IUCN-SSC Crocodile Specialist Group - Student Research Assistance Scheme

Progress Report

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





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Background of the Study

The Nile crocodile (*Crocodylus niloticus*) has been farmed in southern Africa since the 1960s. In this regard, stress in captive crocodilians is a constant concern for the crocodile industry, seeing that stress can negatively affect animal production and the health of crocodiles (Huchzermeyer FW, 2003: Crocodiles: Biology, husbandry and diseases, CABI Publishing, Wallingford, UK). When confronted with a stressor, an individual displays a stress response consisting of a suite of physiological and behavioural alterations to cope with the challenge. In this regard, a typical physiological response is the activation of the hypothalamic–pituitary–adrenal axis and thereby the secretion of glucocorticoids from the adrenal cortex. So far, the assessment of adrenocortical function in crocodilians, as a stress-related physiological marker, is done invasively (e.g. Mahmoud et al., 1996, Comp. Biochem. Physiol.,A 1: 57-62), with the disadvantage of a possible self-induced stress response due to handling (collection of the blood samples).

By establishing a non-invasive technique to monitor glucocorticoid metabolite levels in captive Nile crocodiles based on faecal hormone analysis, in combination with observations of stress-related behavioural changes, this study will provide for the first time a reliable, feedback-free tool to monitor adrenocortical endocrine function in a crocodilian species, thereby contributing to a better understanding of the endocrine correlates of stress in crocodilians and the potential impact due to handling. The applicability of the newly established method will open opportunities to improve the management and welfare of captive crocodiles in terms of more appropriate housing conditions and optimal husbandry practices. However, it would be just as helpful to evaluate potential stressors for wild crocodiles, e.g. rapid degradation and pollution of their environment. Nowadays, rivers are getting more and more polluted all over the world. Coincidently, the large number of crocodiles that died in the upper (2007) and lower parts (2008, 2009 and 2010) of the Olifants River, raised concerns that wild Nile crocodiles could disappear completely from South African rivers in future (Myburgh JG et al., 2010: Pansteatitis associated mortalities in Nile crocodiles from South Africa – a retrospective overview. 20th Working Meeting of the CSG, IUCN/SSC, Manaus, Brazil). In this regard, frequent monitoring of adrenocortical endocrine function on a population level in areas with a high pollution potential could help to identify impacted populations. Finally this project could also be a starting point for developing similar tests for other crocodilian species, thereby assisting conservation efforts and ongoing breeding programs of endangered crocodilian species.

Study set-up and aims

The overall aim of this study was to develop a method to assess adrenocortical activity in captive Nile crocodiles by measuring faecal glucocorticoid metabolite (FGM) concentrations.

To select an enzyme-immunoassay (EIA) for a reliable non-invasive assessment of adrenocortical endocrine function in the Nile crocodile, a physiological validation of the respective test system is necessary. This validation procedure should proof that changes in activity of the hypothalamic-pituitary-adrenal (HPA) axis are reflected in FGM concentrations measured in fecal material of the species of interest by the respective assay. One way to ascertain this is by injecting an animal with adrenocorticotropic hormone (ACTH) which is known to elicit a rise of glucocorticoid levels in the animal. ACTH is produced and secreted by the anterior pituitary gland in response to the corticotropic releasing hormone (CRH) released by the hypothalamus. The elevation in glucocorticoid level should then be detectable in the blood as well as in the faeces,

Another important factor which needed to be evaluated was the stability of faecal glucocorticoid metabolites post defecation. To determine the rate of FGM degradation in excreted faeces under different environmental conditions, freshly collected faecal material was kept for different periods of exposure time under conditions simulating indoors and outdoors housing conditions. This is important to optimise sampling and test the suitability of faecal samples collected under field conditions.

Finally the impact of group size on FGM levels in captive Nile crocodiles was investigated.

Progress of the study

- An ACTH stimulation test was conducted on 18 captive crocodiles at Le Croc crocodile farm.



