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Review Article

Faecal cortisol a non invasive biomarker for stress assessment in wild animals, confounding factors, estimation, quantification, and interpretation.

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Abstract

The biological response elicited when an individual perceives a threat to its homeostasis is stress and this has been constantly a dwindling factor in animal populations playing an important role in performance and population dynamics. During the past decade, measuring faecal steroid hormone metabolites had become a widely used technique, because of its noninvasiveness, this tool has proven to provide important information about an animal population as well as the individual animal as they predict fitness quotient in the individuals. But a lot of variations and co-variations exist in the cortisol excretion patterns, animal variability, feeding habits, functional place in the ecosystem, predator pressure, human manipulation, coexistence of varied fauna and parasites. The variations related to age, sex, seasonal and reproductive stage variations are briefly discussed. The validation of cortisol assessment has also been reviewed to find out the best method of cortisol assessment. This manuscript aims to provide considerable guidelines for cortisol assessment and its confounding factors, techniques of cortisol assessment and the simple physiology of stress and its effect in wild animals.

Keywords: Cortisol, Non- invasive, Stress, Wild animals.

Introduction

The availability of biological samples from wild animals are very difficult and it becomes an important factor that the Non invasive sampling must utilized to the maximum extent to find out the indicative health status of the population. The detailed guidelines of assessing faecal glucocorticoids have been detailed by (Palme 2005). Fowler (1986) opined that "Stress" was a cumulative response-the result of an animal's interaction with its environment through receptors and was an adaptive phenomenon. All responses were primarily directed at coping with environmental change and behavioral repertories may be dependent on the stressful interactions of animals with their environment and the stressors could be classified as somatic, psychological, behavioral and miscellaneous ones. Stress in mammals matched with a complex and multistage syndrome that was orchestrated by the sympathetic nervous system and glucocorticoids. The activation of sympathetic nervous system and release of glucocorticoids comprised the stress responses. Although short term stress responses were thought to help an animal cope with adverse environmental conditions, long term activation of stress response could decrease the health (Sapolsky *et al*. 2000). This paper deals with the various confounding factors that affect the excretion, quantification and the maintenance of stress in wild animals.

Cortisol and stress factors

Hofer and East *et al.* (1998) reported that the increase in environmental pressure due to anthropogenic disturbances might induce physiological stress and affected the resistance to diseases, survival and

reproduction, negatively. Faecal glucocorticoid metabolite concentrations represented a pooled fraction of plasma glucocorticoids, providing an estimate of the adrenal status that smoothed the effect of diurnal and pulsatory variations and translocation was known to enhance glucocorticoid secretion in mammals (Goymann *et al*., 1999).Terio *et al.* (1999) concluded that a variety of acute pharmacological and physiological stressors resulted in the increased faecal cortisol metabolite excretion. Ohe and Servheen (2002) suggested that glucocorticoid concentrations was widely used as an index for stress and discussed about the stress response and non-stress factors affecting the excretion of glucocorticoid metabolites.
Washburn and Millspaugh (2002) linked the elevation in the level of fecal glucocoricoid metabolites with the stress condition. Wingfield and Kitaysky (2002) stated that acute rise in glucocorticoids following perturbations of the environment might actually avoid chronic stress and served primarily as 'anti-stress' hormones. Millspaugh and Washburn (2003) opined that several biotic like diet and abiotic like environmental conditions might influence the measurements of faecal glucocorticoid metabolites. Pride (2005) opined that faecal glucocorticoid levels were useful predictors of individual survival probabilities in wild populations and a powerful tool for non invasive monitoring of wild animals. Cabezas *et al*. (2007) reported that following exposure to long term stress, moderately elevated serum corticosterone and fecal glucocorticoid metabolites levels in wild rabbits were negatively associated with body condition, but positively associated with subsequent survival upon release.

