Non-invasive assessment of adrenocortical function in captive Nile crocodiles (*Crocodylus niloticus*) and its relation to housing conditions

by

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IN LOVING MEMORY OF MY FATHER.

DECLARATION

I, Stefanie B. Ganswindt, do hereby declare that the research presented in this dissertation, was conceived and executed by myself, and apart from the normal guidance from my supervisors, I have received no assistance.

Neither the substance, nor any part of this dissertation has been submitted in the past, or is to be submitted for a degree at this University or any other University.

This dissertation is presented in partial fulfilment of the requirements for the degree MSc in Paraclinical Sciences.

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Signed _____

Stefanie B. Ganswindt

Date _____

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LIST OF ABBREVIATIONS

3α,11oxo-CM:	glucocorticoid metabolites with a 3α,11oxo-aethiocholanolone structure
11,17-DOA:	11,17-dioxoandrostanes
ACTH:	adrenocorticotropic hormone
ANOVA:	Analysis of variance
BSA:	bovine serum albumin
CITES:	Convention on Trade in Endangered Species
CSG:	Crocodile Specialist Group
CSL:	cortisol
CCS:	corticosterone
CV:	coefficient of variance
EIA:	enzyme immunoassay
FGM:	faecal glucocorticoid metabolite
g:	gram
h:	hour
HPA:	hypothalamic-pituitary-adrenal
l:	liter
Lrlc:	Low risk, least concern
m:	meter
PBS:	phosphate buffer saline
RIA:	radio immunoassay
SCC:	Species Conservation Group
SD:	standard deviation
SE:	standard error

ABSTRACT

The Nile crocodile (*Crocodylus niloticus*) is one of 23 extant crocodilian species, and has been farmed in southern Africa since the 1960s. For the crocodile industry, chronic stress and its often negative consequences are a concern, since stressors can negatively affect animal production as well as the health of the crocodiles. When confronted with a stressor, an individual displays a stress response consisting of a suite of physiological and behavioral alterations to cope with the challenge. So far, however, no method for determining stress-related responses in Nile crocodiles has been established.

In other crocodilians, the assessment of physiological responses to stress, like the related alterations in glucocorticoid concentrations, has already been done, but only by using an invasive approach, with the disadvantage of a possible handling-induced stress response. By establishing a non-invasive technique to monitor glucocorticoid levels in captive Nile crocodiles based on faecal hormone analysis, this study not only made an important contribution to a better understanding of stress and related hormonal changes in Nile crocodiles, but also provided a solid basis for developing similar non-invasive tools to collect information on the level of stress experienced by other crocodilians.

Specifically the study aimed 1) to assess adrenocortical activity in Nile crocodiles by measuring faecal glucocorticoid metabolite (FGM) concentrations, and 2) to characterise changes in FGM levels in captive Nile crocodiles in relation to different housing conditions.

An adrenocorticotropic hormone (ACTH) challenge was performed on 10 sub-adult crocodiles at Le Croc crocodile farm, South Africa, resulting in serum corticosterone levels of up to ~1200 %, 1 - 5 hours post-injection, above the pre-injection levels. An additional 8 individuals were exposed to electric immobilisation and handling only (control group), which resulted in a 20 – 2700 % elevation in serum corticosterone concentrations, indicating that handling was already a sufficient stressor. FGM levels in 3 singly housed animals (2 ACTH challenge; 1 handling only) reached peaks of 136 – 380 % above pre-injection levels at about 7 to 15 days following treatment, demonstrating that non-invasive hormone monitoring can be used for assessing adrenocortical function in captive Nile crocodiles based on FGM analysis.

By assessing the impact of group size (n = 1, 2, or 4 individuals) on FGM levels, highest mean hormone values were found in the paired animals. A possible explanation for this finding could be that the necessary re-grouping for the study resulted in an unstable group composition, especially for the paired animals of similar size, which is reflected in comparable higher FGM concentrations. However, future research would be necessary to investigate this potential relationship in more detail.

My study created opportunities to improve the management and welfare of farmed crocodiles in terms of more appropriate housing conditions and husbandry for these animals. Finally, the now established non-invasive method for monitoring adrenocortical function in Nile crocodiles provides a solid basis for further studies focusing on monitoring factors influencing adrenocortical function in populations of Nile crocodiles in the wild.

Chapter 1: INTRODUCTION

The Nile crocodile (*Crocodylus niloticus*; Laurenti, 1768) is one of 23 extant crocodilian species (Merchant et al., 2006), and has been farmed in southern Africa since the 1960s (Luxmoore, 1992). The occurrence of stress-inducing factors in captive crocodilians is an omnipresent concern for the farming industry, because stress can negatively affect animal production and reproduction, as well as the health of the animals (Huchzermeyer, 2003, Morici et al., 1997).

Furthermore, the actual appearance of severe pathologies in free-ranging Nile crocodile populations in South Africa underlines the need for respective tools to be able to monitor animals in afflicted areas (Ferreira and Pienaar, 2011; Ashton, 2010; Myburgh and Botha, 2009). Additionally, environmental contaminants could act as stressors as seen in American alligators (Guillette et al., 1997), which could lead to elevated plasma corticosterone concentrations in animals living in contaminated lakes, when compared to pristine areas (Gunderson et al., 2003). Due to the practical difficulties involved in assessing the state of health in free-ranging crocodiles, non-invasively collected information on the level of stress experienced by crocodiles could help to identify affected populations.

Hormone analysis is a precise and widely accepted tool for monitoring responses to stressors (Ganswindt et al., 2012). Although hormone levels can be measured in various biological matrices, non-invasive methods have gained popularity over the past 30 years as a more practical approach for assessing adrenocortical activity in intractable wildlife species (Ganswindt et al., 2012). The use of faeces as basic material for determining glucocorticoid output in crocodiles would allow a non-handling approach and therefore avoid alteration of the results due to handling stress. Furthermore, such an approach would not affect the well-being of the animal and also increase the safety of the investigator during the monitoring process. So far, however, a non-invasive approach has not been used to monitor adrenocortical activity as a measure of stress in any crocodilian species.

By establishing a non-invasive technique to monitor adrenocorticoid function in captive Nile crocodiles based on faecal hormone analysis, this study aims to provide the necessary tools to further investigate the occurrence of stress in crocodiles, its hormonal correlates and their relationship with management practices. Monitoring glucocorticoid metabolite concentrations on

a regular basis could help to improve the management and welfare of captive crocodiles in terms of more appropriate housing conditions and husbandry for the animals. Finally, a non-invasive method which allows us to reliably monitor adrenocortical function in Nile crocodiles could help to develop similar methods for other crocodilians and therefore subsequently assist conservation and education efforts, as well as ongoing breeding programs of endangered crocodilian species.

Chapter 2: LITERATURE REVIEW

2.1 Crocodiles

The Nile crocodile is a reptile belonging to the family of *Crocodylidae*, which includes three subfamilies: *Crocodylinae* (crocodiles), *Alligatorinae* (alligators and caimans), and *Gavealinae* (gharials). The subfamily *Crocodylinae* consists of three genera: *Crocodylus* (true crocodiles), *Osteolaemus* (dwarf crocodiles) and *Tomistoma* (false gharials). Members of the family *Crocodylidae* occur throughout the tropics in Africa, Asia, the Americas and Australia, where they tend to congregate in freshwater habitats like rivers, lakes, wetlands and sometimes brackish water (Cope, 1869; Huchzermeyer, 2003).

The size of crocodiles varies between species, from the dwarf crocodile (~ 1.5 m) to the saltwater crocodile (> 6 m) and they show pronounced sexual dimorphism with males growing much larger and more rapidly than females (Lane, 1996). Although no reliable technique exists to determine the age of crocodiles, several techniques are used to derive a reasonable guess. The most common method is to measure lamellar growth rings in bones and teeth. By using these methods, larger crocodile species are estimated to live around 20 - 50 years (Huchzermeyer, 2003; Lane, 1996). Crocodiles are opportunistic predators, feeding mostly on vertebrates like fish, birds, mammals and reptiles (including smaller crocodiles), sometimes on invertebrates like molluscs and crustaceans (Lane, 1996). As poikilothermic predators, they have a slow metabolism, and thus can survive long periods without food.

Crocodiles reproduce by laying eggs, usually one clutch of eggs per year. Egg and clutch size is species-dependent and varies from 25 - 80 eggs per clutch for Nile crocodiles. Egg size is also age dependent, with younger females usually laying smaller eggs (Huchzermeyer, 2003). Crocodiles do not have sex chromosomes, and, unlike humans, gender is not determined genetically. Instead, gender is determined by incubation temperature in the egg during a thermosensitive period, e.g. predominantly male American alligators (*Alligator mississippiensis*) are produced between 32°C and 34.5°C, and females produced at slightly lower or higher temperatures (Lang and Andrews, 1994). For Nile crocodiles, at 31 °C and below only females are produced. The threshold temperature for maleness ranges between 31 ° and 34 °C, but

appears to vary between clutches (Hutton, 1987). The average incubation period of the eggs ranges from 40 - 100 days depending on the species and temperature (Lane, 1996).

Crocodilians inhabit tropical and subtropical regions. They are profoundly influenced by their surroundings, by virtue of their reliance on environmental factors for regulation of metabolic activity and body temperature (Rooney and Guillette, 2001). Lance and colleagues (2001) mentioned that the preferred temperature range selected by crocodilians is 25 - 35°C. Animals maintained in captivity, where a selection of temperature ranges is not possible, appear to do better at a constant temperature of around 30 - 32°C (Lance et al., 2001), or 28 - 33°C (Huchzermeyer, 2003). All physiological functions of crocodilians are optimal at this temperature range, but they are impaired at temperatures below, and particularly above this optimum (Huchzermeyer, 2003). Animals maintained for any length of time at temperatures only 4°C outside of this range show evidence of severe stress. Any attempt therefore, to assess the physiological and endocrinological response of a crocodilian to stress must take temperature into consideration (Lance et al., 2001).

2.2 The Nile crocodile

The Nile crocodile (*Crocodylus niloticus*) occurs across Africa, from the Senegal River and the Nile in Egypt to the Okavango Delta and South Africa. They occur in the western and southern parts of Madagascar and have occasionally been spotted in Zanzibar and the Comoros (figure 1).

Given the wide distribution range of Nile crocodiles, a number subspecies have been proposed, but they are not officially recognized (e.g. Schmitz et al., 2003). Suggested subspecies are *C. n. africanus* (East African Nile crocodile), *C. n. chamses* (West African Nile crocodile), *C. n. corviei* (South African Nile crocodile), *C. n. madagascariensis* (Malagasy Nile crocodile, Malagasy alligator, Croco Mada), *C. n. niloticus* (Ethiopian Nile crocodile), *C. n. pauciscutatus* (Kenyan Nile crocodile, Kenya alligator, Kenya caiman), *C. n. suchus* (Central African Nile crocodile).

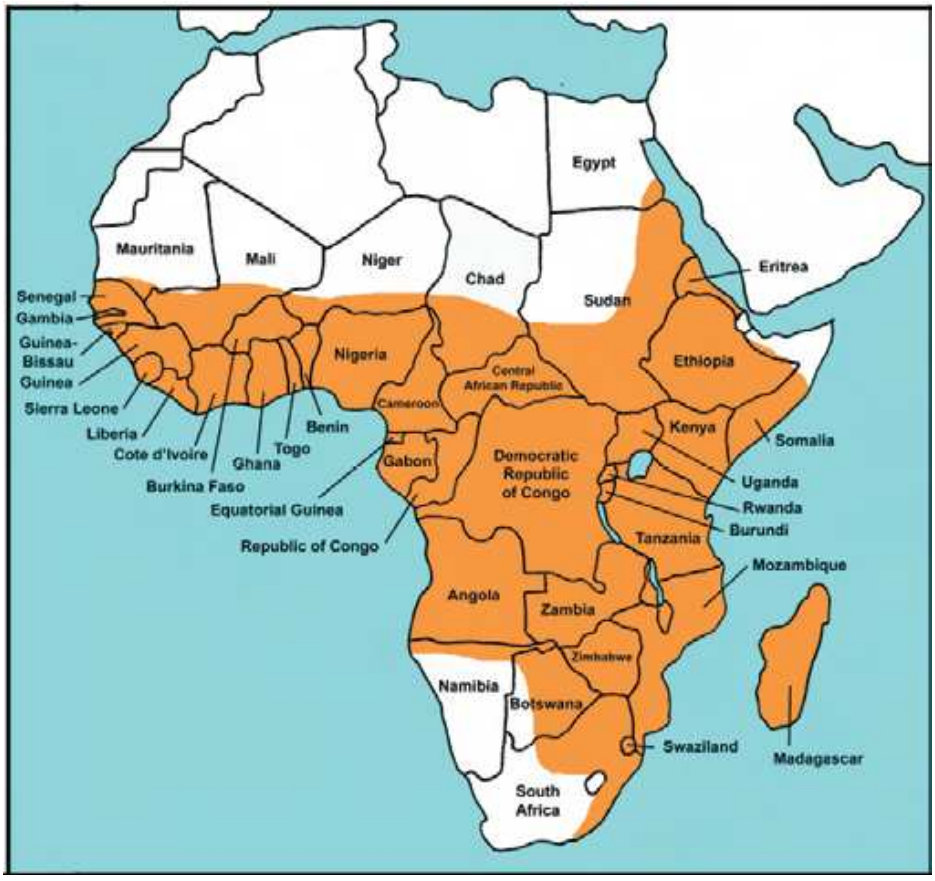

Figure 1: The natural distribution of the Nile crocodile is shown as orange area (Fergusson, 2010).

The Nile crocodile is the largest crocodilian in Africa and can be found in rivers, lakes, marshes and dams, but also in coastal lagoons and estuaries. The male adult crocodile usually measures from 3.5 – 5 metres. Adult males weigh about 200 - 500 kg, but very large specimens can reach up to 900 kg. Like all crocodiles they are sexually dimorphic, with the males up to 30 % larger than the females. Mature female Nile crocodiles measure 2.5 - 4 metres, and typically weigh 200 - 300 kg (Loveridge and Blake, 1972; Flamand et al., 1992).

Nile crocodiles have a dark bronze colouration above, with black spots on the back and a creamy white belly (figure 2). The flanks, which are yellowish green in colour, have dark patches arranged in oblique stripes. In all crocodilians, the nostrils, eyes, and ears are situated on top of their heads, so the rest of the body can remain concealed underwater (Grigg and Gans, 1993).

Figure 2: Nile crocodile (*Crocodylus niloticus*) (photo: Sarah McCans).

The Nile crocodile is an apex predator capable of taking almost any animal that is within attacking range. A significant portion of the diet is fish and other small vertebrates, but adult crocodiles prefer to consume larger prey to conserve energy. The diet of hatchlings consists of small prey, including insects and small aquatic invertebrates, whereas the diet of juveniles includes amphibians, reptiles, and birds (Huchzermeyer, 2003). Nile crocodiles have an ectothermic metabolism and can eat up to 20 % of their body weight at a time (San Diego Zoo Global Library Factsheets).

Nile crocodiles are long-lived (50 to over 80 years), reaching sexual maturity at about 12 - 15 years (Leslie, 1997), over a fairly large size range which is locality-dependent, e.g. in the Okavango region, females reach sexual maturity at 2.3 m total length (Detoeuf-Boulade, 2006). During the mating season, males attract females by bellowing, slapping their snouts in the water, and blowing water out of their noses. Once a female has been attracted, the pair warbles and rubs the underside of their jaws together. Females lay 25 - 80 eggs in November/December about two month after mating. Unlike most other crocodilians, female Nile crocodiles will bury their eggs in sand rather than incubate them in rotting vegetation, and multiple females may nest together. After burying the eggs, the female guards the nest for the three month incubation period (Crocodilian Species List *Crocodylus niloticus*). After hatching, the female may lead the young to water, or even carry them there (Crocodilian Species List *Crocodylus niloticus*). Hatchlings are about 30 cm long at birth and are protected by the female for up to two months (Hutton, 1984).

Nile crocodiles normally slide along on their bellies, but they can also “high walk” with their trunks raised above the ground, briefly reaching maximum speed by galloping over short

distances. They can swim extremely fast by moving their body and tail in a sinuous fashion (Huchzermeyer, 2003). They normally dive for only a couple of minutes, but can hold their breath for up to 2 hours. They have a rich vocal range, good hearing, and their skin has integumentary sense organs that may react to changes in water pressure (Lane, 1996; Jackson and Brooks, 2007).

Nile crocodiles play an important role in the ecosystem in which they occur, e.g. by consuming dead animals that would otherwise pollute the waters or by controlling fish numbers. An example is the sharptooth catfish (*Clarias gariepinus*) in Africa, an omnivorous fish that otherwise could consume fish population that other species, like birds, depend on (Alcala and Dy-Liacco, 1989). The primary threats to free-ranging Nile crocodiles, in turn, are general habitat loss (pressure on resources including water, fish, sand and wetlands throughout much of the species' range), direct conflict with people (uncontrolled killing in response to attacks on humans), indirect anthropogenic effects (e.g. pollutants, poor water quality and dam-building coincided to cause the deaths of most of the Olifants River crocodile population in South Africa during 2008), and uncontrolled hunting for artisanal trade in leather goods (Fergusson, 2010).

