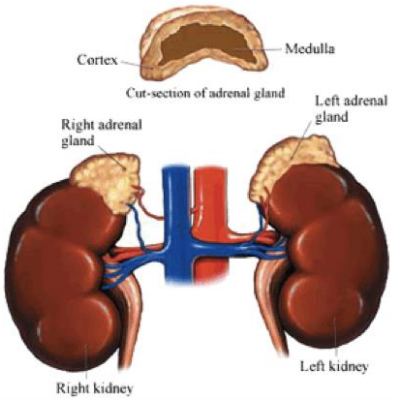

Figure 2. The adrenal glands located on top of each kidney.
Dr. Wilson (2014)

On the metabolic level, cortisol acts to increase blood sugar and provide the organism with adequate substrate to prepare it for reacting to a certain stressor, by activating the fight or flight response. Cortisol does so by increasing blood glucose through gluconeogenesis and decreasing glucose uptake in both skeletal and adipose tissue, mediated by glucose transporters type 4 (GLUT4s). The hormone also provides more substrate available for gluconeogenesis by inhibiting protein synthesis, especially in skeletal muscle tissue. In between digestive activity, cortisol is sparing glucose for energy supply, as cortisol increases the catecholamines' effect of lipolysis, which results in additional free fatty acids available as an energy source, instead of glucose (Koeppen & Stanton 2010).

In spite of the primary function of cortisol, to ensure enough substances to maintain a suitable energy level during physical exercise, a continuously increased level of cortisol due to chronic stress will compromise the functioning of several physical systems.

1.2 Effects of chronically elevated cortisol levels

The maintenance of homeostasis, when facing internal or external challenges, requires constant adjustment of hormonal, behavioural and autonomic systems. Successfully meeting the challenges (or allostasis if these are excessive) can result in cumulative physiological strain. This burden is referred to as 'allostatic load' and accumulates when the body needs to continuously cope with challenges outside the normal operating range. This 'wear and tear' on the organism, caused by high allostatic load, can in time lead to changes in the body, resulting in disease (Miller & O'Callaghan 2002).
Chronic stress has several negative impacts on the body, including atrophy to various tissues, insomnia and depression, ulcers (Koeppen & Stanton 2010), impairing growth (Romero 2004), wear and tear to arteries and blood vessels and atrophy to brain tissue, affecting memory and learning skills (Seaward w.y.). Although it is well accepted that chronic stress has negative impacts on the organism, the neurobiological mechanisms that link chronic stress to development of disease are not well understood (Miller & O'Callaghan 2002).

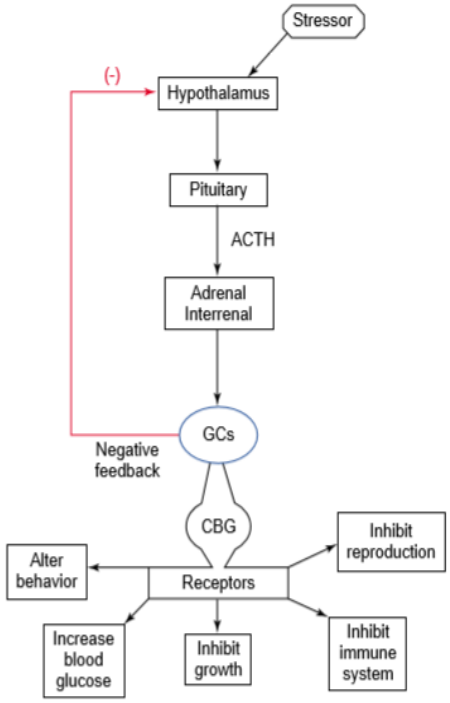

Figure 3. The effects of chronic stress (Romero 2004)

Excessive levels of cortisol, due to chronic stress, inhibits the organism's ability to return to homeostasis. This can cause damage to various systems (see Figure 3) (Seaward w.y.), including immune and inflammatory response, and can affect the way in which the organism responds to an acute stressor, among others cause immune cell glucocorticoid receptor resistance (GCR) (Viena *et al.* 2012). Cortisol is maintaining the immune homeostasis by repressing the production of proinflammatory cytokines and stimulating the production of anti-inflammatory cytokines (Koeppen & Stanton 2010), and therefore

inhibiting the immune response during chronically elevated cortisol levels. In addition, during acute stress responses, immunoglobulin A is commonly secreted for immune activation. This happens to a lesser extent during chronic stress responses, and thus results in an impairment of the body's ability to fight off pathogens when chronically subjected to stress (Viena *et al.* 2012).
It is also noteworthy, that elevated cortisol levels suppress the reproductive functions in mammals due to the anabolic effects of cortisol. The hormone partly inhibits the reproductive axis on both the hypothalamic, pituitary and gonadal levels (Koeppen & Stanton 2010). Therefore, if affected by constantly elevated cortisol levels, the reproductive status of an animal might be impaired.

1.2.1 Acclimation and facilitation

As described previously, a normal functioning stress response is accompanied by secretions of GCs, of which concentrations will increase in accordance to the degree of stress the stressor will induce, and once the perceived stressor either is absent or ceases to induce a stress response, the GCs will return to baseline. This is in contrast to chronically elevated cortisol levels or exposure to repeated ephemeral elevations, which can alter the HPA axis and hence change the stress response of an animal, with alterations in GC responses following as a result. Acclimation and facilitation are two primary changes that are relevant to this project. With acclimation follows, that the animal ceases to respond to a repeated or continuous stressor (i.e. the stress response is less prominent). This alteration is due to the psychological aspect of the stress response, as the animals' perception of the stimuli has changed to seem either less or non-deleterious, and thus the stress response is manifested to a lesser degree. The animal therefore acclimates to the stressor. This results in different responses among individuals, after repeated or constant exposure to a certain stressor, and thus acclimation generally results in lower GC responses. With acclimation follows alterations to the physiology of the HPA axis, in which stress responses to novel stressors are enhanced compared to stress responses of non-acclimated animals. This is due to facilitation, which is the other significant change in the functional stress response. The acclimation process galvanizes the animal's responsiveness to other stressors and thus enhances the elicited stress response. It is not all stressors, and especially not relatively severe ones, that potentially induce acclimation and facilitation (Romero 2004).

2. Welfare and stress - what is the relationship between the two?

2.1 What is stress?

The term 'stress' typically refers to the negative consequences of an animal's failure to cope with stressors in its environment, although there is considerable controversy about what exactly constitutes stress.

The term ‘stress’ as understood in everyday language and as defined in a scientific sense, might be quite different. In a pseudo-scientific understanding, stress is widely used as a mental and physical state one might experience when going through a hectic time. In a scientific understanding, stress is often referred to as physiological stress. This is the nervous and hormonal response to potential harmful or noxious stimuli exhibited by healthy individuals, in an attempt to maintain homeostasis (Romero 2004). In a welfare-oriented perspective, stress is referred to as a chronic and deleterious state, in which the animal is unable to cope with its environment, as opposed to acute stress which is often beneficial as it leads to increased fitness (Keay 2006). Chronic states of stress can lead to a variety of fitness-reducing circumstances such as, apathy, physical illness and mental degeneration (Seaward w.y.; Broom 1991).
In physiology, it is common to assess stress as a hormonal and nervous occurrence, whereas in ethology, it is common to examine the behavioural symptoms as indicators of stress. In ethology, stress is assessed by an alteration in behaviour and is often evaluated by observing individuals through a period, in order to observe certain changes such as apathy, stereotypic behaviour, aggression or frustration (Wielebnowski *et al. 2002*).

2.2 What is welfare?

To assess animal welfare, a clear definition of the term ‘welfare’ that embraces the different aspects on the matter, must exist. When the term ‘animal welfare’ is used in society, it is often used as an indicator of how we should treat animals in our care, based on the ethical obligations humans have toward animals. In scientific use, however, animal welfare refers to the state an animal is in and is thus considered as a characteristic of the animal, rather than something provided by humans (Keeling *et al.* w.y.). With this approach to animal welfare, not only animals in our care can be subjected to bad welfare, but animals in the wild as well, due to external and internal factors, such as loss of habitats, which compromises survival, or disease (Keeling *et al.* w.y.).
Animal welfare is many-sided and official definitions have provided an overview of these throughout time. A well-known definition is the “Five Freedoms”, provided by FAWC (2009), which defines good animal welfare as freedom from hunger and thirst, freedom from discomfort, freedom from pain, injury or disease, freedom from fear and distress and freedom to express normal behaviour (Keeling *et al.* w.y.). It is noteworthy that these are irrelevant to free-living animals, as most of these stressors are inevitable in their natural life. While this provides one with good guidelines of what constitutes animal welfare, contradictions between the different freedoms may arise in practice. Thus, it is of importance to be able to evaluate which freedom weighs more on a welfare scale, compared to others. Unfortunately, there is no scientific approach for this today, which then results in a relatively strong weakness when assessing animal welfare. However, there is a developing scientific approach to assess animal welfare within the different categories based on the “Five Freedoms”, which is fundamental in order to measure animal welfare of captive animals and to implement changes that can improve this (Keeling *et al.* w.y.).

Considering various feelings (e.g. pain, fear and boredom) of an animal, along with common physiology (e.g. health and ability to reproduce) and the ability to perform natural behaviour, is part of assessing animal welfare today in a scientific manner (Keeling *et al.* w.y.). A scientific approach is essential in order to support any assertion regarding the degree of welfare, for the sake of implementing improvements.

