Non-invasive assessment of adrenocortical function in captive Nile crocodiles (*Crocodylus niloticus*)

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Abstract:

The occurrence of stress-inducing factors in captive crocodilians is a concern, since chronic stress can negatively affect animal health and reproduction, and hence production. Monitoring stress in wild crocodiles could also be beneficial for assessing the state of health in populations which are potentially threatened by environmental pollution. In both cases, a non-invasive approach to assess adrenocortical function as a measure of stress would be

preferable, as animals are not disturbed during sample collection, and therefore sampling is feedback-free due to the absence of capture and handling. So far, however, such a non-invasive method has not been established for any crocodilian species. As an initial step, we therefore examined the suitability of two enzyme-immunoassays, detecting faecal glucocorticoid metabolites (FGMs) with a 11β,21-diol-20-one and 5β-3α-ol-11-one structure, respectively, for monitoring stress-related physiological responses in captive Nile crocodiles (*Crocodylus niloticus*). An adrenocorticotropic hormone (ACTH) challenge was performed on 10 sub-adult crocodiles, resulting in an overall increase in serum corticosterone levels of 272% above the pre-injection levels 5 hours post-injection. Saline-treated control animals (n=8) showed an overall increase of 156% in serum corticosterone levels 5 hours post-administration. Faecal samples pre- and post-injection could be obtained from three of the six individually housed crocodiles, resulting in FGM concentrations 136-380% above pre-injection levels, always detected in the first sample collected post-treatment (7-15 days post-injection). FGM concentrations seem comparatively stable at ambient temperatures for up to 72 hours post-defaecation. In conclusion, non-invasive hormone monitoring can be used for assessing adrenocortical function in captive Nile crocodiles based on FGM analysis.

Keywords:

ACTH challenge test; adrenocortical function; corticosterone; enzyme-immunoassay; faecal glucocorticoid metabolites; Nile crocodile; post-defaecation hormone stability

Introduction

Stress can be defined as the generic term for a state of perturbed homeostasis in an individual which evokes diverse adaptive reactions known as the stress response (Wielebnowski, 2003). A typical physiological response to a perceived stressor is the increased secretion of glucocorticoids by the hypothalamic–pituitary–adrenal axis, and although such a response is adaptive in the short term, chronically increased glucocorticoid levels can produce an array of pathologies, including reproductive suppression, ulcers, muscle wasting, and immune suppression (Munck et al., 1984; Liptrap, 1993; Sapolsky, 2002; Dobson and Smith, 2000). Monitoring glucocorticoid levels can be therefore of central importance in a number of disciplines of biological research and animal management.

The Nile crocodile (*Crocodylus niloticus* Laurenti, 1768) is one of 23 extant crocodilian species, and has been farmed in southern Africa since the 1960s (Luxmoore, 1992). The occurrence of stress-inducing factors in captive crocodilians is a concern for the farming industry, because chronic stress can negatively affect animal health and reproduction, and hence production quality and quantity (Huchzermeyer, 2003; Morici et al., 1997). Furthermore, environmental contaminants can act as stressors as seen in American alligators (*Alligator mississippiensis*) (Guillette et al., 1997), which could lead to an elevation in stress-related hormones in animals living in contaminated lakes (Gunderson et al., 2003). In this regard, 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 the state of heath in animals living in afflicted areas (Ferreira and Pienaar, 2011; Ashton, 2010; Myburgh and Botha, 2009).

Hormone analysis is a precise and widely used tool for monitoring responses to stressors, and although hormone levels can be measured in various biological matrices (Sheriff et al., 2011), non-invasive methods have gained popularity over the past 30 years as a

more practical approach for assessing adrenocortical activity in wildlife species (Ganswindt et al., 2012). In this regard, the use of faeces as hormone matrix for determining glucocorticoid output in crocodiles would allow a non-handling approach and therefore avoids induced alteration in stress-related hormone concentration due to handling. Thus, such an approach would not affect the well-being of the monitored animal due to the absence of capture and restraint, and also increases the safety of investigators during the monitoring process. So far, however, such a non-invasive approach has not been established for any crocodilian species.

By validating a respective non-invasive technique for monitoring adrenocortical function in captive Nile crocodiles based on faecal hormone analysis, this study aimed to provide the necessary tool for further investigating stress in crocodiles, its hormonal correlates, and their relationship with management practices. The ability to monitor 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. Furthermore, it could help to develop similar methods for other crocodilian species and therefore subsequently assist in conservation and education efforts, as well as ongoing breeding programs of endangered crocodilian species.