Figure 1: Temporary housing of crocodiles for the ACTH study at Le Croc crocodile farm



Figure 2: Preparation of material for ACTH injection and blood collection

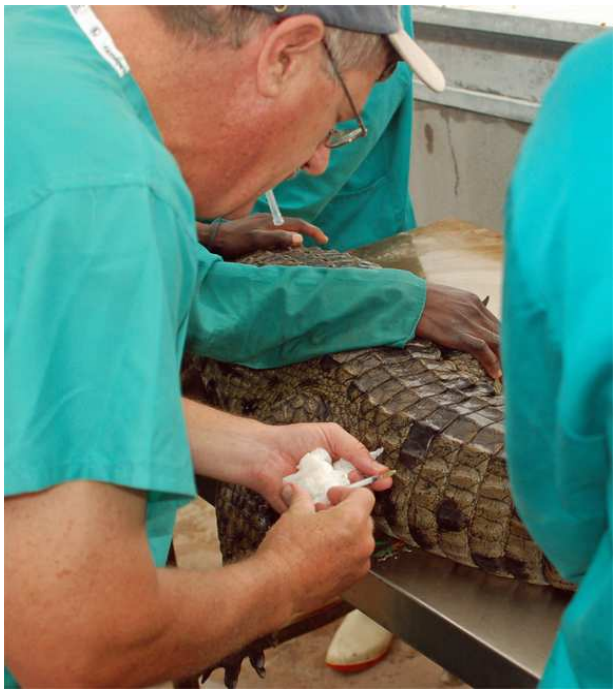


Figure 3: Intramuscular injection with ACTH by Dr. Jan G. Myburgh

- During the period from 26 November 2010 to 31 December 2010 the following data-sets were collected pre and post ACTH/saline injection: 54 serum samples and 115 faecal samples.



Figure 4: Blood collection from the spinal vein by Dr. Jan G. Myburgh

- The serum samples were analysed for quantitative changes in cortisol and corticosterone concentrations at the Veterinary Hormone Laboratory, Section of Reproduction, Dept. of Production Animal Studies, Faculty of Veterinary Science, University of Pretoria.



Figure 5: Processing of blood samples for hormone analysis

- After performing an extraction trial, the protocol for hormone extraction from faecal samples was optimized and subsequently all collected faecal samples were lyophilised and extracted accordingly.

- Quantitative hormone analysis with enzyme immuno-assays (EIAs) measuring cortisol-, and corticosterone-metabolites, and two group specific assay measuring 11,17-dioxoandrostanes (11,17-DOA) and metabolites with a 5β-3α-ol-11-one structure (3α,11oxo-CM), respectively, were performed on a subset of the faecal hormone extracts. Due to the best performance of the 3α,11oxo-CM and corticosterone EIAs, these assays were used to analyse the complete set of faecal hormone extracts.