2.3 Stress as an indicator of welfare

In the assessment of stress and in turn welfare, physiological measurements are essential. Stress can arise due to different stressors that originate either from physical causes (e.g. hunger or thirst) or from emotional causes (e.g. presence of humans or fear). However, stress originated from physical causes and the subsequent physiological responses may influence the emotional state of the animal and thus the two are closely connected. Therefore, it may be challenging to isolate these two types of stress from one another. When measuring cortisol it is important to note that the measures do not reflect the stress response in itself, but instead quantifies the reaction an animal has towards a given stressor. This is due to the stress response, as a whole, consisting of different systems within the organism. Thus, to convert these measures into indicators of animal welfare, it is important to include various physiological and behavioural measurements, along with genetic, environmental and temporal factors (Blache *et al.* w.y.).
Due to variation in HPA activation, resulting from factors such as age, variation in individuals and species, type and duration of stressors, it can be difficult to determine a threshold that, when above, animal welfare is compromised. In addition, a stressor might not only consist of negative stimuli, but also positive ones; for example mating stimuli, that do not have a negative impact on the physiology of the animal per se, which again encumbers assessing welfare from only one or a few measures (Blache *et al.* w.y.).

From the previous sections it is clear, that chronically stressed animals have compromised welfare, as a consequence of the inability to cope with their environment and the subsequent allostatic load, which may result in reduced fitness, and thus chronic stress and welfare are positively correlated.

3. The difference of cortisol levels in captive and free-living felids

Due to problems with welfare and fitness in captive felids, it is beneficial to acquire a thorough understanding of the factors contributing to these. It is assumed that the stressors present in a captive environment, differ from those present in the wild, but are associated to the reduced opportunity to perform natural behaviours. Captive animals are free from many of the stress-inducing factors experienced by free-living animals, such as hunting, mating activities, defending territories and defence from predation or competition, yet free-living felids seem to have no physiological problems reproducing, as

their co-species in captivity often have. In the following section, we examine some of these stressors in order to illustrate what causes elevated cortisol levels and thereby reduced fitness.

3.1 Factors affecting cortisol levels in captive felids

Assessing cortisol levels in captive felids can be an overwhelming task, as the term 'captive felids' covers a variety of living conditions. Furthermore, the species-specific differences among felids may have great impact on stress levels, yet regardless of the species, the scientific literature on stress levels in captive felids show numerous joined problems. Clouded leopards (*Neofelis nebulosa*) (Wielebnowski *et al.* 2002), jaguars (*Panthera onca*) (Montanha *et al.* 2009), tigrinas (*Leopardus tigrinus*), marcays (*Leopardus wiedii*) (Moreira *et al.* 2007), cheetahs (*Acinonyx jubatus*) (Terio *et al.* 2004) and tigers (*Panthera tigris*) (Narayan *et al.* 2013) are all examples of species that are susceptible to reproductive challenges and stereotypic behaviour, which are causes of concern due to their fragile population sizes in the wild.

There are clear differences in the living conditions among captive felids throughout the world. In many South American countries, the smaller felids are kept in barren cages without any enrichment or activation (Moreira *et al.* 2007), whereas in many European and North American countries, legislation and adaptations of animal welfare has been applied (Witham & Wielebnowski 2013). Thus, it is interesting and of importance to investigate what factors are causing distress in felids. The causes of reproductive problems in captive felids is poorly understood, as many of the different species have no difficulties reproducing in the wild, hence the declining population sizes are primarily due to habitat loss and poaching (Brown 2011).

A variety of potential stressors exists in the life of captive felids. Most conspicuous is the sight of and noise from visitors at the zoos, along with the limited size of enclosures with restricted space to roam. Furthermore, there are potential stressors such as lack of activation (causing frustration), management within the zoo, housing arrangements in relation to their social structure in the wild along with their position in the zoo (e.g. if they located close to prey animals or potential enemies). Not all Felidae species are equally affected by these potential stressors. Jaguars, for instance, seem to be minimally affected by audience, yet are very sensitive to changes in routines and management (Montanha *et al.* 2009). Thus, it is necessary to be aware of which stressors are affecting each species, in order to implement the optimal living conditions to increase welfare and fitness.

Montanha *et al.* (2009) examined salivary cortisol levels in seven jaguars; three jaguars in a Brazilian zoo (two of which origins are not provided and one male of unknown origin) and four jaguars in a conservationist breeding facility (three of which were captive-born and one of unknown origin that had been in captivity for at least six years). Results showed that jaguars kept in enclosures with refuge near ground, had higher cortisol levels than jaguars with the possibility of refuge in trees. In addition, jaguars with no possibility of refuge in trees, did in fact show higher cortisol levels when the number of visitors

increased, whereas the jaguars with possibility of fleeing up the trees, exhibited steady cortisol levels throughout an entire week. This stresses the importance of arboreal species having access to elevated refuge.

Carlstead *et al.* (1993) found, that the translocation of four leopard cats *(Felis bengalensis*) (all captive born), to a novel environment with auditory, visual and olfactory contact with each other, but with no other animals in proximity and with a zookeeper visiting once a day, resulted in an increased cortisol response which returned to baseline after one week. However, moving the cats to a novel environment with auditory and olfactory contact to other felids (lions (*Panthera leo)*, tigers, pumas (*Puma concolor*) and jaguars) and zookeepers in the building for eight hours a day, resulted in a prolonged increased adrenal response lasting for 10 weeks. Carlstead *et al.* (1993) believe that the latter response may have been due to the lack of visual contact, combined with significant olfactory and auditory contact with natural enemies, as the natural niche of most small felids overlap with these larger felids that may thus prey upon them in the wild. Especially tigers and leopards (*Panthera pardus*), are both found within the range of leopard cats and are known to prey on other carnivores. Carlstead *et al.* (1993) also argued that the zookeepers' continuous presence during the day acted as a potential stressor, as it might have contributed to additional noises that could be perceived as threatening.

Moreira *et al. (*2007) studied the female tigrina and margay (both captive-born), and found that the tigrina reacted more dramatically to relocation from a large, enriched environment to a smaller, barren enclosure. Increased levels of faecal corticoids and associated agitated behaviour was observed. The behaviour was associated with increased stereotypic movement, characterized with pacing from one side to the other, in the days following relocation. Enrichment of the enclosure caused the increased levels of corticoids to return to baseline, which stresses the importance hereof.

Interestingly, as the corticoid levels of the tigrina rose as a consequence of relocation to a small, barren environment, it exhibited a decrease in overall estradiol concentration, which equals a reduced ovarian activity (Moreira *et al.* 2007). This response did not return to normal following enrichment, which demonstrates how ovarian activity is downsized when the animal is dealing with chronic distress.

When transferred to smaller, barren environments, both the tigrina and margay exhibited more apathetic behaviour after the initial agitated response. The passive reaction is believed to be associated with an increase in HPA activity. This response explains the passive behaviour exhibited by felids in captivity and that high levels of stress is not necessarily associated with increased activity, such as stereotypic behaviour, frustration or aggression.

Terio *et al.* (2004) examined baseline corticoid concentrations in faeces of 20 captive (all captive-born) and 20 free-living cheetahs, and found an increased baseline in captive cheetahs (see Figure 4). According to Terio *et al.* (2004), this suggests that adrenal hyperplasia is associated with chronic stimulation and increase in corticoid production. Despite the variations in corticoid concentrations among individuals, the findings are still significant. Terio *et al.* (2004) also found that cheetahs housed on exhibit, generally had

higher baseline corticoid concentrations than cheetahs housed off exhibit, which suggest that cheetahs are affected by their environment. Due to the small sample size of the cheetahs on exhibit, the findings are not significant, although they are supported by literature on other felids, both wild and captive, eliciting the same responses to public display (Piñeiro *et al.* 2012; Wells *et al.* 2004; Wielebnowski *et al.* 2002).

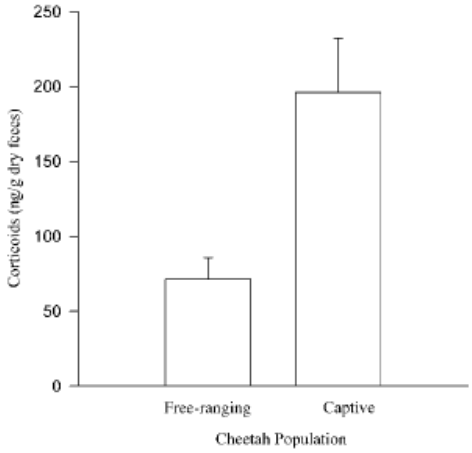

Figure 4. Difference in corticoid concentrations of free-ranging and captive cheetahs (Terio *et al.* 2004)

Wielebnowski *et al.* (2002) studied the effects of different potential stressors on 72 clouded leopards, of unknown origin, in North American zoos. They found a negative correlation between faecal corticoids and enclosure height (hence the opportunity for the leopards to climb), whereas available floor size did not seem to have an impact, as there were no significant relationship between this and faecal corticoid concentrations.
The number of zoo keepers appeared to be of relevance as well, as a higher number of keepers was associated with elevated corticoid concentrations. Rotations with multiple keepers resulted in each keeper spending less time with the animals. This could be the reason why they elicited increased levels of corticoids. Conversely, more time spent by the keeper tending to the animals resulted in lower concentrations.
As Wells *et al.* (2004) and Terio *et al.* (2004) found in other felids, Wielebnowski *et al.* (2002) found that clouded leopards on display and/or with visual contact to potential predators, had higher faecal corticoid concentrations, than animals located where these stressors were absent.

From these findings, it is clear, that the deprivation of the opportunity to perform natural, species-specific behaviour, elicits the greatest increase in stress levels. In order to keep the stress levels near baseline, it is important that felids can perform natural behaviours associated with survival and fitness. When on display, felids need to be provided with hiding places that mimic their natural escape (e.g. jaguars seeking refuge in trees). Furthermore, it is of importance that they are not located to have olfactory, auditory or visual contact with potential enemies.

3.2 Factors affecting cortisol levels in free-living felids

The non-invasive measurement of cortisol levels in free-living felids can be of value in improving both welfare and conservation. The following section describes stressors that are part of the lives of free-living felids, both naturally occurring and anthropogenic, which causes elevations in HPA activity. Although the section does not describe all possible factors that act as stressors in the wild, it outlines various significant factors that have been examined and are specific to free-living animals.

