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Biological validation of enzyme immunoassays for measuring physiological stress in rehabilitated Temminck's pangolins

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Abstract

Monitoring stress physiology through non-invasive techniques is critically important for the effective management of vulnerable wildlife species, particularly those experiencing substantial anthropogenic pressures. This study aimed to validate enzyme immunoassays (EIAs) for accurately quantifying faecal glucocorticoid metabolite (fGCM) concentrations, as indicators of physiological stress in Temminck's pangolin (*Smutsia temminckii*). Due to significant ethical, logistical, and conservation challenges associated with the direct handling and experimental manipulation of this threatened species, the study utilized a biological validation approach, relying on opportunistic collection of faecal samples from pangolins during their rehabilitation after confiscation from illegal wildlife trafficking. Faecal samples collected from multiple individuals at various rehabilitation facilities were analysed using a cortisol, corticosterone, two 11-oxoaetiocholanolone (72a and 72 T), and a 5α-pregnane-3ß,11ß,21-triol-20-one (37e) EIA. Among these assays, both 72a and 72 T detected significant increases (> 150%) in fGCM concentrations in female pangolins following exposure to known stressors. Notably, the 72a assay also exhibited robust performance in male pangolins, providing evidence of its utility across sexes. The study further underscored that direct human interactions, even during rehabilitation, elicited pronounced physiological stress responses in pangolins. These insights are particularly crucial for refining rehabilitation protocols to minimize stress and enhance welfare outcomes for rescued animals. This validation study not only reinforces the applicability and efficacy of non-invasive hormone monitoring techniques but also emphasizes their critical role in assessing animal welfare, rehabilitation success, and broader conservation management strategies for Temminck's pangolin.

Keywords Non-invasive, Welfare, Pangolin, Stress, Validation, Rehabilitation

1 Introduction

Hormone monitoring has become a vital tool in wildlife conservation, providing critical insights into the adrenal physiology of various species [1, 2]. This approach has allowed researchers to deepen their understanding of animal behaviour, population dynamics, health, and survival [3, 4]. The physiological stress response, which primarily involves

the hypothalamic–pituitary–adrenal (HPA) axis and the sympathetic nervous system (SNS), is an evolutionary adaptation that can help an organism survive stressful periods [5, 6]. While the SNS leads to the secretion of catecholamines and the activation of the well-known 'fight or flight' response [7], the activation of the HPA axis results in elevated secretion of the glucocorticoid (GC) steroid hormones [8]. An acute increase in GCs can be adaptive, ensuring that an organism's energy demands are met, enhancing cardiovascular activity, and changing behaviour, all of which contribute to a return to homeostasis and survival [8–10]. However, long-term GC elevations can have several deleterious effects on an individual, including immune and reproductive suppression, as well as a general decrease in fitness [11–13]. As such, researchers often monitor GC concentrations as a proxy for the environmental stress an individual experiences and the likely effects on survival and reproduction.

The collection and analysis of blood have long been the preferred method for monitoring hormone patterns in both captive and free-ranging wildlife species, as it provides real-time measurements of adrenal hormone concentrations [14]. However, utilising blood as a hormone matrix also has several shortcomings. Firstly, capturing and restraining an animal to collect a blood sample poses risks to both the researcher and the animal. Additionally, the capture process itself has been shown to activate the physiological stress response [15, 16], significantly elevating blood glucocorticoids within 3–5 min post-capture [17]. This rapid stress-induced increase can confound presumed baseline hormone concentrations. Moreover, repeated blood collections are impractical for small-bodied species with limited blood volumes, potentially leading to decreased survival rates due to cumulative stress and blood loss [18, 19]. Finally, glucocorticoids assessed in serum or plasma are affected by episodic fluctuations in hormone secretions driven by factors such as pulsatile release patterns, circadian rhythms, and feeding state [20–23]. Due to the invasive nature of endocrine monitoring via blood collection and analyses, this technique has fallen out of favour with many members of the research community, especially those working on threatened and endangered species [14].