The number of Nile crocodiles in the wild is estimated to be 250 000 to 500 000 individuals and their Conservation Status under the 1996 World Conservation Union (IUCN) Red List is "Lower Risk" (Lrlc) (see IUCN Red List). The Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) lists the Nile crocodile under Appendix I (threatened with extinction) in most of its range, and under Appendix II (not threatened, but must be controlled) in the remainder (populations of Botswana, Ethiopia, Kenya, Madagascar, Malawi, Mozambique, Namibia, South Africa, Uganda, the United Republic of Tanzania, Zambia and Zimbabwe), which either allows farming or sets an annual quota of skins from the wild (see CITES). In this regard, one of the most commonly farmed crocodilian species is the Nile crocodile. Their hide is tanned and used to make leather goods such as shoes and handbags, whilst crocodile meat is considered a delicacy.

2.3 Crocodile farming

Alligators and crocodiles have been bred on farms since the early twentieth century, but until the 1960s they were primarily kept as tourist attractions. Commercial crocodile farming was driven by depleted numbers of alligators and crocodiles in the wild, and began in North America and South Africa in the 1960s, and in Australia in the 1980s by either harvesting eggs from the wild or breeding crocodiles on-site. In South Africa, harvesting of crocodile eggs from the wild is not allowed, therefore all crocodiles are bred on the farms. There were only three crocodile farms by 1980 in South Africa, and by the early 1990s the number of farms had increased to 35 (Luxmoore, 1992). It is estimated that between 1 and 2 million crocodilian skins are marketed world-wide every year. Alligators are farmed intensively today, mostly within each species' respective native region. The Nile crocodile is farmed all over Africa, and the saltwater crocodile is mostly farmed in Australia.

The Nile crocodile is one of the most commercially utilized species of crocodilians. In 1993, 80 000 skins were traded worldwide, with the majority coming from Zimbabwe (54 %) and South Africa (15 %), from ranching and captive breeding (Collins, 1995). In comparison, exports of *Crocodylus niloticus* appear to have remained steady at 140,000 - 170,000 skins per year since 2000 with South Africa, Zambia and Zimbabwe being the main suppliers (Caldwell, 2012).

Crocodile farming is a capital intense long-term enterprise that requires sound husbandry techniques and management practises of the highest standard in order to be successful. In this regard, it is economically important to avoid diseases, reduced growing and stress in crocodiles to secure an optimal financial output.

2.4 Current threat for wild Nile crocodiles in South Africa

In South Africa, some Nile crocodile populations are confronted with a new and lethal threat of so far unknown origin, due to *pansteatitis* (Yellow fat disease), a pathological metabolic condition in which the body fat becomes inflamed (Myburgh and Botha, 2009). Afflicted animals have stiffened tails which causes immobilization and subsequently exposure and starvation (figure 3). Additionally, anthropogenic effects like increased pollution, which leads to ecosystem

degradation and more specifically deterioration of water quality, is reflected in the loss of numbers of Nile crocodiles as the top predators in the Olifants River (Ferreira and Pienaar, 2011; Ashton, 2010; Fergusson, 2010).

Due to the practical difficulties involved in assessing a crocodile's state of health in the wild, non-invasively collected information on the level of stress experienced by crocodiles on a population level, could help to identify afflicted areas.

Figure 3: Nile crocodile carcass in Kruger National Park, South Africa (photo: Jan Myburgh).

2.5 Stress

Although "stress" has become an increasingly popular and widely applied term, it is a challenging concept to define (Palme et al., 2005). The term "stress" originated in the engineering field and was introduced into the biological field in the late 1930s by Hans Selye (Wielebnowski, 2003). In a pioneering approach, Selye defined stress as a general syndrome that threatens, or appears to threaten, the homeostasis (or the physiologic and physical integrity) of an individual (Selye, 1936; Wielebnowski, 2003). Although the debate about an overall definition of the term "stress" continues, (e.g. Morgan and Tromborg (2007) define stress as intrinsic or extrinsic demands that exceed an individual's resources for responding to those demands) it is generally accepted that when confronted with a stressor (physical or emotional), the threatened individual

relies on different biological systems (behavioural, autonomic nervous, neuroendocrine, and immune) to elicit stress responses to cope with the situation (Burchfield, 1979; Palme et al., 2005). If an individual has to deal with the consequences of perturbed homeostasis following endangerment, the central nervous system assesses whether a stimulus or a group of stimuli (stressor or stressors) represents a significant challenge to an organism (Ganswindt et al., 2010).

2.5.1 Sources of stress

A stressor is a stimulus or event that provokes a stress response in an organism (Selye, 1936). There is a long list of potential stimuli which can originate from internal or external sources (Saunders Comprehensive Veterinary Dictionary, 3rd ed.). Apart from classical stimuli like injury or intoxication by a chemical or biological agent, circumstances like excessive muscular exercise, pregnancy, or harassment can also act as stressors (Selye, 1936; Reeder and Kramer, 2005; Saunders Comprehensive Veterinary Dictionary, 3rd ed.). Certain social (e.g. group size, mating season) and environmental conditions (e.g. heat/cold, resource availability) can act as a stressor (Millspaugh and Washburn, 2004; Schwarzenberger, 2007). However, such conditions may only lead to a stress response when the challenging condition is unpredictable or uncontrollable (Creel, 2001; Creel et al., 2009).

2.5.2 Stress response

The stress response is usually a cascade of reactions with respective feedback loops (figure 4), and can be broadly divided into three stages, namely the recognition of a stressor, the biological defence against this stressor, and the consequences of the stress response (Moberg, 2000). The way a particular individual responds to stress, however, is influenced by numerous factors like, for example, early experience and genetics (Moberg, 2000). Acute (often short-term) as well as chronic stress responses can lead to distress in an animal (Moberg, 2000), but short-term stress responses can also improve fitness by mobilising energy or mediating physiological and behavioural changes. Chronic stress has often negative consequences on health and therefore may decrease fitness (Möstl and Palme, 2002; Touma and Palme, 2005; Ganswindt et al., 2010; Sheriff et al., 2011) (figure 5).

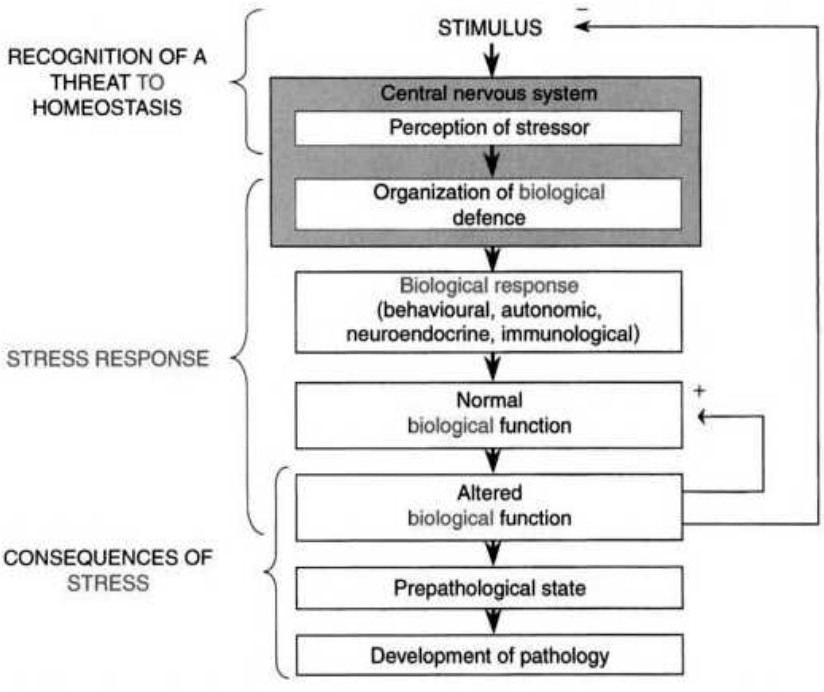

Figure 4: A model of the biological response of animals to stress (Moberg, 2000).

To cope with a stressor and possibly restore homeostasis, different peptides, steroids, and biogenic amines operate within the brain and also after they have been released into circulation (Holsboer and Ising, 2010). From a physiological perspective, an essential component of the response to stress is the activation of the hypothalamic-pituitary-adrenocortical (HPA) axis (figure 5) and the sympatho-adrenomedullary system, which result amongst others in an increase in glucocorticoid and catecholamine secretion (Möstl and Palme, 2002; Sapolsky, 2002; Touma and Palme, 2005). Catecholamines are released within fractions of a second as part of the acute stress response (Palme et al., 2005). The glucocorticoid response can be adaptive in the short term, prolonged periods of elevated glucocorticoid levels due to chronic stress can have an array of disruptive effects, including reproductive suppression, muscle atrophy, and immune suppression (Munck et al., 1984; Möstl and Palme, 2002; Sapolsky, 2002; Touma and Palme, 2005).

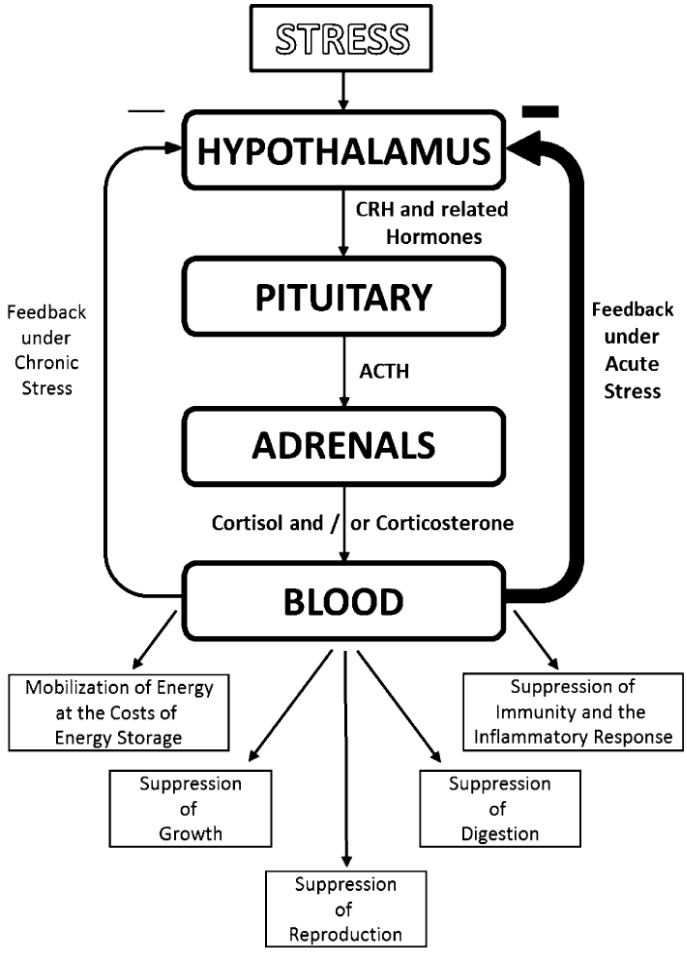

Figure 5: The hypothalamic–pituitary–adrenal axis, the negative feedback response of animals to an acute stressor compared with those under chronic stress, and the major impacts stressors have on bodily processes (Sheriff et al., 2011).

2.5.3 Glucocorticoids

The secretion of glucocorticoids forms one of the front-line responses to stressors in animals (Palme et al., 2005), and can be used as a physiological indicator of stress in wild and captive animals (Millspaugh and Washburn, 2004). Plasma glucocorticoid concentrations are therefore widely used to assess stress responses in various species (Touma and Palme, 2005). The type of glucocorticoid secreted by the adrenal gland differs between species, but cortisol is primarily

secreted in primates, carnivores and ungulates whereas corticosterone is the main hormone in rodents, birds and reptiles (Palme et al., 2005; Touma and Palme, 2005).

The processes regulating the secretion of glucocorticoids are complex, and include circadian and circannual rhythms to regulate energy balances in specific environments, but the severity of the stressor usually influences the amount of glucocorticoids secreted to a certain extend (Palme et al., 2005; Reeder and Kramer, 2005). In the liver, glucocorticoids undergo metabolism and conjugation and if not recycled via the enterohepatic pathway (see figure 6), are excreted via urine and/or faeces (Möstl and Palme, 2002). The main route of excretion, however, might differ between species and sex of a given species (Palme et al., 2005). Therefore, glucocorticoid concentrations can be assessed non-invasively using either urine or faeces as the hormone matrix.

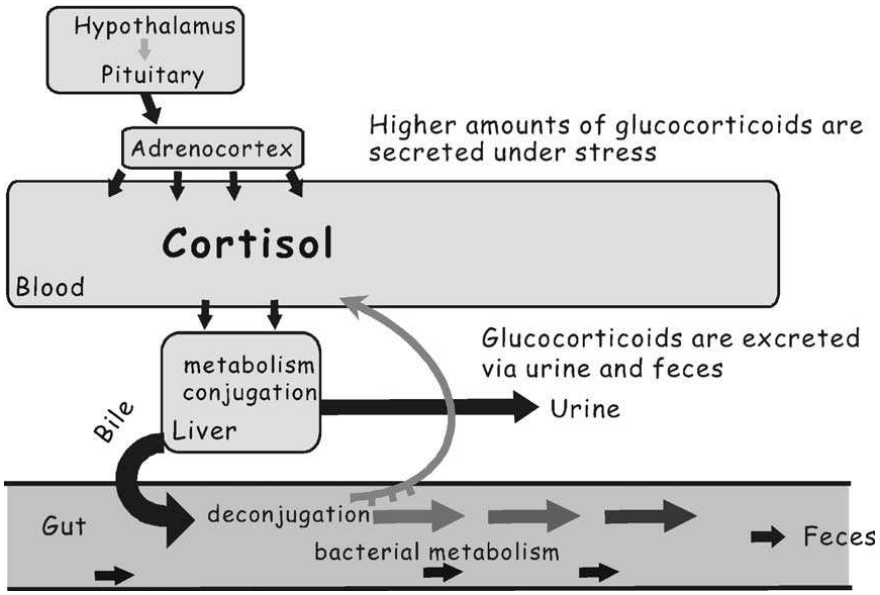

Figure 6: Secretion, metabolism and excretion of glucocorticoids (Möstl and Palme, 2002).

2.6 Stress in crocodilians

Stress in crocodilians has been reported in different contexts, related to feeding, mating, nesting, and living/housing conditions (Huchzermeyer, 2003). For example stress (caused by unsuitable weather conditions, suboptimal nutrition and various stressing incidents and interactions) negatively affects the number of nests produced, as well as the percentage of fertile eggs, and

seriously reduces male sex hormone levels and thus male fertility (Lance and Elsey, 1986). A body core temperature of ≥ 35°C seems to induce stress and may be fatal, while temperatures < 28°C reduce the rate of digestion and assimilation, as well as the metabolic rate and growth (Huchzermeyer, 2003). Behaviourally, farmed hatchlings and juvenile crocodiles confronted with a potential stressor (e.g. loud noise, thunder, visitors, etc.) tend to pile on top of each other in a corner of a pen (Huchzermeyer, 2003). Finally, certain diseases are also related to stress in crocodilians, for example osteoporosis and poor dental calcification which allows diaphanous teeth to be used as clinical indicator of chronic stress in captive and farmed crocodiles (Huchzermeyer, 2003), and it is speculated that chronic stress is the cause of osteo- and arthropathy in the long bones of Nile crocodiles (Huchzermeyer et al., in preparation).

2.7 Stress measurement in crocodilians

So far, the assessment of adrenocortical function in crocodilians has been achieved exclusively by measuring glucocorticoid concentrations in blood. This has been done in three crocodilian species, namely the American alligator (*Alligator mississippiensis*) (Mahmoud et al., 1996, Elsey et al., 1990a,b; Guillette et al., 1997, Lance and Elsey, 1999, Morici et al., 1997), the Estuarine crocodile (*Crocodylus porosus*) (Franklin et al., 2003), and the Australian fresh water crocodile (*Crocodylus johnsoni*) (Jessop et al., 2003).

In American alligators, Elsey et al. (1990a,b) showed sex differences in plasma corticosterone baseline levels, and reported higher plasma corticosterone levels for juvenile and adult American alligators maintained at high stocking densities, whereas plasma corticosterone levels in animals maintained at low stocking density were comparable with those found in wild populations. Furthermore, one of the studies showed that lower nesting success was correlated with higher plasma corticosterone concentrations (Elsey et al., 1990a). A follow up study further demonstrated that high doses of exogenous corticosterone increased mortality and decreased growth (Morici et al., 1997).

Guillette et al. (1997) showed an increase in plasma corticosterone concentrations in juvenile American alligators after handling. The same study found no differences in plasma corticosterone

concentrations of animals living in pristine or contaminated lakes, whereas a similar study by Gunderson et al. (2003) described elevated plasma corticosterone levels in alligators living in lakes with highest contaminant levels. Franklin et al. (2003) compared different handling methods in estuarine crocodiles and found a two-fold elevation in plasma corticosterone levels in manually restrained crocodiles compared to animals immobilized by electro-stunning. There were interactions between ecology, demography, capture stress, and plasma corticosterone concentrations in a free-living population of Australian freshwater crocodiles and the plasma corticosterone levels increased significantly with time post-capture, but was not associated with any ecological or demographic factors including sex and age class (Jessop et al., 2003).

Only two studies so far evaluated the respective test systems used for monitoring of adrenocortical function in crocodilians by performing an adrenocorticotropic hormone (ACTH) challenge test. Both these studies looked at plasma corticosterone levels in American alligators (Mahmoud et al., 1996; Lance and Lauren, 1984).

