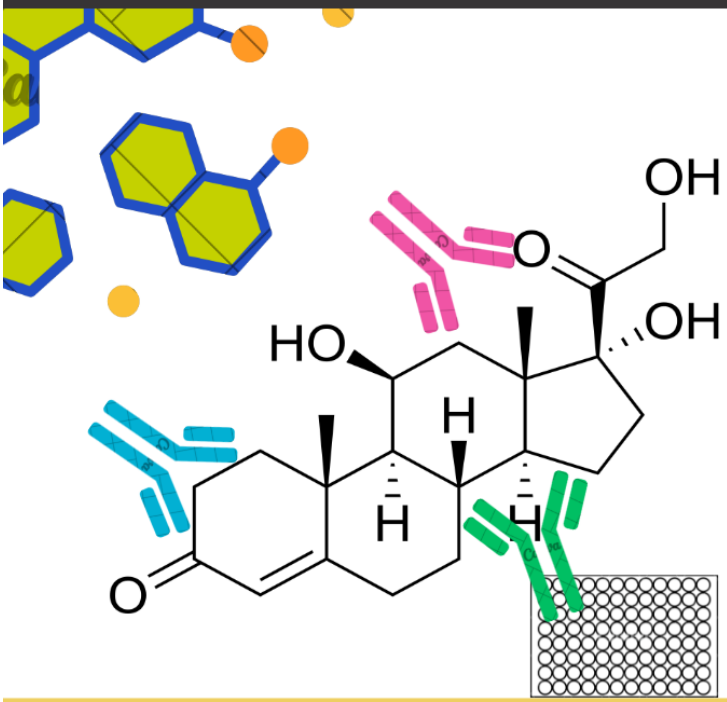
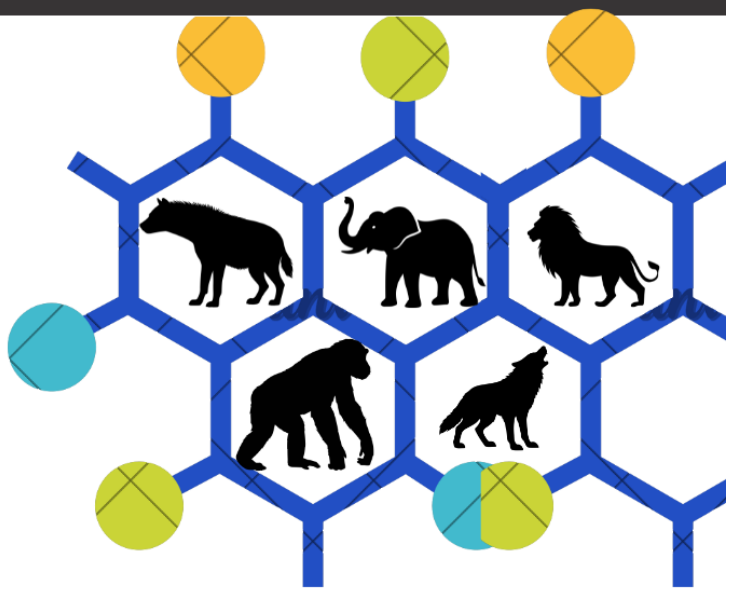
MODERNISATION OF FOREST FORCE
2024-25

PROJECT COMPLETION REPORT

EVALUATION OF STRESS HORMONE IN SELECTED CAPTIVE WILD ANIMALS





TAMIL NADU FOREST DEPARTMENT
ADVANCED INSTITUTE FOR WILDLIFE CONSERVATION
(Research, Training & Education)
Vandalur - Kelambakkam road, Vandalur, Chennai - 600 048.

PROJECT COMPLETION REPORT

MODERNISATION OF FOREST FORCE 2024 – 25

Evaluation of Stress Hormone in Selected Captive Wild Animals

by

ADVANCED INSTITUTE FOR WILDLIFE CONSERVATION
(RESEARCH, TRAINING & EDUCATION)
TAMILNADU FOREST DEPARTMENT
VANDALUR – KELAMBAKKAM ROAD,
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Project sanctioned in G.O. (MS) No.: 95 , Environment, Climate Change and Forests Department, dated 18.06.2024

PROJECT PERIOD: OCTOBER 2024 – JULY 2025

Evaluation of Stress Hormone in Selected Captive Wild Animals

TAMILNADU FOREST DEPARTMENT
ADVANCED INSTITUTE FOR WILDLIFE CONSERVATION
(RESEARCH, TRAINING & EDUCATION)

Declaration:

I (we) declare that this project report has been prepared in accordance with the Department of Science and Technology, Ministry of Science and Technology, Government of India format for project completion report (Reference: accessed on 29 May 2024).

https://dst.gov.in/sites/default/files/PROJECT%2520COMPLETION%2520REPORT.pdf

To cite this report:

Karunyaa R[#], C. Bala Amarnath[#], S. Siva Ranjani[#], K. Sridhar[*], D. Eswaran[#], S. Senbagapriya[#], A. Udhayan[#] (2024). Evaluation of stress hormone in selected captive wild animals. AIWC-MoFF 2024-25/Report.

– Advanced Institute for Wildlife Conservation

* – Arignar Anna Zoological Park

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ACKNOWLEDGEMENT

The Project Scientist and Project Associate of this project place their heartfelt gratitude and acknowledge the continuous help and support provided by:

The Principal Chief Conservator of Forests and the Director, Deputy Director (Admin), Deputy Director (Tech), Forest Veterinary Assistant Surgeons, Forest Range Officers, Foresters, Forest Guards, and all administrative staff of the Advanced Institute for Wildlife Conservation for their constant and kind support.

The Director and Deputy Director of AAZP for providing the necessary permission to collect the samples. The AAZP officials-VAS, Forest Range Officers, Foresters, Forest Guards and Zoo/Animal keepers for their remarkable support rendered during sample collection. The Modernization of Forest Force (MoFF) scheme for providing the necessary financial assistance to the project.

Dr. C. Bala Amarnath
Project Scientist - II
Ms. Karunyaa.R
Project Associate - II

ABBREVIATIONS

HPA	– Hypothalamic Pituitary Adrenal Axis
fGCM	– Faecal Glucocorticoid Metabolite
ACTH	– Adeno Cortico Tropic hormone
HPLC	– High-Performance Liquid Chromatography
ELISA	– Enzyme Linked Immuno Sorbent Assay
AB	– Assay Buffer
WB	– Wash Buffer
RIA	– Radio Immuno Assay
EIA	– Enzyme Immuno Assay
STD	– Standard
OD	– Optical Density
WL	– Wolf
CH	– Chimpanzee
LI	– Lion
HY	– Hyena
EL	– Elephant
Pg	– Picogram

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PROJECT COMPLETION REPORT

1	**G.O. No**	:	G.O. (MS) No.: 95, Environment, Climate Change and Forests Department, dated 18.06.2024
2	**Project Title**	:	Evaluation of Stress Hormone in Selected Captive Wild Animals
3	**Duration**	:	October 2024 to July 2025
4	**Funding agency**	:	Modernisation of Forest Force
5	**Project Budget**	:	Rs. 6, 44, 000 /-
6	**Principal Investigator**	:	The Director
7	**Organisation**	:	Advanced Institute for Wildlife Conservation (Research, Training & Education) Tamil Nadu Forest Department Vandalur – Kelambakkam road, Vandalur, Chennai – 600 048.
8	**Co-Principal Investigator**	:	NIL
9	**Collaborating organisations**	:	NIL

PART A – SUMMARY REPORT

1. Project objectives

Objectives as per the approved project	Fully Achieved / Partially achieved (indicate shortfall)	Reason for partial achievement
To establish feces sample-solvent-based extraction protocol from different captive animals.	Fully achieved	NIL
To optimise the stress hormone estimation assay through EIA (Enzyme Immuno Assay).	Fully achieved	NIL
To validate and analyse the unknown samples and estimation of stress hormone level.	Fully achieved	NIL

2. Deliverables

Deliverables as per the approved project	Fully/Partially/Not Achieved	Reason for partial / non-achievement
Establishment of stress hormone (cortisol) estimation from faecal samples using EIA.	Fully achieved	NIL

3. Specific Benefits/Outcome

i) Patent, if any:

NIL.

ii) Product/Process developed/Technology transferred:

Stress hormone (cortisol) extraction and estimation – Process developed.

iii) Publications

Requires an additional data for six-month continuous estimation of cortisol level.

iv) Linkages developed

NIL

v) Manpower trained

1 No. of Project Associate II

4. Summary of significant Science & Technology achievements:

As a first part of the project, stress hormone extraction from faecal samples of captive wild animals was optimised under various sample processing conditions to extract out the maximum stress hormone (cortisol). The second part was optimisation and validation of the stress hormone (cortisol) level estimation using Enzyme Immuno Assay.

