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Extraction of stress hormones by fecal sampling of big cats, ungulates and mammals in captivity and wild by using enzyme linked immunoabsorbant assay (ELISA)

Jahanzeb Sarwer[1], Saqafat Ahmed[1], Maryam Khan[1], Sikander Hayat[1], Bushra Nisar Khan[2], Arif Malik[1], Saba Shamim[1*]

[1]*Institute of Molecular Biology and Biotechnology, The University of Lahore, Defence Road Campus, Lahore, Pakistan*

[2]*Department of Zoology, University of the Punjab, New Campus, Lahore-54590, Pakistan*

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Abstract

The determination of fecal cortisol and steroids via immunoassay and extraction techniques is a widely accepted and used phenomena with respect to both captive and field study for the provision of the estimation of the regulating concentration of hormones in animals, which was achieved through non-invasive procedures. The reposition of fecal samples is a significant matter of concern due to the metabolism of fecal steroids by bacteria present in the feces of animals only after a few hours of deposition. In this study, the estimation of fecal hormones like estrogen (fE) and glucocorticoid (fGC) metabolites was carried out in big cats of the wild in captivity, such as lions, tigers, African lions, puma, jaguar, few ungulates, mouflon sheep, deer, chinkara, zebra and Punjab urial. The fecal samples (n=106) were collected from these wild animals and were treated with methanol to curb the metabolism of fecal hormones by bacteria. The expression of stress hormone levels in different animals as obtained by ELISA is given as 0.644±0.03 for Punjab urial followed by 0.619±0.02 for deer, 0.614±0.05 for Chinkara, 0.606±0.01 for tiger and Puma, 0.579±0.02 for lion and 0.061±0.04 for Jaguar. Much stress hormone was observed in Punjab urial whereas Jaguar was least affected by the stress. It showed that animals living in small and noisy environment are more affected by any disturbance in their surroundings as compared to the animals living in quiet and less noisy environment.

* **Corresponding Author:** Saba Shamim ✉ sabashamimgentics@gmail.com

Introduction

The animals have been kept in zoos for centuries for entertainment, conservation, education and research purposes. To provide these animals with an environment as close as possible to the wild, is very essential as it stimulate animals to display natural behavior and stay stress free (Schildkraut, 2016). When an animal is in stress, it shows many physiological changes or response. These changes can be of many forms like, increase in heartbeat, elevated stress hormones (cortisol) circulation, increase in metabolic rate and dehydration in severe cases of heat exposure (Bourne and Cunningham, 2019). The environmental disturbances can activate or release the stress hormones (glucocorticoids). The glucocorticoid hormones are important to understand how animals deal with perturbations in their environment and they are related to an individual wellbeing (Dantzer *et al.*, 2014). The glucocorticoids concentration determined from biological matrices, such as blood, saliva, urine or feces can act as a potential indicator for level of stress (Webster *et al.*, 2018). The difficulties in obtaining blood samples and the recognition of the stressor effect of blood sampling are primary drivers for the use of non-invasive sample media (Claxton, 2011). The blood sample collection for measuring the glucocorticoids can bias the stress results in case of captive animals. The non-invasive methods of measuring stress hormones are important especially for free roaming species as capturing of an animal can itself cause stress (Behringer and Deschner, 2017).

The front-line hormones to overcome the stress are glucocorticoids and catecholamines (Grimmett *et al.*, 2008). When glucocorticoids hormone secretes in the blood stream, it is metabolized in the kidneys and liver and then excrete through urine and feces (DeRango *et al.*, 2019). In most sample media, measurement of a specific corticosteroid is a requirement depending on the species, e.g. cortisol in most mammals, or corticosterone in birds. However, in samples involving products of excretion, methodologies that measure a broad range of structurally related compounds are probably optimal. The utility of minimally invasive sample media as biomarkers of stress responses depends on the degree to which the corticosteroid content of the sample represents adrenocortical activity. Commonly, this involves comparisons between corticosteroid concentrations in blood plasma with concentrations in the alternative sample media (Cook, 2012). Measuring fecal cortisol metabolites as an indicator of adrenocortical activity in animals, offers the advantage of a simple sampling technique that will not interfere with the results of the study and enables even long-term and as well as longitudinal studies. Thus, such methods will be a valuable tool in a variety of research fields such as animal welfare (handling, housing and transportation) and also in ethological and environmental studies (Möstl and Palme, 2002).

Materials and methods

Samples collection

A total of 107 animal feces samples of various wild big cats, ungulates, and some other mammals were collected from Lahore zoo, Bahawalpur zoo, Lahore safari zoo and Islamabad zoo. The environmental conditions of the animals and collection were noted.

Samples preservation

The samples were preserved by treating them with methanol. The 2 grams of sample was taken and incubated at 60 °C for 15 minutes. After incubation, the samples were dried. They were crushed and 1 gram was added to 20 ml methanol.