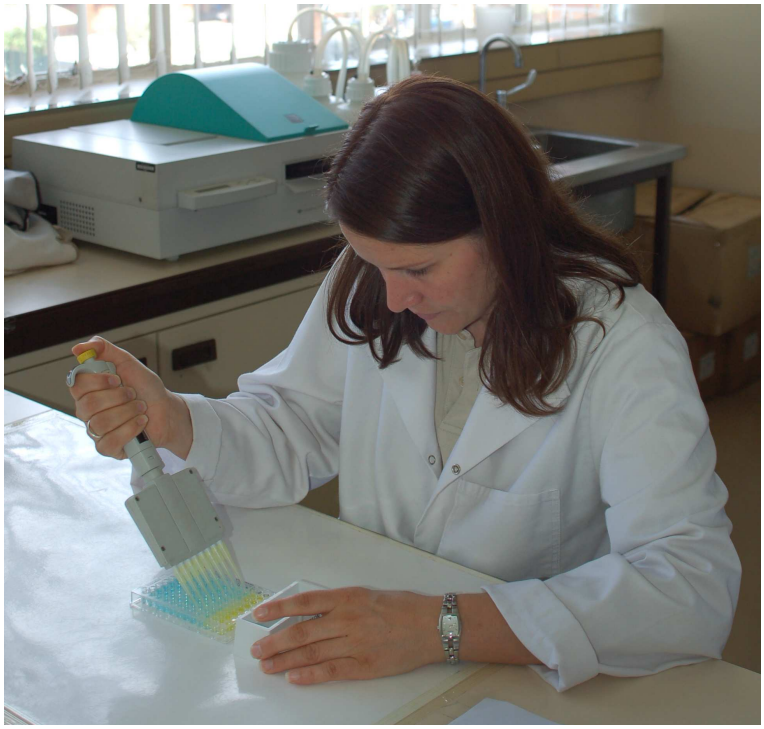


Figure 6: Measurement of FGM concentrations

- In addition, an experiment was conducted to test for the stability of the hormone metabolites post defecation under different storage conditions. Therefore, fresh faecal samples were collected from 45 freshly slaughtered crocodiles at *Izintaba* crocodile farm. The samples were pooled and homogenised, and sub-samples stored for different periods of time (1-72 h) under different conditions (in an incubator at constantly 32℃ and on a windowsill in the sun). After storage, the samples were lyophilized, extracted and the resulting extracts analysed with a corticosterone- and 3α,11oxo-CM EIA, respectively.

- Finally, the impact of group size on faecal glucocorticoid metabolite levels in captive Nile crocodiles.

- The findings of the study were presented at an international conference (8th International Conference on Behaviour, Physiology and Genetics of Wildlife, held from 14th to 17th September in Berlin, Germany), and at the University of Pretoria Faculty day of the Faculty of Veterinary Sciences (held on 6th September 2012 in Onderstepoort, South Africa).

Main findings of the study

The study animals showed a significant increase in serum corticosterone concentrations following ACTH administration (see Figure 7). 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