5. Project Budget: Rs. 6,44,000/-

6. Suggestions for utilisation of project outcome:

The established EIA may support the Arignar Anna Zoological Park's captive animal surveillance stress hormone level. Long term monitoring of the animal may create a record of the stress that the animal undergoes which may be due to any infection, lack of copulation, fight between animals, hinderance by humans, etc. Wildlife managers may utilise this test as part of wildlife health management.

PART B – COMPREHENSIVE REPORT

1. Project title

Evaluation of Stress Hormone in Selected Captive Wild Animals.

2. Outcome of project – Identify beneficiaries

Stress hormone extraction from faecal samples of captive wild animals was optimised under various sample processing conditions to extract out the maximum stress hormone (cortisol). The second phase focused on the optimisation and validation of the stress hormone (cortisol) level estimation using an Enzyme Immuno Assay. Arignar Anna Zoological Park (AAZP) is identified as a key beneficiary. The established EIA protocol can support routine surveillance of stress hormone levels in captive animals at AAZP. Long-term monitoring of the animal may create a record of the stress that the animal undergoes which may be due to any infection, lack of copulation, fight between animals, hinderance by humans, enclosure habitat, etc. Wildlife managers may utilise this test as part of wildlife health management.

3. Scientific Formulation and Methodology description

3.1. Background

Stress is a fundamental biological response that enables animals to cope with challenges, or stressors, that disrupt their internal physiological balance, also known as homeostasis. Such stressors may be environmental (e.g., noise, temperature extremes), social (e.g., isolation or competition), or physiological (e.g., disease or injury). In response, animals activate a complex neuroendocrine system known as the Hypothalamic Pituitary Adrenal (HPA) axis**,** which leads to the secretion of glucocorticoid hormones, primarily cortisol, from the adrenal cortex (Sheriff *et al.*, 2011). These hormones help mobilise energy, regulate the immune system, and prepare the animal to confront or escape the stressor, commonly referred to as the "fight or flight" response (Sapolsky *et al.*, 2000). Traditional techniques for assessing stress hormones, such as blood sampling, are often invasive and can induce stress during collection, thereby influencing cortisol levels (Young *et al.*, 2004). As a result, there has been increasing interest in non-invasive sampling methods that allow for hormone analysis from faeces, urine, saliva, hair, or claws. These approaches offer the dual benefit of

reducing animal disturbance and enabling repeated monitoring over time (Palme *et al.,* 2005; Jewgenow *et al.,* 2020).

Among these, faecal glucocorticoid metabolite (FGM) analysis has gained widespread acceptance due to its practicality and reliability. FGMs represent the metabolised form of cortisol excreted in faeces, providing an integrated measure of hormone activity over the preceding hours or days (Palme *et al.,* 2005). This method is particularly suitable for wildlife studies where minimising animal handling is critical. Furthermore, the biological matrix selected for hormone analysis can reflect different time scales of stress: blood and saliva indicate acute stress responses, while hair, feathers, and claws reflect chronic exposure (Fokidis *et al.,* 2023). Faecal analysis strikes a balance, offering insight into recent stress without the need for intrusive procedures.

Cortisol is widely recognised as a biomarker for stress in mammals due to its role in regulating various physiological systems. Although beneficial in short bursts, chronic elevation of cortisol can have detrimental consequences, including immune suppression, impaired reproduction, cardiovascular disorders, and behavioural dysfunctions (Goymann *et al.,* 1999; Palme *et al.,* 2005). This is particularly relevant in captive wildlife, where artificial housing conditions and management practices may predispose animals to sustained stress.

The effectiveness of faecal cortisol as a stress biomarker also lies in its responsiveness to both environmental and biological conditions. Cortisol levels can vary across species, age, sex, season, as well as environmental factors, such as habitat loss, climate change, poaching, and human-induced disturbances (Sheriff *et al.,* 2011). Regular monitoring of faecal cortisol concentrations therefore provides valuable insights into how animals respond and adapt to their environments, in both wild and captive settings. Such information enables conservationists and wildlife managers to make evidence-based decisions aimed at improving animal welfare, conservation success, and overall population.

3.2. Stress hormones in wild animals

Captive management of wild animals has become a crucial component of modern wildlife conservation efforts, particularly in response to the growing threats of habitat destruction, poaching, and population decline. Zoological parks, conservation breeding centres, and rescue facilities play a pivotal role in species preservation, serving as platforms for education, research, and public engagement. However, shifting animals from their natural

ecosystems to artificial enclosures introduces a range of environmental, physiological, and psychological stressors that can adversely affect their health and welfare (Sheriff *et al.,* 2011; Karaer *et al.,* 2023). In wildlife, especially in captive conditions, stress can result from factors such as restricted movement, inadequate enclosure space, social isolation, lack of environmental enrichment, and frequent human interaction. Such stress can impair reproductive performance, suppress immunity, and lead to behavioural abnormalities (Creel *et al.,* 2013). Therefore, assessing stress levels becomes essential for evaluating animal welfare, health, and conservation success, particularly in captive breeding and rehabilitation programs.

Palme *et al.* (1996) conducted a comparative study to understand the excretion patterns of radiolabeled steroid hormones progesterone, testosterone, cortisol, and oestrone in sheep, ponies, and pigs**.** The study aimed to improve non-invasive steroid monitoring methods, particularly for wildlife and zoo animals. The results revealed species-specific differences in both the route and timing of steroid hormone excretion. The findings highlight the importance of digesta passage rate in determining excretion timing and support the reliability of faecal samples for stress hormone analysis. The study provided a valuable foundation for the development of non-invasive hormone monitoring techniques in both domestic and wild species.

Goymann *et al.* (1999) validated a non-invasive method for monitoring stress in spotted hyenas (*Crocuta crocuta*) by measuring faecal glucocorticoid metabolites**.** The study compared four enzyme immunoassays and found that the ICN-corticosterone assay provided the most accurate and sensitive results. Goymann *et al.* (2001) also investigated the influence of social status, reproductive condition, and environmental factors on faecal corticosteroid levels in free-ranging female spotted hyenas (*Crocuta crocuta*). The findings reinforce that faecal glucocorticoid metabolites are reliable, non-invasive indicators of biologically meaningful stress responses, reflecting both short-term environmental pressures and long-term physiological changes. This supports the broader application of faecal hormone monitoring in wildlife welfare evaluation and behavioural ecology**.**

Young *et al.* (2004) assessed the efficacy of non-invasive faecal hormone monitoring for evaluating adrenocortical activity in eight captive carnivore species, including cheetahs, red wolves, clouded leopards, and black bears. The study compared a cortisol enzyme immunoassay (EIA) and a corticosterone radioimmunoassay (RIA), validating both through

Adenocorticotropic hormone (ACTH) challenge tests, which confirmed significant elevations in faecal glucocorticoid metabolites within 1–2 days post-stimulation. Results demonstrated species-specific differences in glucocorticoid metabolism and excretion, with varying responses to physical restraint, anaesthesia, medical procedures, and environmental stressors.

Palme *et al.* (2005) provided a comprehensive overview of the metabolism, excretion, and non-invasive measurement of stress hormones, particularly glucocorticoids, in mammals and birds**.** This study further supports the utility of faecal glucocorticoid analysis as a reliable, non-invasive tool for monitoring stress physiology across diverse carnivore species.

