Final Report for SRAS and FHVS-SRAS

The biobehavioural stress response in farmed Nile crocodiles, with and without standardised Sceletium tortuosum extract

**This study was completed in fulfilment for the M.Sc. degree of Andre Anton Swanepoel (orcid.org / 0000-0003-0344-9180)*

Abstract:

Crocodylians have received little anxiety-based research which is hindering progress being made in the mitigation of stress and its sequelae. Treatment-based research for strass is scarce, but herbal calmers like *Sceletium tortuosum* hold promise. We aimed to validate stress biomarker levels in Nile crocodiles before and during exposure to a stressor and explore feeding behaviour as a non-invasive stress indicator. Additionally, we aimed to study the anxiolytic effects of *Sceletium tortuosum* administration on stress exposure. Twenty-five grower crocodiles were selected, ten of which underwent treatment with a *Sceletium tortuosum* extract and were subjected to a simulated transport stressor. The control and treatment cohorts were compared using novel and standard plasma biomarkers, as well as feeding behaviour. No significant differences were observed in biomarkers between treatment and control groups, but it was unclear whether the treatment or methodology concerning administration was ineffective. Significant differences in the measured biomarkers were elicited after the stressor, with acute fluctuations observed in the fast-acting catecholamines (adrenaline and noradrenaline) when compared to baseline measurements. Downstream effects of these catecholamines and those of corticosterone appear to be associated with a persistent increase in plasma glucose and HLR. Lactate also showed acute fluctuations over time but returned to baseline by the final measurement. DHEA fluctuated during the stressor but its role in the stress axis remains unclear. The study tests new innovations to address stress in these animals as well as highlights the temporal dynamics of physiological markers under acute stress, contributing to understanding crocodylian stress and potentially informing improved farming practices for conservation and sustainable management.

Introduction/Problem Statement:

While crocodiles hold the title of being the largest extant reptiles on Earth, they remain relatively understudied in terms of neurological, behavioural, and psychological aspects (Ganswindt, 2012). Crocodylians, much like other animals and humans, undergo stress responses crucial for immediate survival (Huchzermeyer, 2003; Lance & Elsey, 1999). Dysfunctional stress reactions can lead to severe consequences (Gangloff & Greenberg, 2023; Moore & Jessop, 2003), particularly impacting conservation efforts and commercial crocodylian farms (CCFs). Substandard conditions in captivity may result in agitation, anxiety, aggression, anorexia, and poor breeding, posing commercial and financial challenges for farmers (van As, 2021). Addressing these issues necessitates a focus on

developing stress mitigation techniques, hindered by an incomplete understanding of the crocodylian stress response and its regulatory biological processes (Kabelik & Hofmann, 2018). The potential use of herbal calmers, such as Sceletium tortuosum (ST), is explored in this context. ST has established mechanisms for regulating mood, anxiety, and stress pathways (Olatunji *et al.*, 2022) and can be incorporated into crocodile feed with minimal stress during administration.

Methods/Activities:

Twenty-five juvenile, farm-raised, Nile crocodiles were screened and subsequently divided into five groups of five and assigned to a treatment (n = 10) or control cohort (n= 15). Following a 4-week acclimation period and baseline (BL) blood collections (earmarked “Day 1”), the animals were held for a seven-day recovery period. The ST extract (Zembrin®) was administered in their feed at a dose of 100 mg/kg every Tuesday and Friday for 4 weeks. Thereafter, all the crocodiles were removed from their holding pens, restrained in hessian bags (simulated transport stress) and subjected to venous blood withdrawal immediately after capture (T0H), two hours after capture (T2H), and seven hours after capture (T7H). Blood plasma was collected and processed for analysis of adrenaline (Adr), noradrenaline (NA), corticosterone (CORT), dehydroepiandrosterone (DHEA), the CORT:DHEA ratio, glucose (GLUC), lactate, serotonin (5-HT) and 5-hydroxy indoleacetic acid (5-HIAA). Finally, the heterophil-to-lymphocyte (HLR) ratio was obtained via differential leucocyte counts from blood smears made at the time of blood collection. The animals were then returned to their experimental enclosures, i.e., holding pens, and monitored until their normal feeding habits returned. The monitoring of feeding habits occurred throughout the study period by measuring the weight of feed consumed within 24 hours after each feeding. Ethical oversight was provided by both NWU-AnimCareREC (NWU-00786-22-A5) and the UPAEC (REC124-22).

Findings:

The most important finding of this work is that the analysed biomarkers are adequate indicators of acute stress. Notably, NA and Adr demonstrate rapid reactivity upon the perception of a stressor, whereas noticeable effects on HLR are only seen over time. The CORT response to acute stress exhibits a vast range of downstream effects with varying response times. These effects lead to correlations with DHEA and lactate, following an acute response, whilst still displaying temporal effects on GLUC and HLR. Finally, acute stress is associated with fluctuations in the CORT:DHEA ratio underscoring its reactivity and swift response in crocodiles. This highlights its crucial role in investigations pertaining to the crocodilian stress response.