2.8 Non-invasive assessment of adrenocortical activity

Although blood sampling has long been the most common method for measuring glucocorticoid concentrations, it is mostly limited to studies on domestic animals (Ganswindt et al., 2010; Sheriff et al., 2011). When using an invasive approach for determining glucocorticoid concentrations, the specific animal needs to be captured, or at least handled and restrained, for sample collection. The stress associated with these procedures can elevate glucocorticoid levels, thereby confounding the results (Ganswindt et al., 2010; Schwarzenberger, 2007; Sheriff et al., 2011). Therefore, the assessment of adrenocortical function using faeces as the hormone matrix is a widely accepted alternative approach for monitoring responses to stressors, because faeces can be collected easily, animals are usually not disturbed during sample collection, and sampling is feedback-free (Touma and Palme, 2005; Ganswindt et al., 2012).

The measurements of hormones and their metabolites are usually carried out by immunological procedures using hormone- or hormone-group-specific antibodies (Hodges et al., 2010). Two main types of immunoassays are available: radio immunoassays (RIA) and enzyme

immunoassays (EIA). RIAs use radioactively labeled hormone as the competitive tracer in the quantification process, whereas EIAs use either enzyme- or biotin- labeled preparations in the process (Hodges et al., 2010). Over the past 20 years, a range of test-systems for the analysis of faecal glucocorticoid metabolite analysis has been established and used for assessing adrenocortical function in various mammal, bird, reptile, amphibian and fish species (Hodges et al., 2010; Schwarzenberger, 2007). Since all immunoassays are highly sensitive, assay performance has to be carefully validated in terms of applicability for the species-specific hormone matrix of interest to ensure a reliable quantification of respective glucocorticoid metabolites (Hodges et al., 2010; Schwarzenberger, 2007). The main criteria of assay validation are: a) sensitivity - minimum amount of hormone that can be detected, b) precision - within- and between- assay repeatability, c) accuracy - ability to detect the correct amount of hormone in the sample, and d) specificity - degree of specificity of the antibody used and the possible influence of interfering substances (Hodges et al., 2010). Prior to analysis, a number of additional factors, such as stability of faecal metabolites post-defaecation and efficiency of steroid extraction, has to be considered and if necessary assessed to ensure a representative result (Millspaugh and Washburn, 2004; Freeman et al., 2010).

As in other species, the invasive assessment of adrenocortical activity in crocodilians includes certain disadvantages, like the possibility of a self-induced stress response due to handling. It was shown that repeated handling required for blood sample collection influenced glucocorticoid levels (Lance and Lauren, 1984; Elsey et al., 1990a), and that the time of blood sample collection is important due to circadian variation in plasma corticosterone levels (Lance and Lauren, 1984). The collection of faeces as basic material for monitoring adrenocortical function in crocodiles as an alternative to blood hormone measurements would provide the opportunity to avoid repetitive handling of animals and therefore distortion of the results by induced handling stress.

There is little information on faecal hormone levels in reptiles. The technique to non-invasively assess glucocorticoid levels in reptile faeces is so far only established and used for a few species, including three-toed box turtles (*Terrapeneca rolinat riungu*) (Rittenhouse et al., 2005), green iguanas (*Iguana iguana*) (Kalliokoski et al., 2012), and two snake species, namely the African house snake (*Lamprophis fuliginosus*) (Berkvens, 2012) and the Eastern Massasauga rattlesnake (*Sistrurus catenatus catenatus*) (Lentini, 2008). Additionally, studies were conducted to non-

invasively assess reproductive hormones in reptile faeces for a few species, including blue-tongued lizards (*Tiliqua nigrolutea*) (Atkins et al., 2002), and veiled chameleons (*Chamaeleo calyptratus*) (Kummrow et al., 2011). Therefore, an appropriate validation of a respective assay system would be necessary before a non-invasive approach would be suitable for assessing adrenocortical activity in crocodilians.

2.9 Aim and specific objectives of the study

The overall aim of the present study was to establish a non-invasive technique to monitor glucocorticoid levels in captive Nile crocodiles based on faecal hormone analysis. In the process, the impact of differences in housing conditions (group size) on faecal glucocorticoid metabolite levels was investigated.

The study has the following specific objectives:

1) Determine the extraction efficiency of glucocorticoid metabolites from crocodile faeces using different concentrations of two solvents.

2) Examine the stability of faecal glucocorticoid metabolites of crocodiles post-defaecation.

3) Identify a reliable enzyme immunoassay for determining stress-related physiological responses in Nile crocodile faeces by performing an ACTH stimulation test.

4) Validate the selected enzyme immunoassay by examining the sensitivity, precision, accuracy, and specificity of the respective assay.

5) Investigate the impact of group size on faecal glucocorticoid metabolite levels in captive Nile crocodiles.

Chapter 3: MATERIALS AND METHODS

3.1 Study sites

The project was carried out in two crocodile farms near Pretoria, South Africa. Izintaba crocodile farm (-25.64643, 27.96275) is located approximately 35 km west of Pretoria, and Le Croc crocodile farm (-25.49247, 27.68045) is situated 20 km north of Brits in the Northwest Province of South Africa (see figure 7).

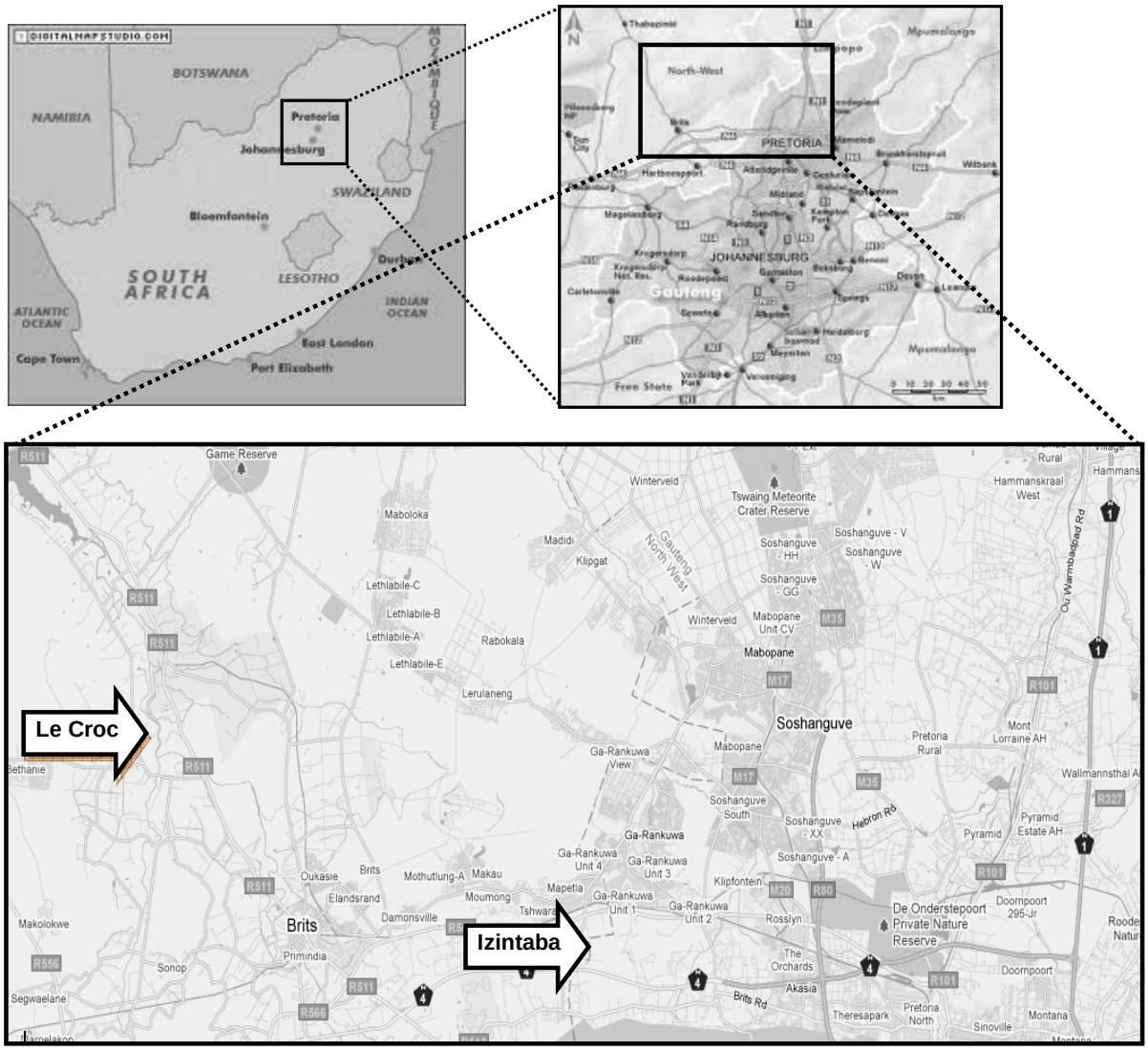

Figure 7: Locations of the two crocodile farms included in the study.

3.2 Study setup

The present study was conducted stepwise in the two facilities. For the degradation experiment, fresh faeces were collected from 36 crocodiles (belly width 34 - 37 cm), which were slaughtered at Izintaba crocodile farm in a routine procedure. At Le Croc crocodile farm, a total of 18 crocodiles (belly width 43 - 61 cm) were housed temporarily in separate enclosures and used for the ACTH stimulation test and further studies.

3.3 Study animals and housing

At Le Croc crocodile farm, 16 male and 2 female crocodiles (n = 18) were used for the ACTH stimulation test (total length of 1.82 - 2.19 m; body mass of 26 - 55 kg). The crocodiles were housed temporarily (from 26 Nov until 31 Dec 2010) in separate enclosures, to ensure controlled conditions and easy and frequent access to the animals (figures 8, 9).

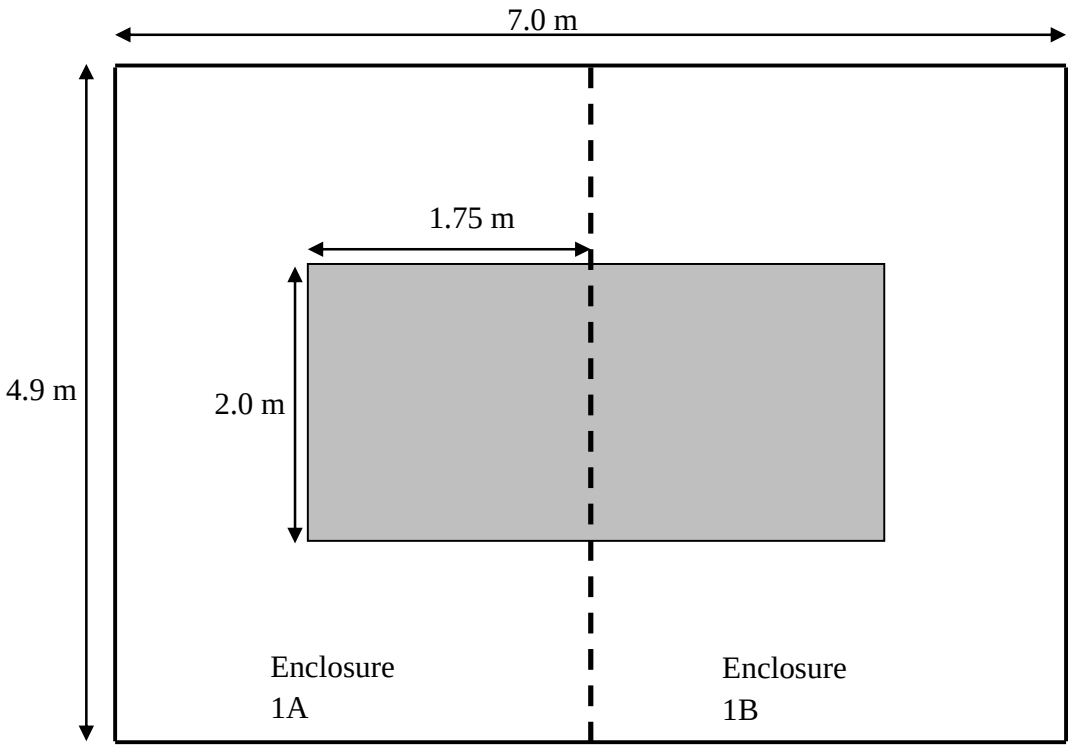

Figure 8: Schematic drawing of two adjacent enclosures at Le Croc crocodile farm.

Six animals were singly housed, 4 animals were housed in groups of two animals each, and 8 animals were housed in groups of four animals each (figure 9). All animals had permanent access to ponds. Enclosures opposite to each other were separated only by mesh wire partitioning (figure 9B), allowing visual contact of the animal(s) housed in these enclosures. Maximum and minimum temperature and humidity were recorded daily.

The crocodiles were fed five times per week (Monday - Friday, except public holidays) with food consisting of minced chicken and supplements, and access to water for drinking and submerging/thermoregulation was provided for each crocodile. The pens were cleaned daily except Sundays and public holidays. Between 13 and 26 Dec 2010 the amounts of food provided as well as removed from the enclosures prior cleaning were weighed to calculate individual food intake. For group housed animals individual food intake was calculated by dividing the total food intake per enclosure by the number of animals.

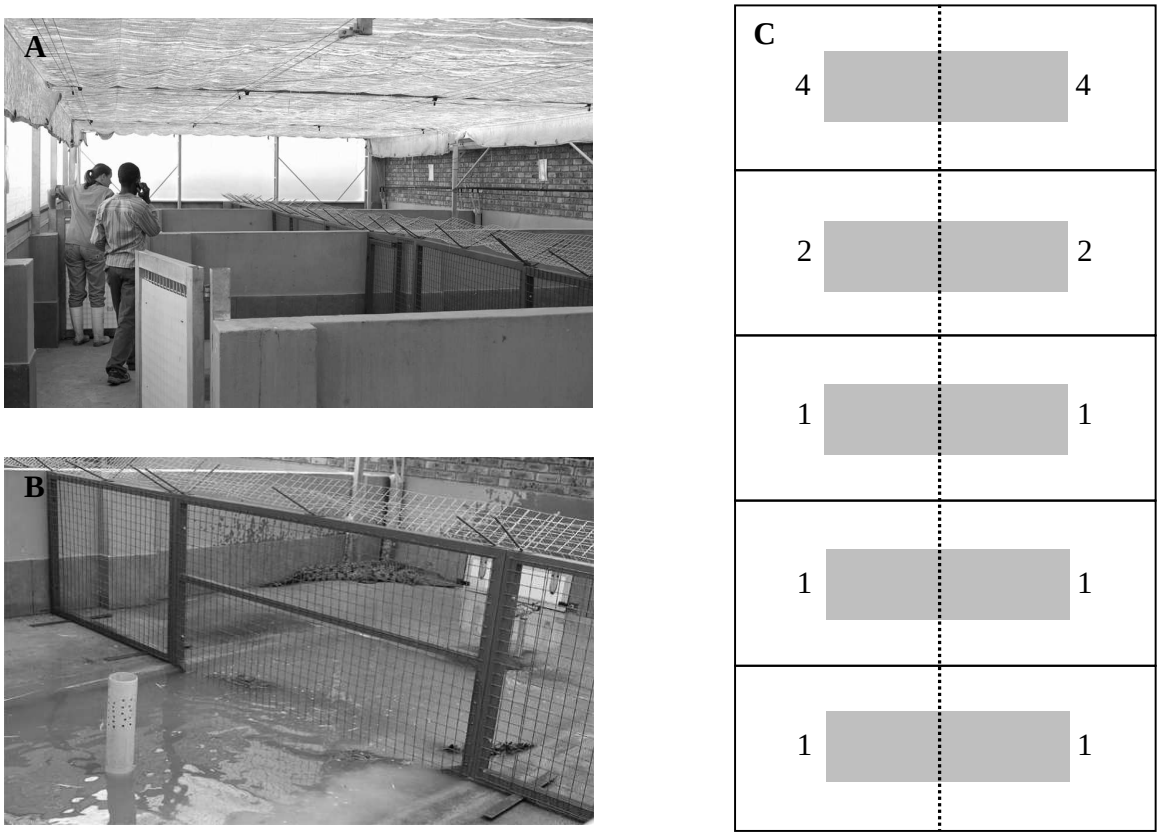

Figure 9: Temporary housing of crocodiles at Le Croc crocodile farm, view of the crocodile house from the entrance (A) and two adjacent enclosures separated by mesh wire partitioning (B), schematic drawing of the crocodile house with animal numbers kept in the respective enclosures (C).

3.4 Experimental procedures

3.4.1 FGM metabolisation post-defaecation

To check stability of faecal glucocorticoid metabolites (FGMs) in a sample once they are defaecated, a study was carried out to determine further metabolisation of FGMs. Fresh faeces were collected from the rectums of 36 slaughtered crocodiles, the faecal samples were pooled, mixed into one ball, frozen immediately, and stored at -20°C until analysis. Subsequently the pooled faeces were thawed, homogenized, and divided into 54 sub-samples. The sub-samples were stored indoors or outdoors for different periods of time up to 72 h. In detail the sub-samples were stored in triplicates at each condition, namely indoors in an incubator set at 32°C (32 - 34 % humidity), or outdoors at 22 - 28°C (6 - 70 % humidity, no precipitation) for each time span (0 h, 1 h, 2 h, 4 h, 8 h, 16 h, 24 h, 48 h, and 72 h), respectively. Finally, all 54 sub-samples were frozen, freeze-dried, extracted and analysed using an 11-oxoetiocholanolone EIA detecting 3α,11oxo-CM, and a corticosterone EIA.