Benhaiem *et al.* (2012) validated a non-invasive enzyme immunoassay (EIA) for measuring faecal glucocorticoid metabolites (fGCMs) in spotted hyenas (*Crocuta crocuta*) to assess physiological stress. The study employed radiometabolism analysis, ACTH challenge tests, and anaesthesia exposure, confirming the assay's sensitivity and specificity. Significant increases in fGCM concentrations following these procedures demonstrated the assay's effectiveness in detecting stress responses. Higher fGCM levels were observed in juveniles under six months of age, attributed to social instability and maternal separation. This supports fGCM monitoring as a reliable tool for evaluating stress in both captive and free-ranging individuals, with age and social environment playing key roles in glucocorticoid variation. Although faeces and blood offer insights into short-term hormone fluctuations (Cook, 2012), they are less suitable for long-term monitoring due to the need for repeated sampling and the stress induced by animal capture (Cook *et al.*, 2000; Romero, 2004). Keratinised tissues like hair and claws have emerged as promising alternatives for assessing chronic stress and reproductive hormone profiles. These studies reinforce the importance of integrating both short- and long-term indicators to better understand stress physiology and welfare in wildlife populations. This study supports the use of fGCM analysis as a reliable tool for monitoring adrenocortical activity in both captive and wild individuals, reinforcing the importance of age and environment in stress evaluation.

Creel *et al.* (2013) investigated how anthropogenic factors including land use, group composition, and proximity to human settlements affect faecal glucocorticoid metabolite (fGCM) levels in African lions in Kenya's South Rift Valley. Using non-invasive ELISA-based hormone assays**,** they analysed 136 faecal samples collected over two years. The study found that stress levels of lions were significantly lower in protected conservation areas compared to buffer zones inhabited by people and livestock. The study emphasises the need

for spatial buffers and effective land-use planning to mitigate stress in large carnivores. The study emphasises the need for spatial buffers and effective land-use planning to mitigate stress in large carnivores.

Ghosal *et al.* (2013) examined the endocrine correlates of musth in free-ranging male Asian elephants (*Elephas maximus*) in Kaziranga National Park, India, using non-invasive enzyme immunoassays to measure faecal testosterone and glucocorticoid metabolites. The study confirmed a significant increase in testosterone during musth, indicating clear androgenic activation. Interestingly, glucocorticoid levels did not differ significantly between musth and non-musth phases, suggesting that musth is not a physiologically stressful condition in wild elephants. This contrasts with findings from captive elephants, where stress hormone levels often increase during musth, likely due to confinement and management pressures. These findings highlight the influence of environmental context on hormone regulation and underscore the value of non-invasive faecal hormone monitoring for understanding both reproductive and stress physiology in wild megafauna.

Hadinger *et al.* (2015) investigated the use of faecal cortisol metabolites (FCMs) as a non-invasive biomarker of stress in free-ranging Alpine chamois (*Rupicapra rupicapra rupicapra*). Using enzyme immunoassays (EIA) validated via high-performance liquid chromatography (HPLC), they analysed 183 feacal samples and found significant seasonal variation, with FCM levels peaking in June, September, and October. Interestingly, even faeces collected on the same day from individuals in the same social group showed considerable differences in FCM concentrations, suggesting the influence of individual physiological variation.

Dhairykar *et al.* (2020) conducted a comparative assessment of faecal cortisol concentrations in captive Asian elephants (*Elephas maximus*) housed at Kanha, Panna, and Bandhavgarh Tiger Reserves in Madhya Pradesh. Using a commercial ELISA kit (DetectX, Arbor Assays), the study measured cortisol concentrations and found relatively consistent concentrations across all three locations—234.2 ng/g at Kanha, 250.8 ng/g at Panna and 232.2 ng/g at Bandhavgarh suggesting similar baseline stress levels among elephants regardless of location.

Jewgenow *et al.* (2020) assessed the reliability of two commonly used enzyme immunoassays (EIAs)—cortisol-3-CMO and cortisol-21-HS—for measuring hair cortisol

concentrations in six mammalian species, including cheetahs, spotted hyenas and Asiatic black bears. By comparing results with the reference method LC-MS/MS, the study found that the 21-HS EIA significantly overestimated cortisol levels, likely due to cross-reactivity with unknown compounds. In contrast, the 3-CMO EIA showed better specificity and closely matched LC-MS/MS values, though slight overestimation persisted. The authors emphasised that assay selection critically affects the accuracy of hormone data and cautioned that the use of unvalidated assays can lead to misleading interpretations.

Despite the breadth of these studies, a critical need remains for species-specific standardisation of hormone extraction methods, assay validation, and context -based interpretation of cortisol levels, particularly within Indian captive settings, where such baseline data are currently limited. Addressing this gap, the present study focuses on evaluating stress hormone levels in selected captive wild animals using non-invasive faecal sampling. Hormone extraction was carried out using a validated alcohol-based protocol, followed by quantification using ELISA. The findings are expected to support improved management decisions on breeding, welfare assessments, and species-specific care protocols in captive wildlife facilities.

3.3. Objectives

1. To establish feces sample-solvent-based extraction protocol from different captive animals.
2. To optimise the stress hormone estimation assay through EIA kits (Enzyme Immuno Assay).
3. To validate and analyse the unknown samples and the estimation of stress hormone level.

3.4. Materials & Methods

3.4.1. Selection of captive animals

The captive animals included in this study were selected from Arignar Anna Zoological Park after having detailed discussion with Zoo veterinarian, taking into consideration species-specific behavioural traits and their potential influence on stress hormone levels. The Lion and spotted Hyena were chosen as they experience breeding related stress and are housed separately. Grey wolves were selected for their aggressive behaviour with conspecifics. Chimpanzees and elephants were selected to understand stress responses

associated with frequent exposure to large numbers of human visitors and varied anthropogenic activities.

3.4.2. Sample collection

The faecal samples were collected from Lion, spotted Hyena, Wolf, Chimpanzee and Elephants on Monday, Wednesday and Friday during the period from February 2025 to March 2025 (for elephants in March 2025). The faecal samples were collected in a 50 mL sample container and immediately transported to laboratory.

3.4.3. Standardisation of cortisol extraction

Dry heat method: The collected faecal samples were evenly spread over a sterile petri plate, covered with aluminium foil and incubated at 60°C for 8 hours. The dried samples were then ground into a fine powder using a sterile mortar and pestle. The powdered sample was either stored at -80°C until further analysis or used for extraction immediately.

Freeze drying method – The collected faecal samples were evenly spread over a sterile petri plate, covered with aluminium foil and incubated at -80°C for 2 hours. The samples were further transferred to freeze drier (lyophiliser) and incubated for 8 hours. The dried samples were then ground into a fine powder using a sterile mortar and pestle. The powdered sample was either stored at -80°C until further analysis or used for extraction immediately.

Extraction

1. Approximately 0.5 g of the powdered sample was weighed into a sterile 15 mL tube.
2. About 5 ml of absolute ethanol was added to each tube.
3. The mixture was subjected to vigorous shaking for 45 minutes using a mechanical vortex or orbital shaker to enhance hormone solubilisation.
4. The tubes were then centrifuged at 5000 rpm for 15 minutes at 4 °C to separate the supernatant.
5. The supernatant was transferred to a clean petri plate.

6. The ethanol was allowed to evaporated completely at room temperature, leaving behind the concentrated hormone residue.