The overall aim of this study was to examine the suitability of two enzyme immunoassays (EIAs) detecting faecal glucocorticoid metabolites (FGMs) with a 11β,21-diol-20-one and 5β-3α-ol-11-one structure , respectively, for monitoring adrenocortical function in *Crocodylus niloticus*. More specifically, the aims of this study were: a) determining stress-related physiological responses in Nile crocodile faeces by performing an adrenocorticotropic hormone stimulation test (ACTH challenge test), b) investigating the effect of storage by determining the rate of metabolism of faecal glucocorticoid metabolites post-defaecation, and c) to characterise changes in FGM levels in captive Nile crocodiles in relation to different housing conditions.

Material and Methods

Study animals and housing

The study was carried out on 18 captive Nile crocodiles (16 males and 2 females; total length 1.8 - 2.2 m; body mass 26 - 55 kg) at the Le Croc crocodile farm, South Africa. During the study, the animals were fed five times per week with minced chicken, supplemented with vitamins and minerals as prescribed by a qualified animal nutritionist, and had permanent access to water for drinking and submerging/thermoregulation. Maximum and minimum temperature as well as humidity was recorded daily. All animals were kept in one house and the enclosures were cleaned six times per week. The study was performed with approval of the University of Pretoria Animal Use and Care Committee (Reference V045-10).

Experimental design

During the 5-week study period, six of the 18 study animals were housed individually (4 males and 2 females), four crocodiles in pairs (all males), and eight animals in two groups of four (all males). After immobilization via electro-stunning, 10 animals (1 group of four, 1 pair, and 1 female and 3 male individually housed animals) were injected intramuscularly with a total of 0.5 IU/kg of synthetic ACTH (Synacthen Depot©, Hoffman La Roche AG), and the remaining 8 animals (1 group of four, 1 pair, and 1 female and 1 male individually housed animal) were injected with 1 ml saline as control. To monitor the actual effects of the challenge on circulating glucocorticoid levels, an initial blood sample was taken from each animal within 3 minutes from electro-stunning, a second blood sample after one hour, and a third sample five hours after administration. Blood samples were collected from the spinal venous sinus according to the technique described by Myburgh et al. (2014).

After administration of ACTH/saline, faeces were collected for 2.5 weeks. In the Nile crocodile, faeces and urine are stored in different parts of the cloaca, namely the rectum and urinary chamber, preventing a mix of defaecated urine and faecal material under normal circumstances (Myburgh et al 2012). The enclosures were inspected three times a day (in the morning, at noon, and in the afternoon), all faeces collected, immediately frozen at -20°C, and kept frozen until hormone analysis. To determine baseline FGM levels, faeces were also collected daily for 2.5 weeks prior to ACTH administration.

The stability of hormone metabolites post defaecation was investigated by collecting fresh faeces from the rectum of 36 slaughtered crocodiles at Izintaba crocodile farm, South Africa. The collected material was homogenized, pooled, and subsequently divided into 2x27 equal subsamples, which were stored either indoors (oven at 32°C, 32-34% humidity) or outdoors (22-48°C, 6-70% humidity). Subsequently, 3 subsamples from both storage regimes were frozen at -20°C after 0 h, 1 h, 2 h, 4 h, 8 h, 16 h, 24 h, 48 h, and 72 h, respectively.

Sample processing and extraction

Blood samples were left to clot for 30-60 min at room temperature, and subsequently centrifuged at 1500 g for 15 min. The serum was transferred into polystyrene tubes and stored at -20°C until analysis.

The frozen faecal samples were lyophilized, pulverized and sieved through a mesh strainer to separate faecal powder from any existing more indigestible material like bone fragments. A weighed amount (100-110 mg) of faecal powder was then extracted with 3 ml of 70% methanol in distilled water by vortexing for 15 minutes. After centrifugation at 1500 g for 10 minutes, the supernatant was transferred into micro-centrifuge tubes, and stored at -20°C until analysis.

Hormone analysis

Serum corticosterone levels were determined by using a Coat-A-Count© Corticosterone Radio-Immunoassay (Diagnostic Products Coat-a-Count Rat-Corticosterone). In brief, 50 µl standards, controls, and samples were transferred in duplicates into coated tubes, respectively. One ml ^{125}I corticosterone solution was added, and the tubes were incubated for 2 h at room temperature. Subsequently, all the liquid was removed, the tubes patted dry and immediately counted for one minute in a gamma counter (Wallac Wizzard^2, Perkin Elmer) using MULTICALC software. Sensitivity of the assay was 5.7 ng/ml, and major cross-reactivities, as given in the manufacturer's pamphlet, were corticosterone, 100%; 11-deoxycorticosterone, 2.86%; progesterone, 0.83%; and cortisol, 0.35%. The inter-assay coefficient of variation (CV), determined by repeated measurements of high and low concentration standards, was 4.0% and 14.9%, respectively.