It is because of such difficulties that researchers have turned to non-invasive endocrine monitoring techniques. Once steroid hormones are secreted by the HPA axis into the bloodstream, they are metabolised by the liver and excreted in bile and faeces as metabolites [24, 25]. As a result, steroid hormone metabolite monitoring can serve as a robust proxy for adrenal function in wildlife species [1]. The use of non- or less-invasive sampling techniques, such as faecal sampling, has several advantages over using blood as a monitoring matrix. Not only do faecal collection protocols eliminate the need for direct human-animal interaction, thereby reducing the risk of injury to both animals and researchers, but they also prevent stress-related feedback that can occur during blood collection events [2]. Furthermore, the pooling effect of metabolites in the gut ensures that hormone metabolite concentrations in faeces are less influenced by episodic fluctuations in hormone secretion [26]. Consequently, hormone metabolite concentrations provide a less erratic and longer-term value that spans several hours. Finally, collecting faeces as a monitoring matrix enables researchers to obtain repeated, longitudinal samples from small-bodied species, a task that is often difficult or impossible when collecting blood samples [27, 28].

Although non-invasive endocrine monitoring offers several advantages, endocrine secretion, metabolism, and excretion are sex- and species-specific [29]. Accordingly, any

enzyme immunoassay (EIA) used to monitor adrenal activity via faecal glucocorticoid metabolites (fGCM) must be validated in each species to ensure reliable quantification. Enzyme immunoassay validation can be achieved through physiological or biological approaches. Physiological validation typically involves administering a compound to transiently activate the HPA axis (e.g., adrenocorticotropic hormone challenge). Accordingly, faecal samples collected before and after the challenge are analysed with carefully selected EIAs to determine which assays detect biologically relevant changes in faecal hormone metabolite concentrations [25]. However, because this procedure is invasive, it is not always feasible, particularly in threatened or endangered species [30, 31]. In such cases, biological validation can confirm an appropriate EIA for measuring stress physiology in the species. For stress-related biological validation, samples are collected before and after a natural or human-induced stressor (e.g., injury, aggressive interactions, capture and handling, transport, or isolation) [25]. An appropriate EIA is then selected for measuring faecal glucocorticoid metabolite (fGCM) concentrations in that species. Monitoring stress physiology in endangered and threatened species provides unique insight into the impacts of environmental stressors, habitat restoration, disease, and breeding or rehabilitation programs on individual well-being and species survival [32].

The Temminck's pangolin (*Smutsia temminckii*) is currently classified as Vulnerable by the International Union for Conservation of Nature [33]. The species faces a range of anthropogenic threats, including poaching for the illegal wildlife and traditional medicine trades, habitat loss, electric fences, and overexploitation [33]. Furthermore, individuals rescued from illegal trade often require extended rehabilitation and careful reintroduction into the wild [33]. In such contexts, a validated EIA is essential for monitoring stress physiology and assessing individual health, rehabilitation outcomes, adaptability, and survival prospects. However, it is not possible to capture, hold, or conduct physiological experiments on pangolins due to the drastic decline in their numbers throughout their distribution [34]. Due to this limitation and the stress-prone nature of the species [35], it is imperative that a non-invasive means for monitoring stress physiology is developed. To our knowledge, no fGCM EIA has been validated for the Temminck's pangolin. Therefore, the aim of this study was to validate an appropriate EIA for quantifying fGCMs in the species.

2 Material and methods

2.1 Study animals

The study was conducted on the Temminck's pangolin. Due to the species' conservation status and its high susceptibility to stress [35], limited information exists on the physiological stress response mechanisms. It must be noted that most rehabilitation centres place emphasis on limiting the interaction between pangolins and people. As such, studies aiming to elucidate the physiological workings in pangolins must make do with short-term, opportunistic sample collection.

2.2 Participating facility and animal care

Samples were collected opportunistically from five Temminck's pangolin admitted to the Johannesburg Wildlife Veterinary Hospital (JWVH) during 2020 and 2021. For the safety of pangolins and staff, all individuals were housed at an undisclosed off-site location. Each pangolin was kept in an individual enclosure of approximately 8 m^2, which

