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Stress evaluation by estimation of fecal cortisol metabolites of free ranging Barasingha (*Rucervus duvaucelii branderi*)

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Abstract
Barasingha also known as Swamp deer are found in the the central India as well as the around the foothills of Himalayan region. Hard ground swamp deer (*Rucervus duvaucelii branderi*) is the state animal of Madhya Pradesh, the site of this study. They are enlisted in Schedule 1 of the Wildlife (Protection) act, 1972, amended in 2022. Poaching and anthropogenic invasion of their natural habitats endanger their survival and extensive conservation efforts are needed to save them. In the present study, estimation of faecal cortisol metabolites (FCM) was performed to compare stress levels of free-ranging Barasingha of Kanha Tiger Reserve (KTR) and Satpura Tiger Reserves (STR). The body condition of Barasingha was also evaluated in both the tiger reserves. Accordingly, faecal samples were processed in following ways *i.e.* (i) for coprodiagnostic analysis to screen gastrointestinal parasitic egg load and (ii) assessment of faecal cortisol metabolites using ELISA diagnostic kit (DetecX, Arbor assay, Ann Arbor, Michigan, USA). The mean FCM level 263.335±43.050 pg/ml and 290.054±40.147 pg/ml were recorded in KTR and STR respectively with non-significant difference indicating acclimatization to new habitat. Significant positive correlation was observed between FCM concentration and parasitic load (OPG) for *Eimeria* spp. in Barasingha of both the parks (p<0.01). Furthermore, significant positive correlation was also observed between FCM level and poor body condition score in Barasingha of both the parks (p<0.01). The study suggests that poor body condition and coccidiosis contribute to the stress levels in Barasingha and affect their foraging behavior.

Keywords: Faecal cortisol metabolite, Barasingha, body condition score, Kanha tiger reserve, Satpura tiger reserve

Introduction
Stressors are environmental factors that stimulate abnormal homeostatic, physiological and behavioral responses. Different stressors are anthropogenic activities; adverse climatic conditions like drought, sudden rain or wind, excessive fatigue due to predator or pain (Radostits *et al.*, 2007) [12]. Wild animals of protected and non-protected forest areas face many stressors such as decrease in wilderness and encroachment of common water resources and grazing lands in and around the protected forest areas. Urbanization contributes not only to increasing stress levels in wild animals but is also responsible for spillage of infectious diseases that are manifested in the form of outbreaks. Stress mechanism comprises the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic-adrenal-medullary (SAM) axis. The HPA axis on being activated leads to the release of glucocorticoid hormones while the SAM axis facilitates the release of norepinephrine and epinephrine. The short-term release of cortisol is helpful in mobilization of energy to cope with stress while chronic release of cortisol leads to muscle wastage, tachycardia and reduced immunity and fertility (Sapolsky, 1990) [14].
Non-invasive method of stress evaluation by estimation of faecal cortisol metabolites was used in the study and compared between two sites-Kanha and Satpura tiger reserves of Madhya Pradesh. In the present study, Barasingha (*Rucervus duvaucelii branderi*) those were translocated from Kanha tiger reserve to Satpura tiger reserves were considered for estimation of faecal cortisol metabolites to identify the status of stress levels along with evaluation of body conditions and intensity of gastrointestinal parasitic load.

Materials and Methods

Selection of animals: The herds of Barasingha of Kanha tiger reserve and Satpura tiger reserves were involved for collection of biological samples using non-invasive techniques**.**

Sample collection and processing: The herds of Barasingha were followed early in the morning and observed from a close distance. When the herd moved from their resting place, the freshly laid faecal samples were collected from the pasture. The faecal samples were collected in containers and therefore divided into two parts, one part kept without any preservative in cold chain for stress analysis and another part kept in 10% buffered formalin for coprodiagnostic investigation. The samples were brought to SWFH laboratory for further processing. The faecal samples stored in cold chain were used. The samples were dried at 60 °C for four hours and then powdered finely. 80% methanol was used to dilute the faecal samples. The samples were vortexed for 30 minutes and centrifuged for 15 minutes at 5000 rpm. The supernatant is collected and stored at-2 °C (Palme *et al.*, 2013) [10]. The supernatant was diluted using assay buffer. The reconstituted diluted samples were run in the assay as per standard protocol.

The formalin preserved faecal samples were processed using standard techniques as per Soulsby (1982) [17] and Sloss *et al.* (1994) [15] including qualitative and quantitative techniques-sedimentation, floatation and McMaster techniques.

The animals were photographed with the close vicinity and the body condition was judged based on body evaluation scheme as proposed by Meetei *et al.* (2021) [7]. SPSS Statistics software version 30.0 was used for analysis of results (Snedecor and Cochran, 1994) [16].

Results

The visual body condition of Barasingha revealed non-significant difference ($p>0.05$) in body condition of Barasingha in KTR and STR. The visual examination of both the populations revealed maximum Barasingha in healthy body condition indicative of optimum environment (Table 1).