3.4.2 ACTH stimulation test

To select a reliable enzyme immunoassay (EIA) for non-invasive assessment of adrenocortical function in Nile crocodiles, a physiological validation (*in vivo*) of the respective test system is needed. This validation procedure should prove that changes in activity of the hypothalamic-pituitary-adrenal (HPA) axis are reflected in FGM concentrations measured by the respective assay, e.g. a high increase in FGM levels following an adrenocorticotropic hormone (ACTH) challenge. ACTH is an important component of the HPA axis and is often produced in response to perceived or real endangerment. It is produced and secreted by the anterior pituitary gland in response to the corticotropic releasing hormone (CRH) released by the hypothalamus (figure 5).

Ten animals (9 males and 1 female) were injected intramuscularly with 1 ml ACTH solution (Synacthen® Depot, Novartis; 5 µg/kg body weight; see figure 10). Seven males and one female were injected with 1 ml saline (control group), see table 1.

Figure 10: Intramuscular injection with ACTH by Dr. Myburgh.

Table 1: Calculation of ACTH doses injected into the study animals.

Pond No.	Group	Sex (M/F)	Total Length (cm)	Weight (kg)	ACTH Dose (µg)
A1	ACTH	M	182	27	135
A2	ACTH	M	184	30	150
A3	Saline	M	218	54	0
A4	Saline	M	206	39	0
A4	Saline	M	193	35	0
A5	Saline	M	199	36	0
A5	Saline	M	209	42	0
A5	Saline	M	200	41	0
A5	Saline	M	190	33	0
B1	Saline	F	183	26	0
B2	ACTH	F	205	50	220*
B3	ACTH	M	212	52	260
B4	ACTH	M	217	48	240
B4	ACTH	M	213	45	225
B5	ACTH	M	198	36	180
B5	ACTH	M	219	55	275
B5	ACTH	M	204	38	190
B5	ACTH	M	202	36	180

* less ACTH was injected (= 4.4 µg / kg)

Collection of blood samples was carried out to confirm the successful administration of ACTH. On the day of the ACTH stimulation test three blood samples from each animal were collected, the first sample immediately before ACTH/saline administration, the second sample one hour after administration, and the third sample 5 hours after administration. Accurate times of individual blood collection were recorded.

Ponds that housed experimental (ACTH) and control (saline) animals were inspected daily and all faeces collected when available for 36 days in total between 26 Nov and 31 Dec 2010. To be able to determine baseline faecal glucocorticoid metabolite (FGM) levels, faeces were collected for about two weeks prior to ACTH/saline administration.

3.4.3 Blood sample collection

Blood was collected from the spinal venous sinus with a 20 gauge needle into plain serum tubes, according to the procedure described by Myburgh and colleagues (in preparation; see figure 11). One blood sample collected was visibly contaminated with cerebrospinal fluid and therefore excluded from the analysis. Blood samples were left to clot for 30 - 60 min at room temperature, and subsequently centrifuged at 1500 x g for 15 min. The serum was transferred into polystyrene tubes and stored at -20°C until analysis. An example of the blood sample record sheet is attached (Appendix A).

Figure 11: Blood collection from the spinal vein by Dr. Myburgh.

3.4.4 Faecal sample collection

The enclosures were inspected three times per day, in the morning (5:30 - 10:00), at noon, and in the afternoon (14:00 - 17:30), and all faeces were collected in individually labelled zip-lock bags using gloves (figure 12). In this regard, individual sampling was only possible for singly housed individuals. An example of the faecal sample record sheet is attached (Appendix B). The samples were frozen at -20°C immediately after collection, and kept frozen until analysis.

Figure 12: Collection of individual crocodile faeces during the study at Le Croc crocodile farm.

3.5 Sample analysis

3.5.1 Radio immunoassay

Serum samples were analysed using commercial radio immunoassays (RIAs) for cortisol and corticosterone (Diagnostic Products, Coat-a-Count Cortisol and Coat-a-Count Rat-Corticosterone) according to the manufacturer's instructions. Briefly, 25 µl (for the cortisol RIA) or 50 µl (for the corticosterone RIA) standards, controls, and samples were transferred in duplicates into coated tubes, respectively. 1 ml tracer was added, and the tubes were incubated for 45 min in a 37°C waterbath (for the cortisol RIA) or 2 h at room temperature (for the corticosterone RIA). All the liquid was removed and the tubes were patted dry. The tubes were

measured for one minute in a gamma counter (Wallac Wizzard^2, Perkin Elmer) using the MULTICALC software. The results are given in nmol/L (cortisol RIA) or ng/ml (corticosterone RIA). Sensitivities of the assays are 2.0 ng/ml for the cortisol RIA and 5.7 ng/ml for the corticosterone RIA, respectively. Cross-reactivity for the cortisol and corticosterone RIAs, as given in the manufacturer's pamphlets, is shown in table 2.

Table 2: Cross-reactivity (%) of cortisol and rat-corticosterone Coat-A-Count RIAs according to the manufacturer's pamphlets (shown only > 0.05 %; n.d. = not denoted).

Compound	Cortisol RIA	Corticosterone RIA
Corticosterone	0.94 %	100 %
Cortisol	100 %	0.35 %
Prednisolone	76 %	n.d.
Methylprednisolone	12 %	n.d.
11-Deoxycortisol	11.4 %	n.d.
Prednisone	2.3 %	n.d.
Betamethason	1.6 %	n.d.
Cortisone	0.98 %	n.d.
Tetrahydrocortisol	0.34 %	n.d.
11-Deoxycorticosterone	0.26 %	2.86 %
Triamcinolone	0.13 %	n.d.
Progesterone	0.02 %	0.83 %
Aldosterone	0.03 %	0.22 %

3.5.2 Faecal sample processing

Faecal samples were lyophilized for 72 hours until dry at -54°C in an Instruvac freeze-dryer (Air and Vacuum Technologies; Model No. VFDT02.50) with vacuum set at approximately 8 mTorr. Following lyophilization, samples were pulverized by hand and sieved through a mesh strainer to separate faecal powder from any existing fibrous material like bone fragments (Ganswindt et al., 2002; 2003). A weighed amount (± 100 mg) of faecal powder was extracted with 3 ml of solvent, either ethanol or methanol (see 3.5.3).

3.5.3 Steroid extraction from crocodile faeces

Steroid extraction from crocodilian faeces has not been published previously, and so no validated extraction method was available. To achieve the highest extraction efficacy of steroids from crocodile faeces, various solvents were tested for steroid extraction. Three random faecal samples were used for the extraction trial. After collection, the samples were stored at -20°C. Each faecal sample was subsequently lyophilized, pulverized, sieved and a certain amount of faecal powder extracted using two different alcohols (methanol and ethanol), in different concentrations (0 % - 100 % in 10 %-increments), respectively. For each extraction, 100 mg of faecal powder was weighed, and 3 ml of the respective solvent added. The samples were mixed on a multitube-vortex for 15 min and centrifuged at 1500 x g for 10 min. The supernatants were decanted into labeled micro test tubes, and stored at -20°C until analysis.

The faecal extracts were diluted between 1:10 and 1:50, and analysed using four different assays (see 3.5.4). The resulting extraction efficiencies were compared (see 4.3), and 70 % methanol as the chosen solvent was used for all subsequent extractions.

3.5.4 Enzyme immunoassay

Microtiter plates were coated with 150 µl coating solution per well (66 µl Protein A solution (3.47 mg) + 1.59 g Na_2CO_3 + 2.93 g $NaHCO_3$ ad 1 L H_2O; pH 9.6). Following the coating process, the plates were saturated with 250 µl bovine serum albumin (BSA) solution per well (2.42 g Trishydroxyaminomethane + 17.923 g NaCl + 10 g BSA + 1 g NaN_3 ad 1 L H_2O; pH 7.5). For the analysis, four assays were tested regarding their suitability, with antibodies raised in rabbits and labels according to table 3. The major cross-reactivities of the assays are listed in table 4.

Recovery of exogenous hormone was determined three times by adding three different concentrations of respective steroid standards (see table 3) to three faecal extracts containing different concentrations of endogenous FGMs.

Table 3: Enzyme immunoassay specificities (standards, antibodies, labels, sensitivities, intra- and inter-assay variances, and first description of the assays in the literature).

EIA	3α,11oxo-CM	11,17-DOA	CCS	CSL
Standard:	11-oxoethiocholanolone (5β-androstane-3α-ol-11,17-dione)	11-oxoethiocholanolone (5β-androstane-3α-ol-11,17-dione)	Corticosterone (4-pregnene-11β,21-diol-3,20-dione)	Cortisol (4-pregnene-11β,17α,21-triol-3,20-dione
Antibody raised against:	11-oxoethiocholanolone-17-CMO:BSA	11-oxoethiocholanolone-3-HS:BSA	Corticosterone-3-CMO:BSA	Cortisol-3-CMO:BSA
Biotin-label:	11-oxoethiocholanolone-17-CMO-biotinyl-3,6,9-trioxaundecanediamin	11-oxoethiocholanolone-3-glucosiduronate-DADOO-biotin	Cortisol-3-CMO-DADOO-biotin	Cortisol-3-CMO-DADOO-biotin
Sensitivity:	3 pg/well	3 pg/well	5 pg/well	2 pg/well
Intra-assay- CV*:	4.2-9.1 %	5.2-7.6 %	4.7-8.7 %	6.7-11.6 %
Inter- assay- CV*:	12.1-15.7 %	9.3-16.6 %	12.0-14.2 %	-
Reference:	Möstl et al., 2002	Palme and Möstl, 1997	Palme and Möstl, 1997	Palme and Möstl, 1997

* values represent intra- and inter assay coefficients of variation, determined by repeated measurements of high and low value quality controls (no inter assay CV analysis was necessary for the cortisol EIA).

Dilutions of each sample ranged from 1:200 to 1:500 of extract to assay buffer solution for the 3α,11oxo-CM assay, and were 1:20 in all extracts in the corticosterone, cortisol, and 11,17-DOA EIA (assay buffer solution: 8.5 g NaCl + 1 g BSA + 5.96 g Na_2HPO_4; ad 1 L H_2O; pH 7.2). Initially, 50 µl aliquots of the diluted faecal sample, standards and quality controls were pipetted in duplicates into each of the coated microtiter plate wells, and subsequently 50 µl biotin-label solution and 50 µl antibody solution were added into the wells. The plates were incubated overnight at 4°C, then washed four times with 300 µl washing solution per well (1.92 L H_2O + 1 ml Tween 20 + 80 ml PBS stock solution (0.399 g NaCl + 5.752 g Na_2HPO_4 + 1.005 g KCl + 1.028 g KH_2PO_4; ad 1 L H_2O; pH 7.2). 150 µl of streptavidin-peroxidise solution in assay buffer

was added and subjected to 45 minute incubation period on a plate shaker. Subsequently the plates were washed four times with 300 µl washing solution per well, and 150 µl tetramethylbenzidine peroxide substrate solution per well was added and subjected to another 30 - 60 minute incubation period on a plate shaker. The addition of 50 µl H_2SO_4 (2 mol/L) irreversibly stopped the enzyme reaction. Optical density was measured at dual wavelength of 450 nm and 620 nm. To adjust for variations in water content, faecal hormone concentrations were expressed as mass/g dry weight.

Table 4: Cross-reactivities (%) of relevant steroids in the various assays (Palme and Möstl, 1997; Möstl et al., 2002), shown only > 1 % (3a,11oxo-CM) and 0.01% (11,17-DOA, CCS, CSL); n.d. = not denoted.

Steroid	**3α,11oxo-CM**	**11,17-DOA**	**CCS**	**CSL**
4-Pregnene-				
11β,21-diol-3,20-dione	n.d.	< 0.01	6.2	100.0
11β,17α,21-triol-3,20-dione	n.d.	< 0.01	100.0	5.0
5α-Pregnane-				
11β,17α,21-triol-3,20-dione	n.d.	< 0.01	4.6	< 0.01
3α,11β,17α,21-tetrol-20-one	n.d.	< 0.01	0.8	0.15
5β-Pregnane-				
3α,11β,17α,21-tetrol-20-one	< 1	< 0.01	0.1	0.2
17α,21-diol-3,11,20-trione	n.d.	< 0.01	< 0.01	< 0.01
3α,11β,21-triol-20-one	< 1	< 0.01	< 0.01	0.25
3α,17α,21-triol-11,20-dione	n.d.	< 0.01	< 0.01	< 0.01
3α,11α,20α,21-tetrol-11-one	n.d.	< 0.01	< 0.01	< 0.01
3α,11β,17α,20α,21-pentol	< 1	< 0.01	< 0.01	< 0.01
3α-ol-11,20-dione	37.0	n.d	n.d.	n.d
3β-ol-11,20-dione	< 1	n.d.	n.d.	n.d.
3α,11β-diol-20-one	< 1	n.d.	n.d.	n.d.
3α,20α-diol	< 1	n.d.	n.d.	n.d.
11β,17α,21-triol-3,20-dione	< 1	n.d.	n.d.	n.d.
5α-Androstane-				
3α-ol-11,17-dione	< 1	5.7	< 0.01	< 0.01
3β-ol-11,17-dione	n.d.	6.7	< 0.01	< 0.01
3,11,17-trione	n.d.	14.7	< 0.01	< 0.01
5β-Androstane-				
3α,11β-diol-17-one	3.3	0.6	< 0.01	< 0.01
3α-ol-11,17-dione	100.0	100.0	< 0.01	< 0.01
3,11,17-trione	1.2	84.0	< 0.01	< 0.01
3α-ol-17-one	< 1	n.d.	n.d.	n.d.

3.6 Data analysis

Differences in individual food intake between week 1 and 2 post ACTH/saline administration were determined using paired t-test.

Serum corticosterone concentrations were expressed as ng/ml. Each data set was tested for normality using the Kolmogorov-Smirnov test. For each sampling point (t = 0 h; 1 h; 5 h), differences in serum corticosterone concentrations between the two treatments (ACTH and saline) were examined by t-test. For each treatment group, differences in hormone concentrations between the different sampling points were examined using one-way analysis of variance (ANOVA), followed by Tukey test post-hoc analysis, or Kruskal-Wallis ANOVA on ranks. The majority of the serum cortisol concentrations (ng/ml) determined fell below the sensitivity threshold of the RIA used, therefore no further statistical analysis was conducted.

The relative extraction efficiency (in %) was calculated for each of the three subsets separately using the highest hormone concentration determined as 100 % (see table 6). Subsequently mean extraction efficiency for each of the three EIAs was determined. The relationship between two variables (e.g. between the different extracting agents, or between two different EIAs used) was examined by Pearson product-moment correlation test.

Faecal glucocorticoid metabolite (FGM) concentrations were expressed as µg/g dry faeces. The effect of the ACTH stimulation was determined in singly housed individuals from which samples pre- and post treatment could be collected. To determine hormone baseline levels for the ACTH stimulation test, FGM concentrations of all samples collected before ACTH/saline administration were averaged for each assay and each individual (100 % of initial hormone concentration). FGM levels of the 2 - 3 samples collected post ACTH/saline administration were compared with individual and assay specific baseline levels and the respective increase post administration was calculated according to the following formula:

$$\%\ increase = \left(\frac{peak\ FGM\ concentration}{mean\ baseline\ concentration} - mean\ baseline\ concentration \right) x\ 100\%$$

The relative FGM metabolism rate post-defaecation (%) was calculated for each of the three sample subsets separately, using the mean hormone value determined at $t = 0$ as 100 %. Differences in the distribution of FGM concentrations between sampling subsets were determined using Friedman's rank sum test.

For the comparison of housing types (single, pair, group of four), baseline values of FGM concentrations were determined for each housing type using an iterative process where all values greater than the mean plus 2 standard deviations (SD) were removed (Brown et al., 1999). The averages were subsequently recalculated and the elimination process was repeated until there were no values greater than the means plus 2 SD. The remaining values yielded the baseline FGM concentrations. Baseline FGM concentration data sets for each housing type were tested for normality using the Kolmogorov-Smirnov test. Differences in hormone concentrations between the three different housing types were examined using one-way analysis of variance (ANOVA), followed by Tukey test post-hoc analysis, or Kruskal-Wallis ANOVA on ranks followed by Dunn's Method.

For all statistical analyses significance was assumed when $P < 0.05$. In case of all pairwise multiple comparison procedures, the alpha level of significance was adjusted applying Bonferoni adjustment using t-test and Mann-Whitney U test. Statistical analyses were performed using the software programs SigmaStat (Version 2.0) and KyPlot (Version 2.0 beta 13). Data are presented as means ± SD.

Chapter 4: RESULTS

4.1 Housing conditions

To account for changes in housing conditions, minimum and maximum temperatures, as well as minimum and maximum humidity were recorded on a daily basis during the study period (see figures 13 and 14).

Variability in maximum temperatures from 27 Nov to 31 Dec 2010 ranged from 25.7 - 44.6°C. Maximum temperatures showed two distinct dips beginning at 1 Dec and 13 Dec, and lasting one and 5 days, respectively. Minimum temperatures remained more constant during the entire study period, and ranged between 19.6 - 26.3°C.