7. The dried extract was then reconstituted in 400 to 1000 µL of absolute ethanol.

8. The reconstituted samples were stored at −20 °C until further analysis by ELISA.

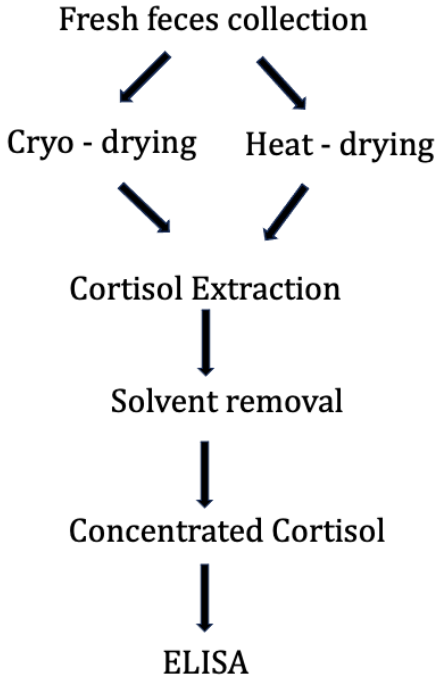


Fig. 1: Overview of the cortisol estimation

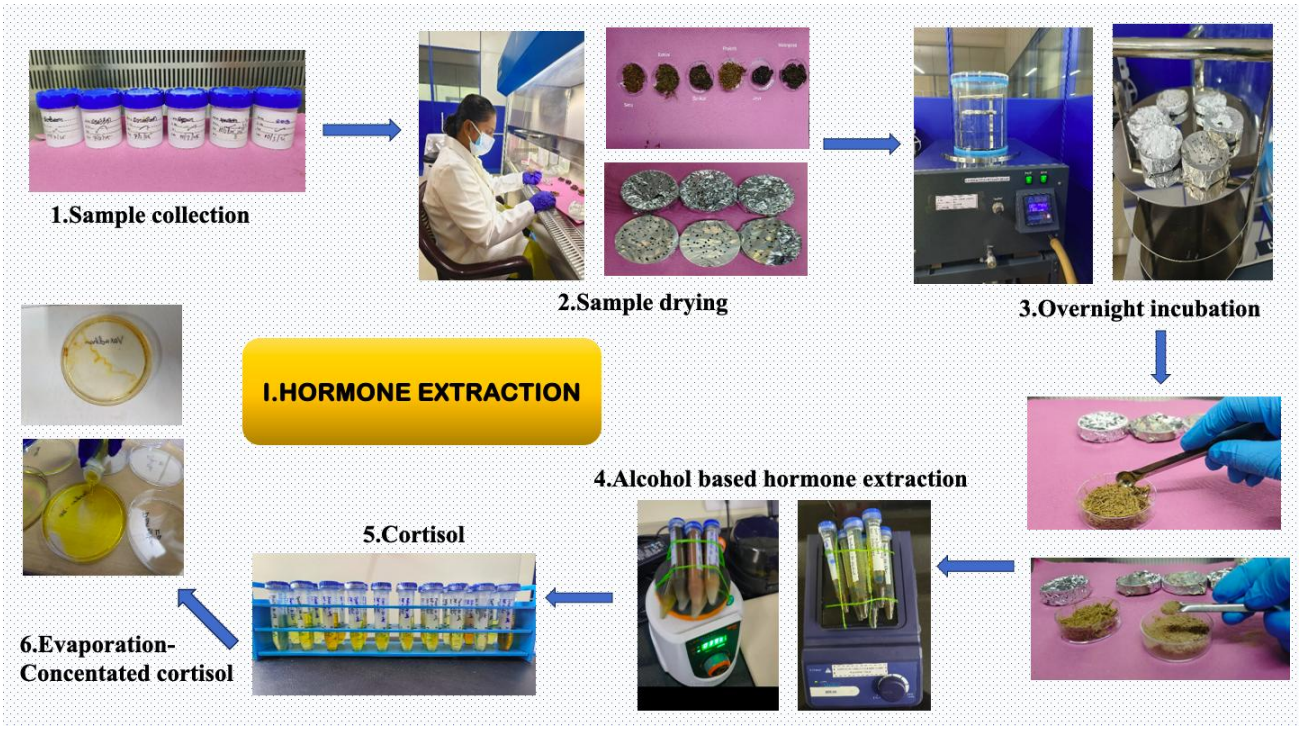


Fig. 2: Stages of hormone extraction

Table 1: Sample collection details from Chimpanzees

Chimpanzee	Sex	Age	Date	Day
Comby	Male	32 years	24/02/25	Monday
			10/03/25	Monday
			12/03/25	Wednesday
			17/03/25	Monday
			19/03/25	Wednesday
			21/03/25	Friday
Gowri	Female	27 years	24/02/25	Monday
			10/03/25	Monday
			12/03/25	Wednesday
			17/03/25	Monday
			19/03/25	Wednesday
			21/03/25	Friday
Adithya	Male	4 years	24/02/25	Monday
			10/03/25	Monday
			12/03/25	Wednesday
			17/03/25	Monday
			19/03/25	Wednesday
			21/03/25	Friday


Comby

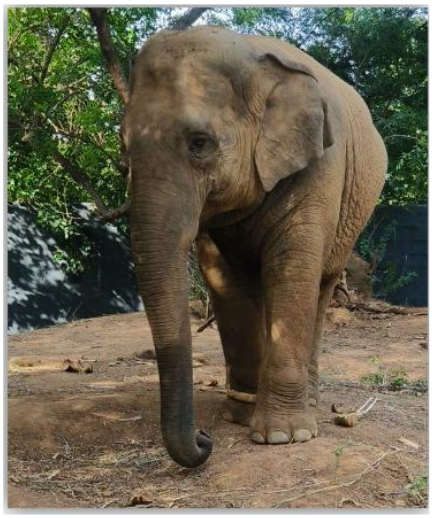

Gowri


Adithya

Table 2: Sample collection details from Elephants

Elephant	Sex	Age	Date	Day
Prakriti	Female	8 years	03/03/25	Monday
			05/03/25	Wednesday
			07/03/25*	Friday
			10/03/25	Monday
			12/03/25	Wednesday
			14/03/25	Friday
			17/03/25	Monday
			19/03/25*	Wednesday
			21/03/25*	Friday
Rohini	Female	10 years	03/03/25	Monday
			05/03/25	Wednesday
			07/03/25	Friday
			10/03/25	Monday
			12/03/25	Wednesday
			14/03/25	Friday
			17/03/25	Monday
			19/03/25	Wednesday
			21/03/25	Friday

* - sample not collected



Prakriti

Rohini

Table 3: Sample collection details from Hyenas

Hyena	Sex	Age	Date	Day
Jothi	Female	7 years	24/02/25*	Monday
			26/02/25*	Wednesday
			28/02/25	Friday
			03/03/25	Monday
			05/03/25*	Wednesday
			07/03/25*	Friday
			10/03/25*	Monday
			12/03/25	Wednesday
			14/03/25	Friday
			17/03/25	Monday
			19/03/25	Wednesday
			21/03/25	Friday
Deepa	Female	7 years	05/02/25	Wednesday
			26/02/25	Friday
			28/02/25	Monday
			03/03/25	Wednesday
			05/03/25*	Friday
			07/03/25	Monday
			10/03/25	Wednesday
			12/03/25	Friday
			14/03/25	Monday
			17/03/25	Wednesday
			19/03/25	Friday

* not collected

Deepa

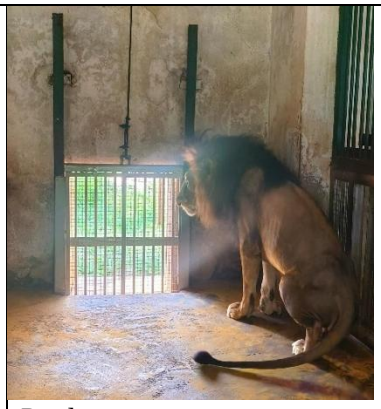
Jothi

Table 4: Sample collection details from Lions

Lion	Sex	Age	Date	Day
Seru	Male	6 years	26/02/25	Wednesday
			28/02/25	Friday
			03/03/25	Monday
			05/03/25	Wednesday
			07/03/25	Friday
			10/03/25	Monday
			12/03/25	Wednesday
			14/03/25	Friday
			19/03/25	Monday
			21/03/25	Wednesday
Pradeep	Male	7 years	26/02/25	Wednesday
			28/02/25	Friday
			03/03/25	Monday
			05/03/25	Wednesday
			07/03/25	Friday
			12/03/25	Wednesday
			21/03/25	Friday
Jaya	Female	7 years	26/02/25	Wednesday
			03/03/25	Monday
			05/03/25	Wednesday
			07/03/25*	Friday
			10/03/25	Monday
			12/03/25	Wednesday
			17/03/25	Monday
			19/03/25	Wednesday
			21/03/25	Friday

* not collected

Seeru



Pradeep

Jaya

Table 5: Sample collection details from Wolves

Wolf	Sex	Age	Date	Day
Darshan	Male	7 years	26/02/25	Wednesday
			28/02/25	Friday
			03/03/25	Monday
			05/03/25	Wednesday
			07/03/25	Friday
			10/03/25	Monday
			12/03/25	Wednesday
			19/03/25	Wednesday
			21/03/25	Friday
Varadhan	Male	13 years	28/02/25	Friday
			07/03/25	Friday
			10/03/25	Monday
			12/03/25	Wednesday
			14/03/25	Friday
			17/03/25	Monday
			19/03/25	Wednesday
Praveen	Male	7 years	26/02/25*	Wednesday
			28/02/25	Friday
			03/03/25	Monday
			05/03/25*	Wednesday
			07/03/25	Friday
			12/03/25	Wednesday
			19/03/25	Wednesday
			21/03/25	Friday
Satya	Male	6 years	03/03/25	Monday
			05/03/25	Wednesday
			07/03/25	Friday