Faecal extracts were measured for immunoreactive FGM concentrations using two different enzyme-immunoassays (EIAs), detecting FGMs with a 11β,21-diol-20-one structure (corticosterone EIA) and 5β-3α-ol-11-one structure (11-oxoaetiocholanolone EIA), respectively, according to the procedure described by Ganswindt et al. (2002). Serial dilutions of faecal extracts gave displacement curves that were parallel to the respective standard curve in both assays. Sensitivities of the assays were 3.0 ng/g faeces for the corticosterone EIA and 1.5 ng/g faeces for the 11-oxoaetiocholanolone EIA. Intra- and interassay CV, determined by repeated measurements of high and low concentration pool samples, ranged between 4.7% and 14.2% for the corticosterone EIA and 4.2 and 14.7% for the 11-oxoaetiocholanolone EIA. Recovery of added standard was 107.5 ± 12.4% (mean ± standard error [SE]) for the 11-oxoaetiocholanolone assay and 110.0 ± 14.0% for the corticosterone assay. Details of the two EIAs, including cross-reactivities are described by Palme and Möstl (1997) for the corticosterone EIA and by Möstl et al. (2002) for the 11-oxoaetiocholanolone EIA.

Data analysis

Individual serum corticosterone concentrations were compared regarding sampling time (0 h; 1 h; 5 h) as well as between groups (ACTH and control). After testing for normality using Shapiro-Wilks test and equal variance using F-test, differences in hormone concentrations between two sets of data were examined by t-test. Differences in hormone concentrations between more than two sets of data were examined using either one-way ANOVA, followed by a post hoc analysis using Tukey test, with the application of Bonferroni correction or by Kruskal-Wallis one-way ANOVA on ranks.

Baseline values of FGM concentrations were determined for each housing type (individual, pair, group of four) using an iterative process where all values greater than the mean plus 2 standard deviations (SD) were removed (Brown et al., 1999). For that, all FGM concentrations of an individual data set exceeding the mean plus 2 SD were excluded, the average then recalculated, and the elimination process repeated until no values exceeded the mean plus 2 SD. Baseline FGM values were subsequently compared between the housing types using either one-way ANOVA (post hoc analysis using Tukey test), or by Kruskal-Wallis one-way ANOVA on ranks (post hoc analysis using Dunn's method). A parametric or non-parametric approach was chosen after testing for normality and equal variance in advance using Shapiro-Wilks and F-test.

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.

Statistical significance was assumed when $P < 0.05$ and analyses performed using the software programs Jandel Sigma Stat (Version 2.0) and KyPlot (Version 2.0 beta 13).

Results

Housing conditions and defaecation rates

Maximum temperatures ranged from 26-45°C (40°C ± 5°C (mean ± SD)) during the study, with two distinct dips in temperature after 5 and 16 days, lasting one and five days, respectively. Minimum temperatures remained more constant during the entire study period, ranging between 20-26°C (23°C ± 2°C (mean ± SD)). Maximum humidity varied between 57-95%, with a distinct 5-day peak after two weeks. Minimum humidity varied between 0-62%, with highest humidity levels (45-62%) recorded at the same time.

A total of 112 faecal samples were collected during the 35-day study period from the 18 study animals. Defaecation rate ranged from 0 to 0.50 faecal sample per individual per day with an overall average defaecation rate of 0.18 ± 0.16 faecal sample per individual per day (mean ± SD). This rate varied per housing type, with 0.14 ± 0.17 samples per individual per day for individually housed animals, averaged 0.21 ± 0.27 samples per individual per day for pair housed crocodiles, and averaged 0.19 ± 0.18 samples per individual per day for group housed animals. Two notable periods of overall lower defaecation rates (0.06 ± 0.05 faecal sample per individual per day) were also recognized during the study, which lasted 4 and 8 days, respectively.

ACTH challenge

Animals showed a significant increase in serum corticosterone concentrations following ACTH administration ($F = 11.04$; $p = 0.0004$; post hoc (Tukey test) 0h vs 5h; $p < 0.001$; see figure 1A). Overall baseline serum corticosterone concentration was

25.3 ng/ml ± 14.5 ng/ml (mean ± SD), which increased by 121% (55.8 ng/ml ± 28.3 ng/ml) one hour after treatment, and by 272% (94.1 ng/ml ± 44.2 ng/ml) 5 hours after treatment.

No significant differences were found when comparing serum corticosterone levels collected at 0 h, 1 h, and 5 h following saline administration ($H = 4.409$; $p = 0.1103$; see figure 1B). However, serum corticosterone levels were 31.2 ng/ml ± 18.0 ng/ml (baseline), which increased by 33% (41.6 ng/ml ± 26.2 ng/ml) one hour post-injection, and by 156% (79.9 ng/ml ± 74.4 ng/ml) 5 hours after saline administration.