Table 1: Body condition scoring in Barasingha at Kanha and Satpura tiger reserves

Place of work	Number of animals screened	Body condition Number of animals (Percentage)			χ^2, p
		Good	Fair	Poor	
Kanha tiger reserve	n = 36	18 (50.00%)	16 (44.44%)	02 (05.55%)	0.95, 0.61
Satpura tiger reserve	n = 36	14 (38.88%)	19 (52.77%)	03 (08.33%)	
Total	n = 72	32 (44.44%)	35 (48.61%)	05 (06.94%)	

$p>0.05$, Non-significant

Coprodiagnostic investigation

The qualitative coprodiagnostic investigation revealed the occurrence of *Amphistome* spp., Strongyles and *Eimeria* spp. The surveillance of parasitic disease in Barasingha was conducted in the present study with relation to stress evaluation. Statistical analysis revealed no significant difference in gastrointestinal parasitic occurrence between the two populations at KTR and STR which indicates similar habitat quality in both tiger reserves (Table 2). The study terrain of KTR and STR is interspersed with marshy lands and ponds where large population of water snails thrive that serve as intermediate host for diseases caused by flukes. That is why, high occurrence of trematode *i.e. Amphistome* spp. infection was observed in the study. The hot and humid weather during period of study acts conducive for parasitic larval survival and growth (Mir *et al.* 2016) [8].

The quantitative coprodiagnostic investigation revealed mean oocycst per gram of faeces (OPG) of *Eimeria* spp. similar in Barasingha of KTR and STR ($p>0.05$) (Table 3). The mean eggs per gram of faeces (EPG) for *Amphistome* spp. and Strongyles in Barasingha of STR was significantly higher than Barasingha of KTR ($p<0.05$).

Soulsby (1982) [17] emphasised that the gradient of infective load (EPG/OPG) depends on the seasonal variation. This gives explanation for the variations in the reported values. During field sampling, the presence of stagnant water resources beside grazing lands was found higher in STR than KTR. Grazing near such water bodies increases the chances of picking up gastrointestinal parasites and further studies in this regard can help in better understanding the gastrointestinal parasitism in Barasingha.

Table 2: Occurrence of gastrointestinal parasites in Barasingha of Kanha and Satpura tiger reserves

Place of work	Number of positive samples (Percentage)				χ^2, p
	Amphistome spp.	Strongyles	*Eimeria* spp.	Mixed Infection	
Kanha tiger reserve (n = 36)	12 (33.33%)	04 (11.11%)	02 (05.55%)	18 (50.00%)	1.69, 0.63
Satpura tiger reserve (n = 36)	09 (25.00%)	02 (05.56%)	03 (08.33%)	22 (61.11%)	

$p>0.05$, Non-significant

Table 3: EPG/OPG of gastrointestinal parasites in Barasingha in Kanha and Satpura tiger reserves

Place of work	Gastrointestinal parasitic specie		
	Amphistome spp. (EPG)	Strongyles (EPG)	*Eimeria* spp. (OPG)
Kanha tiger reserve (n = 36)	1477.42^{a}±161.09	355.55^{a}±81.69	1033.33±185.59
Satpura tiger reserve	2068.57^{b}±202.12	1113.89^{b}±239.74	1205.56±192.14

Mean values with different superscripts between tiger reserves differ significantly ($p<0.05$)

Stress Evaluation

In the present study, FCM in Barasingha were measured for the first time in tiger reserves of Madhya Pradesh to compare stress level in populations in both the tiger reserves and understand the impact of habitat-change due to translocation.

Mean FCM level in Barasingha was estimated to be 263.33±43.05 pg/ml in Kanha tiger reserve (KTR) while 290.05±40.15 pg/ml in Satpura tiger reserve (STR) with non-significant difference (p>0.05). Effect of translocation for both translocated and resident animals depend on the availability and type of habitat (Beaman *et al.* 2023) [1]. In the present study, similarities in FCM level in both the populations of Barasingha may be attributed to similarities in the geographical and meteorological parameters of both tiger reserves and subsequent acclimatization of Barasingha in STR.

The differences in the mean FCM level in this study compared to others is self-explanatory that FCM varies with multiple factors.

Correlation of FCM level and gastrointestinal parasitic EPG

Gastrointestinal parasites lead to reduced vigour and immunity in the animal as the nutrients are absorbed by parasites in gut rather than metabolized by the animal's body (Cizauskas *et al.*, 2015) [3].

Statistical correlation of individual animal's FCM values and EPG/OPG of gastrointestinal parasites was analysed in the present study and FCM level was found significantly correlated to load (OPG) of *Eimeria* spp. as signified by the positive Pearson coefficient of 0.73 in Kanha tiger reserve and 0.72 in Satpura tiger reserve (p<0.01) (Table 5 and Table 6). It can be explained by the fact that *Eimeria* spp. exhibits high host specificity and cause pathogenic effects only when the parasitic load is high and the host has low immunity (Lopez-Osorio *et al.*, 2020) [6]. The pathogenic effects compound to trigger stress level in animals.