Maximum humidity varied between 57 - 95 % during the five week study period. A distinct peak in maximum humidity (92 - 95 %) was detected after two weeks (14 to 17 Dec 2010). Minimum humidity varied between 0 - 62 %, with the highest percentage of 45 - 62 % in the period of 13 to 16 Dec 2010.

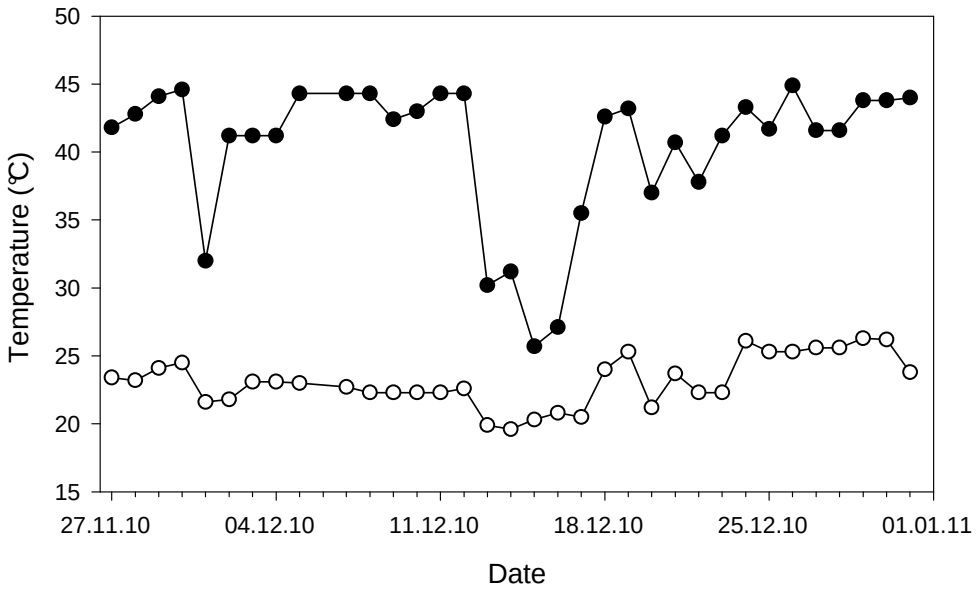

Figure 13: Maximum (●) and minimum (○) temperatures in the experimental crocodile enclosure at Le Croc crocodile farm during the study period in Nov and Dec 2010.

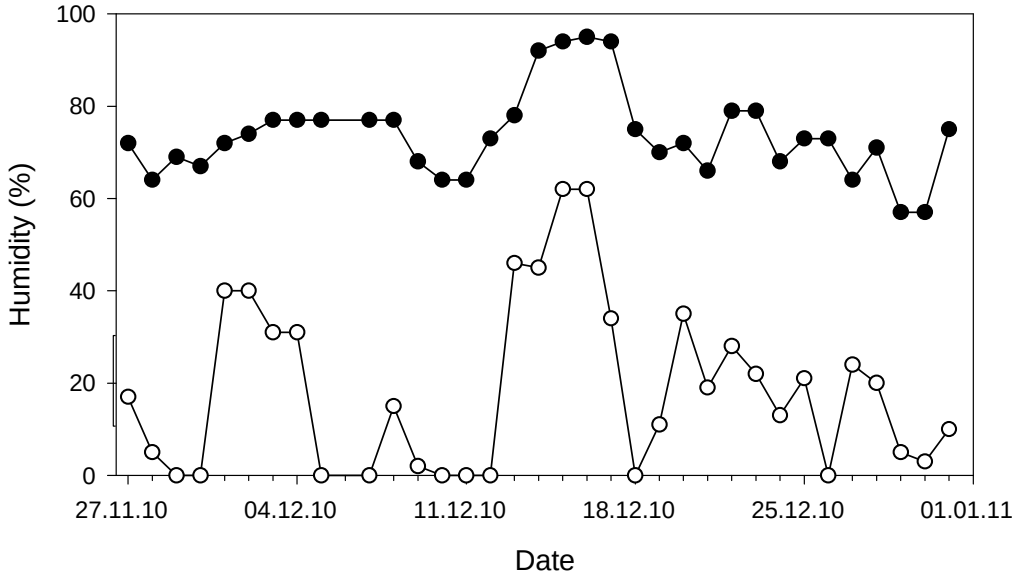

Figure 14: Maximum (●) and minimum (○) humidity in the experimental crocodile enclosure at Le Croc crocodile farm during the study period in Nov and Dec 2010.

4.2 Feeding rate

Amounts of food intake (daily amount of food fed minus leftover) were recorded post ACTH/saline administration for a period of 2 weeks (13 to 26 Dec 2010). In the first week the average amount of food intake per animal ranged from 0.44 kg to 1.58 kg. Although the lowest values were recorded for three singly housed individuals (0.44 kg, 0.44 kg, 0.49 kg), no overall trend between food intake and group size was evident. In comparison the average amount of individual food intake increased significantly in the second week ($t = -6.94$; $df = 9$; $p = 0.00007$) and ranged between 1.39 kg and 4.35 kg. Compared to week one, the increase in food intake in the week two ranged from 27 % to 804 % (see table 5). Inaccuracies in the measured food amounts could have occurred due to food that might have fallen into the water and was lost from removing, resulting in a potential over-estimate of food intake.

Table 5: Average individual weekly food intake per crocodile at Le Croc crocodile farm.

group size	single	single	single	single	single	single	pair	pair	group of four	group of four
treatment	ACTH	ACTH	ACTH	ACTH	saline	saline	ACTH	saline	ACTH	saline
food intake week 1 (kg)	1.54	0.44	0.49	1.09	1.12	0.44	0.98	1.00	1.58	1.31
food intake week 2 (kg)	4.35	3.94	3.54	1.39	3.47	2.75	3.07	2.68	2.91	2.67
increase in food intake (%)	183	804	623	27	211	524	212	170	85	103

4.3 Optimisation of steroid extraction efficiency

Extraction efficiency was determined using two different alcohols, namely ethanol and methanol. Subsets of three different faecal samples (n = 2 for 11,17-DOA analysis) were extracted using the respective alcohol in different concentrations (0 - 100 %; see figure 15), and analysed using initially four different enzyme immunoassays (EIAs) (table 3). Measurements with the cortisol enzyme immunoassay gave concentrations below the sensitivity threshold of the assay, even when the samples were only diluted 1:10. As the use of lesser diluted faecal extracts might cause influencing matrix effects during analysis, this assay was not taken into further consideration for any subsequent analyses.

The respective curves determined by the remaining three assays are significantly correlated when using ethanol (see table 7) as well as methanol (see table 8) as extracting agent. Furthermore, for each particular assay the two curves from the ethanol and methanol extraction are significantly correlated (see table 9). Due to a higher comparability of the three extraction curves for methanol extraction (see tables 7 and 8), this extracting agent was used for all subsequent extractions. Acceptable extraction efficiencies were revealed by using 60 - 90 % methanol (see figure 15B), therefore an intermediate concentration within this range of 70 % methanol was chosen for all subsequent extractions.

Table 6: Absolute FGM levels (ng/g DW) set as 100% extraction efficiency for comparison between assays and solvents.

Solvent	Extract	Corticosterone (ng/g DW)	11,17-DOA (ng/g DW)	3α,11oxo-CM (ng/g DW)
Ethanol	E1	776	85	2046
	E2	321	23	582
	E3	816	402	2665
Methanol	E1	625	96	2051
	E2	242	22	658
	E3	749	400	2363

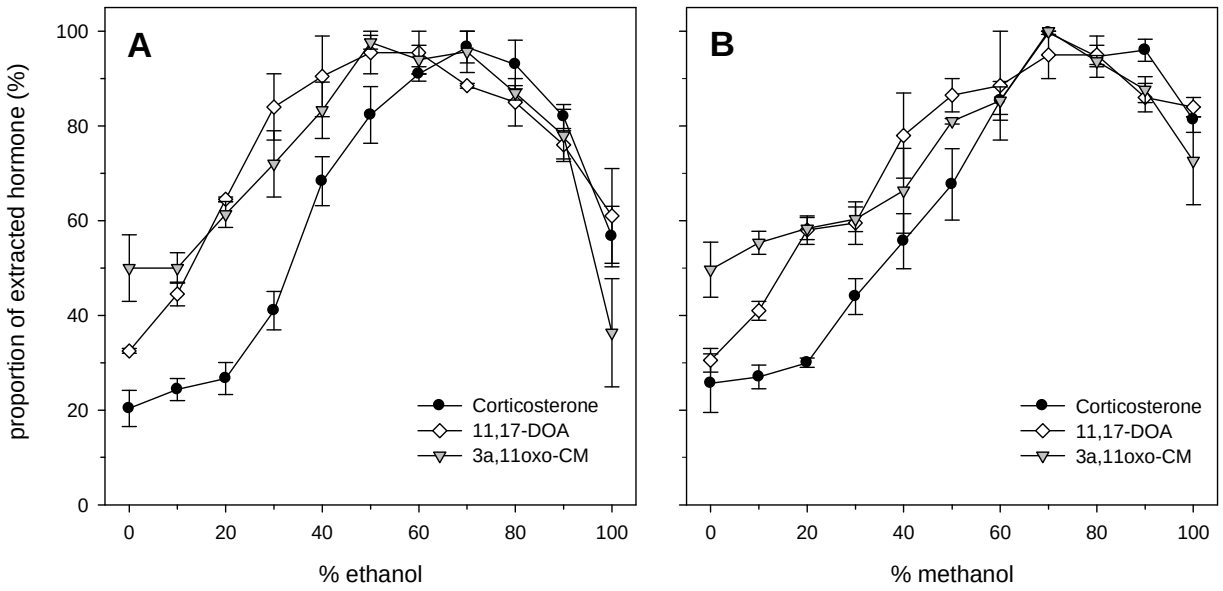

Figure 15: Percent of extracted immunoreactive steroid concentration (mean ± SE) using ethanol (A) or methanol (B) as extracting agent. Extracts were analysed using three different EIAs (corticosterone, 11,17-DOA, and 3α,11oxo-CM).

Table 7: Pearson Product Moment correlation between immunoreactive hormone concentrations determined with three different EIAs (corticosterone, 11,17-DOA, and 3α,11oxo-CM) using ethanol as extracting agent.

Compared EIAs	Correlation coefficient	Probability value
Corticosterone – 11,17-DOA	r = 0.813	p = 0.00236
Corticosterone – 3α,11oxo-CM	r = 0.796	p = 0.00339
11,17-DOA – 3α,11oxo-CM	r = 0.865	p = 0.00059

Table 8: Pearson Product Moment correlation between immunoreactive hormone concentrations determined with three different EIAs (corticosterone, 11,17-DOA, and 3α,11oxo-CM) using methanol as extracting agent.

Compared EIAs	Correlation coefficient	Probability value
Corticosterone – 11,17-DOA	r = 0.927	p = 0.000040
Corticosterone – 3α,11oxo-CM	r = 0.957	$p = 4.1 \times 10^{-6}$
11,17-DOA – 3α,11oxo-CM	r = 0.918	p = 0.000068

Table 9: Pearson Product Moment correlation between respective immunoreactive hormone concentrations determined with either the corticosterone, 11,17-DOA, or 3α,11oxo-CM EIA, comparing two different extracting agents (methanol and ethanol).

EIA	Correlation coefficient	Probability value
Corticosterone	r = 0.929	p = 0.000036
11,17-DOA	r = 0.821	p = 0.00194
3α,11oxo-CM	r = 0.702	p = 0.01597

4.4 EIA identification

To identify reliable EIAs for assessing adrenocortical function using faeces as hormone matrix, the performance of each tested assay has to be carefully examined. In this regard, the following four main criteria of validation were taken into account for the EIAs used in this study: sensitivity (minimum amount of hormone than can be detected), precision (within- and between assay repeatability), accuracy (ability to detect the correct amount of hormone in the sample), and

specificity (i. specificity of the antibody, ii. possible influence of interfering substances; matrix effects).

4.4.1 ACTH stimulation test

4.4.1.1 Serum corticosterone concentrations

Animals showed a significant increase in serum corticosterone concentrations following ACTH administration (one-way ANOVA; $F = 11.04$; $p = 0.0004$; see figure 16A). Post hoc analysis for the ACTH treated group revealed significant differences between 0 h and 5 h (Tukey test; $p < 0.001$). No significant differences were found between 1 h and 5 h (Tukey test, Bonferoni adjustment; $p = 0.04$) and between 0 h and 1 h (Tukey test, Bonferoni adjustment; $p > 0.05$). No significant differences were found when comparing serum corticosterone levels collected at 0 h, 1 h, and 5 h following saline administration (Kruskal-Wallis ANOVA on ranks; $H = 4.409$; $p = 0.1103$; see figure 16B). No significant differences were found when comparing serum corticosterone levels between the ACTH and saline groups collected at 0 h (t-test; $p = 0.48$), 1 h (t-test; $p = 0.30$), and 5 h (t-test; $p = 0.62$), respectively.

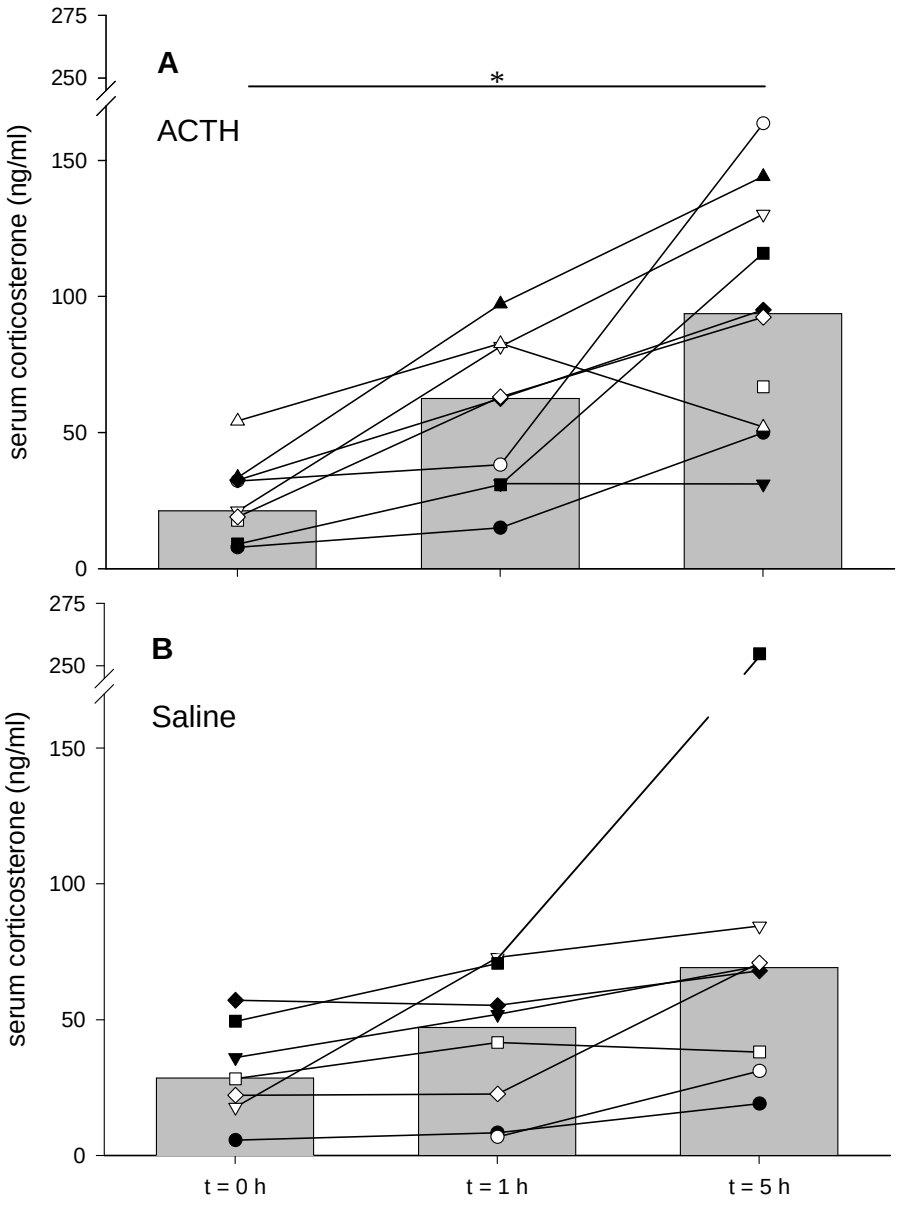

Figure 16: Symbol-bar plots of serum corticosterone concentrations (ng/ml) of captive Nile crocodiles following ACTH (A) or saline (B) administration. Each symbol represents the individual hormone concentration of an animal, either before (t = 0 h), one hour (t = 1 h), or five hours (t = 5 h) after administration, respectively. Concentrations of the same animal are connected by a line. Each bar shows the resulting median concentrations of all treatment / time combinations of the respective group of individuals. Asterisks indicate statistically significant differences between groups.