No significant differences were found when comparing serum corticosterone levels between the ACTH and saline groups collected at 0 h ($t = -0.73$; $df = 14$; $p = 0.48$), 1 h ($t = 1.07$; $df = 15$; $p = 0.30$), and 5 h ($t_{A\text{-}W} = 0.48$; $df = 10.8$; $p = 0.62$), respectively.

Faecal samples pre- and post-injection were only obtained from three of the six individually housed crocodiles (two animals treated with ACTH (1 female and 1 male) and one control male treated with saline). FGM concentrations determined using the corticosterone as well as the 11-oxoaetiocholanolone EIA showed a distinct increase (> 100%) in the first sample collected post treatment in all three cases, with the group-specific 11-oxoaetiocholanolone EIA detecting larger overall quantities. Baseline FGM concentrations were 0.69 µg/g ± 0.10 µg/g (mean ± SD) dry faecal powder (11-oxoaetiocholanolone EIA) and 0.23 µg/g ± 0.03 µg/g dry faecal powder (corticosterone EIA), whereas FGM concentrations post treatment were 2.18 µg/g ± 0.73 µg/g dry faecal powder (11-oxoaetiocholanolone EIA) and 0.60 µg/g ± 0.14 µg/g dry faecal powder (corticosterone EIA) (see figure 2). Peak samples were collected between 7 and 15 days post ACTH administration.

Stability of FGM post-defaecation

The analysis with the 11-oxoaetiocholanolone EIA showed no significant changes in FGM distribution across sampling subsets for both storage regimes (indoors: $\chi^2 = 7.73$, $p = 0.46$; outdoors: $\chi^2 = 3.56$, $p = 0.89$). FGM concentration at t = 0 h was 3.60 µg/g dry faecal powder ± 0.44 µg/g (mean ± SD), maximum decrease in mean hormone levels for samples stored indoors was 21% after 72 h, (2.83 µg/g ± 0.19 µg/g), and for samples stored outdoors 8% after 48 h (3.29 µg/g ± 0.35 µg/g) (see figure 3A).

Likewise, hormone concentrations analysed with the corticosterone EIA showed no significant changes in FGM concentration across sampling subsets for indoors ($\chi^2 = 7.49$, $p = 0.49$) and outdoors ($\chi^2 = 7.84$, $p = 0.45$). Compared to the 11-oxoaetiocholanolone results, FGM concentrations determined with the corticosterone EIA showed a more variable pattern with an increase and decrease in hormone concentrations over time. FGM concentration at t = 0 h was 0.49 µg/g dry faecal powder ± 0.02 µg/g (mean ± SD), maximum decrease in mean hormone levels (6% after 48 h) was found in samples stored indoors (0.46 µg/g ± 0.05 µg/g), whereas a maximum increase of 8% after 24 h was found in samples stored outdoors (0.52 µg/g ± 0.02 µg/g) (see figure 3B).

Group size

Baseline FGM concentrations determined with the 11-oxoaetiocholanolone as well as the corticosterone EIA detected significant differences between individually, pairwise, and group housed animals (11-oxoaetiocholanolone: $H = 7.68$; $p = 0.022$; see figure 4A; corticosterone: $F = 13.35$; $P < 0.00001$; see figure 4B).

Post hoc analysis showed a significant difference between individually and pairwise housed animals (Dunn's Method; $p < 0.05$) for the 11-oxoaetiocholanolone data, but no

significant differences were found between individually and group housed crocodiles, as well as between pairwise and group housed animals (Dunn's Method; $p > 0.05$).

For the corticosterone data set, post hoc analysis showed significant differences between individually 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 individually and group housed animals ($p = 0.32$).

Discussion

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 respective influencing events. Furthermore, infrequent defaecation can complicate data interpretation. Defaecation rates varied considerably between individuals, and two periods of overall lower average defaecation rates were identified. A possible explanation for the first recognized drop 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 treatment but also overlapped with a period of low ambient temperatures. Therefore it is unclear, if this reduction in food intake might be a result of the conducted physiological treatment and handling, the change in housing conditions, as it is known that such changes could act as a severe stressor (Lance et al., 2001), or a combination of both. Overall, the average individual defaecation rate of 0.18 sample per day found in this study is e.g. comparable to the defaecation rate of 0.24 sample per day described for three-toed box turtles

(Rittenhouse et al., 2005). As it seems almost impossible to collect indvidual samples on a daily basis in reptiles like e.g. turtles or crocodiles, it might be advisable to interpret cause-and-effect relationships by using larger time intervals (weeks or months).