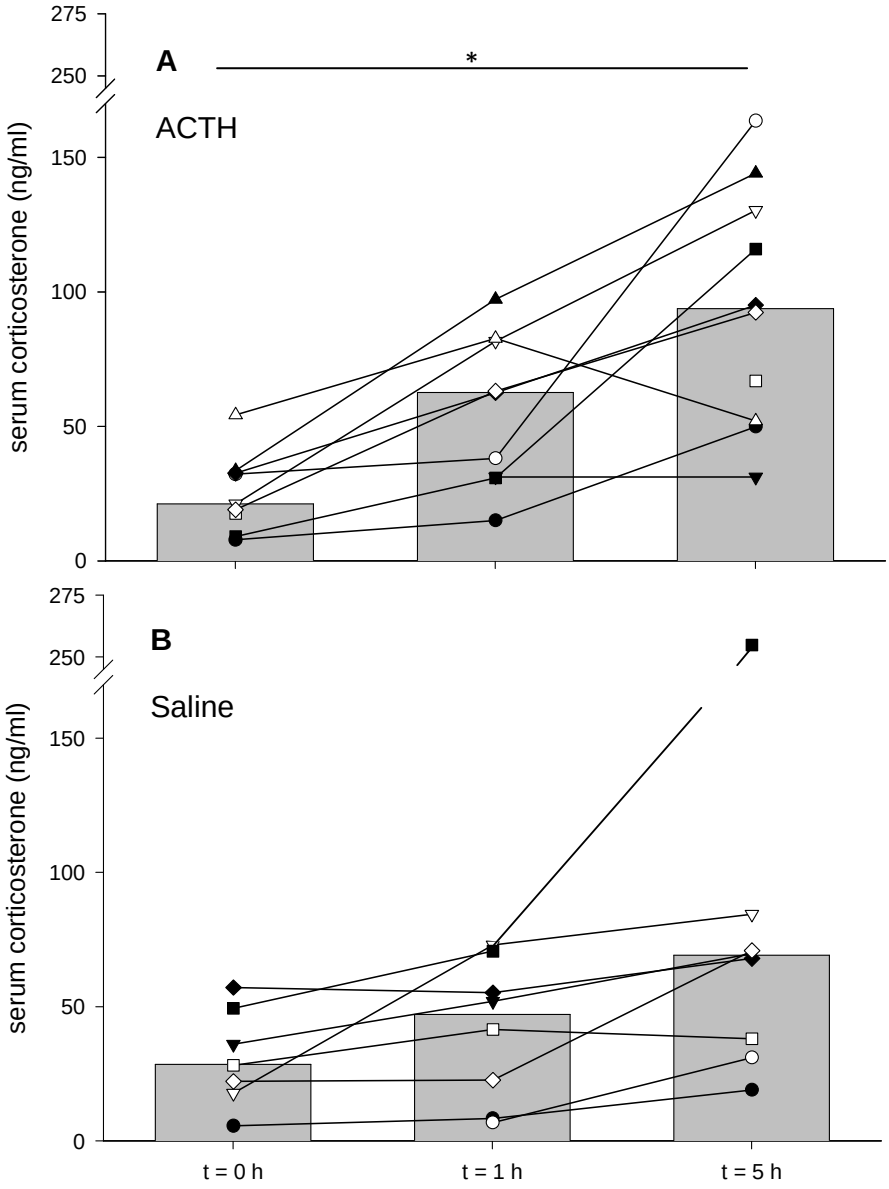


Figure 7: Symbol-bar plots of serum corticosterone concentrations (ng/ml) of captive Nile crocodiles following ACTH (A) or saline (B) administration. Each symbol represents the individual hormone concentration of an animal, either before (t = 0 h), one hour (t = 1 h), or five hours (t = 5 h) after administration, 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.

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 8).

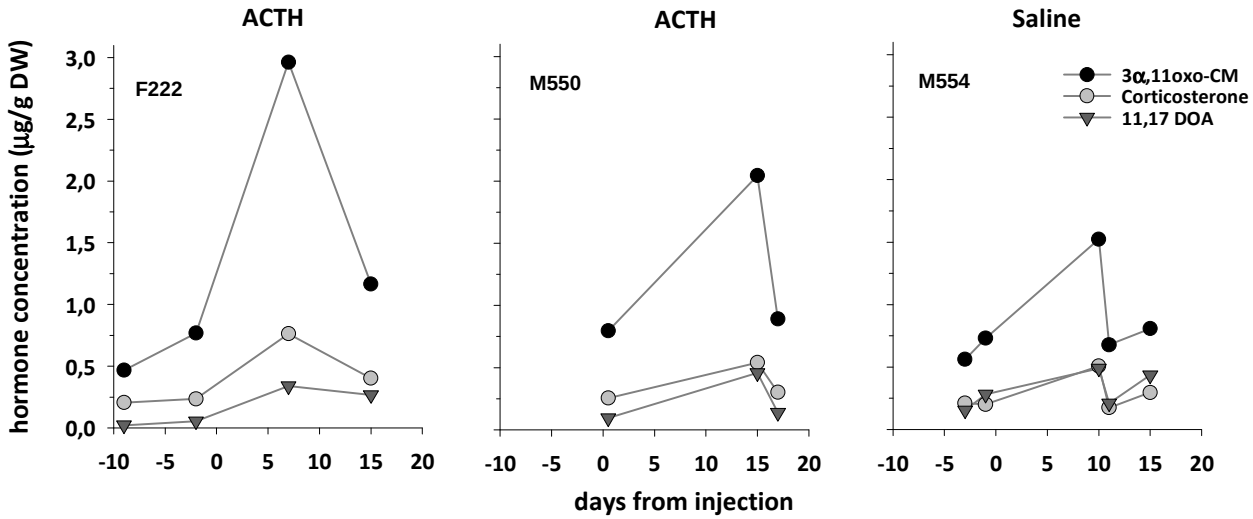


Figure 8: Faecal hormone metabolite concentrations in mass per g dry weight of samples from three singly housed crocodiles treated with either ACTH or saline. Samples were analysed using three different EIAs (corticosterone, 11,17-DOA, and 3α,11oxo-CM; F222 and M550 received ACTH administration and M554 saline administration).

To account for changes in hormone concentration post-defaecation, homogenized sub-samples of freshly collected faeces were stored and analysed as described above. The analysis with the 3α,11oxo-CM EIA and corticosterone EIA revealed no significant changes in FGM distribution across sampling subsets for both storage regimes (see Figures 9 A + B).