No statistically significant differences were evident across all the measured biomarkers and behavioural markers, between the control and Zembrin® treatment (“the extract”) cohorts, ascribed to possible brumation in the crocodiles. This was supported by consistently reduced feeding over the treatment period and the lack of effect of ST on plasma 5-HT and 5-HIAA, known to describe the

mechanism of action of ST. Taken together, the extract may have been consumed at sub-therapeutic levels, and thus the findings are inconclusive.

Considering the non-treated cohorts, Adr, NA and DHEA rapidly increased following the perception of a stressor, compared to baseline (BL vs. T0H). CORT, CORT:DHEA ratio, lactate, GLUC and HLR, showed no significant differences between BL and T0H, possibly indicating that chronic stress was not a factor between Day 1 vs. Day 32. Furthermore, Adr and NA not only showed a strong positive correlation (r = 0.67) on Day 32 but also correlated with significant hyperglycaemia at this time point. Interestingly, GLUC was further influenced by the temporal effects of CORT, revealing a sustained increase on Day 32. Increased plasma CORT was accompanied by a sustained increase in the HLRs on Day 32, suggesting immunosuppression. Lactate analysis showed a positive correlation with CORT (r = 0.40). Here the corresponding fluctuations in CORT and lactate, with their levels returning to near baseline levels by T7H after having peaked at T2H, suggest a temporal switch indicative of effective HPA axis negative feedback over time.

CORT produced more acute and statistically significant increases and decreases in its levels over the collection points, whereas DHEA showcased a gradual decrease between T0H and T7H. DHEA, a glucocorticoid antagonist, induced a sustained decrease following T0H, indicative of its metabolic and immunosuppressive effects in the presence of dwindling DHEA levels where the latter is unable to effectively suppress the effects of CORT. CORT and DHEA did, however, reveal a moderate positive correlation (r = 0.32), thus, DHEA rises as CORT rises, while the downstream effects of CORT on lactate, GLUC and HLR, confirm this ratio to be an indicator of HPI axis dysfunction.

Conclusion:

An acute capture-like stressor evokes a unique association between acute and late biomarkers of stress such as the catecholamines and HPI axis hormones. This observation highlights their temporal change within the context of stress and why their combined dysregulation is deleterious to the welfare of these animals. This study has produced an in vivo platform to assess established and novel physiological systems involved in the crocodilian stress response, as well as to test novel anxiolytic treatments and interventions.

Despite the lack of success in the proof-of-concept study involving ST and the incorporation of a behavioural stress marker, i.e., feeding habits, attributable to methodological constraints, valuable insights have been gleaned, offering potential contributions to subsequent investigations. In conclusion, this research not only contributes to a deeper understanding of the stress response mechanisms in crocodilians but also facilitates the exploration of enhanced farming practices. Ultimately, this contributes to the conservation viability and sustainable management of the species.

Follow-Up Work:

Climate influences crocodylians' feeding habits, impacting behavioural markers like feeding habits, necessitating validation in various scenarios for potential treatment options and welfare advancements (Huchzermeyer, 2003; Ganswindt, 2012; Gangloff & Greenberg, 2023). Recommendations include repeating the study in warmer climates to ensure baseline stress levels and reduce environmental influences, whilst recognising the importance of stress studies during colder seasons due to crocodylians' heightened sensitivity. These studies could shed light on previously unknown stress response factors and offer potential avenues for researchers to develop mitigation methods. Further recognising a limitation of the current study, i.e., the low number of collection points, future recommendations involve increasing points while avoiding complications associated with repeated blood collection. Routine blood analysis is suggested for a comprehensive pharmacokinetic and target engagement analysis, with further investigation into treatment administration routes and the slow metabolism of crocodylians/reptiles for improved treatment outcomes and reduced stress during procedures.

Literature Cited:

Ganswindt, S.B. 2012. Non-invasive assessment of adrenocortical function in captive Nile crocodiles (*Crocodylus niloticus*) and its relation to housing conditions. University of Pretoria.

Grigg, G. & Kirshner, D. 2015. Biology and evolution of crocodylians. 1. Australia: Comstock Publishing Associates a division of Cornell University Press Ithaca and London.

Huchzermeyer, F.W. 2003. Crocodiles: Biology, husbandry and diseases. CABI Publishing, 44 Brattle Street, 4th Floor, Cambridge, MA 02138, USA: CABI.

Kabelik, D. & Hofmann, H.A. 2018. Comparative neuroendocrinology: A call for more study of reptiles! Hormones and Behavior, 106:189-192. Doi: .1016/j.yhbeh.2018.10.005

Lance, V.A. & Elsey, R.M. 1999. Plasma catecholamines and plasma corticosterone following restraint stress in juvenile alligators. Journal of Experimental Zoology, 283(6):559-565. Doi: 10.1002/(sici)1097 010x(19990501)283:63.0.co;2-4

Olatunji, T.L., Siebert, F., Adetunji, A.E., Harvey, B.H., Gericke, J., Hamman, J.H. & Van der Kooy, F. 2022. Sceletium tortuosum: A review on its phytochemistry, pharmacokinetics, biological, pre-clinical and clinical activities. Journal of Ethnopharmacology, 287:114711. Doi: 10.1016/j.jep.2021.114711

van As, S. 2021. Sa crocodile farmers must adapt to survive. Farmer's Weekly, 2021(Issue):32-33. Doi:10.10520/ejc-farmweek-v2021-n21016-a9

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