Table 5: Correlation of faecal cortisol metabolite level (pg/ml) with gastrointestinal parasitic load (EPG/OPG) in Kanha tiger reserve

Parameters		FCM	*Amphistome* spp.	Strongyles	*Eimeria* spp.
FCM	Pearson Correlation	1.00	-0.02	0.27	0.73**
Amphistome spp.	Pearson Correlation	-0.02	1.00	-0.18	0.05
Strongyles	Pearson Correlation	0.27	-0.18	1.00	-0.36*
Eimeria spp.	Pearson Correlation	0.73 **	0.05	0.36*	1.00

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Table 6: Correlation of faecal cortisol metabolite level (pg/ml) with gastrointestinal parasitic load (EPG/OPG) in Satpura tiger reserve

Parameters		FCM	*Amphistome* spp.	Strongyles	*Eimeria* spp.
FCM	Pearson Correlation	1.00	-0.11	0.33*	0.72**
Amphistome spp.	Pearson Correlation	-0.11	1.00	-0.10	-0.09
Strongyles	Pearson Correlation	0.33*	0.10	1.00	0.41*
Eimeria spp.	Pearson Correlation	0.72**	-0.09	0.41*	1.00

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

In the present study, statistical analysis revealed significant positive correlation between Strongyle infection load and FCM level in Barasingha in Satpura national park (p<0.05) indicated by a Pearson correlation coefficient of 0.33 (Table 6). This might be due to higher parasitic load as Sapkotal *et al.* (2022) [13] and Paul *et al.* (2020) [11] have emphasised that Strongyles infection with higher EPG leads to anorexia, inappetance, watery diarrhoea and weight loss in host and in conjunction with *Eimeria* spp. infection, it may lead to gastroenteritis, anaemia and loss of body condition. As mentioned, maximum animals had mixed infection in this study. Co-infecting pathogens lead to modified impact on infected host's immune system and identifying their interaction is important. Defolie *et al.* (2019) [4] found positive parasite-cortisol relationship for all parasite types in mammals on meta-analysis of records of parasite-glucocorticoid relation in mammals.

However, no significant effect of *Amphistome* spp. infection was observed on FCM level in Barasingha. This may be due to the fact that, mature Amphistomes are usually non-pathogenic (Soulsby 1982) [17]. In agreement to that, Mondal *et al.* (2003) [9] found animals in thriving condition despite high infection load of *Amphistome* spp. and conjectured the same to be due to chronic exposure and development of immunity.

The relation of FCM and gastrointestinal parasitism is affected by the proportion of bound glucocorticoid metabolites and released in faeces which is altered with variation in gut microbiota (Hickmott *et al.*, 2022) [5] and hence, might be associated with blood-glucocorticoid levels and other factors that need further exploration.

Correlation of FCM level and body condition score of Barasingha

In the present study, a significant positive correlation was observed between incidence of poor body condition and FCM concentration in Barasingha signified by a positive Pearson correlation coefficient of 0.57 calculated in KTR (Table 7) and positive Pearson correlation coefficient of 0.46 in STR (p<0.01). While, in STR, good body condition was found to be significantly negatively correlated to FCM level in Barasingha (p<0.05) signified by a Pearson coefficient of-0.35 (Table 8).

Table 7: Correlation of faecal cortisol metabolite level (pg/ml) with body condition score in Kanha tiger reserve

Parameters		FCM	Good	Fair	Poor
FCM	Pearson Correlation	1.00	-0.23	0.03	0.57**
Good	Pearson Correlation	-0.23	1.00	-0.88**	-0.17
Fair	Pearson Correlation	0.03	-0.88**	1.00	-0.16
Poor	Pearson Correlation	0.57**	-0.17	-0.16	1.00

**. Correlation is significant at the 0.01 level (2-tailed).

Table 8: Correlation of faecal cortisol metabolite level (pg/ml) with body condition score in Satpura tiger reserve

Parameters		FCM	Good	Fair	Poor
FCM	Pearson Correlation	1.00	-0.35*	0.14	0.46*
Good	Pearson Correlation	-0.35*	1.00	-0.89**	-0.24
Fair	Pearson Correlation	0.14	-0.89**	1.00	-0.21
Poor	Pearson Correlation	0.46**	-0.24	-0.21	1.00

****.** Correlation is significant at the 0.01 level (2-tailed).
*. Correlation is significant at the 0.05 level (2-tailed).

The results indicate deterioration of body condition coincides with increase in animal's FCM level. Elevated cortisol levels lead to mobilization of energy and also suppress protein synthesis, thus reducing body condition. Therefore, body condition was found to affect stress levels in Barasingha with higher FCM levels in animals in poor body condition and lower FCM levels in animals with good body condition.

Conclusion

Stress in free ranging animals is a.fected by a myriad of factors including feed intake and environmental conditions. Coccidian parasites known to affect the health and foraging behaviour of Barasingha also contribute to higher FCM level in Barasingha. Non-significant difference noted in the mean cortisol level in the two sites, thus revealing no adverse impact of translocation. Poor body condition affects stress level as observed in both the tiger reserves.

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