Serum corticosterone concentrations were already determined in a number of crocodilian species in context with different stressors (reviewed by Lance et al., 2001). For wild Nile crocodiles mean serum corticosterone levels exceeded 100 ng/ml after capture by noose trap and extended handling (Lance et al., 2001), which is comparable to hormone levels found in this study 5 hours post-ACTH treatment. In *Alligator mississippiensis*, adrenocortical function has been evaluated in two studies by performing an ACTH stimulation test (Lance and Lauren, 1984; Mahmoud et al., 1996). 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. These findings are comparable with the results of the present study as an overall 3.7-fold increase (272% above baseline) was found 5 h post-ACTH administration. Although no significant differences were found for the control group, mean serum corticosterone levels also distinctively increased post-treatment, again indicating that repeated handling (electro-stunning) and blood collection alone can act as an explicit stressor in Nile crocodiles. This is partly in line with findings of Franklin et al. (2003), who showed a significant increase in plasma corticosterone concentrations following restraint (noosing) but not following immobilization (electro-stunning) in estuarine crocodiles (*Crocodylus porosus*). In contrast to Lance and Lauren (1984) and Mahmoud et al. (1996), who found significant differences in serum corticosterone concentrations in alligators treated with saline or ACTH, no significant differences in serum corticosterone concentrations were found between the ACTH and saline control groups in the present study. However, future more detailed comparative studies would have to confirm this species specific difference.

FGM levels markedly increased in the first sample collected post treatment (7-15 days following ACTH or saline administration), demonstrating that non-invasive hormone monitoring can be used for assessing adrenocortical function in captive Nile crocodiles using faeces as hormone matrix. The obtained hormone profiles provide good evidence that the two EIA systems used in this study sufficiently detect stress-related changes in circulating glucocorticoid levels in both male and female Nile crocodiles. The monitored single-housed female showed a greater elevation in FGM levels post treatment (380% and 246% elevation determined with the 11-oxoetiocholanolone and corticosterone EIA, respectively) compared to respective hormone values from the males (ACTH treatment: 157% and 111% elevation; saline treatment: 136% and 147% elevation; determined with the 11-oxoetiocholanolone and corticosterone EIA, respectively). These differences might be a result of sex-related differences in hormone metabolism as shown for mice (*Mus musculus* f. *domesticus*) and discussed elsewhere (Goymann, 2012; Touma et al., 2003).

As literature for reptiles in terms of non-invasive hormone measurement 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. One study determined the effect of radio transmitters on FGM levels of three-toed box turtles, in which no change in FGM levels was determined in relation to fitting the animals with radio transmitters (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, as an induced change in glucocorticoid concentration could not clearly be shown (Berkvens, 2013). A third study showed that handling and changes in housing conditions in green iguanas (*Iguana iguana*) resulted in a significant increase in FGM levels (Kalliokoski et

al., 2012), which generally supports our findings of a 111%-157% elevation in FGM levels 10 days after handling for individually housed animal treated with saline.

Experiments to determine the stability of FGMs post-defaecation were already conducted on several mammal species with various results, and modification in the composition of faecal hormone metabolites by bacteria has been discussed to be responsible for the alterations in steroid metabolite levels found (Möstl et al., 1999; Lexen et al., 2008; Hulsman et al., 2011, Palme et al., 2013). Although not significantly different, the changes in FGM levels found in the present study when using the 11-oxoethiocholanolone EIA seem to follow the same decreasing trend as seen e.g. in sheep (Lexen et al., 2008) or brown hyena (Hulsmann et al., 2011), where the same EIA was used. Although still not conclusively explained, the bacterial-driven modification of the FGM composition seems to result in a lower signal, presumably due to a lower cross-reactivity of the new metabolites with the respective antibody used. When using the corticosterone EIA, FGM concentrations were comparatively stable at ambient temperatures for up to 72 hours post-defaecation. As this would be a favourable combination of the specificity of the respective EIA and the occurring FGMs in Nile crocodiles faeces at given times, a further not mutually exclusive 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).

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 depends 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., 1990). From an endocrinological perspective, a recent study on Saltwater crocodiles (*Crocodylus porosus*) by Isberg and Shilton (2013) shows no difference in plasma corticosterone concentrations between individually and communally housed animals. In the present study the impact of group size on stress-related hormone levels was also investigated, and highest mean FGM concentrations were found in pairwise kept animals. A possible explanation for this result could be that all study animals lived in established group compositions of 4 - 7 animals prior to the 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, it might be that a previous established dominance hierarchy has been overruled in the pairwise housed crocodiles, resulting in an unstable group composition, reflected in comparatively higher glucocorticoid concentrations (Creel, 2001; Creel et al., 2013). A possible explanation for the comparatively low FGM concentrations found for individually housed animals could be that the animals had audio-visual and olfactory contact to other crocodiles, but faced no competition for space and food during the experimental period.