4.4.1.2 Serum cortisol concentrations

Analysis of serum cortisol concentrations revealed hormone levels below the sensitivity threshold of the RIA used (< 2.0 ng/ml) for all animals before treatment, and for all crocodiles treated with saline 1 h after administration (see table 10). In 7 of 10 animals treated with ACTH, hormone concentrations remained below the sensitivity threshold 1 h after treatment. In contrast, serum cortisol levels above the detection limit were found in 7 of 10 animals treated with ACTH and in 4 of 8 animals treated with saline, 5 h after administration.

Table 10: Serum cortisol concentrations (ng/ml) of captive Nile crocodiles following ACTH or saline administration.

animal ID	treatment	$c_{cortisol}$ [ng/ml]		
		t = 0 h	t = 1 h	t = 5 h
F222	ACTH	< 2.0	< 2.0	< 2.0
M550	ACTH	< 2.0	< 2.0	3.16
M555	ACTH	< 2.0	< 2.0	< 2.0
M556	ACTH	< 2.0	2.49	2.26
M551	ACTH	< 2.0	< 2.0	2.96
M552	ACTH	< 2.0	< 2.0	2.25
M553	ACTH	< 2.0	3.28	3.72
M226	ACTH	< 2.0	2.71	2.88
M202	ACTH	< 2.0	< 2.0	3.17
M445	ACTH	< 2.0	< 2.0	< 2.0
F224	control	< 2.0	< 2.0	< 2.0
M554	control	< 2.0	< 2.0	< 2.0
M444	control	< 2.0	< 2.0	< 2.0
M446	control	< 2.0	< 2.0	2.44
M441	control	< 2.0	< 2.0	< 2.0
M442	control	< 2.0	< 2.0	2.38
M443	control	< 2.0	< 2.0	3.23
M225	control	< 2.0	< 2.0	3.57

4.4.1.3 Faecal glucocorticoid metabolite (FGM) concentrations

Faecal samples pre- and post-injection were obtained from three singly housed crocodiles (two animals treated with ACTH and one control animal treated with saline). Faecal glucocorticoid metabolite (FGM) concentrations were determined using three different EIAs, detecting immunoreactive corticosterone, 11,17-dioxoandrostanes (11,17-DOA) and metabolites with a 5β-3α-ol-11-one structure (3α,11oxo-CM). All three assays detected a sufficient increase (> 100 %) in FGM concentrations 7 - 15 days post treatment (see figure 17). Due to exceptionally low 11,17-DOA baseline levels in the ACTH treated animals this assay showed the highest relative increase after ACTH administration (see table 11). However, by comparing absolute quantities of immunoreactive FGM measured, the 3α,11oxo-CM EIA performed best, detecting on average values 3 - 4 times higher than the corticosterone and 11,17-DOA assays (see figure 17). Therefore, the 3α,11oxo-CM EIA as group specific assay and the corticosterone EIA as compound specific assay were selected for all subsequent analyses.

Table 11: Responses of immunoreactive FGM concentration (%) following ACTH or saline treatment.

Animal ID	Treatment	3a,11oxo-CM	Corticosterone	11,17-DOA
F222	ACTH	380.1 %	245.6 %	780.5 %
M550	ACTH	156.8 %	111.3 %	382.1 %
M554	saline	136.1 %	147.2 %	125.3 %

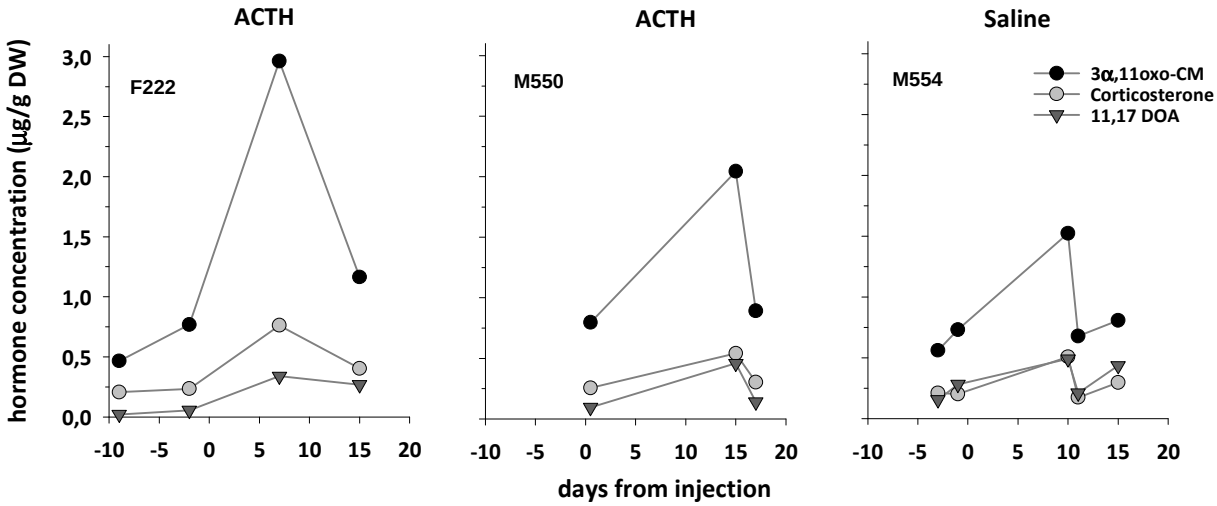

Figure 17: Faecal hormone metabolite concentrations in mass per g dry weight of samples from three singly housed crocodiles treated with either ACTH or saline. Samples were analysed using three different EIAs (corticosterone, 11,17-DOA, and 3α,11oxo-CM; F222 and M550 received ACTH administration and M554 saline administration).

4.4.2 Assay validation

The two assays used for further analysis (3α,11oxo-CM and corticosterone) were validated with regard to reproducibility (intra- and inter assay variances, table 3), specificity (cross-reactivity, table 4), recovery (figure 18), and parallelism.

For determining the accuracy of the 3a,11oxo-CM and corticosterone assays, mean and standard deviations were calculated for the respective recovery rates. In addition differences in measured hormone concentrations and endogen hormone concentrations were plotted (figure 18).

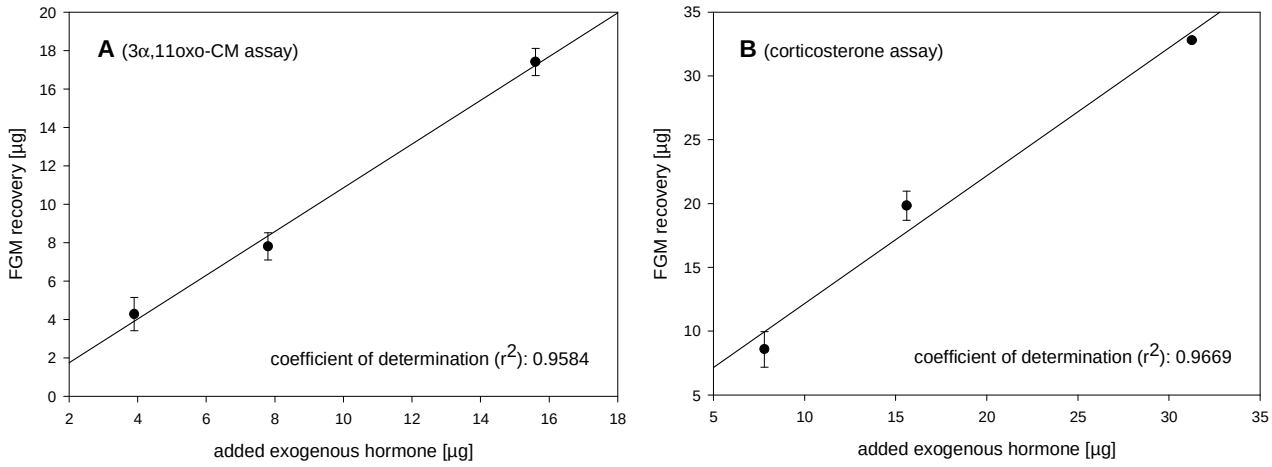

Figure 18: Recovery of FGMs in faecal extracts determined by two different EIAs, 3α,11oxo-CM (A) and corticosterone (B). The linear regression of added and subsequently measured (recovered) FGM concentrations is shown as mean +/- standard error.

The percentage of recovery (mean ± SE) was 107.51 ± 12.43 %, with the 3α,11oxo-CM assay and 109.95 ± 14.01 % with the corticosterone assay. The accuracy was rated as correct, if the recovery was in the range of 80 - 120 %. Therefore the determined results from faecal extracts could be considered to be accurate.

To test, if matrix components of the samples would lead to interferences in the hormone analysis, it was examined, if serial dilutions of samples would yield a parallel slope to the linear range of the standard curve (analysis of parallelism). Because different dilutions of the hormone extracts were used for analysis in only one assay (3α,11oxo-CM), only this assay was tested for parallelism. Serial dilutions of extracted faecal samples gave displacement curves which were parallel to the standard curve in this assay (2.2 % - 7.3 % deviation).

4.4.3 Stability of FGM post-defaecation

To account for changes in hormone concentration post-defaecation, homogenized sub-samples of freshly collected faeces were stored indoors at 32°C, 32 - 34 % humidity and outdoors at 22 - 28°C, 6 - 70 % humidity for different periods of time up to 72 h, and subsequently analysed using the 3α,11oxo-CM and corticosterone EIAs (see figure 19A and B). The analysis with the 3α,11oxo-CM EIA revealed no significant changes in FGM distribution across sampling subsets for both storage regimes (indoors: $\chi^2 = 7.73$, $p = 0.46$; outdoors: $\chi^2 = 3.56$, $p = 0.89$). Maximum decrease in mean hormone levels for samples stored indoors was 21 % after 72 h, and for samples stored outdoors 8 % after 48 h (see figure 19A).

Likewise, hormone concentrations analysed with the corticosterone EIA showed no significant changes in FGM distribution across sampling subsets for indoors ($\chi^2 = 7.49$, $p = 0.49$) and outdoors ($\chi^2 = 7.84$, $p = 0.45$). Compared to the 3α,11oxo-CM results, FGM concentrations determined with the corticosterone EIA showed a more variable pattern with an increase and decrease in hormone concentrations over time. Maximum decrease in mean hormone levels (6 % after 48 h) was found in samples stored indoors, whereas a maximum increase of 8 % after 24 h was found in samples stored outdoors (see figure 19B).

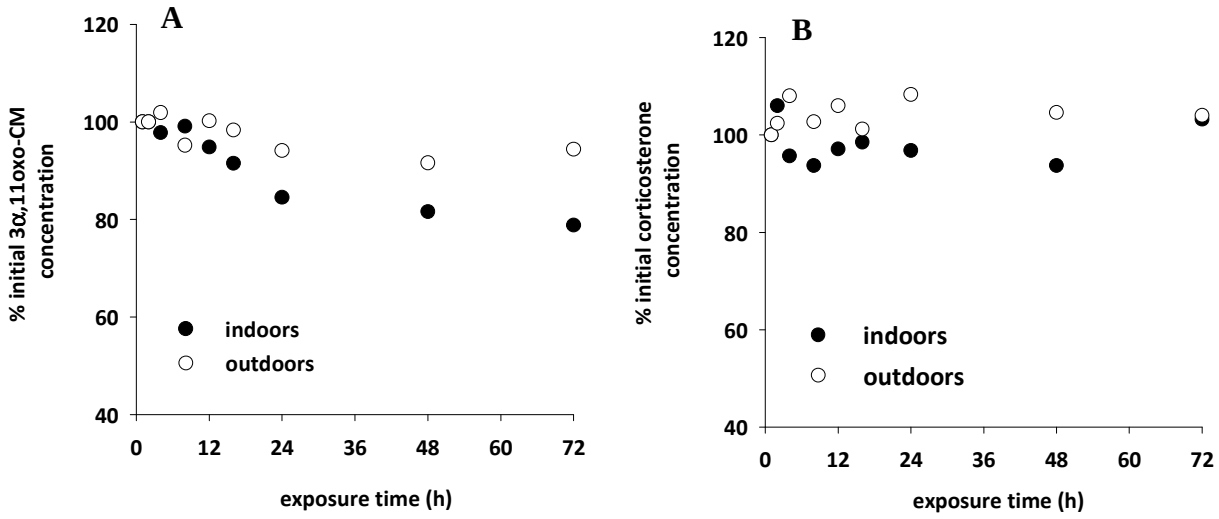

Figure 19: Changes in mean 3α,11oxo-CM (A) and corticosterone (B) concentrations (%) over time for faeces stored indoors at 32°C, 32 - 34 % humidity and outdoors at 22 - 28°C, 6 - 70 % humidity.

4.5 Faecal sample collection rates

At Le Croc crocodile farm a total of 112 faecal samples were collected during the 35-day study period from 18 study animals (average of 1.2 faecal samples per individual per week). Defaecation rates varied throughout the study period and showed two periods of lower average defaecation rates of 0.4 faecal sample per individual per week, which lasted from 27 to 30 Nov and 15 to 22 Dec, respectively (see figure 20). Throughout the study period, average individual defaecation rates varied per housing type, with 1.9 samples per individual per week for singly housed animals, 2.9 samples per individual per week for pair housed crocodiles, and 2.5 samples per individual per week for group housed animals. The water content of the collected faecal samples ranged from 38 - 85 % with an average of 61 %, and the pH (n = 3 tested) ranged between 6 - 6.5.

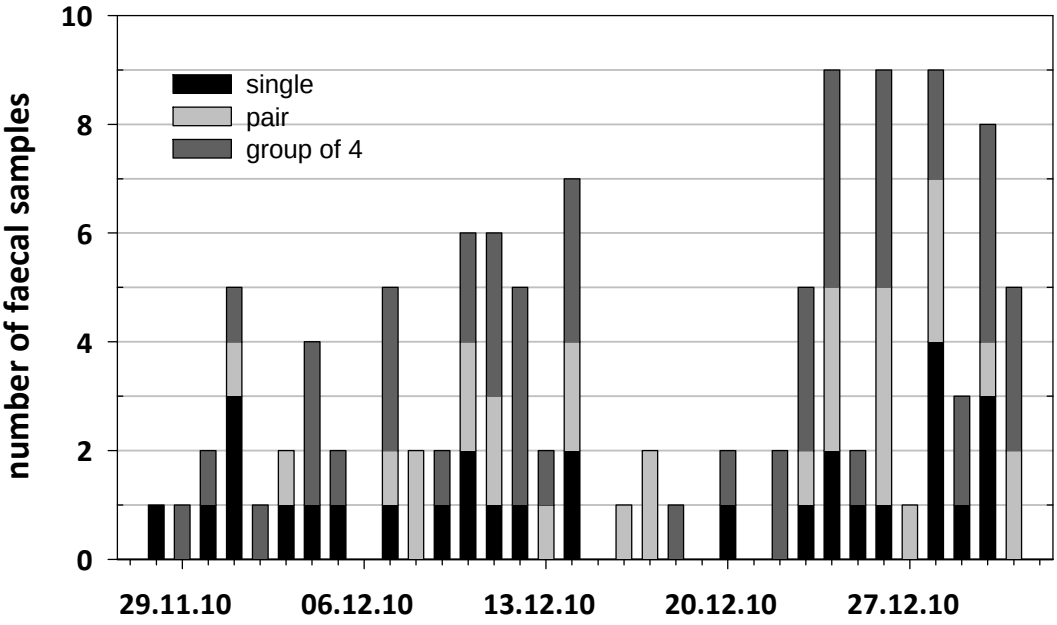

Figure 20: Number of faecal samples collected from 18 study animals (singly, pairwise, group-housed) at Le Croc crocodile farm.

4.6 Long term profiles

Faecal samples could be frequently collected only from one of six individually housed crocodiles (13 samples in 35 days). The respective 3α,11oxo-CM profile (figure 21A) revealed three clear peaks above baseline. The first peak (29 Nov) appeared three days after separation, and the third peak (23 Dec) nine days after handling and saline administration. No event could be related to the second peak, detected on 9 Dec. The corticosterone profile (figure 21B) revealed a similar pattern, showing the three peaks described above.

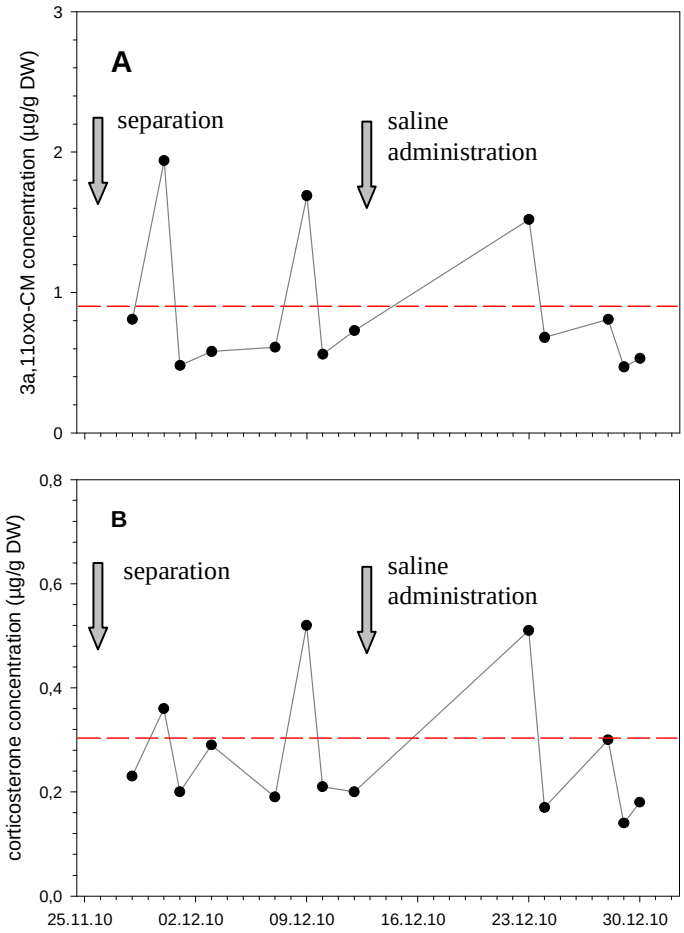

Figure 21: Longitudinal concentrations of FGMs of a singly housed crocodile subjected to saline administration measured with (A) 3α,11oxo-CM EIA, and (B) corticosterone EIA. The dotted horizontal lines show respective baseline concentrations, vertical arrows indicate recorded potentially stressful events.