3.2.1 Seasonal changes in cortisol levels

The cortisol levels of free-living felids varies throughout the year, by a number of stressors that are an inevitable part of the life of a free-living animal. Studies have shown that in numerous mammals, GC levels often varies according to season, in which an increase or decrease can arise due to factors associated with a particular season (Romero 2002).

Various seasonal changes can cause elevated GC levels. For example, changes in weather and temperature, food availability, reproductive season and migrations can act as the inducing stressors.

Different explanations to these seasonal variations of GCs has been proposed: the effects of GCs on metabolism, the effect of CGs on behaviour and the role of CGs in preparation to a following stressor.

3.2.1.1 The energy mobilisation hypothesis

The hypothesis concerning GCs metabolic effects focuses on CGs influence on energy metabolism. Since cortisol has a sparing effect on energy metabolism, it is reasonable to assume that during times when energy demands are either high or energy availability is low, the concentration of CGs will consequently be elevated. Certain times of the year are accompanied by factors that causes alterations in energy demands and utilization (e.g. cold winters or mating season), hence the annual variation of the GCs concentrations. Thus, according to this hypothesis, it is the energy costs of the stressors that causes the annual variations in GC levels (Romero 2002). This can explain the findings in a study by Naidenko *et al.* (2011), where HPA activity in free-living Siberian tigers (*Panthera tigris altaica*) peaked in the months of November to January. This is a time of year that is characterized by low average daily temperatures and significantly deep snow covering the landscape in the habitat of the Siberian tiger. These factors can encumber hunting and thus energy availability may be scarce due to reduced feed intake and more movement required to find feed (which increases metabolism), hereby causing elevated cortisol levels (Naidenko *et al.* 2011). In the same study, comparisons of HPA activity of the Siberian tiger in captivity and in the wild have been conducted. The HPA activity in the free-living Siberian tigers was significantly higher compared to tigers in captivity in each analysed month. These findings might be related to the increased metabolism of the free-living animals (Naidenko *et al.* 2011), in addition to various other stressors that can occur in the wild, which is reviewed in the following sections.

3.2.1.2 The behaviour hypothesis

The hypothesis concerning the behavioural aspect of GCs, proposes that the animal will be in need of expressing or repressing certain GC-dependent behaviours that are specific to a given season, in order to achieve a goal (e.g. relocating due to climatic changes). This suggests that the most vital role of GCs in free-living animals is behavioural, which is based on the discovery of a membrane-bound GC receptor that activates a G-protein, and is supported by evidence that there are fast changes in behaviour after GC administration. According to this, it is the desired acute effects of GCs that cause the annual variation in GC levels (Romero 2002).

3.2.1.3 The preparative hypothesis

The preparative hypothesis suggests that seasonal changes in GC levels, act to modulate the priming of the different stress pathways during periods when several potential stressors might occur. Thus, this serves as a preparation mechanism to certain times of the year, such as seasons of migration, when the animals are not only exposed to the elevated stress levels from exhaustive relocation, but also to subsequent stressors that might occur, such as acquiring new territories.

Following this, GC concentrations might be elevated although the animal is not actually subjected to a stressor, and the baseline may be higher due to potential adverse situations. Thus according to this hypothesis, annual GC variations are an evolutionary result of seasonal alterations in potential exposures to stressors (Romero 2002).

It is assumable, that free-living felids will have more prominent alterations in GC concentrations due to seasonal effects compared to captive felids, as many of the factors causing these variations partly will be eliminated in captivity.

3.2.2 Reproduction

In some animals, the seasonal changes in cortisol levels are related to reproduction (Naidenko *et al.* 2011). The stressors that arise with reproduction are numerous, in which the act of mating is not the only one as it coincides with pre- and post-breeding behaviour, along with the potential subsequent risk factors that follows raising and protecting offspring. In a study by Romero (2002), findings demonstrate that various animals have higher GC concentrations during breeding season. Although this study only examined seven different mammalian species, excluding felids, this follows the general understanding of felids' stress levels during breeding season. Piñeiro *et al.* (2012) conducted a study on wildcats (*Felis silvestris*), and found a general pattern of elevated faecal cortisol metabolites (FCMs) during times related to reproduction. FCMs concentrations were prominent during times of elevated progesterone levels in spring, during gestation period and during the dispersion of the young in autumn (Piñeiro *et al.* 2012). Thus, one must account for reproduction as contributing factor, which elevates cortisol levels among Felidae in the wild.

It is conceivable that free-living felids may have more pronounced elevations of FCMs throughout the breeding season, compared to captive felids, due to differences in the behaviour of pre- and post-mating. In the wild, animals must search for and pursue a mate, which might act as a stressor, and additionally, the offspring are at higher risk of predation by other species or a competing male. Thus, as these factors are partly eliminated in captivity, there might be less stressors associated with reproduction in captivity, compared to in the wild.

3.2.3 Anthropogenic disturbances

In addition to the above, various anthropogenic disturbances can act as stressors and cause elevated GC levels in free-living felids.

Human contact or predator abundance (which in the case of felids is often limited to humans), causes increase in stress responses, which can be important to consider when measuring cortisol levels of felids that are subjected to poaching (Busch & Hayward 2009). Exposure to tourism represents another factor, which might contribute to increased HPA activity. In general, free-living animals exposed to tourism has elevated GC concentrations, although the response acclimates with repeated exposures over time (Busch & Hayward 2009). It is noteworthy, that while some studies have shown a down-regulated response to regular exposure to tourism, others find that the animals will have higher initial stress responses (Busch & Hayward 2009). Piñeiro *et al* (2012) found that tourism acts as a stress agent on wildcats, as FCM concentrations were elevated at forest roads that crossed the natural park in frequent visitation zones, which supports the hypothesis that anthropogenic disturbances are causes of stress in wild felids.

Whether habitat changes are due to deforestation or due to habitat fragmentation (which make the animals more susceptible to human disturbance), studies point toward these disturbances as causes of elevated GC concentrations of various animals involved (Busch & Hayward 2009).

Some disturbances may give rise to multiple stress factors that are intertwined, with both ecological and physiological origins, and thus it may be important to have an understanding of these linked stressors. An example hereof are the stressors arising with habitat fragmentation (Busch & Hayward 2009), which may not only cause elevated GC levels due to factors such as anthropogenic disturbances, but also a decrease in food abundance, due to habitat loss which also serves as a stressor. Whether or not the multiple factors are positive or negative, it is important to be aware of and account for the interaction between factors, when measuring cortisol levels in animals affected.

3.3 How do social structures and behavioural differences affect cortisol levels in captive felids?

3.3.1 Social status and stress

As some Felidae species (e.g. lions and cheetahs) live in group structures in the wild, it is essential to account for potential stressors that may arise in these social groups. In spite of

being part of a social group provides many advantages, for example group hunting and protection from competitors, it may also result in disadvantages in forms of social conflicts and insider competition. In addition, being part of a social group comes with a potentially low-ranked status in which the animal might be susceptible to feeding last or to the risk of getting banished from the group, but it also follows that being dominant can increase cortisol levels. A study by Wolfgang & Wingfield (2003) concerning various species of animals, shows that there is a significant positive correlation between allostatic load from being dominant and GC concentrations, and when allostatic load was higher as a result of being subordinate, so was GC concentrations. The allostatic load that follows being either dominant or subordinate, depends on interactions in the group. For instance, dominant members might repeatedly be challenged for their rank. However, if a subordinate member constantly has to challenge others or defend itself, elevations in GCs will follow (Wolfgang & Wingfield 2003).
It is assumable that the more stable groups implemented in captivity, contributes to less fluctuations in the GCs concentrations, compared to in the wild. In addition, competition among group members may also be less prominent. Thus, when comparing basal concentrations of free-living and captive felids that live in group structures, one must consider these factors when analysing data.

3.3.2 Social patterns and stress in captivity

In addition to inhibiting the naturally occurring group alterations that can occur in the wild, felids in captivity are often housed in arrangements that are unnatural to the social structure of the species. It is feasible that the imposed social interactions, or the lack hereof, might act as a stressor and thus cause elevated GCs levels. These stressors could be due to enforced solitary for an otherwise social animal or vice versa, forced interactions between animals that exclusively live unaccompanied in the wild. One of the species of Felidae that are subject to this is the female cheetah, which in its natural habitat in Africa lives a solitary life. Due to intra-species aggression is rarely occurring in captivity, cheetahs are often housed with other cheetahs (Brown 2011). A study by Wielebnowski and Brown (1998), found that although serious confrontations rarely happened, the occurrence of subtle antagonistic behaviours increased along with stereotypic behaviour and reduced auto-grooming. In addition, average faecal estrogen concentrations were reduced in paired cheetahs. When separating the pairs, normal behaviour and ovarian activity rapidly followed. Although no connection between ovarian activity and increased adrenal corticoid activity was found in this study, Jurke *et al.* (1997) found a possible connection between elevated faecal corticoid concentration and acyclicity. Thus, the ovarian suppression of these animals may have arose due to increased corticoid concentration that, in turn, may have been due to inappropriate social housing (Brown 2011). However, several other studies on felids in captivity found that animals kept in social groups exhibited less stereotypic behaviour, although unnatural in regards to their social pattern, which could be due to social interactions that are only possible in group-housed animals (Quirke *et al.* 2012).

It is generally accepted, that stereotypic behaviour and stress are closely related. Numerous studies, including one conducted on clouded leopards, found that an increase in stereotypic behaviour follows an elevation of stress hormones (Quirke *et al.* 2012). Thus, due to the inconsistencies in the previous findings, additional studies are recommended in order to further assess how social interactions affect the GC concentrations in captivity.