			10/03/25	Monday
			12/03/25	Wednesday
			14/03/25	Friday
			19/03/25	Wednesday
			21/03/25	Friday
Sandya	Female	6 years	26/02/25	Wednesday
			03/03/25	Monday
			07/03/25	Friday
			10/03/25	Monday
			12/03/25	Wednesday
			17/03/25	Monday
Rohit	Male	6 years	28/02/25	Friday
			03/03/25	Monday
			05/03/25	Wednesday
			07/03/25	Friday
			10/03/25	Monday
			12/03/25	Wednesday
			14/03/25	Friday
			17/03/25	Monday
			19/03/25	Wednesday
			21/03/25	Friday
Karupan	Male	7 years	26/02/25	Wednesday
			28/02/25	Friday
			03/03/25	Monday
			05/03/25	Wednesday
			07/03/25	Friday
			10/03/25	Monday
			12/03/25	Wednesday
			19/03/25	Wednesday
			21/03/25	Friday
Arun	Male	7 years	26/02/25	Wednesday
			28/02/25	Friday
			03/03/25	Monday
			05/03/25	Wednesday
			07/03/25	Friday
			10/03/25	Monday
			12/03/25	Wednesday
			21/03/25	Friday
Keerthi	Female	13 years	26/02/25	Wednesday
			28/02/25	Friday
			03/03/25	Monday
			05/03/25	Wednesday

			07/03/25	Friday
			10/03/25	Monday
			17/03/25	Monday
			19/03/25	Wednesday

* not collected

Karuppan, Praveen, Satya, and Arun

Darshan and Varathan

3.4.4. Optimisation of ELISA

DetectX – Cortisol Enzyme Immunoassay Kit (K003, Arborr Assays, USA) was used for detection and estimation of faecal cortisol in this study. All the reagents were thawed to room temperature, standards and samples were run in duplicates. The protocol is as follows:

1. A plate layout sheet should be prepared to aid in proper sample and standard identification.
2. Thaw only the required number of wells and return the unused wells to the foil pouch with desiccant. Seal the ziplock plate bag and store it at 4˚C.
3. 50 µL of samples or standards added into wells in the plate (columns A to H).
4. 75 µL of 1X Assay Buffer added into the non-specific binding (NSB) wells.
5. 50 µL of 1X Assay Buffer added into the maximum binding (B0 or Zero standard) wells.
6. 25 µL of the DetectX® Cortisol Conjugate was added to each well.
7. 25 µL of the DetectX® Cortisol Antibody was added to each well, except the NSB wells.
8. The plate was covered with the plate sealer and shaken at room temperature for 1 hour at 700–900 rpm.
9. Discard the plate content and each well washed 4 times with 300 µL 1X Wash Buffer. The plate was tapped dry on clean absorbent towels.
10. 100 µL of the TMB Substrate was added to each well.
11. The plate was incubated at room temperature for 30 minutes without shaking.
12. 50 µL of the Stop Solution was added to each well.
13. Optical density was read in a plate reader capable of reading at 450 nm.

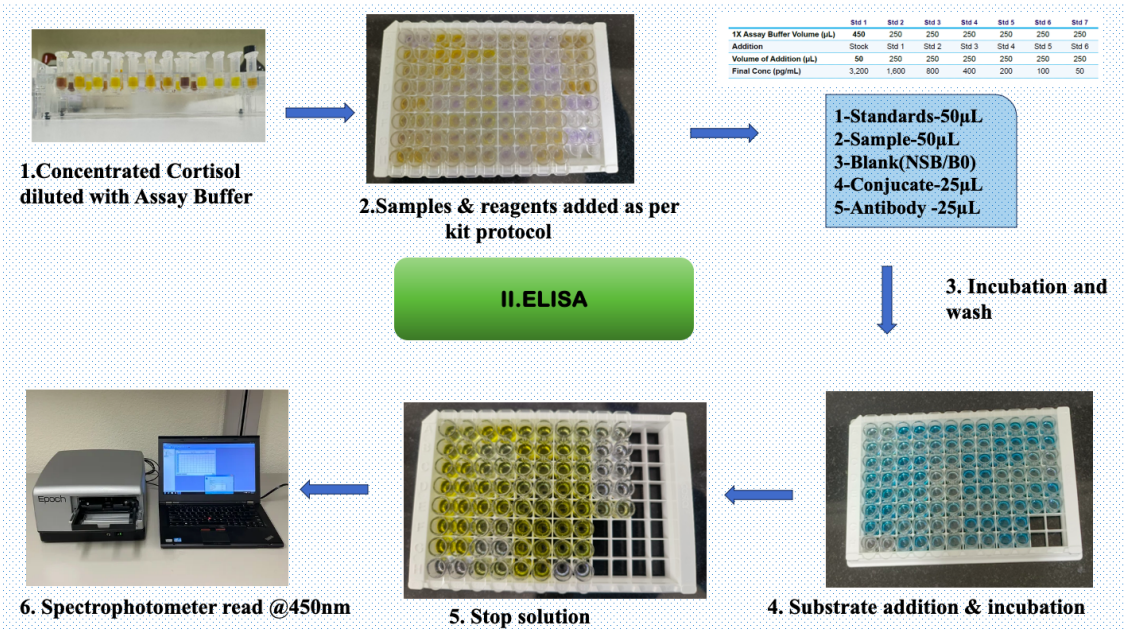


Fig. 3: Stages of cortisol detection in ELISA

3.4.5. Estimation of cortisol

The cortisol concentrations were estimated using a four-Parameter Logistic model (sigmoidal or hill model), a statistical tool using Origin Pro v2024 software. Briefly, the mean optical density (OD) values for each standard and sample were calculated. Net OD was calculated by substracting mean standard and sample OD from non specific binding (NSB) mean OD. %B/B_0 was calculated by dividing net OD by B_0 OD and multiplying by 100. 4 PL model was fitted to generate a standard sigmoidal curve using Standard %B/B_0 and standard concentration. Using this standard curve, cortisol concentration of unknown samples were calculated and multipled with dilution factor to obtain the neat sample concentration.

3.5. Results

Samples were collected from Chimpanzees, Elephants, Hyenas, Wolves and Lions from AAZP on Monday, Wednesday and Friday for a period of ten days. The hormone extraction was standardised and optimised for freeze drying of faecal samples and ethanol based extraction. Hormone samples were concentrated by evaporation and used either directly or diluted 100 times and used in the ELISA for detection of cortisol hormone. The estimation of cortisol hormone was performed through the generation of a standard curve fitting by 4-PL regression model using optical density values of known cortisol and the unknown sample cortisol was estimated.