was furnished with a designated resting site to promote comfort and minimize stress. Feeding and care were managed by trained facility personnel in accordance with established protocols for pangolin rehabilitation and husbandry. Information on animal care and location cannot be disclosed for security reasons. Upon arrival at the centre, all confiscated pangolins underwent thorough veterinary assessments and received immediate medical care as required. Ongoing health monitoring was conducted through scheduled check-ups or as clinically indicated. For this study, the primary focus was on individuals recently confiscated from illegal trade and those undergoing routine veterinary examinations (see Table 1 for details). To adhere to the veterinary hospital's animal health and welfare practices and policies, a strict faecal sample collection regime could not be implemented. As such, a focus was placed on collecting the first three excreted samples post-stressor, supplemented by any samples the hospital staff could collect without disturbing the animals. Individuals did not defecate frequently, with samples excreted days apart. With gut passage times spanning ~ 20 h for the white-bellied pangolin (*Phataginus tricuspis*) [36], 43 h for the Chinese pangolin (*Manis pentadactyla*) [37, 38], and ~ 35 h in the Sunda pangolin (*Manis javanica*) [39], our collection regime should account for peak fGCM levels in the Temminck's pangolin. However, calculated excretion times could not be determined for Temminck's pangolin during this study.

2.3 Biological validation process and sample collection

In addition to the inability to hold individuals for research, we were also unable to conduct a physiological validation to determine the most appropriate EIA for monitoring stress physiology in the species. As a result of this, we opted to conduct biological validation.

All study individuals (F: 3, M: 2) were confiscated from the illegal wildlife trade and transported to the JWVH. It should be noted that for Female 1, Male 1, and Male 2, the initial samples were collected shortly after their arrival at the centre. Any elevated fGCM levels observed at these time points may therefore reflect stress associated with the confiscation process, rather than veterinary handling. In addition, no two individuals are treated the same throughout the rehabilitation process. Individuals were monitored from arrival at the respective rehabilitation centers and faecal samples collected whenever possible, focusing on periods following the animal's arrival and handling (including veterinary care), as direct human contact has been shown to activate the physiological

Table 1 The faecal sample distribution (n = 28) for the five confiscated individuals used as part of the biological validation for determining the most ideal enzyme immunoassay for measuring faecal glucocorticoid metabolite concentrations in *S. temminckii*

	Events correlated to collected samples					
	Sample 1	**Sample 2**	**Sample 3**	**Sample 4**	**Sample 5**	**Sample 6**
Female 1	*Arrival*	Baseline	Baseline	Baseline	Baseline	Baseline
Female 2	Baseline	Baseline	Baseline	Baseline	*Decrease in health*	*Decrease in health*
Female 3	Baseline	Baseline	*Veterinary check-up*	Baseline	Baseline	Baseline
Male 1	*Arrival*	Baseline	*Veterinary check-up*	Baseline	Baseline	
Male 2	*Arrival*	*Veterinary procedure*	Baseline	Baseline	Baseline	

"Baseline" samples refer to faecal samples collected during periods with no known acute stressor or clinical intervention (i.e., outside arrival, veterinary procedures/check-ups, or documented health decline)

stress response [40]. Staff members opportunistically collected fresh faecal samples; however, due to the nature of the facility and a focus on animal care and recovery, recurring or long-term sampling was not possible. Instead, the focus was on collecting samples before and after a stressful event. Samples collected during periods where no animal handling occurred were marked as 'baseline' samples (Tab. 1).

We collected a total of 28 faecal samples (5–6 samples per individual) from three female and two male Temminck's pangolin confiscated from the wildlife trade. Although there is a predetermined protocol in place for Temminck's pangolin brought into rehabilitation centres, each animal is assessed on an individual basis. All fresh faecal samples were placed into a sample collection bag, sealed and stored at -20 °C until transport to the SANBI Wildlife Biobank in Pretoria, South Africa.

2.4 Steroid extraction and analyses

All frozen faecal samples were extracted at the Biobank following the protocol of Scheun et al. [41]. Samples were lyophilised, pulverised, and passed through a fine mesh to remove non-faecal material. Subsequently, 1.5 mL of 80% ethanol was added to 0.050–0.055 g of faecal powder and the mixture was vortexed for 15 min, then centrifuged at 1500 × g for 10 min. The supernatant was transferred to clean, clearly labelled 2.5 mL microcentrifuge tubes and stored at – 20 °C until EIA analysis at the Endocrine Research Laboratory (ERL), University of Pretoria, South Africa.