3.3.3 Differences in sexes

The literature suggest that life in captivity is more stressful for female felids than for males. Wielebnowski *et al.* (2002) studied the effects of different stressors on 72 clouded leopards in North American zoos and found a significant difference in faecal corticoid concentrations, in which females had higher concentrations than males. This is consistent with other studies on felids and other non-human mammals (Kudielka & Kirschbaum 2005; Narayan *et al.* 2013; Zavala *et al.* 2011), and is assumed to be an evolutionary adaptation to increase vigilance to protect and rear cubs and to avoid aggression from dominant males.

However, Conforti *et al.* (2012) found that male jaguars had an overall higher concentrations of FCMs than females when measuring pre- and post ACTH challenge, yet did not see a significant difference in the magnitude of the response to ACTH. They observed lower baseline concentrations of FCM in captive-born females and found a significant interaction between gender and origin, and thus the effect of gender on mean baseline FCM concentrations should not be considered separately from origin. Conforti *et al.* (2012) suggest that the higher baseline of wild-born males can be due to these adapting well to captivity once they are acclimated, because their mean baseline FCM concentrations did not differ from captive-born males.

The scarce information and conflicting results, offer a foundation for conducting further studies on the difference in baseline FCM concentrations between sexes.

The factors affecting captive and free-living felids are quite distinct and the effects of many of these are uncertain, as previous studies offer conflicting results. This encumbers comparison of the two groups and the approach applied by the majority of the articles from this chapter, using the GC levels of the animals themselves as baselines, seems attainable and valid.

Analysing the data from non-invasive samples requires several techniques in order to obtain a true depiction of the cortisol levels as a measure of stress and the uncertainties following these techniques should be acknowledged.

4. The methodology of cortisol sampling and stress assessment

This chapter is a review of the considerations that are necessary when assessing cortisol levels as a reflection of stress in non-invasive sampling, including the techniques used for analysing and what factors to take into account when sampling and analysing. The pros

and cons of using different non-invasive media and what considerations should be applied when using cortisol to assess welfare, are also reviewed.

4.1 Techniques for determining coherence and analysing glucocorticoids in samples by ACTH challenge, EIA, RIA and HPLC immunogram

In the following, the most commonly used techniques and practical applications used to measure GC concentrations are briefly described.

4.1.1 ACTH challenge

In order to further the use of the stress hormones that are accessible in various emitted matters, the degree an animal reacts to a given stressor at the adrenocortical level should be experimentally demonstrated, displaying how the faecal glucocorticoid metabolite (FGCM) concentrations increase in response to the stimuli. The most common technique used for this is ACTH challenge, which stimulates the activity of the adrenal cortex (Ludwig *et al.* 2013). After application of ACTH challenge, a distinct increase in FGCM concentrations should emerge, with a lag time proportional to defecation rate henceforth, displaying this upsurge in adrenocortical activity (Ludwig *et al.* 2013). Sample pre- and post-administration should be collected to assess a pattern of the adrenocortical activity in various stressful situations.

4.1.2 Enzyme immunoassay (EIA) and radioimmunoassay (RIA)

Biochemical procedures for validation must test for accuracy, specificity (cross-reactivity), sensitivity and precision. When analysing GC levels, immunoassays are commonly used and there are especially two techniques applied when analysing steroid hormones in mammals: radioimmunoassay (RIA) and enzyme immunoassay (EIA) (Wielebnowski & Watters 2007).

RIAs rely on radioactive isotopes, such as tritium or iodine, to generate a radioactive signal in order to quantify GC levels, whereas EIAs rely on enzymes to generate a colorimetric signal (Sheriff *et al.* 2011).

Both immunoassays are competitive binding assays, as immunoassays rely on the characteristic ability of an antibody to bind to a specific structure of a molecule. The radioisotope or enzyme generates a colour signal proportional to the amount of target antigen existing in the original sample (Immunochemistry Technologies 2014).

4.1.3 High Performance Liquid Chromatography (HPLC) immunogram

High Performance Liquid Chromatography (HPLC) immunograms are conducted to characterise and quantify the immunoreactive metabolites present in biological samples. When characterised, the metabolites can be evaluated for immunoreactivity and then selected for an immunoassay (e.g. RIA or EIA), to measure different corticosteroids and metabolites (Keay *et al.* 2006).

HPLC immunogram can be conducted in combination with a radiolabel infusion on pooled sample extracts, collected from different individuals and species (Wielebnowski & Watters 2007). To describe the species-dependent FGCMs and the affinity of the of the antibody hereof, injection of ^{3}H labelled cortisol along with HPLC immunogram, is a useful approach. Comparison of the excreted radiolabeled metabolites and the metabolites, which are products from the ACTH administration, will disclose the major GCs that are specific to a given species, along with the fit of the specific immunoassay (Ludwig *et al.* 2013).

4.2 Advantages and disadvantages of using non-invasive cortisol sampling in urine, hair, saliva and faeces

The benefits of measuring cortisol levels non-invasively are many (some are mentioned briefly, earlier in this project). Most pronounced is the opportunity to collect samples without stressing the animal in the process and to avoid biased results caused by handling-induced stress responses. Although non-invasive sampling seems to be a valid and practical method for measuring stress, there are considerations to take into account, some of which are common for all the media for FGCMs and some are specific to a given media.

4.2.1 Urine

Urine sampling can be conducted on many species non-invasively by collecting samples when excreted, without disturbing the animal, which makes it efficient for free-living as well as captive animals. A disadvantage of sampling urine is that many felids void by spraying, which makes it difficult to collect (Moreira *et al.* 2007). Furthermore, the primary excretion of cortisol metabolites is through faeces (Conforti *et al.* 2012).

An advantage of this media, is that GCs excreted through urine are protein-bound corticosteroids from the kidney filtrate, and thus are a direct reflection of the free hormone in plasma during the time between voiding (Cook 2012). As urinary cortisol is a product of adrenocortical output and hydration, two individuals with the same plasma levels of cortisol can excrete different urinary cortisol concentrations, due to differences in the total urine output throughout the sampling period. Thus, it is necessary to correct for the effects of dilution. A common way to do so, is by expressing urinary cortisol as a ratio to urinary creatinine (i.e. a breakdown product of creatine phosphate, which is a skeletal muscle peptide). Because creatinine is produced at a relatively constant rate with almost no reabsorption by the kidneys, this is relatively unaffected by urine volume (Cook 2012).

A beneficial aspect of measuring cortisol in urine, is that there are no special requirements for the collection and storage of the samples. However, as with other media, urine should be frozen as soon as possible after sampling to avoid bacterial decay. Hormones in urine are measurable in most immunoassays and assay sensitivity is rarely a problem, as long as sample volumes are not too small. It is necessary to be aware of assay specificity, because the presence of cross-reacting and structurally similar conjugated compounds is the major contributor of the variation in cortisol measurements between assays (Cook 2012).

4.2.2 Hair

Measuring cortisol in hair usually requires some handling of the animal. This makes it best suitable for captive animals, as it can be difficult to access free-living animals. Although hair sampling is considered to be minimally stress inducing, it often entails handling or even fixation of the animal (Cook 2012).

A great advantage of hair sampling is the accumulation of cortisol over time, which provides a timeline of the level of stress the animal has experienced during a period of weeks or months, depending on rate of hair growth (Sheriff *et al.* 2011). This also entails that it is not necessary to collect samples regularly, as necessary with other non-invasive media, although it will create more accurate results if the hair is re-clipped in order to display GC exposure between samplings. Re-clipping entails considerations of shedding and anatomical locations, as there are differences in rate of hair growth (Sheriff *et al.* 2011; Cook 2012). Hair sampling is thus a valid technique for assessing chronic stress, yet is a less useful approach for assessing more transient stressors (Sheriff *et al.* 2011). However, this can to an extent be overcome by clipping the hair sample in shorter pieces, provided the rate of hair growth is known (Cook 2012).

The study of GCs in hair needs significantly more empirical data. So far, few studies have used this media and most of them are conducted with a small number of samples and in addition, few have made attempts to establish whether hair GC levels accurately reflect long-term plasma GC levels (Sheriff *et al.* 2011). Due to the relatively scarce knowledge of how GCs are incorporated into hair, the contribution from various sources is not yet known. As several wild animals secrete scents onto the fur in certain areas of the body, which vary seasonally or with dominance, these secretions along with GCs from saliva from grooming, may contribute to variations in measured GC levels. Some researchers recommend washing the hair before analysing it, although this contains the risk of the sterilization products penetrating the hair and removing incorporated GCs (Sheriff *et al.* 2011).

Hair samples seem to be containable at room temperature for a longer duration, which make them practical to store. Assay sensitivity is a key issue for measurement of hair cortisol content, as it is generally low. Typically, 7-50 mg of hair is necessary when using commercial kits (Cook 2012).

4.2.3 Saliva

Saliva offers a non-invasive sampling medium that reflects free GC concentrations in plasma, and the high positive correlation between salivary GC levels and free serum GC levels remain high throughout the circadian cycle (Sheriff *et al.* 2011).

Sampling saliva is usually done by obtaining an object chewed or licked on by the animal, which offers the opportunity of collecting samples without being in close proximity. This enables the opportunity of collecting saliva samples from free-living animals as well, although it might be more difficult to obtain samples in regular intervals. When using saliva sampling as a technique in free-living animals, it does offer the issue of providing

suitable chewable or lickable objects along with ensuring that only a single, known animal deposits its saliva on the object.
When sampling from animals by handling (e.g. swabbing saliva directly from the mouth) the increase of cortisol does not occur until 20-30 minutes later, in contrast to three minutes in plasma, and thus is not subject of biasing the results (Sheriff *et al.* 2011). Although from a welfare point of view, handling is still potentially stressful for the animal.
The samples can easily be stored at room temperature for two days or up to four weeks, as GCs are highly stable in saliva. Furthermore, by cooling or freezing the samples, they can be stored for 3 months or 1 year respectively. A variety of commercial sample analysing kits are available, which makes it fairly inexpensive to analyse while making it possible to use sample sizes as small as 2-10µL (Sheriff *et al.* 2011).