Conclusions

The ability to non-invasively assess adrenocortical function in Nile crocodile now provides a solid basis to further examine endocrine responses to putative stressful impacts, e.g. acid mine drainage, even in free-ranging crocodile populations. After assessing regional variability in Nile crocodile glucocorticoid output, this approach could finally function as a viable aquatic systems biomonitoring tool by looking at stress responses in this apex predator.

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Figure captions

Fig. 1: 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) post-injection, respectively. Hormone values belonging to 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.

Fig. 2: Faecal glucocorticoid metabolite (FGM) concentrations in mass per g dry weight (DW) of samples from three individually housed crocodiles treated with either ACTH (F222 and M550) or saline (M554). Samples were analysed using a corticosterone and and 11-oxoaetiocholanolone EIA.

Fig. 3: Changes in mean FGM concentrations over time (%) measured with the 11-oxoaetiocholanolone EIA (A) and corticosterone EIA (B) for faeces stored indoors (black dots) at 32°C, 32-34% humidity or outdoors (white dots) at 22-48°C, 6-70% humidity.

Fig. 4: Boxplots of baseline FGM concentrations of individually, pairwise, or group housed crocodiles measured with the 11-oxoaetiocholanolone EIA and corticosterone EIA. Boxes show median, 25 and 75 percentiles, whiskers show 10/90 percentiles, and dots show outliers. Asterisks indicate statistically significant differences between groups.