In contrast to the individually housed crocodiles, faecal samples pre- and post-administration were obtained from all pairwise and group housed enclosures (total n = 82). Grouped 3α,11oxo-CM levels (intervals of 5 days) from these animals showed an average elevation 90 % (ACTH treated animals) and 108 % (saline treated animals; based on a single value) above baseline (0.84 µg/g DW for ACTH group; 0.93 µg/g DW for saline group) within the first five days after separation (-14 to -10 in figure 22), and an average elevation of 110 % (ACTH) and 90 % (saline) above baseline six to ten days post ACTH or saline administration (see figure 22A and B).

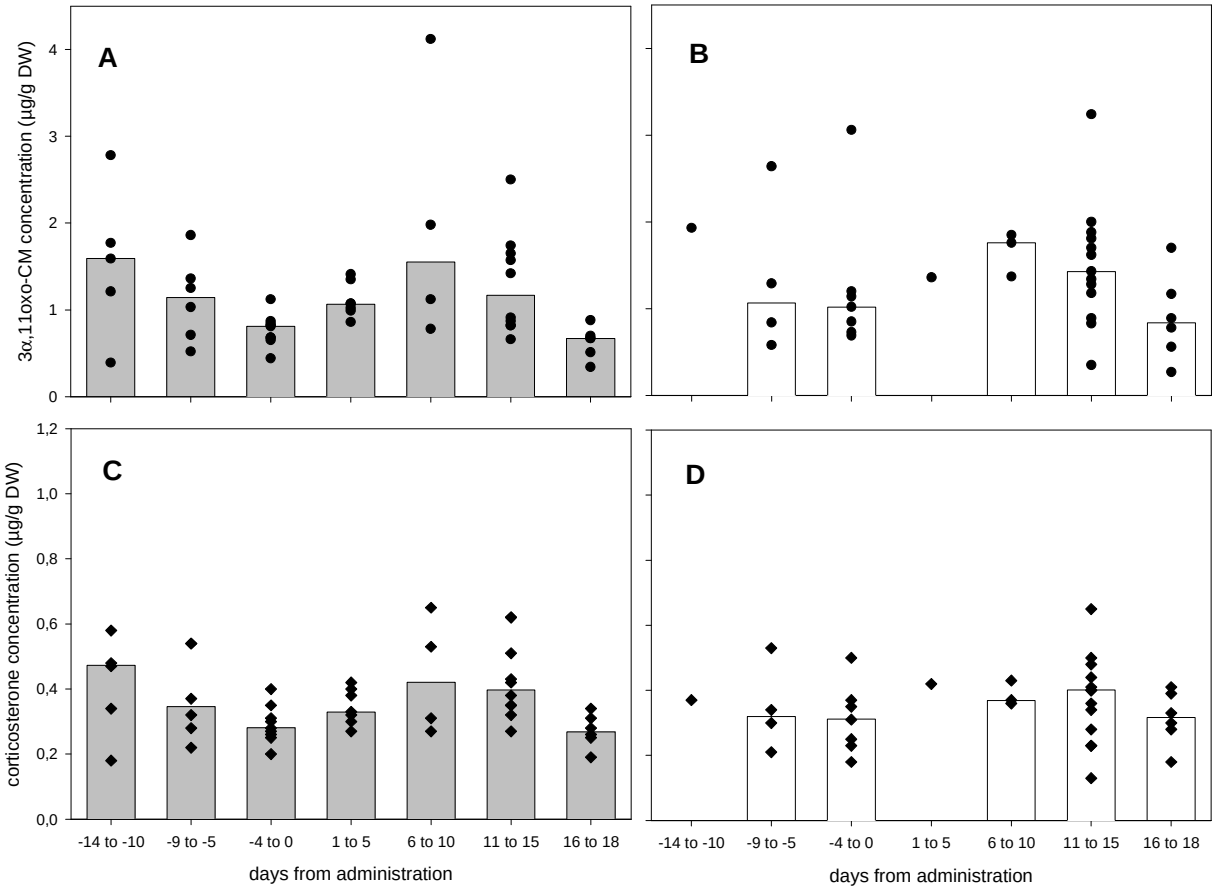

Figure 22: Grouped FGM levels (interval of 5 days) of samples from group-housed animals pre- and post ACTH (A+C) and saline (B+D) administration measured with 3α,11oxo-CM EIA (A+B) and corticosterone EIA (C+D). Black dots and diamonds represent individual concentrations, boxes show median hormone levels of the respective time interval.

The described elevations are less pronounced in the corticosterone profile. Grouped corticosterone levels from these animals showed an average elevation of 35 % (ACTH treated animals) and only 12 % (saline treated animals; based on a single value) above baseline (0.35 µg/g DW for ACTH group; 0.33 µg/g DW for saline group) within the first five days after separation. An average elevation of 21 % above baseline was detected six to ten days post ACTH administration, as well as 11 to 15 days post saline administration (see figure 22C and D).

4.7 Comparison of baseline FGM levels for different group sizes

Baseline faecal glucocorticoid metabolite (FGM) concentrations of singly housed animals, pairwise housed animals and animals housed in groups of four were compared. To eliminate the influence of known and unknown stressors, an iterative approach was applied to eliminate elevated FGM concentrations and to subsequently reveal overall baseline FGM levels for the individuals of each housing type (figure 23).

Baseline FGM concentrations (between singly, pairwise, and group housed animals) determined with the 3α,11oxo-CM EIA were significantly different (Kruskal-Wallis ANOVA on ranks; $H = 7.68$; $p = 0.0215$; see figure 23A). Post hoc analysis revealed a significant difference between singly and pairwise housed animals (Dunn's Method; $p < 0.05$). No significant differences were found between singly and group housed (Dunn's Method; $p > 0.05$), as well as between pairwise and group housed animals (Dunn's Method; $p > 0.05$).

Baseline FGM concentrations determined with the corticosterone EIA revealed a significant difference between singly, pairwise, and group housed animals (one-way ANOVA; $F = 13.35$; $P < 0.00001$; see figure 23B). Post hoc analysis revealed significant differences between singly and pairwise housed animals (Tukey test, Bonferoni adjustment; $p = 0.0001$), as well as between pairwise and group housed animals ($p = 0.0004$). No significant difference was found between singly and group housed animals ($p = 0.32$).

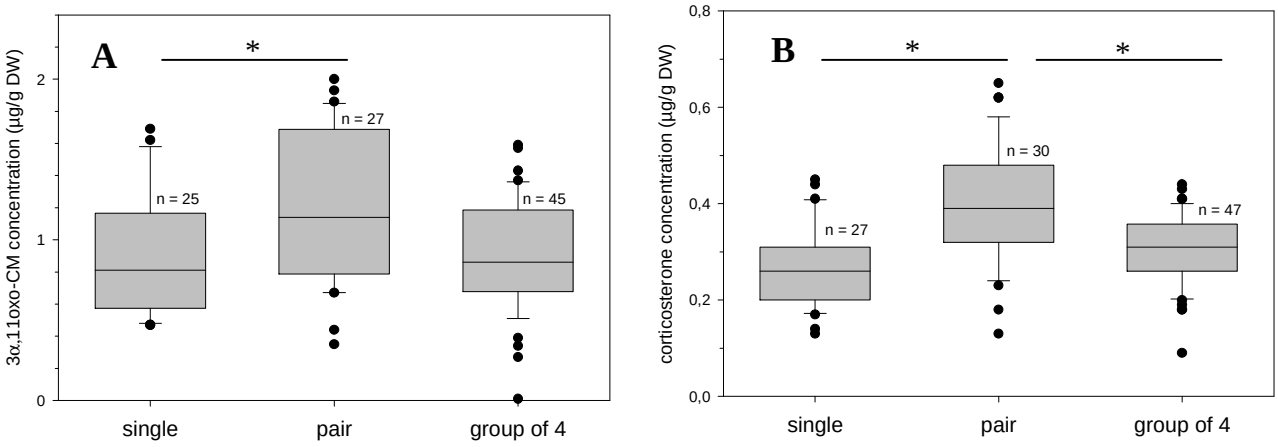

Figure 23: Boxplots of baseline FGM concentrations of singly, pairwise, and group housed crocodiles at Le Croc crocodile farm measured with 3α,11oxo-CM EIA (A) and corticosterone EIA (B). Boxes show median, 25 and 75 percentiles, whiskers show 10/90 percentiles, and dots show outliers. Asterisks indicate statistically significant differences between groups.

To exclude a possible gender-effect, samples from the two females were omitted (n = 7) and the group size comparison repeated.

Baseline FGM concentrations (between singly, pairwise, and group housed animals) determined with the 3α,11oxo-CM EIA were significantly different (Kruskal-Wallis ANOVA on ranks; $H = 6.21$; $p = 0.04$). Post hoc analysis revealed a significant difference between singly and pairwise housed animals (Dunn's Method; $p < 0.05$). No significant differences were found between singly and group housed ($p > 0.05$), as well as between pairwise and group housed animals ($p > 0.05$).

Baseline FGM concentrations determined with the corticosterone EIA were significantly different between singly, pairwise, and group housed animals (Kruskal-Wallis ANOVA on ranks; $H = 24.9$; $p = 0.000004$). Post hoc analysis revealed a significant difference between singly and pairwise housed animals (t-test; $p = 0.00001$), between singly and group housed (t-test; $p = 0.0004$), as well as between pairwise and group housed animals (Mann-Whitney U test; $p = 0.001$).

Chapter 5: DISCUSSION

The main objective of the present study was to establish a non-invasive technique to monitor adrenocortical function in Nile crocodiles by analysing FGMs using EIAs. As the measurement of steroids in faeces usually requires an extraction step before the assay (Hodges et al., 2010), two organic solvents (ethanol and methanol) containing various amounts of water were tested to ensure good steroid recoveries. To demonstrate that the available EIAs reliably measure FGM levels as an accurate reflection of physiological events, an experiment was performed using a pharmacological stimulation of the adrenal cortex (ACTH stimulation test). As an additional biological validation, the effect of handling on FGM output was monitored in a control experiment. Subsequently, respective assay performance was assessed in terms of assay sensitivity, precision, accuracy, and specificity. Stability of FGMs post-defaecation was also determined to assess possible changes in measured hormone concentrations in unpreserved faecal material. To be able to interpret cause-and-effect relationships between e.g. physiological changes and excretion of hormone metabolites, defaecation rates were closely monitored as these can clearly vary due to diet, gut microbial metabolism, and environmental conditions in reptiles (e.g. Kummrow et al., 2011). As adult Nile crocodiles are known to be gregarious animals (Huchzermeyer, 2003), the potential influence of variation in group size on faecal hormone levels was finally investigated.

5.1 Faecal steroid extraction

Numerous extraction procedures are described, with the choice dependent on the hormone being measured, the method of sample storage, or personal preference (e.g. Hodges et al., 2010; Sheriff et al., 2011). In most species, steroids are excreted in the free (unconjugated) form into the faeces, therefore a simple extraction with organic solvents (ethanol or methanol) containing 5 – 20 % water generally results in good steroid recoveries (Hodges et al., 2010; Sheriff et al., 2011). The use of dry faecal powder for extraction is a common approach (Hulsman et al., 2011; Ganswindt et al., 2010; Ahlers et al., 2012) to control for the variation in water content, as faecal samples can vary considerably in their consistency (Hodges et al., 2010).

In the present study two solvents were used for faecal steroid extraction, and the resulting extracts were subsequently analysed using four different EIAs detecting FGMs (CCS, CSL, 3α,11oxo-CM, and 11,17-DOA). The solvents were tested in different concentrations, as former studies have shown that the type and concentration of alcohol in the solvent influences the amount of steroids extracted from the faecal matrix (e.g. Möstl et al., 1999; Palme and Möstl, 1997; Freeman et al., 2010). Three of the four EIAs tested (CCS, 3α,11oxo-CM, and 11,17-DOA) showed similar results for both types of alcohol used (ethanol and methanol). As demonstrated for other species (Palme et al., 1997; Freeman et al., 2010), the alcohol concentration in water seems to be more critical in terms of extraction efficiency than the type of alcohol used. A high percentage of alcohol is required to get higher extraction rates (Sheriff et al., 2011), but by using 100 % alcohol, more polar steroids are often lost from the extract (Möstl et al., 1999; Palme and Möstl, 1997), which is especially important for the present study, as glucocorticoid metabolites are the steroid class of interest, and if still bearing a functional group at C_{11} they are usually more polar compared to e.g. androgen metabolites (Ganswindt et al., 2003).

5.2 Defaecation rates

In mammals, the gastrointestinal passage time is a relevant estimate of the delay of steroid metabolites excretion into faeces (Palme et al., 1996). However, in reptiles the lag time between circulating hormone levels and excretion of respective metabolites in faeces can vary considerably (Kummrow et al., 2011), which makes it difficult to interpret reliably the changes in faecal hormone levels in relation to physiological events. Furthermore, infrequent defaecation can complicate data interpretation, if, for example, insufficient individual sample collection pre and post treatment occurs.

The defaecation rate for the 18 monitored individuals varied considerably throughout the study period, with an overall average of 3.2 collected faecal samples per day. Two periods of lower average defaecation rates of one faecal sample per day were identified, which lasted 4 and 8 days, respectively. A possible explanation for the first recognized drop in overall defaecation rate (27 - 30 Nov) could be a reduction in food intake following the regrouping process of the animals for

the study, as it has been reported that anorexia can be triggered in crocodiles by handling and transport (Huchzermeyer, 2003).

The second observed period of low defaecation rate was recorded after the treatment with ACTH or saline (15 – 22 Dec) and also overlapped with a period of low ambient temperatures (13 – 17 Dec). Therefore it is unclear, if the period of reduced defaecation rate, as an indicator of reduced food intake and thus stress experienced by the animals (Huchzermeyer, 2003) might be a result of the physiological treatment or due to the change in housing conditions, as it is known that crocodilians maintained for any length of time at temperatures only four degrees outside of their optimal range show evidence of severe stress (Lance et al., 2001). All animals fed less in the first week after treatment than the second, indicating that the reduction in defaecation rate found might be due to the actual physiological treatment. However, temperatures were also lower in the first week after administration of ACTH/saline than in the second, supporting the explanation of low ambient temperature decreasing defaecation rate, as it has been reported that animals stop eating when ambient temperature drops below 16°C in alligators (Lance, 2003).

The relativly low average individual defaecation rate of 1.24 samples per week found in this study is comparable to the defaecation rate of 1.65 samples per week found in three-toed box turtles (Rittenhouse et al., 2005). As it seems almost impossible to collect indvidual samples on a daily basis over longer periods of time, data interpretation in terms of cause-and-effect relationships have to take place by grouping data into longer time intervals (weeks or months).

5.3 Enzyme immunoassay selection

To class an EIA as to measure FGM levels as an accurate reflection of adrenocortical function, the assay should reveal a substantial increase in FGM levels after administration of a pharmacological agent (e.g. ACTH) known to stimulate glucocorticoid production. Furthermore, the respective EIA should also detect changes in FGM concentrations associated with occurring putative stressful events. Finally, the assay should fulfil the four main criteria of validation, which are sensitivity (minimum amount of hormone than can be detected), precision (within- and between assay repeatability), accuracy (ability to detect the correct amount of hormone in the

sample), and specificity (i. degree of specificity of the antibody itself, ii. possible influence of interfering substances, e.g. matrix effects) (Hodges et al., 2010).

To provide a control that the administration of ACTH resulted in the expected increase in circulating glucocorticoid levels, blood samples were taken before ACTH administration as well as 1 h and 5 h after the injection. Even though blood corticosterone is known to be the main glucocorticoid in crocodilians (Tort and Teles, 2011, Touma and Palme, 2005), the collected material was analysed using a corticosterone and a cortisol RIA as Mahmoud et al. (1996) showed, that circulating cortisol is also present in alligators (although in low amounts) and increases in response to stress.

For captive-reared Nile crocodiles mean baseline serum corticosterone concentrations were reported to be in a range of 5 ng/ml (Balment and Lovedridge, 1989). In comparison, the results of the present study showed about 5-fold higher mean baseline serum corticosterone levels (25.1 ng/ml). However, a direct comparison of absolute hormone values among studies should be conducted with caution, as long as different storage protocols for the samples and most importantly different assays for hormone measurement were used. Instead biologically important changes in hormone levels should be noted for both analyses and the respective trends compared (Millspaugh and Washburn 2004). In addition, a potential explanation for the differences in baseline serum corticosterone concentrations could be also the difference in size/age of the study animals used, or differences in housing and handling conditions between the studies.