ELISA performance

The ELISA kit used in this study was comprised of the following items: ELISA well plate coated with cortisol with MAb, cortisols standards vials (0.5 ml), Enzyme conjugate 20X (1.2 ml), TMB substrate (12 ml), Stop solution (12 ml), 20X wash concentrate (25 ml) and Assay Diluent (24 ml). The experiment was performed in two batches.

Batch-I

In the first phase, the number of samples were 49 and numbers of standards were 7 which were provided with the ELISA Kit. A total prepared conjugate

solution for 56 samples was diluted with distilled water in 1:21 ratio. For 56 samples, 7 wells were set on a holding stand. Every strip contained 8 wells in a single column. For each well, 112 µl of enzyme-conjugate solution was prepared. The total conjugate solution prepared for 56 samples was 11,200 µl. Hence by dividing 11200 µl with total 56 number of samples including standards, a value of 200 µl came for a single well. The 560 µl Wash buffer (1X) was prepared for the required samples. Prior to assay process, the reagents were allowed to stand at room temperature for some time. They were gently mixed before use. Seven strips of coated wells were fixed in a stand holder. The 200 µl of enzyme-conjugate solution was added to each well. Afterward 25 µl of cortisol standard was added to the first 7 wells using a micropipette. The solution was gently mixed with the help of pipette for 10 second. The 25 µl of sample was added in the remaining wells with the help of pipette and gently mixed. The ELISA plate was left for incubation at room temperature for an hour. After incubation, the plate was washed with 1X wash buffer (300 µl) and blotted on an absorbent paper. TMB substrate (100 µl) was added in all plates and incubated for 15 minutes at room temperature. Stop solution (50 µl) was added to all wells. The solution was mixed gently. Within 20 minutes of adding the stop solution, the absorbance was read on ELISA reader.

Batch-II

In the second phase, the number of samples were 58 and the numbers of standards were 6 as provided with the ELISA Kit. Hence the total prepared conjugate solution was diluted with the distilled water (1:21). For 64 samples, 8 wells were set on a holding stand. For each single well, 128 µl of enzyme-conjugate solution was prepared. The total conjugate solution prepared for 64 samples were 12,800 µl or 200 µl for a single well. According to the procedure, 640 µl wash buffer (1X) was prepared for the required samples. Prior to the assay process, the reagents were allowed to stand at room temperature for some time. They were gently mixed before use. Eight strips of coated wells were fixed in stand holder. The 200 µl of enzyme-conjugate solution was added to each well. Afterward 25 µl of cortisol standard was added to the first 6 wells using micro pipette. The solution was gently mixed with the help of pipette for 10 second. The 25 µl of sample was added in the remaining wells with the help of pipette and gently mixed. The ELISA plate was left for incubation at room temperature for an hour. After incubation the plate was washed with 1X wash buffer (300 µl) and blotted on absorbent paper. TMB substrate (100 µl) was added in all plates and incubated for 15 minutes at room temperature. Stop solution (50 µl) was added to all wells. The solution was mixed gently. Within 20 minutes of adding the stop solution, the absorbance was read on ELISA reader.

Results

The fecal samples (106) were collected from different zoos; Lahore zoo, Bahawalpur zoo, Lahore safari zoo and Islamabad zoo. Maximum fecal samples were collected from lions followed by tiger and deer. Only two samples were collected for jaguar and zebra (Table 1).

Table 1. Collection of fecal samples from different captivities/ zoos.

Sr. no.	Animal	Bahawalpur zoo	Safari Lahore	Lahore zoo	Islamabad zoo	Total
1	Lion	8	22	10	2	42
2	Tiger	4	10	8	0	22
3	Deer	0	0	0	13	13
4	Sheep	0	0	0	10	10
5	Chinkara	0	0	0	6	6
6	Punjab Urial	0	0	0	5	5
7	Puma	2	2	0	0	4
8	Jaguar	0	2	0	0	2
9	Zebra	0	0	0	2	2
Total		14	36	18	38	106

It can be clearly seen in Table 1 that Islamabad zoo provided maximum number of samples for each animal followed by Safari zoo of Lahore. The stress hormone levels of all the animals were estimated by ELISA (Table 2). It was observed Punjab Urial showed significant expression of stress hormones while minimum was recorded in Jaguar.

Discussion

The different species of animals showed effective physiological and behavioral responses in case of fluctuated environment conditions. The level of stress hormones was checked in wild big cats and animals by the analysis of their fecal samples. The fecal samples of various ungulates and wild big cats were collected from different regions of Punjab, Pakistan including Lahore zoo, Bahawalpur zoo, Lahore safari zoo and Islamabad zoo. *P. leo* fecal samples were collected from Bahawalpur zoo, Lahore safari zoo, Lahore zoo and Islamabad zoo to check level of stress hormone. According to Putman *et al.* (2019), 21 male African Lion (captive) fecal samples were collected at 9-16 years of age at different time intervals to analyze the stress hormone patterns but no significant change was observed in stress hormone level which does not agree with the current study. Another study conducted by Rimbau, (2019) on Lions also suggested that there was no significant change observed in the stress levels of lions in enclosures which also disagrees with the results of the current study. *P. tigris* fecal samples were collected from Bahawalpur, Safari zoo, Lahore zoo and Islamabad zoo to check level of stress hormone. This study proved that the level of stress hormone fluctuates in captive environment which was in agreement with a study by Vaz *et al.* (2017) conducted in Indian captivities.