Figure 1

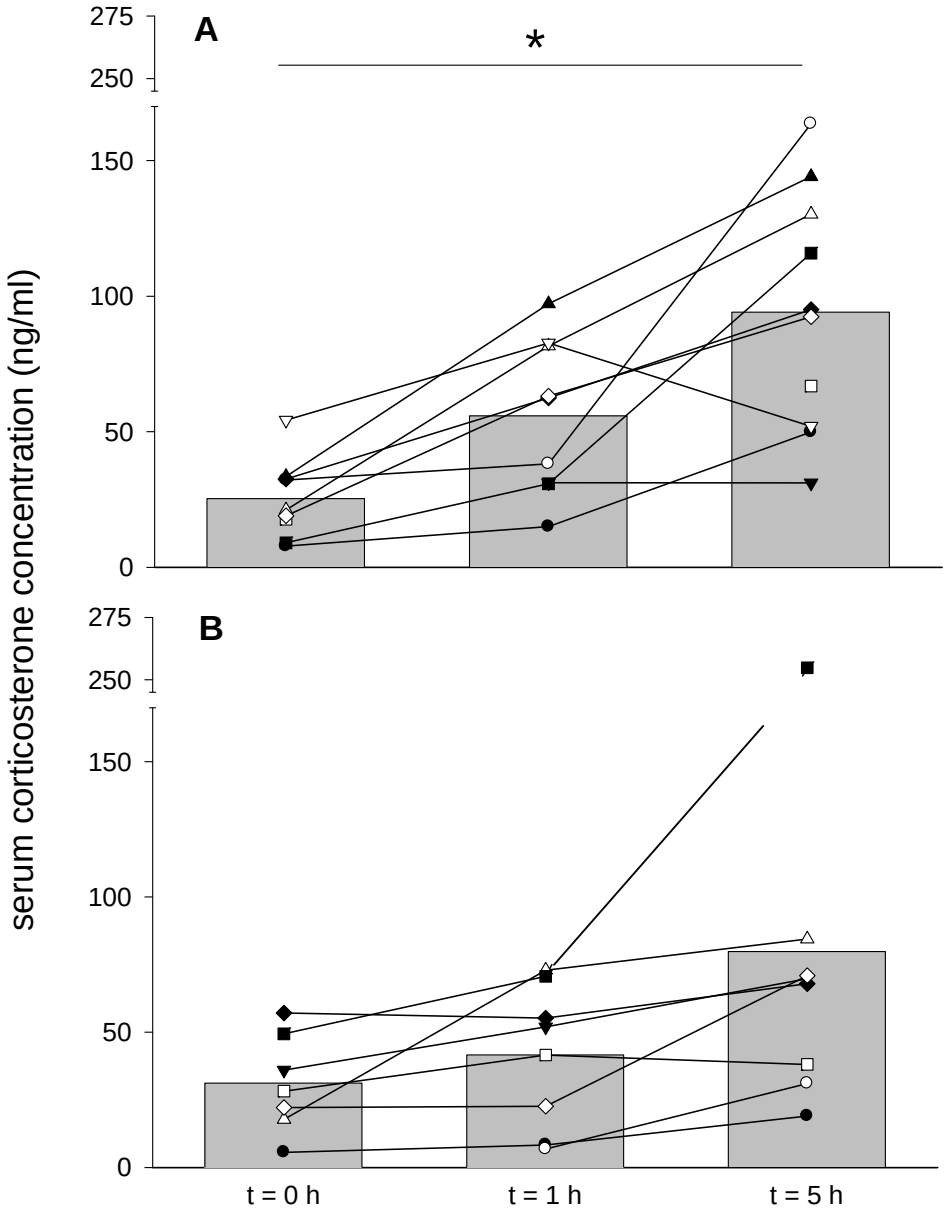

A
B
*
275
250
150
100
50
0
serum corticosterone concentration (ng/ml)
t = 0 h
t = 1 h
t = 5 h

Figure 2

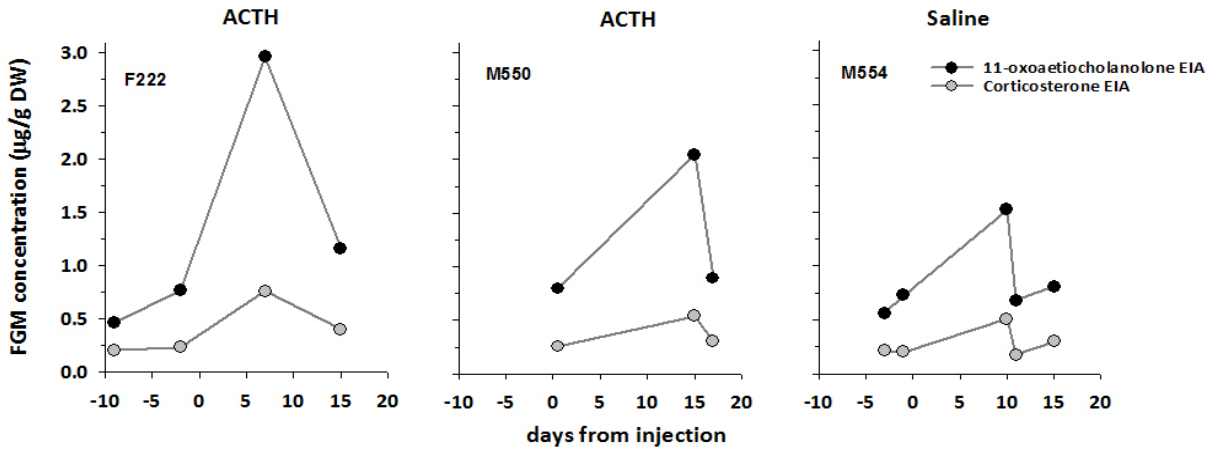

ACTH
ACTH
Saline
F222
M550
M554
11-oxoaetiocholanolone EIA
Corticosterone EIA
FGM concentration (µg/g DW)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-10 -5 0 5 10 15 20
days from injection