4.2.4 Faeces
The methodological part of measuring cortisol in faeces requires theoretical experience and a general understanding of the excretion routes of cortisol. The majority of the literature used in this project focuses on faecal sampling, as the logistical aspect of faecal sampling is less troublesome in the field in comparison to urine. Furthermore, metabolism studies on the domestic cat (*Felis catus*) have shown that adrenal metabolites are excreted primarily in faeces rather than in urine. In addition, because only the free GC fraction from plasma is available for metabolism and excretion, FGCM concentrations reflect the biologically active portion more accurately (Palme *et al.* 2005).
Sampling of faeces is practical and fairly easy, as it is not necessary to be in close proximity to the animal. Thus, it can be used with both captive and free-living animals without stressing the animal. Furthermore, sampling can be done by untrained personnel and samples can be collected regularly through time.
Faecal sampling has the disadvantage of not providing as many physiological indicators of stress as blood samples does and it can be difficult to link the excreta to the individual animal.
In addition to being non-invasive and minimally stress-inducing, faecal sampling offers the advantage of not causing biased results introduced by restraint or handling or caused by short-term fluctuations of GCs, as a result of natural pulsatile changes. This is so, due to the time from the initial release of GCs, as a response to a stressor, to the marker appears in faeces, is far longer than a potential handling or restraint time (Sheriff *et al.* 2011). The steroid metabolite concentrations represent a pooled value of steroids during the previous 12-24 hours, and thus are less affected by episodic fluctuations and the pulsatility of hormone secretion (Ludwig *et al.* 2013; Wielebnowski *et al.* 2002).
When collecting faecal samples, it is of importance to consider that FGCM levels can be affected by environment, age of the sample along with bacterial and microbial degradation post excretion, as samples often cannot be preserved instantly and the time between defecation and collection is often unknown (although both wet and dry faeces can be used for analysis). Thus, samples should be preserved as soon as possible after collection, preferably by freezing (Sheriff *et al.* 2011).

4.3 Factors to consider when sampling and analysing faecal glucocorticoid metabolites

Various factors may apply to the experimental assessments of stress, which can be problematic to the interpretation of data.
Measuring FGCMs in faecal samples provides a useful technique for quantifying GCs, yet there are many factors to consider with application. For example, several biotic and abiotic factors may cause disturbances in
FGCM concentrations, along with factors such as individualism, methodological issues, such as storage techniques or sampling factors, such as age of sample (Millspaugh & Washburn 2003; Millspaugh & Washburn 2004).
The following section is describing the various factors that may cause alterations in the FGCMs and thus cause biased results. The focus is on faecal samples, as this is the most commonly used sampling technique among Felidae in captivity and in the wild.

4.3.1 Experimental factors

4.3.1.1 Handling of faecal samples
There are several techniques for storing faecal samples. Before processing, faeces can be stored as frozen on wet or dry ice, frozen in liquid nitrogen or stored in ethanol, without significantly altering the steroid hormone concentration. In some instances, the samples are heat-desiccated prior to processing. Frozen storage has been the most commonly used technique under controlled laboratory conditions, as this prevents bacterial metabolism of steroids. However, as the conditions required are not always accessible in studies on free-living felids, and storage from remote locations is cumbersome, this may not be an option in the field.
Laboratories often dry faecal samples prior to analysis to adjust for water content, and thus sample desiccation in the field could be a potential preservation technique, allowing transportation of the sample at ambient temperatures, although further studies are needed to validate this technique (Terio *et al.* 2002). Terio *et al.* (2002) found that the concentration of steroid hormones in faecal samples of cheetahs, were not significantly altered when preserved in ethanol and stored at ambient temperature for at least 14 days prior to lyophilisation (i.e. freeze-drying) and extraction.
In spite of the above, storage and processing of samples can induce alterations in steroids in faecal samples and thus caution should be applied when interpreting data. As shown in the study by Terio *et al.* (2002), samples that were dried, in either a solar or conventional oven had variations in steroid hormone concentrations, in which the solar oven caused the most variation in comparison to the control processing technique. The samples in this study, that were stored in ethanol and later dried in a solar oven, showed lower cortisol concentrations. The variations that occur when samples are exposed to heat and light, may result from biochemical alterations that change the immunoreactivity of the steroids and

cause degradation. In addition, the alterations occurring in the samples that had been stored as frozen and then oven-desiccated, may be due to bacterial metabolism during the drying process. To eliminate this risk, storage in ethanol is advised due to its antibacterial effects (Terio *et al.* 2002).
It is not always possible to apply the most advantageous techniques, as shipping regulations in certain countries, may require treatment of samples in order to import these, to eliminate the risk of importing potential pathogens. However, these treatments can cause alterations in the FGCM levels and thus inaccurate measures may follow.
Under normal environmental conditions, faeces will be exposed to ultraviolet light and undergo heat desiccation, which stresses the importance of collecting the faecal samples soon after defecation, in order to avoid steroid degradation and alterations (Terio *et al.* 2002). Thus, the increased time frame of which faecal samples are collected after defecation along with the different storage and processing techniques, serve as a risk of getting inaccurate results.
The collecting of fresh faecal samples is also important, as older samples are susceptible to other environmental conditions, such as rainfall, which may cause artificially increased GC concentrations (Millspaugh & Washburn 2003). It is suspected that the additional moisture added by rainfall, may create a favourable environment for growth of microbes and detritivores. This is based on findings of fungi being present in samples exposed to rainfall, where these microbes metabolized the metabolites, causing an augmented relative affinity towards the antibody used in the study, resulting in elevated FGCM concentrations (Millspaugh *et al.* 2002; Millspaugh & Washburn 2004).
Consistency when collecting faecal samples is also of importance, as infrequent sampling or failure to collect samples, may result in less valid FGCM concentrations (Young *et al.* 2004).

4.3.1.2 Assays

Different assays utilizes certain antibodies with variations in the affinity for FGCMs and in addition, different techniques for preparation of samples are often used. Thus, it is not appropriate to compare data of these across different studies when different assays are used. Without standardized conditions in the field and laboratory, such as storage technique, age of sample and extraction technique, the direct comparison of studies should be conducted with caution, although biologically important trends in the different assays can be compared (Millspaugh & Washburn 2004). Therefore, application of the non-invasive techniques for assessing stress hormones need validation of each species and each hormone, as the antibodies used are often specifically generated to react against the native steroid hormone and thus can have unknown cross-reactives with the excreted faecal metabolites (Ludwig *et al.* 2013). Also, appropriate assays need to be broad spectrum, cross-reacting with the variety of corticoid metabolites found in faeces, yet these possible cross-reactives should be known (Brown 2006). Findings stresses this point, as results from a study on the spotted hyena (*Crocuta crocuta*), demonstrated that cortisol-3-CMO EAI is well-suited for this species, but Ludwig *et al.* (2013) proved it to be inapplicable on

cheetahs. In addition, another study found that cortisol-3-CMO is suitable for cheetahs, whereas yet another found it unsuitable for clouded leopards (Ludwig *et al.* 2013). The techniques of RIA (with application of antibodies to corticosterone) and EIA (with application of antibodies to 11,17-dioxyandrostans or cortisol), has proven to be useful in the Felidae family, with RIA most commonly used (Brown 2006). However, additional data is needed, involving the use of antibodies in laboratories, as different laboratories use antibodies from different manufacturers. The study by Ludwig *et al.* (2013) stresses this, as findings show that similar EIAs, derived from different laboratories, may provide different results. This does not allow comparisons of absolute values and thus, for future application, it requires determination of the basal concentration of different hormones in each laboratory on the basis on existing data (Naidenko *et al.* 2011).
During the transit of steroids through the intestinal tract, enzymatic activities of bacterial flora facilitates changes to the steroid metabolites and creates a vast variety of metabolites with different structures, even in closely related species (Ludwig *et al.* 2013 ; Young *et al.* 2004). Thus, because steroid metabolites are catabolized before excretion, immunoassays with antibodies that are specific to plasma GCs, may not be appropriate for measuring FGCMs and thus, group-specific antibodies that cross-react with a family of metabolites that are derived from a single parent steroid, is preferred for a more valid indication of adrenocortical activity (Young *et al.* 2004). Additionally, it is important to note that the metabolism of stress hormones and gonadal hormones may result in several faecal metabolites that have similar structures, as the native hormones are similar in structure. Thus, if not accounted for, these metabolites with similar structures, yet of different origins, will display higher stress levels than initially induced (Ludwig *et al.* 2013). This issue is displayed in the study by Ludwig *et al.* (2013), who found that the conducted radio-metabolism study displayed a high amount of polar metabolites and the radioactive peak that occurred, might display a cluster of polar metabolites whose steroid origins are unidentified. (Ludwig *et al.* 2013).

Based to the previously described factors that may interfere with the accuracy of measuring of FGCM concentrations, and perhaps additional factors that have yet to be described or discovered, it is obvious that a broad awareness of how the various factors alter the FGCM concentrations and interact is needed and must be accounted for when interpreting the results, along with great caution on the methodological performance.
As mentioned earlier, findings from studies indicate that faecal samples that are not collected while fresh may contain erroneous GCs concentrations. Thus, the sample should be collected soon after defecation, ideally less than two hours later. Although this may be difficult to achieve in the field, it should be a priority as FGCMs may change relatively rapidly (Millspaugh & Washburn 2004). In addition, if the faecal samples are collected with no relation to the animal, individual factors that may affect GC levels will not be considered. This can occur in studies conducted in the field, as tracing the sample back to the animal may be difficult.