All faecal extracts resulting from the biological validation process (n = 28) were measured for immunoreactive fGCM concentrations using five EIAs, namely a (1) cortisol, (2) a corticosterone, two 11-oxoaetiocholanolone ((3) 72a, detecting 11,17 dioxoandrostanes and (4) 72 T, detecting fGCMs with a 5β-3α-ol-11-one structure) and a (5) 5α-pregnane-3ß,11ß,21-triol-20-one (37e) EIA. The inclusion of EIAs using antibodies designed to measure both cortisol and corticosterone, as well as widely used group-specific EIAs, should be sufficient to identify a reliable assay for monitoring GC metabolites in Temminck's pangolin [42, 43]. Details of the assays, including cross-reactivities, are described for the first three EIAs by Palme and Möstl [44], 72 T by Möstl et al. [45], and 37e by Touma et al. [31]. Serial dilutions of extracted samples produced displacement curves parallel to the respective standard curves in all assays (< 5% deviation in slope). The intra- and inter-assay coefficient of variance (CV), determined by repeated measurements of high- and low-value quality controls, as well as assay sensitivities, are shown in Table 2.

2.5 Data analysis

No analytical statistics were conducted due to the low number of samples collected during both validation stages. Mean fGCM concentrations were calculated from all collected samples (Table 1) for each EIA and individual; these mean values were used as

Table 2 The intra- and inter-assay coefficient of variance (CV), as well as the assay sensitivity for the five fGCM enzyme immunoassays tested

Enzyme immunoassay	Intra-assay CV	Inter-assay CV	Assay sensitivity ng/g faecal dry mass
Cortisol	4.89% and 5.83%	6.49% and 10.04%	0.3
Corticosterone	2.81% and 6.31%	4.48% and 7.85%	0.8
72a	4.95% and 7.46%	7.58% and 8.61%	0.4
72 T	5.57% and 6.58%	7.62% and 10.81%	0.25
37e	4.71% and 5.71%	5.26% and 5.61%	0.8

baseline values and set to 0%. All samples were then compared to this value, and the difference was noted as a positive or negative percentage. Baseline variability for each study animal and EIA was reported as the standard deviation (SD) of baseline fGCM values. The calculated SD values provide a robust indication of within-individual dispersion across samples collected when no handling occurred, assisting in contextualising the stability or variability of calculated baseline fGCM concentrations during opportunistic sampling. In combination with assay performance metrics, such as intra- and inter-assay CVs, baseline SD values can provide important information on the consistency of measurements within an individual and assist in interpreting peak adrenal responses. For each individual and assay, all peak fGCM values were expressed as a percentage change relative to the baseline mean calculated ((peak-baseline mean)/baseline mean × 100)). An EIA was considered reliable if it could provide a biologically relevant signal, such as peak fGCM values exceeding a 150% increase above mean baseline values. Several studies have used a 100% increase as a standard threshold [46–48]. As 100% has often been used as a conservative and practically suitable threshold, a more stringent 150% criterion was used in this study to reduce the likelihood of false positives in the collected data.

3 Results

Four of the five EIAs tested in female 1 showed a considerable increase in fGCM concentrations following a stressful event compared to calculated baseline levels. Both the 72a (982%; SD: 2) and 72 T (763%; SD: 4) EIAs had the highest observed increase, followed by the cortisol (481%; SD: 1) and 37e (317%; SD: 32) EIAs (Fig. 1). The standard deviation calculated for all baseline values were similar between all EIAs for female 1.

Similar to female 1, three of the tested EIAs detected a considerable increase in fGCM levels in female 2, including the 37e (325%; SD: 49), 72 T (221%; 92) and 72a (201%; SD: 8) EIAs (Fig. 1). The standard deviation was the lowest for 72a, followed by 37e and 72 T (Fig. 1). The secondary increase in fGCM levels observed at sample 5 and 6 (22/10/2026 and 23/10/2026) corresponded with a move to a holding facility (20/10/2020) prior to animal release (Table 1).

Both the 72a (339%) and 72 T (251%) EIAs detected an increase in fGCM levels in female 3 exceeding the 150% set. Both EIAs had comparatively low baseline standard deviation values (72a: 31; 72 T: 68).