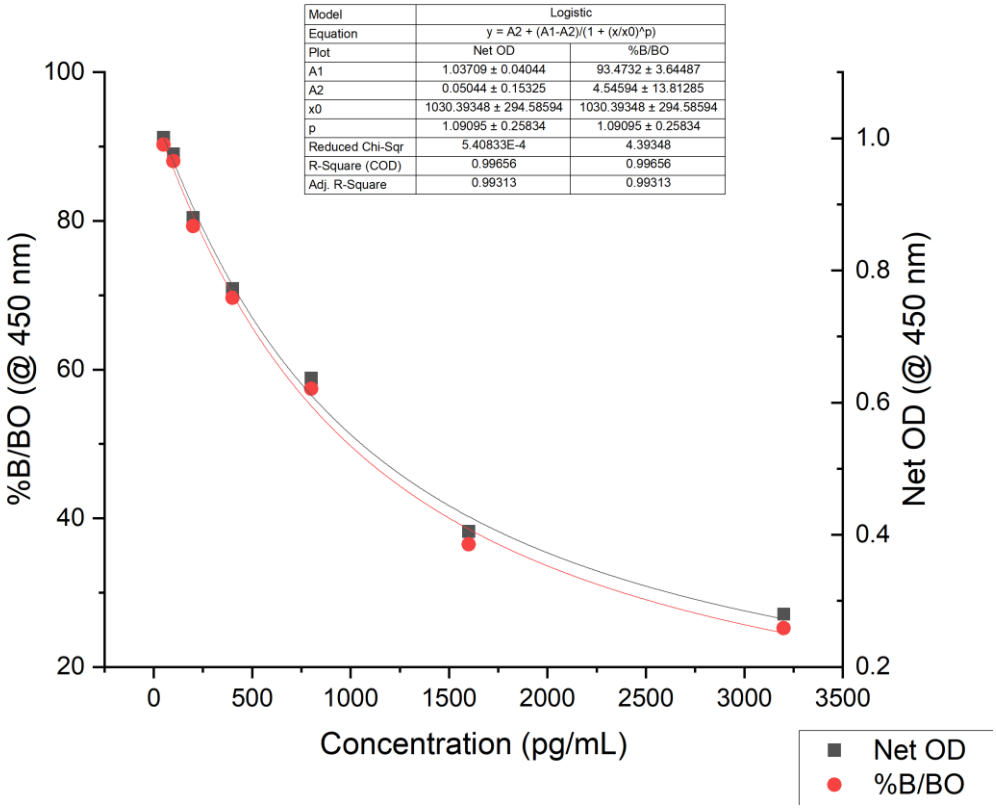


Fig. 4: 4PL-Standard curvefitting

Table 6 Assay standards 4-PL logistics stamdard curve fitting

STD Concentration	OD 1	OD 2	Mean OD	Net OD	%B/B0
3200	0.329	0.332	0.33	0.28	25.24
1600	0.459	0.452	0.46	0.41	36.50
800	0.728	0.647	0.69	0.64	57.41
400	0.848	0.798	0.82	0.77	69.63
200	0.909	0.952	0.93	0.88	79.32
100	1.064	0.99	1.03	0.98	88.01
50	1.083	1.021	1.05	1.00	90.27
NSB	0.051	0.082	0.07	0	0
B0	1.226	1.14	1.18	1.12	100