Physiology of Cortisol

Bahr *et al.* (2000) reported that only small amounts of cortisol were present in urine from Common marmosets (*Callithrix jacchus*), Long-tailed macaques (*Macaca fascicularis*) and Chimpanzee (*Pan troglodytes*) and quantitative levels the excretion of urinary cortisol was lower in the Old World monkey and great apes, when compared to the New World monkeys. Ohe and Servheen (2002) quoted that the release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary gland stimulated the adrenal cortex to increase the synthesis and secretion of glucocorticoids such as cortisol and corticosterone synthesis of specific glucocorticoids and the pattern of their release varied among the species. Barrett *et al.* (2002) quoted that the cortisol was primarily involved in carbohydrate metabolism and maintenance of homeostasis. Palme *et al.* (2005) stated that the glucocorticoids and catecholamines were the hormones to overcome stressful situations in mammals and birds. Ostner *et al.* (2008) opined that the short term elevations of glucocorticoids were an adaptive response to short term stressors and chronic increase in glucocorticoids secretion were due to prolonged stressors, leading to detrimental effects on body functions of Assamese macaques (*Macaca assamensis*). Petrauskas *et al.* (2008) mentioned that the corticotropin-releasing hormone activated the anterior pituitary gland and released adrenocorticotropic hormone (ACTH) which stimulated the adrenal cortex to secrete glucocorticoids in Steller sea lions. Setchell *et al.* (2010) quoted that the vertebrates responded to stress by activating the hypothalamus pituitary-adrenal axis and released glucocorticoids into the blood stream. Sheriff *et al.* (2010) stated that differences in fecal glucocorticoid metabolites levels in Snowshoe hares (*Lepus americanus*) revealed accurate reflection of their physiological state and their ability to a stressor as a response.

Hamalainen *et al.* (2014) reported that the animals age and water content of the fecal samples significantly influenced baseline fecal glucocorticoid metabolite levels in wild gray mouse lemurs (*Microcebus murinus*). Quispe *et al.* (2014) suggested that differential regulatory mechanisms between basal and stress–induced cortisol levels were dependent of cortisol modulation in South-American Caviomorph Rodents (*Octodon degus*).

Factors Influencing Cortisol

Kalin *et al.* (2000) opined that in Rhesus macaque, individuals with extreme right frontal asymmetric brain had high levels of trait-like fearful behavior increased plasma cortisol concentrations physiologically further got associated with this. Mostl and Palme (2002) quoted that the samples collected at every spontaneous defecation, after a short term stress showed that elevated levels of cortisol metabolites were present only in less than three to four consecutive fecal samples. Millspaugh and Washburn (2004) described that the interpretation of fecal glucocorticoids metabolites (FGM) measures might be confounded by the length of time animals were held in

captivity, normal seasonal and daily rhythms, body condition, sample storage and treatment techniques, diet of the animal, assay selection, animal status like social ranking or reproductive status, sample age, condition and sample mass.

Davis *et al.* (2005) reported that an increase in the number of visitors in the zoo was associated with the increase in cortisol level in Spider monkeys. Downs *et al.* (2008) opined that levels of cortisol increased with age in males, but no age-related change was observed in the rhythm of old females of Rhesus macaques.

Rangel-Negrin *et al.* (2009) observed that spider monkeys living in large tracts of conserved habitats had lower cortisol levels than individuals living in fragmented habitats and in captivity. Foerster and Monfort (2010) reported that both temporal and individual variations in fecal glucocorticoids indicated differential metabolic stress among the female Sykes's monkeys (*Cercopithecus mitis albogularis*).

Setchell *et al.* (2010) reported that the glucocorticoid levels were higher in subordinate males under stable conditions, but under conditions of instability higher ranking males had higher glucocorticoid levels.

Chelini *et al.* (2010) opined that the comparison of adrenocorticol response of males and females to an Adrenocorticotrophic hormone (ACTH) challenge and the amplitude and timing of response were revealed and the excretion of glucocorticoids was found in both sexes of Syrian hamsters. Clinchy *et al.* (2013) found that the exposure to predators or predator cues induces "sustained psychological stress" in many wild animals and that was consequently often accurate to be referred as "ecology of fear".

Smith *et al.* (2012) suggested that the fecal cortisol metabolites levels were lower in captivity than in the wild and the natural environment in which animals existed was generally more challenging or less predictable than the life in captivity. Marcilla *et al.* (2012) reported that the rhythms of cortisol secretion might vary annually and thus, it was necessary to take into account these rhythms during evaluation of the physiological significance and fluctuations of this hormone throughout the year as stress indicators in captive Asian elephants (*Elephas maximus*). Carlitz *et al.* (2014) stated that body region did not have a significant influence in hair cortisol concentration of Orangutans.