Table 2. Estimation of stress hormone levels in faeces by ELISA.

Sr. no.	Animal	Temperature (°C)	Humidity (%)	Mean stress hormones value
1	Punjab urial	35 °C	47 %	0.644±0.03
2	Deer	35 °C	47 %	0.619±0.02
3	Chinkara	35 °C	47 %	0.614±0.05
4	Puma	30 – 41 °C	30 – 41 %	0.606±0.02
5	Tiger	30 – 41 °C	30 – 41 %	0.606±0.01
6	Lion	30 – 41 °C	30 – 41 %	0.579±0.02
7	Zebra	35 °C	47 %	0.570±0.01
8	Sheep	35 °C	47 %	0.516±0.03
9	Jaguar	41 °C	30 %	0.061±0.04

The authors of the study reported that the change in environment effected the level of stress hormones in feces of tigers. The study of Mittal *et al.* (2019) conducted a study in India on captive tigers and reported that the animal living in an open field like safari or nature parks have less stress levels compared to the animals living in rather closed cages and cells. A study conducted by Ivanov *et al.* (2017) in Russia reported that the stress levels of tigers are also affected by the temperature fluctuation. For captive tigers, Vaz, (2015) reported that the fecal cortisol level was high in the animals living in more closed cell while the value decreases if the animal is living in a big cell. These results suggested that the stress levels were greatly affected by the area of the confinement. These results are also in agreement with the current study. The study of Sajjad *et al.* (2011) on tigers in captivity in Lahore zoo and Lahore zoo safari suggested that there was no significant increase in stress levels in the both captivities which does not agree with our results. The deer fecal samples were collected from Islamabad zoo to check level of stress hormone in captivity. Jachowski *et al.* (2018) suggested that the stress hormone level in fecal sample increased with an increase in stress condition like quality of food and living conditions. These findings are in agreement with the current study. Caslini *et al.* (2016) also reported that the condition

of living highly affected the level of stress in the deer population in Italy, while the study of le Saout *et al.* (2016) reported dissimilar results. In another study (Zbyryt *et al.*, 2017), an observation was made that the stress in ungulates was high in the area where they were in constant contact with humans suggesting that the animals in zoos were more prone to stress which supported our findings.

The Mouflon sheep (*Ovis aries*) is a wild sheep that is found in the Mediterranean islands of Sardinia and Corsica. The works of Fischer *et al.* (2017) and Yalçintan *et al.* (2018) suggested that the mouflon sheep are quite immune to harsh and stressful conditions and are less likely to get stressed which is in agreement with our results. On the other hand, Ciliberti *et al.* (2017) reported otherwise. No increase of stress levels in Mouflon sheep was because of the reason that these animals were always used as domestic animals and kept in captivity regularly (Marino and Merskin, 2019). The Chinkara (Indian Gazelle) is found in Afghanistan, Iran and India (Din *et al.*, 2020). Aziz *et al.* (2018) concluded that all species of Chinkara showed normal behavior as wild Chinkara given the suitable gender relation and no environmental disturbance was faced in the captive or zoo environment, these results were in agreement with the current study. Khatak *et al.* (2019) conducted a research on the Punjab urial herds in captivity and concluded that the captive environment of the facilities was insufficient for the herds to thrive properly as they would in the wild. These results collaborated with the current study results as the captive animals have a higher stress levels in the captivity. The study of Conforti *et al.* (2012) on Jaguars reported fecal sample was an effective way to measure the stress hormones level in the animal, this supported the measuring methods of stress of the current study. Mesa-Cruz *et al.* (2016) on different species, including Jaguar, reported no increase in stress levels of Jaguar living in the semi-captive nature park. Seeber *et al.* (2018) while working on zebras, it was revealed that the stress level concentrations was higher in large aggregations and in band stallions than in smaller groups and in bachelor males, respectively. Another study by Fourie, (2012) also reported that the free ranging small groups have low fecal stress hormones levels than the ones living in large groups or in close confinements. Conte, (2014) worked on zoo-captivated zebras of USA, determined that such animals were not disturbed or stressed by the presence of human visitors so it was a positive result for the zoos.

Conclusion

The results of the present study concluded that the values of fecal hormones including estrogen (fE) and glucocorticoid (fGC) metabolites are different in all the animals. Hence, the observation tells us that animals in captive conditions near the noisy or road are more affected by the stress as indicated by the rise in fecal cortisol metabolites. While the animals inhabited in less rushy and noisy areas, have less stress levels. In the same way, the area in which the animal is kept in, also plays a major and significant role in it.

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