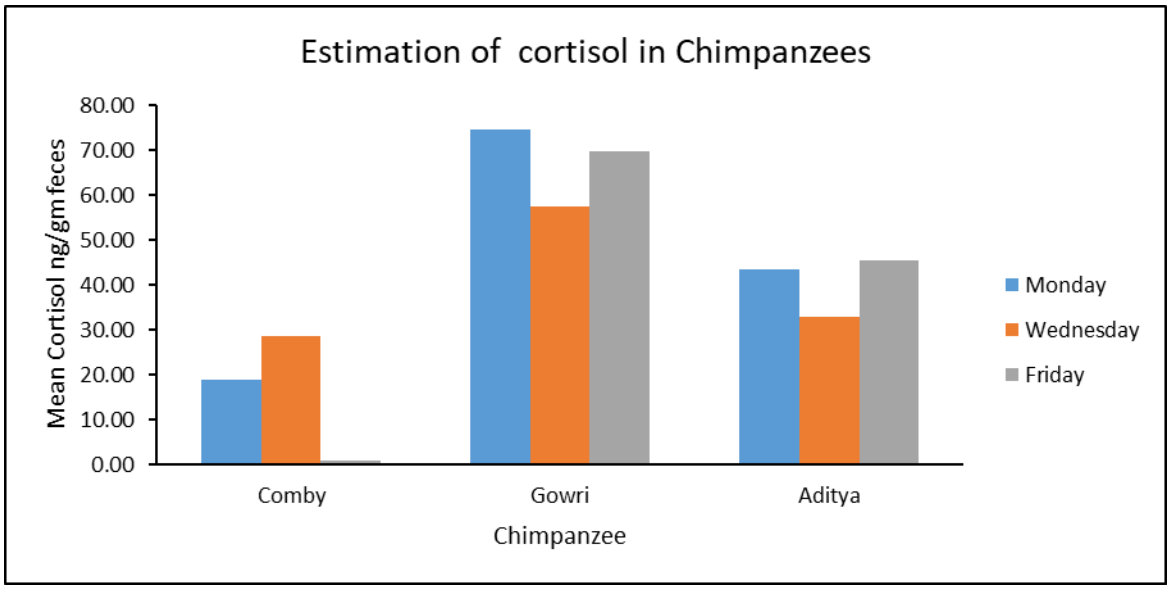


Fig 5: Estimation of cortisol level in Chimpanzees

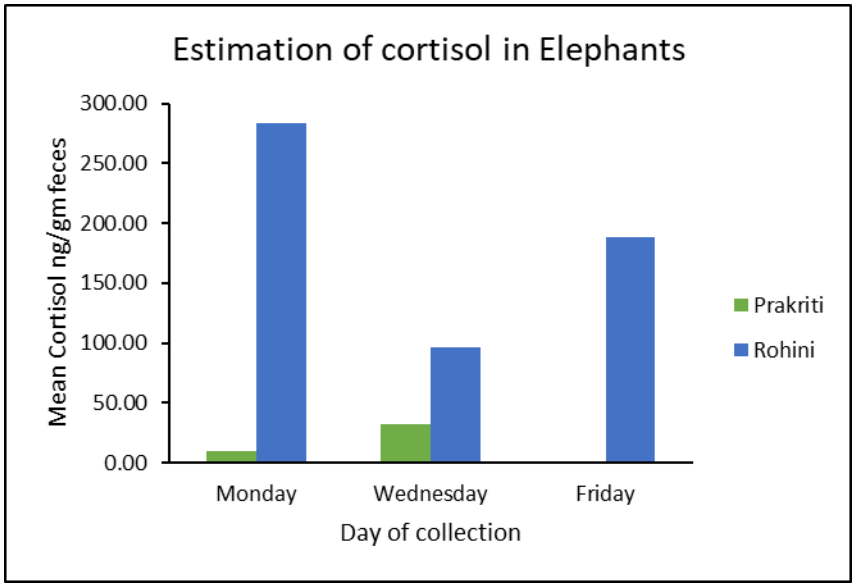


Fig 6: Estimation of cortisol level in Elephants

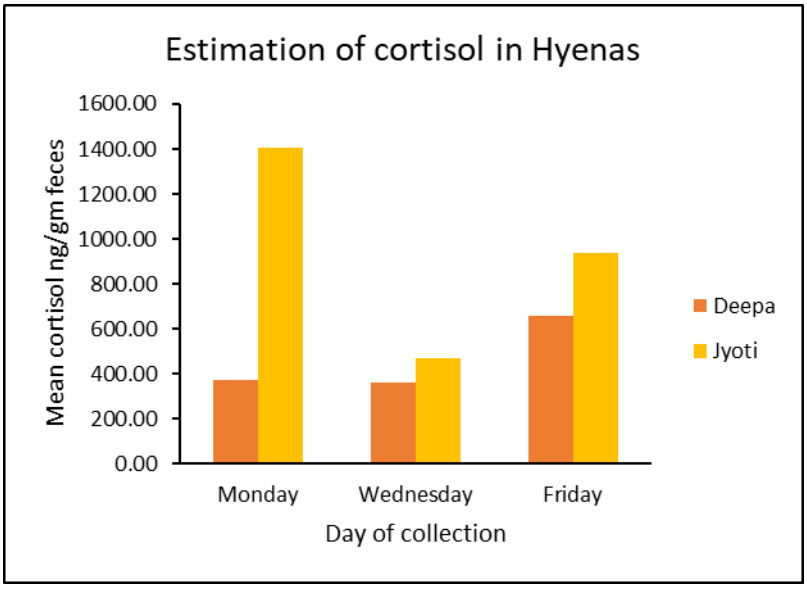


Fig 7: Estimation of cortisol level in Hyenas

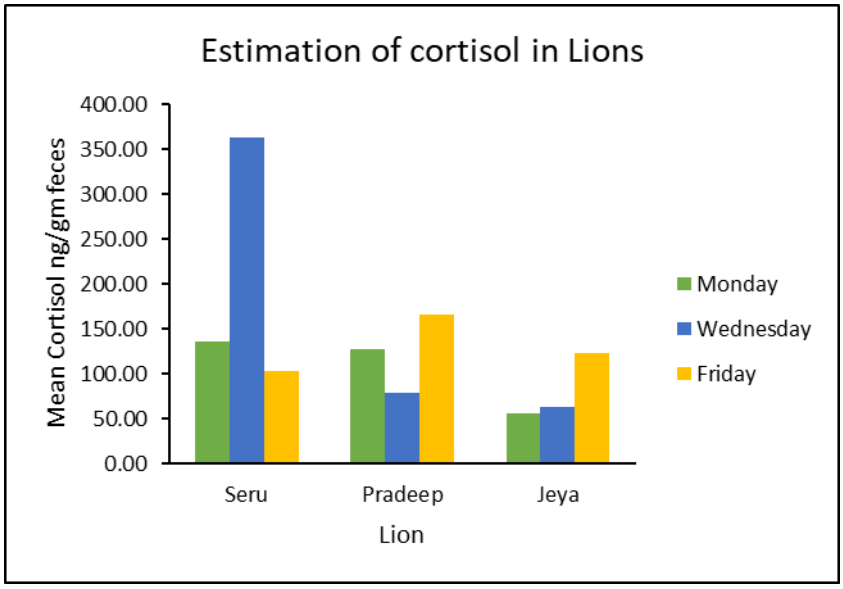


Fig 8: Estimation of cortisol level in Lion

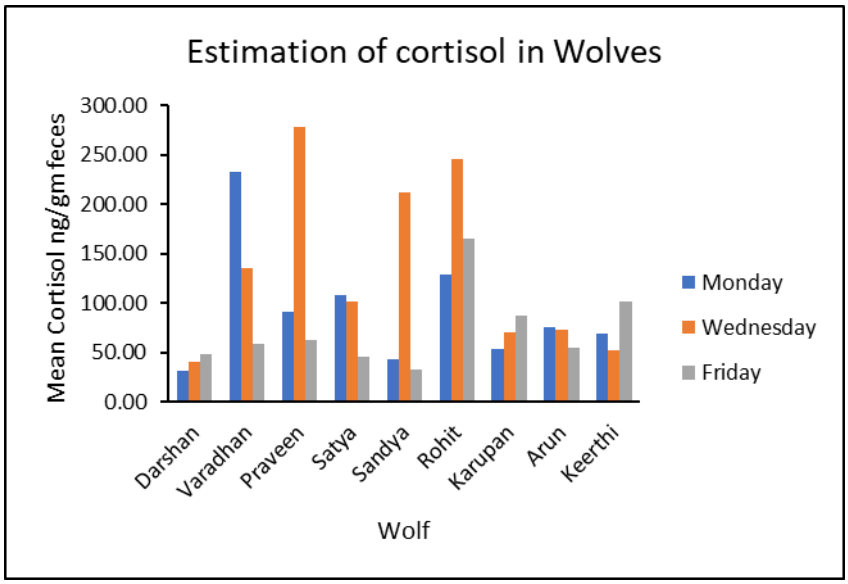


Fig 9: Estimation of cortisol level in Wolf

3.6. Discussion

A substantial body of research supports the use of FGM analysis across diverse taxa. Palme *et al.* (1996) and Benhaiem *et al.* (2012) standardised protocols in livestock and carnivores, while Nishanth *et al.* (2023) and Dhairykar *et al.* (2020) demonstrated cortisol variation among captive elephants based on management differences. Similarly, Ahmad *et al.* (2022) linked elevated cortisol in female blackbucks to summer heat and inadequate enclosure design. In addition, Yamanashi *et al.* (2016) and Jewgenow *et al.* (2020) explored the utility of hair cortisol for monitoring chronic stress, and Fokidis *et al.* (2023) introduced claw cortisol as an alternative marker of chronic stress. The influence of individual traits—such as sex, temperament, and reproductive status—has also been recognised in hormone response patterns (Majelantle *et al.*, 2023; Vaz *et al.*, 2022).

This present study was conducted to understand the influence of weekly holidays (Tuesdays), where no visitors are allowed and how it changes the level of stress in captive animals such as Chimpanzee, Elephants, Hyena, Lion and Wolf. In the present study with chimpanzee, it was observed that chimpanzee Gowri and Adithya had lower levels of cortisol on Wednesday, the day after holiday when compared to Monday and Friday cortisol levels. Cortisol levels were highest on Monday, likely reflecting increased stress associated with the increased number of human visitors on Sunday. A study by Yamanshi *et al.* (2016) reported the utlisation of hair cortisol as a non-invasive biomarker of chronic stress in captive chimpanzees. They demonstrated that the body site of hair collection and storage did not affect the cortisol estimation but sample processing steps, particularly grinding the sample to a fine powder and extraction methods had significantly affected cortisol concentrations. Similary, in our study, it was observed that the faecal samples contains lots of undigested seeds and green leaves, which were removed before extraction of hormone to minimise variability and ensure more accurate cortisol estimation.

The captive elephants Prakriti and Rohini at AAZP, showed lower levels of cortisol on Wednesday when compared to Monday and Friday cortisol levels in this preliminary study. Elevated Cortisol concentrations observed on Monday, proabably due to increased number of human visitors on Sunday. It was also observed the dung samples had many straw like substances which were difficult to grind into powder. These observations highlight the need for long-term monitoring of cortisol levels to better understand stress dynamics in captive elephants. Dhairykar *et al.* (2020) reported a comparative assessment of faecal

cortisol levels in captive Asian elephants housed at Kanha, Panna, and Bandhavgarh Tiger Reserves in Madhya Pradesh. He reported the baseline stress among elephants regardless of location and sex was similar, but significant differences were observed in age based variations. In another study, Nishanth *et al.* (2023) assessed and reported faecal cortisol levels were higher in temple elephants when compared to camp elephants in Mudumalai and Anamalai. The

For the captive Striped Hyenas Deepa and Jothi at AAZP, it was observed that cortisol levels were elevated on Mondays followed by Fridays and lowest level of cortisol on Wednesdays. The faecal samples of hyena were highly dried and after extraction, the solution was found to be viscous that requires repeated dilutions to measure the cortisol concentrations. Goymann *et al.* (2001) reported the influence of social status, reproductive condition**,** and environmental factors on faecal corticosteroid levels in free-ranging female spotted hyenas, and results demonstrated that reproductive status strongly affects stress hormone levels, particularly during periods of social stability. Benhaiem *et al.* (2012) validated a non-invasive enzyme immunoassay (EIA) for measuring faecal glucocorticoid metabolites (fGCMs) in spotted hyenas to assess physiological stress and reported that higher fGCM levels were observed in juveniles under six months of age, attributed to social instability and maternal separation. Jewgenow *et al.* (2020) assessed the reliability of two commonly used enzyme immunoassays (EIAs)—cortisol-3-CMO and cortisol-21-HS—for measuring hair cortisol concentrations in six mammalian species, including cheetahs, spotted hyenas, and Asiatic black bears. Based on their two assays (EIAs and LC-MS/MS) to detect cortisol, they reinforced the need for species- and matrix-specific validation. In another study, Ashish *et al*. (2023) evaluated faecal glucocorticoid metabolites (fGCMs) as stress indicators in wild striped hyenas (*Hyaena hyaena*) in Southern India. Using non-invasive sampling and ELISA, the study found slightly higher cortisol levels in adult and female hyenas, potentially linked to reproductive or territorial stress. Hyenas near human settlements also showed elevated fGCMs, suggesting anthropogenic disturbance as a stressor.

Asiatic Lions housed at AAZP, had expressed differential pattern of cortisol levels when compared to other captive animals under this study. Lion Pradeep and Lioness Jaya showed higher levels of cortisol on Fridays than Mondays, and the levels were lower on Wednesdays. Interestingly, Lion Seru showed much higher levels of cortisol on Wednesdays followed by Mondays and Fridays. It was observed that Lion Seru was always having an

aggressive behaviour irrespective of human presence. Moreover, visitors are not allowed to view Lions in cages like other captive animals in AAZP, rather visitors are taken to the Lion safari in a caged vehicle inside the Lion enclosure where they roam freely. Creel *et al.* (2013) reported fGCM in African lions in Kenya's south rift valley were significantly lower in protected conservation areas compared to buffer zones inhabited by people and livestock. In another study, Vaz *et al.* (2022) examined and reported the relationship between personality traits and stress physiology in captive African lions by measuring faecal glucocorticoid (cortisol) levels and assessing behaviour through a standardized personality checklist. The study identified two primary personality dimensions: dominance and agreeableness, these influencing the physiological stress responses.