The storage techniques of faecal samples also poses a great risk of inducing misleading results. In order to reduce the alterations in FGCMs, immediate freezing and no chemical treatment of the sample should be practiced, in order to slow microbial metabolism of the metabolites and reduce problems with immunoreactivity (Millspaugh & Washburn 2004). Liquid nitrogen or dry ice should be used soon after sampling, although this may be difficult while working in the field. If chemical treatment is unavoidable, the effects of these on the FGCMs are necessary to understand and even if the use of these are avoided, lyophilisation and extraction to avoid effects due to freezing alone, is the best approach. Duration of storage also needs to be accounted for when comparing faecal samples that have been stored for different durations (Millspaugh & Washburn 2004). Thus, as recommended by Millspaugh & Washburn (2004), species-dependency, storage duration in chemicals and whether the sample has been frozen or not, should be accounted for when analysing data.
As different studies may use assays with different antibodies, that have different affinities for metabolites, and different preparation techniques may be used, it may not be appropriate to compare the findings of these different studies.

4.3.2 Non-experimental factors

4.3.2.1 Species-specific factors
A study by Young *et al.* (2004) compared cortisol EIA with corticosterone RIA for the monitoring of adrenocortical activity in a number of carnivore species (including felids), and found a variation between suborders, but not among felids. Among felids, the elution patterns were similar, which is pointing towards a conservation of the GC metabolism within the family. However, the assays found different GC metabolites that was species-specific. Regardless of different metabolites, the longitudinal profiles proved to be qualitatively comparable and the data obtained were highly positive correlated, proposing that both techniques were correspondingly effective in monitoring HPA activity (Brown 2006).
The adrenal cortex synthesizes GCs following ACTH administration, and plasma concentrations of these hormones will rapidly elevate. However, the appearance of these in faeces are much slower and will emerge in a time equivalent to the passage time of which it takes digesta to pass from the duodenum to the rectum. This varies within species, and thus it is a factor to take into consideration when measuring faecal stress hormones, in order to obtain accurate results (Young *et al.* 2004). This assessment of delay must be conducted on every species, in order to quantify the lag time to use as an indicator on what factors are affecting rises in cortisol levels, as the lag time can range from less than 30 minutes to more than a day inter- and intra-species, depending on the activity of the individual (Palme 2005). By implementing this, it is possible to determine the factors affecting stress levels and potential fluctuations can be accommodated to specific events (Narayan *et al.* 2013).

4.3.2.2 Within-sample variation and individualities

Data suggest that there is a variability of the corticoid concentrations excreted in faeces among individual animals. Thus, it is of importance to establish baselines for each animal and draw comparisons of these, in order to conduct comparisons of cortisol levels across individuals (Graham & Brown 1996). In the study by Ludwig *et al.* (2013), findings demonstrate this variability among individuals. As the study can exclude the possibility of a biased result due to infrequent faecal collecting, the study suggests that the deviations in faecal metabolite concentrations among individuals are caused by individual variances in liver metabolism, individual rate of gut passage along with quantitative and qualitative variations in composition of gut bacteria. Thus, when measuring stress hormone concentrations within species, this intra-individuality should be accounted for and studies should therefore base the results on large sample sizes.

Within-sample variation may also occur and act as a factor that can cause misleading results. One variation can occur due to unevenly distributed GC metabolites throughout faecal mass, that in turn can lead to high intra-sample discrepancy, which may affect the ability to detect stress-induced effects (Millspaugh & Washburn 2003). This have been demonstrated on other hormones in a study by Brown *et al.* (1994), who reported unevenly distributed faecal estrogens and progestins within individual faecal samples from cheetahs, clouded leopards and snow leopard (*panthera uncia*) (Millspaugh & Washburn 2003).

The intra-species variation in GC metabolites in faecal samples may also vary among species. Dietary sources may influence FGCM distribution within samples and so the metabolites may be more unevenly distributed in faecal excreta from carnivores than herbivores. As suggested by Brown *et al.* (1994), faecal hormone variability among felids may be due to uneven distribution of hair residue in the samples (Millspaugh and Washburn 2003). It is also suggested that animals may have elevated circulating GC concentrations following the consumption of an animal-based diet (Millspaugh and Washburn 2004).

To avoid these irregular distributions of GC metabolites, it is common to homogenize the entire faecal mass and use subsamples for analysis, although this may be difficult to apply in the field. In addition, some faecal samples may not be intact and thus may not represent the entire faecal mass (Millspaugh & Washburn 2003). Therefore, in order to validate whether smaller portions display accurate measurements, experiments should account for variability among repeated GC metabolite measurements from the same faecal sample, in which a high variability implicates the need for additional samples to achieve statistical indication (Millspaugh & Washburn 2003). It is noteworthy, that Millspaugh & Washburn (2003) also found variations among estimates derived from mixed sub-samples.

Some studies display an absence of elevations in GC concentrations in animals, when otherwise elevations might be expected. In the study by Young *et al.* (2004), various carnivores failed to elicit a stress response when experiencing stressors such as relocation, social tension, breeding and restraint. Of course, this could be due to the animals perceiving these as non-stressful, but it could also be due to individualities among the animals in general. Adrenocortical responses are known to vary among animals, which

reflects the difference in how stimuli are perceived. In addition, the responses that are evoked to react to a stressor, depends on several factors, such as previous experiences, age, genetics and physiological state (Young *et al.* 2004). It is also possible that the elevations in GCs are not detectable, although the stimuli are in fact causing a stress response, as small or brief elevations in GCs in plasma can be concealed by the assembling of metabolites in bile and faeces and thus will not display a stress response measured experimentally (Young *et al.* 2004).
When comparing individuals, the assumption that elevated GC levels in one compared to another, is due to the individual with lower levels being less stressed, might be faulty, as previous stressors may have altered HPA axis function. A possible explanation for situations with elevated GC levels in one individual compared to another (when otherwise equal GC levels might be expected), is that both are chronically stressed, but the one with lower GC levels have acclimated, whereas the other have not. Another situation could arise due to acclimation followed by facilitation occurring in one individual, whereas the other individual has not acclimated and thus will elicit lower GC responses. As both of these individuals will have similar baselines, it is the facilitation that is causing the difference in GC levels. Lastly, only one of the individuals may be suffering from chronic stress and as a result cannot mount an appropriate HPA response, and so the GC elevations will be minor (Romero 2004).

4.3.2.3 Early neonatal stressors

Early neonatal exposure to stress may cause variations in stress responses among species, as exposure to certain stressors has proven to affect the stress response, which persists throughout adult life. The alteration in stress response can either occur due to maternal exposure to chronic stress prepartum, or due to neonatal stress exposure. The exposure to stressors early in life results in significant life-long changes in the HPA axis. A study on rat pups found that exposure to moderate stressors (i.e. handling of the rat pups 15 minutes each day) in early life, caused 40 – 50 % lower GC responses in adult life, in comparison to pups not exposed to moderate stressors, yet no variation occurred in basal levels. It is suggested that this is a mechanism, which causes an increase in the efficacy of negative feedback in stressed pups. Conversely, early neonatal exposure to stronger stressors (e.g. extended maternal separation), resulted in life-long hypersecretion of GCs to moderate stressors. In addition, whether the neonates were exposed to these stressors or not, they all exhibited similar GC elevations in response to more severe stressors as adults (Romero 2004).
These factors can be relevant in studies on both captive and free-living animals, as early stressors can arise in both cases. For example, a study on kittiwake chicks (*Rissa tridactyla*) found that decreased parental provisioning, due to foraging being encumbered by habitat loss, resulted in elevated GC levels, and thus may cause decreased GC levels later in the adult life of these chicks. This tendency is presumably also seen in felids and thus, knowledge of applied conditions around birth is important in order to interpret the results correctly, as result may vary inter-species (Romero 2004).

Early neonatal experiences concerning stress may act as a confounding factor when measuring cortisol levels in free-living felids, as the occurrence of these most likely are unknown. It is thus plausible that stress responses induced by moderate stressors, of free-living animals, may cause biased data as these animals are eliciting lower responses and hence their stress levels might faulty be interpreted as lower. This also contributes to potentially biasing the results in felids in captivity, in cases when the early neonatal experiences are either unknown or not taken into account.
Recent studies have found that pathophysiological effects are not restricted to the first generation of offspring and that there is a transgenerational memory of fetal experience, which persists in multiple subsequent generations. Thus, early neonatal conditions occurring in the fetal stage, not only alter the biobehavioural response to stress in the offspring, but also in future generations (Matthews & Phillips 2013).
Most studies conducted on stress assessments in both captive and free-living felids, do not seem to account for this potential risk factor when interpreting the results and thus it may prove a useful to implement in the data to clarify possible bias.

4.3.2.4 Pathology and stress

Studies have found a positive correlation between medical conditions, such as illness and infections, and GCs. In the study by Young *et al.* (2004), results revealed a close association between elevated excretion of metabolites and medical treatment or abnormal defecation, in the female sloth bear (*Melursus ursinus*), which suffered from a persistent nematode infection. This suggests that infectious stages like this or the treatment itself (e.g. deworming) and possible side effects (e.g. diarrhoea), may induce a stress response in the infected animal. Other studies conducted on horses (*Equus ferus caballus*) and dogs (*Canis familiaris*), displayed elevated cortisol levels associated with abdominal illnesses of which required surgery, also emphasizing the positive correlation between stages that are harmful to the organism and stress responses (Young *et al.* 2004). The endogenous and exogenous stimuli that act as stress factors, can especially be prominent in free-living felids compared to felids in captivity as the risk of parasite infections and disease are increased. Thus, it is of relevance that these factors are accounted for as they can distort data when measuring both stress-induced responses and baseline cortisol levels.

4.4 Factors to consider when using non-invasive sampling of cortisol as a welfare indicator

When measuring cortisol levels as a mean of assessing distress, there is a variety of things to consider. There is a risk of biasing the results when measuring GCs in plasma, as capturing and handling animals can cause a momentary increase in plasma cortisol levels. As everyday stressors affecting the animals are of primary interest, this will cause an increase not related to these. Thus, non-invasive sampling offers an alternative to this and seems to be a valid method for assessing welfare by measuring cortisol levels, yet one must consider the relationship between behaviour and physiology, along with the factors

affecting the fluctuations in cortisol, that are not necessarily decreasing welfare in captive animals.