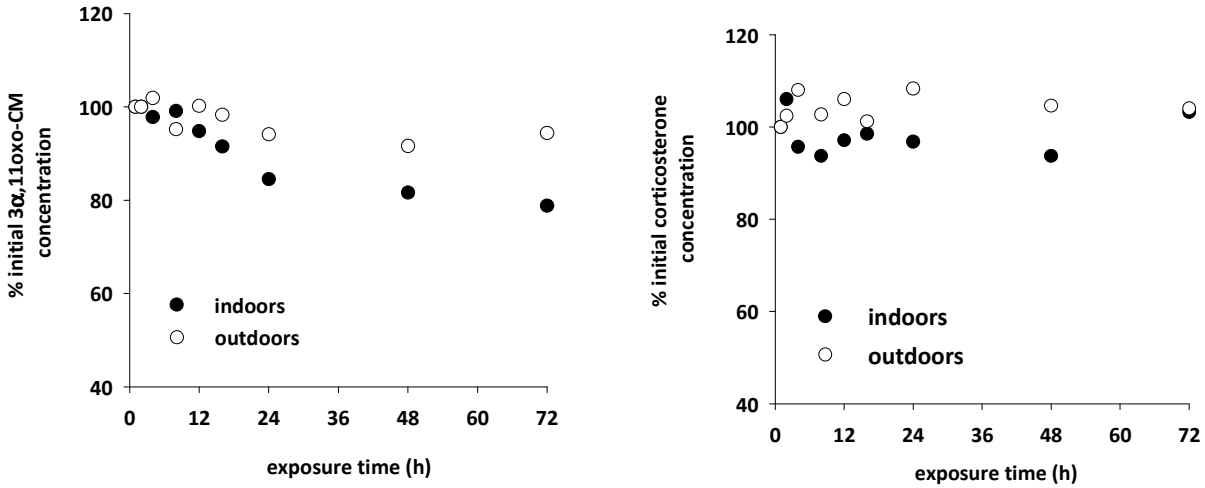


Figure 9: Changes in mean 3α,11oxo-CM (A) and corticosterone (B) concentrations (%) over time for faeces stored indoors at 32℃, 32 - 34 % humidity and outdoors at 22 - 28℃, 6 - 70 % humidity.

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 10).

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 10A). 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 10B). 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 ($p = 0.32$).

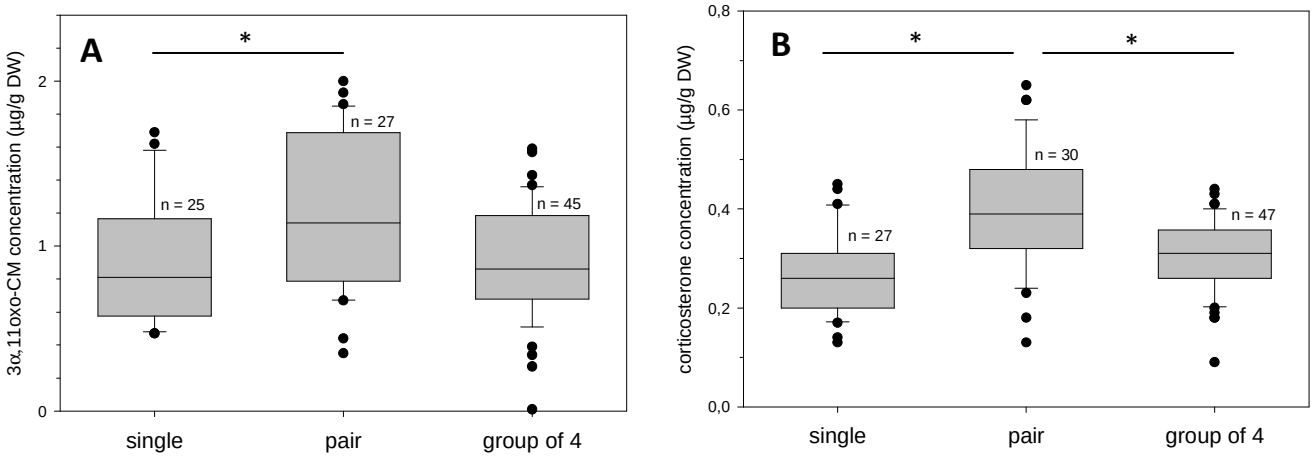


Figure 10: Boxplots of baseline FGM concentrations of singly, pairwise, and group housed crocodiles at Le Croc crocodile farm measured with 3α,11oxo-CM EIA (A) and corticosterone EIA (B). Boxes show median, 25 and 75 percentiles, whiskers show 10/90 percentiles, and dots show outliers. Asterisks indicate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

Conclusion

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.