The 37e assay showed a considerable increase in fGCM levels post-stressor in male 1 (160%), although the baseline standard deviation calculated using this assay was high

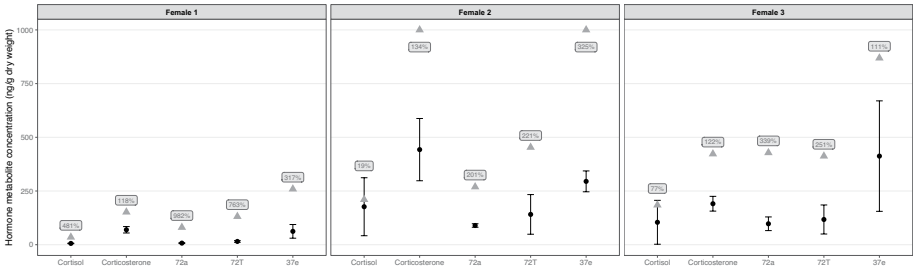

Fig. 1 Mean faecal glucocorticoid metabolite (fGCM) concentrations (ng/g dry weight) for three females across five enzyme immunoassays (5a, Cortisol, Corticosterone, 72a, 72 T). Black circles indicate the mean concentration and error bars show ± SD. Grey triangles indicate the peak concentration observed for each assay/individual. Boxed values represent the percentage change from the mean to the peak [(peak − mean)/mean × 100]. Where peaks exceeded the plotting range, peak markers were capped at the upper axis limit and the corresponding maximum percentage increase is displayed adjacent to the peak marker

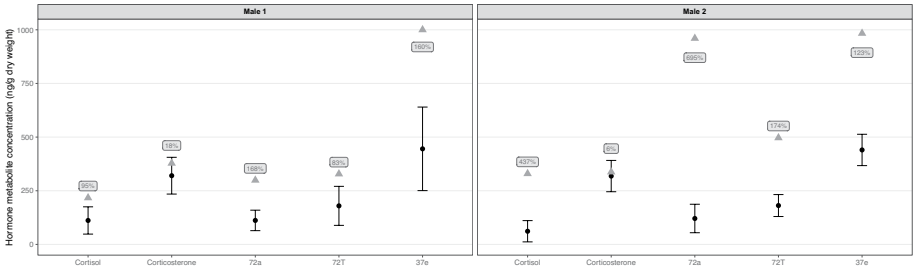

Fig. 2 Mean faecal glucocorticoid metabolite (fGCM) concentrations (ng/g dry weight) for two males across five enzyme immunoassays (5a, Cortisol, Corticosterone, 72a, 72 T). Black circles show the mean concentration with ± SD (error bars), and grey triangles show peak values per assay/individual. Boxed labels indicate the percentage change from mean to peak ((peak – mean)/mean × 100). For peaks exceeding the plotting range, peak markers were capped at the upper axis limit and the maximum percentage increase is shown near the capped marker; for Male 2 (5a and 72a), percentage labels were positioned below the peak marker for clarity

(195). The 72a assay had the highest fGCM increase (168%; SD: 66) in response to the handling stressors (Fig. 2). The remaining three assays had percentage increases of less than 100%.

Three EIAs were able to monitor an increase in fGCM levels post-stressor in male 2 (Fig. 2). While the 72 T assay detected an fGCM increase exceeding the 150% threshold set, it was the cortisol (437%) and 72a (695%) that showed the highest percentage increase from baseline to peak fGCM levels. The baseline standard deviation values were similar for all three of these EIAs (50–66).

4 Discussion

To our knowledge, this is the first study to successfully validate an EIA to reliably monitor fGCM patterns in the Temminck's pangolin. The 72a EIA was the only tested assay able to detect an increase in fGCM levels exceeding 150% in all study animals. The use of animal handling and restraint, as part of the biological validation, proved effective in eliciting a physiological stress response in Temminck's pangolin.

Biological validations such as these have been performed on a multitude of mammal species over the last decade [49, 50]. Similar to those studies, the response observed in Temminck's pangolin allowed us to select the most appropriate EIAs for monitoring fGCM levels in the species. Cortisol metabolites were the primary fGCMs found within faecal samples of our study species. Interestingly, Arora et al. [51] found faecal corticosterone concentrations were significantly higher than faecal cortisol metabolite concentrations in Taiwanese pangolin (*Manis pentadactyla pentadactyla*). It should be noted that the mentioned study used mass spectrometry analyses, thereby monitoring actual hormone concentrations rather than their metabolites. By contrast, the current study quantified immunoreactive fGCMs. The addition of mass spectrometry analyses to Temminck's pangolin would help further determine the active, circulating GC hormones present in the species.