4.4.1 Stereotypic behaviour

It is generally accepted that high levels of cortisol is correlated with high levels of stress, and thereby equated to compromised welfare. But what if the animal is coping with its environment? According to the definition of chronic stress, it is the animal's inability to cope with its environment, and if unable to do so, it will experience distress. As stereotypic behaviour is hypothesised as being a coping mechanism (Broom 1991), resulting from an unfavourable environment (Sajjad *et al.* 2011), the animal must previously have experienced distress. Assumed that stereotypic behaviour is helping the animals to cope with their environment, the baseline cortisol levels should be lower in these, compared to distressed animals that do not perform stereotypic behaviour. However, according to Svendsen *et al.* (2013), stereotyping mink (*Neovision viso*) had higher baseline cortisol levels than non-stereotyping mink, although other studies, with conflicting results, found that individuals spontaneously developing high levels of stereotypic behaviour seemed to cope better with adverse situations than non-stereotyping individuals, living in the same conditions.

Wielebnowski *et al.* (2002) found that clouded leopards performing stereotypic pacing, had higher cortisol levels and Wells *et al.* (2004) found a correlated decrease in stereotypic behaviour and cortisol concentrations when captive cheetahs were provided with more hiding places. Quirke *et al.* (2012) refer to conflicting results concerning the cortisol levels of mammals performing stereotypic behaviour: two studies found a negative correlation between cortisol levels and stereotypic behaviour, yet several other suggest that an increase in stereotypic behaviour is correlated with a subsequent increase in cortisol levels. Likewise, Moreira *et al.* (2007) found that high cortisol levels in tigrinas and margays were associated with increased agitated behaviour, and most pronounced was the stereotypic pacing from one side of the enclosure to the other.

The above indicates that stereotypic behaviour does not decrease baseline cortisol levels in captive felids and that, albeit a coping mechanism, is not a welfare-increasing behaviour and is not likely to cause bias in the results of stress assessment.

4.4.2 Apathy

As stereotypic behaviour seem to cause an increase in cortisol levels, the same seem to apply for apathy. After a period of initial agitation, Moreira *et al.* (2007) found that both species examined exhibited more passive behaviour when housed in small barren environments. This is due to chronic stress and is a behavioural characteristic of helplessness when adapting to an adverse situation, and is often associated with an increase in HPA activity. Moreira *et al.* (2007) suggests that as anxiety increased, active coping shifted to a more passive manner. The same was found in the study by Wielebnowski *et al.* (2002), who reported that sleeping, hiding and generally acting fearful, along with self-mutilating behaviour, was associated with increased faecal corticoid

excretion. Also, a study on the domestic cat, found that generally decreased activity is associated with elevated serum cortisol levels, a finding supported by Carlstead *et al.* (1993).

4.4.3 High levels of cortisol do not equal bad welfare

Another consideration one must take into account when using cortisol as a welfare indicator is that, according to our definition of welfare in chapter 2, high levels of cortisol does not necessarily equal bad welfare. As seen in Naidenko *et al.* (2011), some free-living felids have higher cortisol baselines than captive felids, yet they do not seem to have physiological problems reproducing, nor do they perform stereotypic behaviour. This may imply that their welfare is not compromised to an extent that challenges their fitness. Although free-living felids do experience significantly stressful situations, for example stress occurring as a consequence of mating, hunting, defending territories or escaping potential danger, this is generally accompanied by the subsequent opportunity to perform natural behaviours.

As previously mentioned in chapter 3.2, the seasonal changes are also associated with varying levels of FGCMs (Romero 2002). The difference between stress experienced by captive felids and by free-living felids respectively, is that free-living felids usually have the opportunity to cope with the situation (e.g. remove themselves from a potential threatening situation; seek shelter, cool down etc.), along with the fact that these stressors are transient and thus do not elicit chronic stress, whereas captive felids does not have this opportunity. Thus, it is assumable that high cortisol levels do not necessarily equal bad welfare. When assessing stress, and thereby welfare, by measuring cortisol levels, it is important to take the circumstances regarding the animal into account (e.g. living conditions, reproductive cycles or temperatures outside the thermal neutral zone), in order to establish what stressors are contributing to reduced welfare and what stressors are naturally occurring and fitness-increasing.

4.5 Is measuring cortisol levels a valid method for assessing stress and welfare in non-domestic felids in captivity?

In order to establish whether non-invasive sampling is a valid method for assessing stress and welfare in captive felids, one must consider the potential risk factors reviewed in the preceding sections. When conducting welfare assessments on captive felids using non-invasive sampling, one must consider the most appropriate sampling media for the species in question. In addition, it is of importance to consider the experimental factors, including handling of faecal samples and assays; and non-experimentally factors such as species-specific factors, individualities and pathology, possibly distorting the data.

It is evident that the results from measuring FGCM concentrations will not provide on-point values, and the accuracy depends on the numerous factors outlined in the above. With every faecal sample and the information provided hereby, follows several sources that are potential causes of error, yet when these risk factors are well considered and great

caution is taken when interpreting the data, measuring cortisol levels in faecal samples is an approach that provides a good indicator of the stress level and thereby the welfare of animals.
Finally, the theoretical frame on how stress and cortisol correlates, and which behavioural stress responses elicit increases in cortisol levels must be considered in order to establish whether this reduces welfare. When taking these reviewed considerations into account, non-invasive sampling seems to be a valid approach for measuring cortisol levels, as well as for assessing welfare in captive felids.

5. Discussion

During this project, we have reviewed the physiology of stress responses, the physical consequences of chronic stress and briefly, why cortisol is the appropriate hormone to measure, in order to understand the basic mechanisms of the stress response. We have looked at the term 'welfare' and the term 'stress' and how these two are connected. This is relevant as stress is often used as a measure to assess welfare, and in order to conclude whether the animals experience bad welfare, one must establish exactly what this entails. As the frames of welfare differ between captive and free-living felids, with numerous factors affecting each group differently, one might question whether it is appropriate to use cortisol levels of free-living felids as a baseline for comparing cortisol levels to captive felids, in order to assess welfare of these. In chapter 3, we review the different factors affecting both groups, based on available literature, in order to examine whether the two groups offer an appropriate basis for comparisons. It is evident that the everyday stressors affecting each group are so distinct, that great caution must be taken when comparing these and an in-depth understanding of how the different factors interact and influence GC levels, is needed.
The advantages and disadvantages of cortisol measurements in different non-invasive sampling media are looked upon in section 4.2 and the many benefits of faecal sampling forms the basis for section 4.3. In this section, the techniques for determine coherence between stress responses and GC excretion through faeces are reviewed, along with the techniques for analysing these hormones in samples and the factors that are important to take into account when sampling, analysing and using cortisol as a measure of welfare, in order to get a clear understanding of what factors can cause potential biased results.
Finally, important behavioural responses to chronic stress, along with our arguments that high levels of cortisol do not necessarily equal bad welfare, are examined in order to establish if these are in fact prone to distort the interpretation of data. The factors reviewed in chapter 4 lead us to the discussion on whether non-invasive sampling is a valid approach for assessing stress and welfare in captive felids. Our findings imply that this is indeed a valid method.

We have not limited our project to involve only a specific species of Felidae; instead, we have focused on non-domestic felids in general, as the literature available is relatively scarce. Due to several variations among Felidae species, such as social structures, seasonal variables, hunting behaviour, mating systems and to some extent physiology and stress responses (i.e. species may elicit stress responses differently), it is of importance to incorporate these when interpreting data and comparing across species. In order to assess stress levels intra- and inter-species, it is evident that obtaining additional data on baselines, differences among species and intra-species stress responsiveness is also needed.

To assess cortisol levels in a specific species, it is evident that obtaining additional data on baselines and intra-species stress responsiveness is crucial in order to get valid results, and if one is to compare with another species, all the variations in regards to the species must be examined and incorporated into the interpretations.

We started this project with the assumption that we would be able to assess the welfare of captive felids, by comparing their baseline cortisol levels to the levels of free-living felids. It has since become evident, that the factors affecting each group are so distinct that these encumber the comparison of baseline cortisol concentrations. Free-living felids are subjected to numerous naturally occurring stressors, a lot of which captive felids do not experience as they are generally provided with food, mates, veterinary control, and guaranteed shelter, yet are living in limited space with reduced opportunity to perform natural behaviour. This emphasizes the difficulties of making comparisons between the two groups, which is a tendency reflected in the available literature, as only few articles focuses on this approach.

Despite the larger amount of GCs in free-living felids described by Naidenko *et al.* (2011), and the various potential stressors experienced by free-living felids, reproductive problems rarely occur in the wild. As captive animals do not experience the same stressors as free-living animals do, and that the reduced opportunity to perform natural behaviour seem to be the most prominent stressor, it is plausible that the reduced reproduction can be linked to this kind of stress.

Although difficult to compare, it is evident that the stressors affecting captive and free-living felids are very different and have different impacts on cortisol levels. Thus, it is of importance to acknowledge the factors affecting captive felids, in order to increase the quality of ex situ conservation, welfare and reproduction.

Instead, using the baseline of the animals themselves as a comparison for cortisol concentrations seems to be more applicable, as it is then possible to look at the fluctuations in GCs when the animal is subjected to potential stressors. This does still give rise to difficulties, as the baseline is then from an animal that is probably already stressed and reproductively challenged (as it is the onset for the research). Furthermore, the animal could be acclimated and facilitated or under other influences, hence biasing the result. Thus, it is of importance to consider the factors that could possibly affect the results, as we have throughout this project. Taking these considerations into account when using the GC levels of the animals themselves as baselines, will provide an indication of what factors

alter cortisol levels, and this can therefore be used to improve living conditions, in hope of a subsequent reduction in baseline cortisol levels.
Establishing the baseline cortisol levels, based on and limited to the animal itself, may be both time consuming, expensive to apply in practice and come with logistic difficulties. Collecting samples (e.g. faeces) from specific individuals can be cumbersome when specifying samples for more than one individual in a large sample group. Thus, in order to achieve the most precise assessments combined with the practical implications, using a representative for the species might be an applicable approach. So far, the domestic cat have been used as a generally accepted model for felids, but due to their multiple ways of living, using a model for each species within this family may offer more accurate and obtainable results.