In the captive wolf population housed at AAZP, nine individuals were taken for the study namely, Darshan, Varathan, Praveen, Satya, Sandya, Rohit, Karuppan, Arun and Keerthi. Of these wolves, Praveen, Satya, Sandya and Rohit displayed higher cortisol levels on Wednesdays when compared to Mondays, Fridays and other wolves. Darshan, Varathan, Karuppan, Arun and Keerthi displayed slightly lower levels of cortisol on Wednesdays but not significantly different from Mondays or Fridays. It was observed that the faecal samples of Karuppan, Praveen and Varathan were dark in colour, while samples from Karuppan and Arun appeared liquid in consistency. Rohit had a tail injury and was in the recovery phase; and the extracted hormone from his sample was highly viscous and required dilutions. Varathan's faecal samples found to have tendon-like sheets, while Rohit was always placed inside the enclosure itself and not allowed outside. Roffler *et al.* (2022) validated the use of keratinized tissues—specifically guard hair, undercoat hair, and claw samples—to measure stress and reproductive hormone levels (cortisol, progesterone, and testosterone) in Alexander Archipelago wolves in Alaska. Hormone concentrations were consistently detectable across all tissue types using enzyme immunoassays (EIAs), demonstrating the potential of these matrices for long-term endocrine monitoring.

3.7. Conclusion

The present study reported the stress hormone cortisol levels in selected captive wild animals such as Chimpanzee, Elephants, Hyena, Lion and Wolf. The study aimed at analysing the stress level influenced by human vistors using non-invasive sampling methods. The primary objective was to assess the influence of human visitor pressure on stress levels, with particular emphasis on the weekly "no-visitor" day (Tuesday). Cortisol concentrations

were derived from the samples collected over a ten-day period on Mondays, Wednesdays and Fridays, enabling evaluation of the carry-over effect of the no-visitor day (Tuesday reflected in Wednesday samples) in comparison with the high visitor pressure days (Sunday reflected in Monday samples) and regular visitor days (Friday). Overall, it was observed and the data was very clear to show lower levels of cortisol concentrations on Wednesdays when compared to Mondays and Fridays for Chimpanzee, Elephants, and Hyena. In contrast, Lion and Wolf exhibited elevated levels of cortisols on Wednesdays than on Mondays and Fridays. In addition to hormonal variations, notable differences in animal behaviour were observed, and faecal samples frequently contained undigested materials that adversely affected the hormone extraction efficiency.

This study is a pilot study aimed at standardisation of hormone extraction process and ELISA optimisation along with hypothesis testing of no visitor day (Tuesday) vs Higher visitor day (Sunday) Vs other days and conducted only for 1.5 months (~10 samplings). Although, the data showed lower level of cortisol estimation but statistical observation was not able to be inferred because the sample size and study period was very low. Hence, a minimum of three-month study period has to be planned and executed. Grunwald *et al.* (2024) provided an in-depth review of various methods used to measure glucocorticoid (GC) stress hormones such as cortisol and corticosterone in wildlife species. The study emphasized the importance of selecting the appropriate biological matrix (blood, saliva, faeces, urine, hair, or feathers) based on the type of stress being assessed (acute vs. chronic), the biology of the species, and field practicality. Blood and saliva offer insights into acute stress responses, while faeces and hair are more suitable for assessing long-term or chronic stress**.** The authors also highlighted the need for understanding species-specific hormone metabolism and excretion patterns, as well as the technical limitations of each sampling method, such as hormone degradation in blood or environmental contamination in faeces. This review supports the use of non-invasive sampling techniques—particularly faecal and hair cortisol analysis—for reliably evaluating stress in wild and captive animals, provided that assay validation and sampling protocols are carefully adapted to the species of interest. The findings of the present study suggested that individual behavioural profiles can influence physiological stress responses, highlighting the importance of integrating personality assessment with endocrine markers to enhance welfare practices in captive management and conservation breeding programs.

4 Further work

The Cortisol detection and estimation were validated through optimised extraction and ELISA protocols. However, a longer study period of atleast 3 months is required further to generate conclusive baseline cortisol data along with information on other influencial factors such as behaviour, enclosure characteristics and faecal texture/ingredients. Such an extended assessment would enable the development of a species-specific reference chart for evaluating stress levels in captive animals.

5 Recommendations

Continuous monitoring of stress level significantly plays an important role in assessing the health and welfare of the captive animals and may support the Arignar Anna Zoological Park's captive animal surveillance of physiological stress responses within enclosures and during captive breeding programs. As this approach is non-invasive, it can be readily incorporated into routine wildlife health management practices. Accordingly, AAZP has been identified as a key beneficiary of this study.

6 Output:

A. Technology development

i) Technology developed at lab scale/pilot scale/commercial scale: LAB SCALE

ii) Technology demonstration in field setup – NO

iii) Technology transfer to industry – NO

iv) Buyers/ end users: NIL

v) **Creation of improved product:** REFER TO FURTHER WORK.

vi) Affordability: MOST ECONOMICAL

B. Knowledge creation

i) Publications: Baseline data study is required – INADEQUATE

ii) Patent filings: NIL

iii) Conference/workshops attended: NIL

C. Capacity building

i) Officers/faculty: AWARENESS INCLUDED IN REGULAR TRAININGS.

ii) Temporary manpower recruited/trained: ONE – PA-II.

D. Added value of project outcomes: AAZP CAPTIVE WILD ANIMAL SCREENING.

E. Achievements of the project: NON-INVASIVE FAECAL SAMPLE BASED ESTIMATION..

F. Shortfalls/constraints faced: LONGER PERIOD OF MONITORING IS REQUIRED

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ANNEXURE – 1

STANDARD OPERATING PROCEDURE

Extraction

1. Approximately 0.5 g of the powdered sample was weighed into a sterile 15 mL tube.
2. 5 ml of absolute ethanol was added to each tube.
3. The mixture was subjected to vigorous shaking for 45 minutes using a mechanical vortex or orbital shaker to enhance hormone solubilisation.
4. The tubes were then centrifuged at 5000 rpm for 15 minutes at 4 °C to separate the supernatant.
5. The supernatant was transferred to a clean petri plate.
6. The ethanol was allowed to evaporated completely at room temperature, leaving behind the concentrated hormone residue.
7. The dried extract was then reconstituted in 400 to 1000 µL of absolute ethanol.
8. The reconstituted samples were stored at −20 °C until further analysis by ELISA.

ELISA

1. A plate layout sheet should be prepared to aid in proper sample and standard identification.
2. Thaw only the required number of wells and return the unused wells to the foil pouch with desiccant. Seal the ziplock plate bag and store at 4˚C.
3. 50 µL of samples or standards added into wells in the plate (columns A to H).
4. 75 µL of 1X Assay Buffer added into the non-specific binding (NSB) wells.
5. 50 µL of 1X Assay Buffer added into the maximum binding (B_0 or Zero standard) wells.
6. 25 µL of the DetectX® Cortisol Conjugate was added to each well.

7. 25 µL of the DetectX® Cortisol Antibody was added to each well, except the NSB wells.

8. The plate was covered with the plate sealer and shaken at room temperature for 1 hour at 700–900 rpm.

9. Discard the plate content and each well washed 4 times with 300 µL 1X Wash Buffer. The plate was tapped dry on clean absorbent towels.

10. 100 µL of the TMB Substrate was added to each well.

11. The plate was incubated at room temperature for 30 minutes without shaking.

12. 50 µL of the Stop Solution was added to each well.

13. Optical density was read in a plate reader capable of reading at 450 nm.

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Established in October 2017, Advanced Institute for Wildlife Conservation (AIWC) is one of its kind research institution set up by the forest department of Tamil Nadu primarily to provide scientific solutions to conservation problems. The institute conducts multidisciplinary wildlife research, capacity building in wildlife conservation, training programs and advisories in wildlife research by partnering with leading research institutions in India. The AIWC conducts captive wildlife research in close coordination with Arignar Anna Zoological Park and other zoological parks of Tamil Nadu. The Institute is actively engaged in research across the length and breadth of Tamil Nadu on biodiversity related issues.

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