Despite considerable levels of individual variability, both 11-oxoaetiocholanolone EIAs consistently detected fGCM increases greater than the 150% threshold set in all three female Temminck's pangolins. As secondary ("back-up") options, we considered EIAs that produced at least a 100% increase above baseline in most individuals, even if they did not consistently exceed 150%. Accordingly, the 37e EIA showed an increase

exceeding 100% in all study females, suggesting that this EIA can be used as an alternative when the 72a and 72 T assays are unavailable.

In our male study animals, only the 72a EIA detected fGCM increases above 150% in both male individuals, suggesting that it is the most appropriate assay for use in males. This finding also supports the use of the 72a EIA as the preferred assay for monitoring fGCM levels in Temminck's pangolin. While the cortisol EIA showed a strong response in male 2 (437%), male 1's response did not reach a 100% increase; the cortisol EIA should, therefore, be treated as an equivocal "back-up" pending further validation with additional individuals and stricter post-stressor sampling regimes. As this study was conducted on a limited number of individuals, the recommendations may be applicable only to the animals included in the current study, thereby limiting the generalisability of the results.

While not the primary focus of this study, the findings also underscore the physiological response of Temminck's pangolin during the rehabilitation process. Direct human-pangolin interaction led to a considerable increase (>100%) in fGCM levels across all study animals; these increases were acute and expected for animals undergoing often-intense interventions. Similar fGCM patterns have been observed other species undergoing rehabilitation including African penguin (*Spheniscus demersus*) [52] and koala (*Phascolarctos cinereus*) [53]. Surprisingly, female 2 in this study showed a considerable increase in fGCM levels before a decline in health that led to her readmission to the rehabilitation centre. This finding highlights the importance of conducting a physiological assessment of individuals prior to release to improve survival rates and rehabilitation outcomes. Moreover, the discerned pattern in female 2 not only provides valuable data but also reinforces arguments advocating the use of fGCM monitoring in wildlife to evaluate the health and welfare of endangered species, a theme frequently underscored in the concluding sections of research articles [46, 52].

4.1 Study gaps and future research

It must be noted that, due to the design of this study, we were unable to determine the excretion time lag for Temminck's pangolin, thereby limiting temporal inferences. With average gut passage times in other pangolin species varying considerably [20–60 h; 36, 38, 39], it is important that future studies on Temminck's pangolin establish the temporal dynamics of hormone excretion. Another topic that must be highlighted is variation in how individuals perceive and respond to stressors. In the present study, stressors likely included confiscation events, veterinary handling, and transportation and movement to new facilities. The magnitude of adrenal response to stressors might well be individual-specific [54] and should be considered when such studies are designed. Despite this, all individuals of this study showed an increase in fGCM levels in response to various study stressors.

5 Conclusion

This is the first study to successfully validate an EIA for non-invasive measurement of physiological stress in any pangolin species, using a biological method. The 72a EIA provides researchers, conservationists, and rehabilitation managers with a tool to monitor the physiological responses of Temminck's pangolins not only in captive settings but also in the wild. This is especially true in the rehabilitation setup, where the release of healthy

individuals is of utmost importance to ensure release success and long-term survival. Furthermore, the validated assays are crucial for free-ranging populations, especially given that external factors such as climate change are anticipated to have substantial impacts on Temminck's pangolin in its natural habitat [55, 56]. Long-term monitoring programs can leverage validated techniques like those discussed here to track individual well-being and readiness for release.

Author contributions
J.S.: Conceptualization, resources, funding acquisition, data curation, methodology, formal analysis, visualization, writing (original draft preparation, review and editing). K.L.: Resources, supervision, methodology, writing (origi- nal draft preparation, review and editing). R.J.: Resources, methodology, data curation, writing (orig- inal draft preparation, review and editing). A.G.: Conceptualization, resources, methodology, writing (original draft preparation, review and editing). C.L.: Methodology, writing (original draft preparation, review and editing).

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Data availability
Data is provided within the manuscript or supplementary information files.

Declarations

Ethics approval and consent to participate
The study was performed with the approval of the University of South Africa's Animal Research Ethics Committee (2020/CAES_AREC/109). The Johannesburg Wildlife Veterinary Hospital provided consent to participate in the study.

Consent for publication
All authors provided consent to publish.

Competing interests
The authors declare no competing interests.

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