Comparison of stress levels between populations or individuals, might be troublesome if some animals involved are acclimated or facilitated to stress, due to occurrence of continuously repeated or chronically elevated cortisol levels. Therefore, elevated GC levels may not necessarily be a direct indicator of increased stress levels in one population or individual compared to another. Thus, for future studies we recommend to account for acclimation and facilitation in interpretation of data whenever possible, although this can prove difficult, especially in free-living felids, as previous exposure to stressors most likely is unknown. To assess whether animals have been acclimated or facilitated, ACTH administration may be an option in order to examine whether stress responses have been altered, although this approach may only be an option to conduct on felids in captivity, as free-living felids are not easily accessible.

It is plausible that free-living felids that have down-regulated GC responses (either naturally occurring or induced by chronic stress), might undergo an evolutionary decline (Romero 2004) and thus these individuals will not be included in data on free-living felids, to the same extent as they do in captivity. These animals may have reduced survivability as a result of poor coping mechanisms, caused by failure to elicit proper stress responses (Busch & Hayward 2009). In captivity, these individuals may not be excluded from breeding programs, as this impaired responsiveness may either not be observable, perceptible or due to poorly controlled breeding programs, and this can therefore lead to an unintentional conservation of this unwanted trait. Thus, it is feasible, that the altered responses of these animals will be incorporated into data and perhaps bias results, when comparing felids in captivity and in the wild. This is especially problematic, as these individuals in nature most likely will be limited, if not absent, and thus this may be a factor to incorporate into interpretations of data in future studies. Additionally, this could potentially compromise welfare of felids in captivity, that have inferior responsiveness to stressors, as these may have issues coping with stress and in turn have reduced fitness. A point stressing this claim, and its link to reduced welfare, is that these felids most likely will not survive in the wild due natural selection, and thus these are not functional individuals.

The lack of concern for down-regulated GC responses becomes especially important in a conservation context, where the affected felids either are released into the wild or serve as ambassadors for the re-introduction of the species in a given habitat, and will pass on traits to future generations. Thus, with future studies, it is worth examining whether this is a problem in practice and if so, include it in breeding programs.

This project has focused on GCs as a measure of stress, yet it seems as though stress has significant effects on the reproductive hormones as well. The connection seems logical, as stress does affect reproduction in captive felids. Therefore, it would be interesting to examine how stressors are affecting the reproductive part of the animal endocrinology. This was partly done in Moreira *et al. (*2007), who found that a rise in corticoid concentrations was correlated with a decrease in overall estradiol concentrations, which equals a reduced ovarian activity. Since the concentrations did not return to normal as the corticoid concentrations did, this could indicate that the reproductive hormones are highly affected by stressful environments. As reproduction often is part of the reason for evaluating stress in captive felids, it seems logical to base assessments on these hormones and the interaction of these and GCs, in order to assess whether the population fails to reproduce due to stress or due to unfavourable environmental conditions.
If reproductive hormones are incorporated into future studies, it will be possible to differentiate more between males and females, in order to assess what causes the lack of reproduction. It is generally assumed, that reproductive failure is caused by stress from inadequate living conditions, although it seems, as there is need of additional experimental data, in order to properly connect the two. For example, this could be achieved by examining exactly what causes a decrease in estradiol concentrations (as found in Moreira *et al. (*2007)).
In the assessment of the effects of stress on reproductive hormones, it could possibly prove efficient to incorporate Felidae species that do not have problems reproducing in captivity, to gain an understanding of the factors causing the differences in reproductive success between species. These factors could possibly be related to physiology or the animals' ability to cope with a captive environment.
If future studies were to incorporate assessments of reproductive status through non-invasive methods, it would be possible to identify reproductive problems and thus develop effective solutions.

Data on cortisol concentrations in free-living felids is limited and only few comparisons to captive felids have been made, yet several studies have described cortisol concentrations in captive felids. Thus, it proves difficult to compare cortisol levels of captive and free-living felids, and to draw conclusions based on previous studies in the field. In addition, the studies have been executed under different conditions and the results are presented in different measurements and units, which encumbers comparisons.
One of the few studies comparing cortisol levels between captive and free-living felids is Naidenko *et al.* (2011), who compared levels in the Siberian tiger throughout the year and

found that free-living tigers in general had higher cortisol levels than captive tigers. Naidenko *et al.* (2011) suggest that these higher GC levels may be seasonally induced. However, we suggest that these findings may be affected by methodological circumstances, as sample groups and knowledge of these in the wild are scarce. The study used three males and three females from captivity and regularly collected faeces a few hours after defecation, which offers relatively controlled settings with plenty of opportunity to replicate, yet in the free-living sample group, the conditions were less controlled. There are no available details of number of animals used and thus we assume this was unobtainable. In addition, the duration of the timeframe between defecation and sampling is not specified. The only information provided is that 61 samples were used and collected within one week of defecation. Thus, it is not known whether the samples came from one or several animals, which reduces accuracy when analysing, nor is it known whether the samples had been exposed to microbial or bacterial degradation, as a consequence of rainfall or other climate conditions previously described in section 4.3.1.1.
The lack of information makes the experiment difficult to replicate and we do not believe this is a valid comparison.
Contrary to Naidenko *et al.* (2011), Terio *et al.* (2004) found that captive cheetahs did have higher baseline corticoid concentrations compared to free-living cheetahs. Terio *et al.* (2004) collected faecal samples from 20 captive cheetahs and 20 free-living cheetahs throughout a period of five years (1994-1999), in the months of March - December. Sampling is described in the article as collected for 14 consecutive days. As frequency is not specified, we interpret this as have been done more than once. The knowledge of the captive animals is quite extensive as there is a well-known history of rearing, origin and gender, and there is a protocol of housing and management. The faecal samples were frozen immediately after sampling, although knowledge of the timeframe between defecation and sampling is unknown.
Regarding the sampling from free-living cheetahs, the study by Terio *et al.* (2004) is more informative than the study by Naidenko *et al.* (2011), as the number of cheetahs used is provided. The collection of faecal samples were collected from playtrees (i.e. sites where cheetahs deposit faeces and urine for territory marking). Twenty samples were collected during the same years and months as the captive cheetahs, and selected for analysis based on radio tracking of movements, spoor or video surveillance, to optimize the likelihood of collecting from different animals. Although gender of the cheetahs is unknown, playtrees are mostly visited by males, hence it is assumed that most samples are derived from males. Faecal samples were stored frozen and shipped in ethanol, yet no information on the frequency of sampling is provided in the field either.
Whether the GCs in the samples collected in the field had been degraded was accounted for in this study, as they examined whether other steroid hormones (estradiol and testosterone) was degraded as well, assuming all steroid hormones degrade at the same rate. Results showed a lack of correlation between GCs and estradiol and testosterone, suggesting that the lower GCs in free-living cheetahs was not due to environmental degradation and thus supporting their hypothesis.

Storage and transportation of faecal samples frozen and in ethanol, is the safest technique of handling samples in order to avoid any alterations in GC levels, as done in this study. However, additional information on sampling frequency is useful for potential future replication of the study, although the investigation of the correlation between GCs and other steroid hormone degradation is useful, when frequent and consistent sampling is not possible in the field.
The extensive information in the methodological section in Terio *et al.* (2004), offers a greater opportunity to replicate the experiment and because the two references disagree on their results, it would be ideal to pursue to gain more knowledge on the comparison of GC levels of captive and free-living felids. However, there are still a lot of disadvantages to acknowledge, for example, the lack of information of the origin of free-living felids is naturally very limited. Thus, it could possibly be a useful approach to tag felids from infancy and monitor them throughout their upbringing, as neonatal experiences can affect stress responses later in life.
To identify each individual and link it to the collected samples, implementing some of the techniques used in Terio *et al.* (2004) is a good approach in order attain more accurate data. Due to the qualities described in section 4.2.4, faecal sampling is a useful approach when measuring cortisol concentrations in free-living felids and we recommend this for future studies. When comparing groups, it is a necessity to use the same media in both groups, why faecal sampling should also be used in captive felids. It seems advantageous to collect samples throughout several months, in order to examine the fluctuations caused by factors, such as climate and reproduction, and whenever possible, to collect samples at the same time each sampling. Of course, this complicates sampling in free-living felids, as feeding is not regular and is highly affected by hunting success.
Further studies that are taking these consideration into account are needed in order to establish whether comparisons between captive and free-living felids is a useful approach, in spite of the other factors affecting each group reviewed throughout this project.

Conclusion

The use of non-invasive techniques is still a fairly new approach and requires further studies, as well as acknowledgement and inclusion of the various factors reviewed throughout this project, yet it seems to be valid when assessing stress and welfare in felids. Comparing cortisol concentrations in captive and free-living felids cause many implications, which make it difficult to conduct. Very few studies have applied this approach and the ones that have, found conflicting results. Thus, we have not been able to conclude whether captive felids have higher levels of cortisol, although there are several indications hereof. Future studies focusing on this approach are highly recommended.
Using the GC levels of the animals themselves as baselines is more common and this does seem to be a valid approach, as it is more manageable to account for the various exogenous and endogenous factors causing alterations in GC levels.

All indicators are pointing towards captive felids having chronically elevated cortisol levels, caused by stressors related to life in captivity; this, while having extensive reproductive challenges, leads to the conclusion that captive felids experience distress to such an extent, that it causes deleterious effects on their physiology and welfare.
Further studies, examining the use of non-invasive methods for assessing stress in felids, will broaden the knowledge of stress assessment and the coherence between captivity, welfare and reproductive difficulties, which will help the ex situ conservation of non-domestic felids as well as increase welfare for captive felids.

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