Figure 3

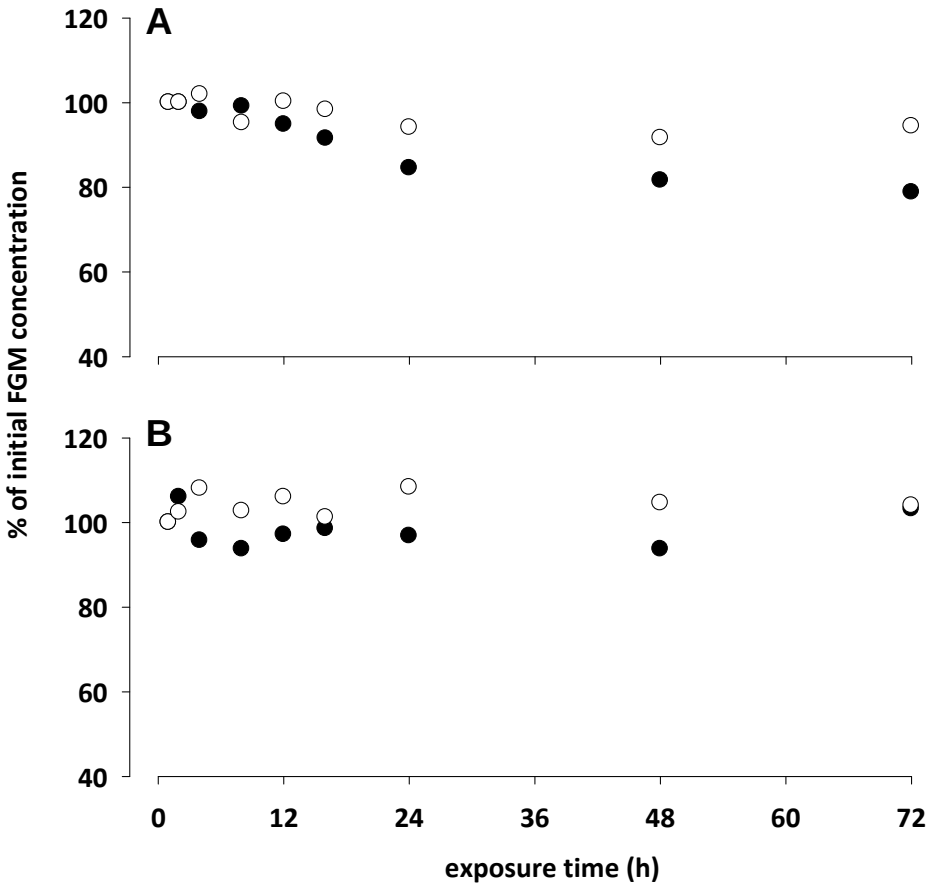
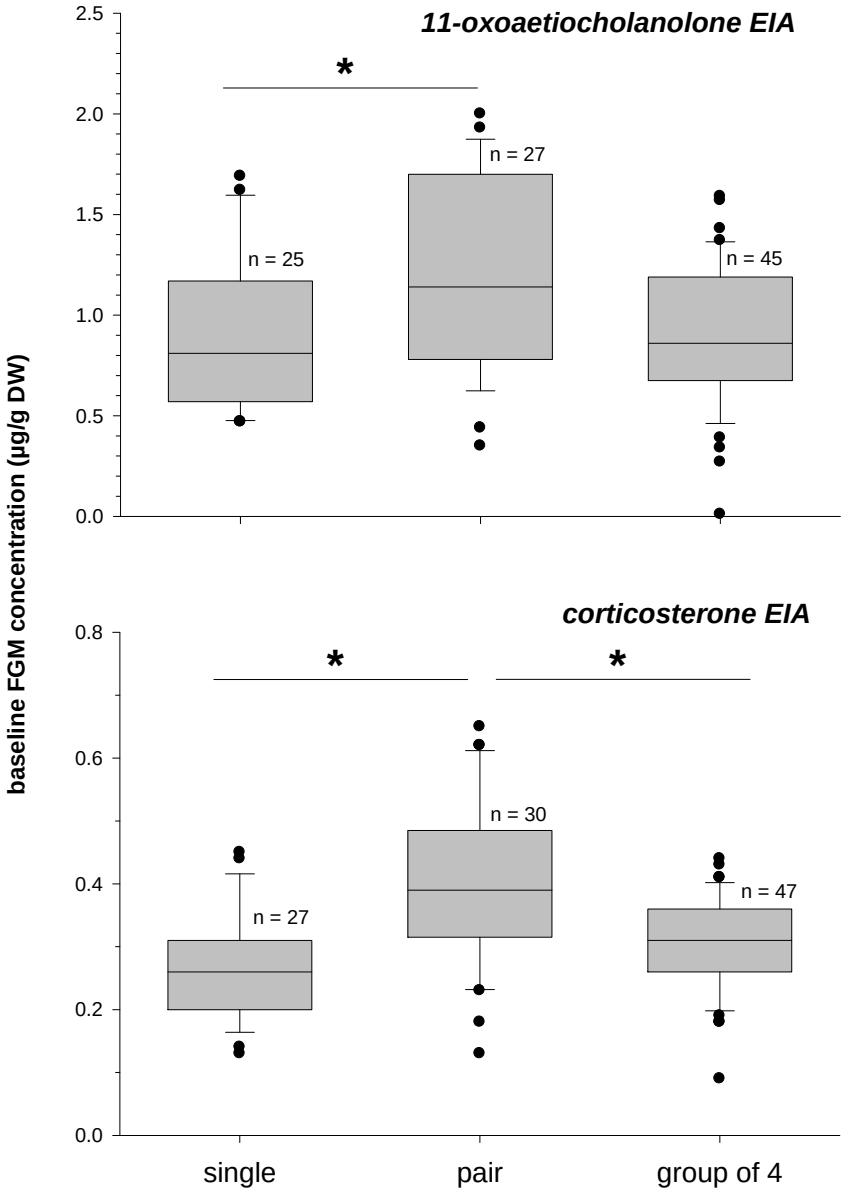

A
B
120
100
80
60
40
% of initial FGM concentration
0
12
24
36
48
60
72
exposure time (h)

Figure 4


11-oxoaetiocholanolone EIA
corticosterone EIA
baseline FGM concentration (µg/g DW)
2.5
2.0
1.5
1.0
0.5
0.0
0.8
0.6
0.4
0.2
0.0
*
*
*
n = 25
n = 27
n = 45
n = 27
n = 30
n = 47
single
pair
group of 4