Serum corticosterone concentrations were already determined in a number of crocodilian species in context with different stressors (reviewed by Lance et al., 2001). For a wild Nile crocodile the mean serum corticosterone level exceeded 100 ng/ml after capture by noose trap and extended handling (Lance et al., 2001). In comparison, the results of the present study showed a 2-fold increase (55.8 ng/ml ± 28.3) 1 hour after treatment and a 4.5-fold increase (94.1 ng/ml ± 44.2) 5 hours post-injection. These concentrations are comparable to the hormone levels reported by Lance and colleagues (2001); however, comparing absolute serum hormone concentrations should generally be treated with caution, as mentioned above. In crocodilians, more specifically in *Alligator mississippiensis*, adrenocortical function has so far been evaluated in two studies by performing an ACTH stimulation test (Lance and Lauren, 1984; Mahmoud et al., 1996). After intraperitoneal ACTH administration, Lance and Lauren (1984) reported a significant 4-fold

increase in serum corticosterone concentrations 4 h post-application, and Mahmoud et al. (1996) reported a significant 7-fold increase in serum corticosterone levels 6 h post-injection, when compared with the baseline concentrations. These findings are comparable with the results of the present ACTH stimulation study in Nile crocodiles as an overall 4.5-fold increase was found 5 h post-ACTH administration. The differences in the allocated signal between the studies might be partly due to species specificity, but more likely due to the different size and therefore age of the animals used, the dosage of ACTH administered, time of blood samples drawn, and different handling techniques of the animals involved. Lance and Lauren (1984) used much smaller animals (41.5 - 45.0 cm; 4 month post-hatch), compared to Mahmoud and colleagues (1996) (30 - 50 kg; 172 - 206 cm total length); the latter comparable with the size of animals used in the present study (27 - 55 kg; 182 - 219 cm total length). Further, Lance and Lauren (1984) administered 8 IU of ACTH per animal, whereas in the study of Mahmoud and colleagues (1996) 25 IU of ACTH per alligator were injected. In the present study 13.5 - 27.5 IU of ACTH per crocodile were administered. The dose per kg used by Lance and Lauren (1984) was much higher, when compared to the other two studies; additionally ACTH of a different source was used, which could have affected the result. Blood was collected by Lance and Lauren (1984) 1, 4, 24 and 48 hours post-injection and after 0.5, 1, 1.5, 2 and 6 hours by Mahmoud et al., (1996). In the present study, blood samples were only collected before and after 1 and 5 hours. No information regarding handling was given by Lance and Lauren (1984). A noose snare was used by Mahmoud and colleagues (1996), whereas animals were electro-stunned just prior to each bleeding in the present study.

For captive alligators, baseline serum cortisol concentrations were in the range of 0.05 - 0.1 ng/ml, and a peak in cortisol levels of about 1.4 - 1.6 ng/ml (30-fold increase) was found 6 h post-ACTH administration (Mahmoud et al., 1996). In comparison, the results of the present study revealed mean baseline serum cortisol concentrations below the sensitivity threshold (≤ 2 ng/ml) of the RIA used. Therefore the existence of similar circulating baseline cortisol levels cannot be proven nor excluded due to the lower sensitivity of the assay used in the present study. Peak cortisol concentration reached up to 3.7 ng/ml, but 7 of the 18 animals showed cortisol levels ≤ 2 ng/ml 5 h after ACTH administration.

The increase in serum corticosterone levels after ACTH administration suggests that the dose of the agent was sufficient to elicit a response of the HPA axis. Circulating glucocorticoids should be, however, determined by using a corticosterone assay, as the tested cortisol RIA failed to detect sufficient amounts of the respective hormone, and therefore at least this particular assay is not suitable for determining changes in adrenocortical activity in Nile crocodiles.

In addition to the administration of ACTH, a control group of animals received only saline, to be able to investigate the effect of repeated handling (electro-stunning) and blood collection on serum glucocorticoid levels. Although mean serum corticosterone levels increased 1 h and 5 h after the saline treatment, respectively, no significant increase in serum corticosterone concentrations was found. Also no significant differences in serum corticosterone concentrations between the respective ACTH and saline groups were found, indicating that handling / repeated handling alone can act as a stressor in Nile crocodiles and cause an elevation in glucocorticoid concentrations. However, future studies would have to confirm this hypothesis, as challenges on alligators revealed significant differences in serum corticosterone concentrations between saline and ACTH groups by 4 and 6 h post-treatment, respectively (Lance and Lauren, 1984; Mahmoud et al., 1996).

Mahmoud et al. (1996) demonstrated significant differences in serum cortisol levels between ACTH and saline administered alligators 6 h post-injection. Although all samples collected prior and 1 h post-treatment revealed serum cortisol levels below the detection limit in the present study, 50 % of the control animals showed hormone levels of 2.4 - 3.6 nmol/L, thereby indicating an increasing trend in line with the findings of Mahmoud et al. (1996).

To reliably determine the corresponding increase in FGM levels post-treatment, faecal samples from singly housed crocodiles only could be used, because in group housed animals an individual identification of faecal samples was not possible. Of the six singly housed animals (4 ACTH and 2 saline treated), faecal samples pre- and post-injection could be only obtained from three individuals (2 ACTH and 1 saline treated). In total, 12 samples were obtained from the three individuals (5 pre- and 7 post-treatment), which were analysed using the CCS, 3α,11oxo-CM, and 11,17-DOA EIA, respectively.

All three tested EIAs detected a sufficient increase in FGM concentrations 7 - 15 days post-treatment, indicating that all three assays would be suitable to determine adrenocortical function using Nile crocodile faeces. The highest relative increase after ACTH administration was found using the 11,17-DOA EIA. However, when comparing absolute FGM levels, the 3α,11oxo-CM EIA showed best results, detecting on average values 3 - 4 times higher than the corticosterone and 11,17-DOA EIAs. For comparison, all three tested EIAs yielded adequate responses to an ACTH challenge in mammals, e.g., corticosterone EIA in mice (Touma et al., 2004); 11,17-DOA EIA in cattle and sheep (Palme et al, 1999), and 3α,11oxo-CM EIA in African elephant (Ganswindt et al., 2003). Because the different EIAs recognise different groups of glucocorticoid metabolites, it is not surprising that their suitability varies across species, as hormone metabolism is highly species-specific. It seems that for some species, e.g. mice (Touma et al., 2003); Common marmoset *Callithrix jacchus* (Heistermann et al., 2006) only one out of several tested EIAs was suitable to reflect adrenocortical activity accurately, whereas for others, e.g. Barbary macaque *Macaca sylvanus* (Heistermann et al., 2006); goats (Kleinsasser et al., 2010), including the Nile crocodile, as shown in the present study, more than one EIA appears to be suitable to monitor adrenocortical function.

As literature for reptiles is limited, no direct comparison is possible with studies carried out in crocodilians or other reptile species. However, effects of extrinsic stressors were studied in a few reptile species, using the non-invasive technique of measuring FGMs. One study determined the effect of radiotransmitters on FGM levels of three-toed box turtles, in which no change in FGM levels was determined in relation to fitting animals with radiotransmitters (Rittenhouse et al., 2005). A second study attempted an ACTH challenge and compared corticosterone levels in faeces and shed skin of African house snake (*Lamprophis fuliginosus*), and found a positive association between shed skin and faecal corticosterone concentrations in these snakes, but the results of the ACTH challenge were difficult to interpret (Berkvens, 2012). A third study showed that handling and changes in housing conditions in green iguanas (*Iguana iguana*) resulted in a significant increase of FGM levels (Kalliokoski et al., 2012). In the present study a similar result was found, as the singly housed animal treated with saline revealed a 1.5 fold elevation in FGM levels 10 days after handling and the saline injection.

The ACTH challenge demonstrated that three of the tested assays would be suitable to provide information of the stress level experienced by captive Nile crocodiles. As the 3α,11oxo-CM EIA detected higher absolute FGM levels compared to the 11,17-DOA EIA this assay was chosen as group-specific EIA for all subsequent analyses. The performance characteristics (recovery of exogenous corticosterone, intra- and interassay precision, parallelism, and assay sensitivity) of the two subsequently used corticosterone and 3α,11oxo-CM EIAs revealed that they accurately and precisely determine FGM output. The two assays showed appropriate ranges of sensitivity and demonstrate linearity under dilution all similar to those shown in other studies (e.g. Berkvens, 2012; Rittenhouse et al., 2005).

5.4 Stability of FGMs post-defaecation

Experiments to determine the stability of FGMs post-defaecation were conducted on several mammal species with various results, for example a distinct decrease in FGMs levels occurs within a few hours post-defaecation, 2 - 24 h in sheep (Lexen et al., 2008), 5 - 32 h in brown hyena (Hulsman et al., 2011), an increase in FGM levels within a few hours, (4 - 24 h in cattle, horses and pigs, (Möstl et al., 1999) and sheep (Lexen et al., 2008), and a rather negligible change in FGM levels over several days, e.g. baboons (Beehnder and Whitten, 2004).

As possible changes in measured FGM concentrations in unpreserved samples depends on the species (Hunt and Wasser, 2003), they seem also to depend on the assay used (see e.g. Lexen et al., 2008).

Although not significantly different, the changes in FGM levels determined with the 3α,11oxo-CM EIA in post-defaecation samples stored unpreserved indoors or outdoors for up to 72 h seem to follow the same decreasing trend as seen in other studies (Lexen et al., 2008; Hulsmann et al., 2011). By using the 3α,11oxo-CM EIA, a distinct decrease in hormone levels 4 - 8 hours post-defaecation was observed in faeces from sheep and brown hyena (Lexen et al., 2008; Hulsmann et al., 2011), and although still not conclusively explained, bacterial enzymes present in the faeces seem to further metabolize FGMs (Millspaugh and Washburn, 2004; Möstl et al., 2005),

which are then showing in some cases more or less cross-reactivity with the respective antibodies used.

When using the corticosterone EIA, faecal FGM concentrations in Nile crocodiles were relatively stable post-defaecation for up to 72 hours at ambient temperatures. As this could be a favourable combination of the specificity of the respective EIA and the occurring FGMs in Nile crocodiles faeces at given times, a further explanation might be the rather low pH found in the samples compared to e.g. the pH determined in faeces of African buffalos (*Syncerus caffer*) stored unpreserved at ambient temperatures (A. Ganswindt, personal communication), as the pH is known as one factor to influence bacterial enzyme activity (e.g. McDermid et al., 1988) including metabolism of colonic bacteria (Edwards et al., 1985).

5.5 FGM profiles

Even though it is possible to monitor adrenocortical activity in Nile crocodiles using faeces as hormone matrix, showing the temporal relationship between the occurrence of a stressor and the respective stress response is difficult. Due to low and irregular defaecation rates, varying lag times skew association of observed stressful events and responding hormone alterations. For example, three obvious peaks were identified in the only individual in which a long term profile was obtained, two of them occurred shortly after noted stressful events (separation and physiological treatment), but a third elevation in FGM levels also occurred between the two events, and no potential stressor could be determined from farm records to explain the elevation. On a group level, the generation and subsequent interpretation of detailed hormone profiles seems even more difficult, as no individual sampling is possible, and therefore temporal allocation of single peak values can be misleading. To overcome this problem, pooling of the samples over a certain time period might be advisable (Palme et al, 1999) to diminish individual variation in defaecation rate. However, this approach can flatten a profile so that single events can be overlooked. By compiling profiles for group-housed individuals using intervals of 5 days, highest mean FGM levels were found shortly after separation (-14 to -10 days) and 6 - 10 days after ACTH administration. In contrast, no clear pattern could be identified in the profile of the saline treated group-housed crocodiles. A potential explanation could be that the single sample

collected for the saline treated group-housed crocodiles -14 to -10 days is not representative for the respective time interval. Also the missing additional stimulation of the HPA axis through ACTH administration may account for the absence of a clear elevation in FGM concentrations 6 - 10 days after treatment in the saline group. Due to the difficulties experienced in compiling and interpreting hormone profiles of Nile crocodiles, monitoring of adrenocortical function might be most applicable for comparing cross-sectional data on a group or population level, especially when applied for animals in the wild.

5.6 Group size comparison

In the wild, adult Nile crocodiles are gregarious, with large dominant males usually holding a territory in which they are hardly ever challenged, as they get right of way by younger subordinate animals, or chase off smaller (probably male) crocodiles, if they enter their territory (Modha, 1967; Huchzermeyer, 2003). However, information about an optimal setup in terms of group size and composition would be speculative as it would depend on various environmental factors which are highly variable. In captivity, very low stocking rates are usually not the norm due to the cost of space, while high stocking densities are avoided to reduce fighting and therefore injuries (Huchzermeyer, 2003). Crowding of juvenile crocodilians also inhibits maximum growth and this behaviour is associated with chronically elevated plasma corticosterone levels (Elsey et al., 1990b).

In the present study the impact of group size (n = 1, 2, or 4 captive individuals) on faecal glucocorticoid metabolite levels was investigated, with an expectation of higher levels of FGMs in singly housed and/or densely housed individuals as these conditions rather reflect an unnatural or potential stressful setup, as indicated above. However, the results were opposite, with highest mean FGM concentrations in the pairwise kept animals. A possible explanation for these results could be that all study animals lived in established group compositions of 4 - 7 animals prior to the present study and were re-grouped only shortly before the experiment. In this regard, each of the two groups of four animals consisted of one large animal, which was the largest animal in the previous group, respectively, and could have remained the dominant individual in the new group because of its size and physical presence. In the case of each of the pairwise housed animals, two

crocodiles of approximately similar size, which were originally housed in the same groups together with larger crocodiles, were combined. Therefore, a possible explanation for the higher FGM concentrations in the pairwise housed crocodiles could be that a previous established dominance hierarchy has been overruled, resulting in an unstable group composition, which could be reflected in elevated glucocorticoid concentrations (Creel, 2001). A possible explanation for the low FGM concentrations in singly housed animals could be that although kept alone, they had audio-visual and olfactory contact to other crocodiles, but faced no competition for space and food during the experimental period.

Chapter 6: CONCLUSIONS

This study is the first to demonstrate adrenocortical activity in captive Nile crocodiles by using serum as well as faeces as hormone matrix, thereby underlining the value of non-invasive endocrine monitoring as a tool to provide information on the level of stress experienced by more intractable species like the Nile crocodile.

The present study showed that after capture and restraint as well as additional ACTH stimulation, serum corticosterone concentrations and faecal glucocorticoid metabolite (FGM) levels were distinctively elevated compared to respective baseline hormone concentrations. Therefore, it can be concluded that stimulation of the adrenal cortex is reflected accurately by corticosterone concentrations in serum and FGM measurements in captive Nile crocodiles. Furthermore, FGM analysis not only proved sensitive enough to detect effects of the ACTH treatment, but also naturally occurring fluctuations in FGM levels in response to environmental challenges. Thus, the results confirm that FGM measurement offers a practical alternative for investigators wishing to non-invasively monitor adrenocortical activity in farmed Nile crocodiles for improving their health and wellbeing.

Space restriction and crowding can act as stressor for crocodilians in captivity (Elsey et al, 1990a,b). Therefore, a non-invasive approach to assess adrenocortical activity in crocodiles kept under suboptimal stocking densities might be beneficial, because sampling is usually feedback free due to the absence of capture and handling. However, infrequent defaecation rates must be considered when designing studies using faecal matter as matrix for hormone analysis.

Regrouping, group size and composition in terms of stable hierarchies were identified as not mutually exclusive factors potentially impacting FGM levels in farmed Nile crocodiles. However, due to low animal numbers available in the present study, future research would be necessary to investigate these factors in more detail.

While the technique of FGM measurement has immediate applications for the assessment of welfare in captive Nile crocodiles, a non-invasive approach could also open new perspectives for monitoring the impact of ecological and anthropogenic factors on adrenocortical function in Nile crocodile populations in the wild.

Much like previous studies in other vertebrate species, this study demonstrated that it is possible to accurately quantify glucocorticoid metabolites in Nile crocodile faeces, opening new avenues for non-invasive stress research in this and possibly others crocodilian species.

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Appendix A: Blood and Urine Sample Record Sheet.

Recording Sheet: ACTH, Blood and Urine Collection

Croc ID	Pond No.	Time stunned	Time blood coll.	Vol. blood (ml)	Time Inject	Time urine coll.	Vol. urine (ml)	Time release	Comments

Appendix B: Food and Faecal Sample Record Sheet.

Recording Sheet: Food and Faecal Samples

Date	Time	Food in [g]	Food out [g]	No. of scats	Weight dung [g]	Freshness	Comments
26.11.2010							
27.11.2010							
28.11.2010							
29.11.2010							
30.11.2010							
01.12.2010							
02.12.2010							
03.12.2010							
04.12.2010							
05.12.2010							
06.12.2010							
07.12.2010							
08.12.2010							
09.12.2010							
10.12.2010							
11.12.2010							
12.12.2010							
13.12.2010							
14.12.2010							
15.12.2010							
16.12.2010							
17.12.2010							
18.12.2010							
19.12.2010							
20.12.2010							
21.12.2010							
22.12.2010							
23.12.2010							
24.12.2010							
25.12.2010							
26.12.2010							
27.12.2010							
28.12.2010							
29.12.2010							
30.12.2010							
31.12.2010							