Variations of cortisol level

Age-Wise Variations

Saltzman *et al.* (1998) reported a rank mediated cortisol difference in female marmosets. Socially subordinate females had markedly low plasma cortisol levels than dominant females. Cavigelli (1999) mentioned that high index-dominance individuals had high cortisol values and low index-dominance animals had low cortisol values. Dominance indices were reliable predictor of cortisol variability and significantly correlated with age. Goymann *et al.* (1999) reported a significant correlation between allostatic load of dominance and relative glucocorticoid concentrations in both females and males. When allostatic load was higher in dominants than in subordinates, dominants expressed higher levels of glucocorticoids; when allostatic load was similar in dominants and subordinates, there were only minor differences in glucocorticoid concentrations; and when allostatic load was lower in dominants than in subordinates, subordinates expressed higher levels of glucocorticoids than dominants. This consistently explains rank differences in glucocorticoid concentrations. Goymann and Wingfield (2003) recorded a substantial increase in fecal cortisol metabolites in a social stressful situation in which two animals fought with each other. Younger free-ranging birds did not exhibit a well defined adrenal reaction in response to capture whereas older birds did. (Nilsson *et al*., 2008). Novakova e*t al.* (2008) stated that significant differences were absent between fecal cortisol metabolite levels in adults and sub adults of spiny mice, although adults were socially dominant.
Ostner *et al.* (2008) reported about the significant effects of age and dominance rank on fecal glucocorticoid levels. Starling *et al*. (2010) opined that social instability, such as intermediate rank, could strain resources of animals that were already physiologically stressed and resulted in the elevated faecal glucocorticoid concentrations over animals' in stable dominance positions, thus indicative of age related variations in the glucocorticoid concentration. Poessel *et al*. (2011) suggested that adrenocortical activity in male black-footed ferrets could be decreased through the provision on environment

enrichment, but enrichment provided to female ferrets might produce the reverse effect by increasing such activity, and that these results were found to be mediated by an age effect.

Sex-Wise Variations

Saltzman *et al.* (1998) suggested that estrogen could elevate glucocorticoid levels and reliable changes across the ovarian cycle were noticed with levels in mid to late follicular, peri-ovulatory and early luteal phases higher than those in the remainder of the cycle. Kenagy and Place (2000) opined that circulating glucocorticoid levels were significantly elevated in female yellow pine chipmunks that were lactating and this elevation was likely influenced in their fecal glucocorticoid metabolites as well. Romero *et al*. (2000) reported that male and female European starlings varied in the adrenocortical response to capture and handling during their breeding season. Boonstra *et al.* (2001) concluded that arctic ground squirrels were markedly affected by stress, but during the mating period reproductive adult males already had high free cortisol concentrations independence of the stressor and were less capable of mounting a stress response than females. Lynch *et al.* (2002) recorded an elevated cortisol level in both adult and sub adult males during the peak of sexual activity of adult females in Capuchin monkeys. Huber *et al*. (2002) opined faecal glucocorticoid levels did not differ between males and females; neither lactation nor rut seemed to affect fecal cortisol excretion. Touma *et al*. (2003) observed that male mice excreted higher amounts of fecal corticosteroid metabolites than the female counterparts. Palme *et al*. (2005) reported that sex played an important role in the metabolism and excretion of fecal glucocorticoids, and this was the probable reason why some assays yielded good results in one sex and not in the other. Petrauskas *et al.* (2006) estimated that in stellar lions, males had a significantly higher fecal corticosterone concentration in the breeding season, while females significantly higher concentration in between molting and breeding season. Wittig *et al.* (2008) predicted that during unstable periods following a predator attack or the immigration of an infanticidal male, females who had a stable and focused grooming network should experience less stress and mentioned that glucocorticoid levels in female baboons were strongly influenced by events that directly affect their reproductive success. Chelini *et al.* (2010) quoted that in Syrian hamsters the sex of the animal influenced the excretion of fecal glucocorticoid metabolites. In males, the concentrations were almost four times higher than the females.

Forester and Monfort (2010) recorded temporal and individual variations of fecal glucocorticoids which indicated existence of differential metabolic stress, among female Sykes monkeys. Starling *et al*. (2010) reported that in ring tailed lemurs males, experienced their greatest stressors during the breeding season and females showed significantly greater values during pregnancy and lactation. Brent *et al.* (2011) recorded that in rhesus macaques high ranking females having smaller proximity networks had significantly lower cortisol levels.

Seasonal Variations

Cavigelli (1999) stated that fecal cortisol levels were relatively high corresponding with the end of dry season when high intensity anti predatory behavior and estimates of feeding effort were high. Harper *et al*. (2001) observed a clear temporal pattern variation in fecal glucocorticoid levels and it was lowest during October, which was coincident with shortening day length and decreasing ambient temperatures. Romero (2002) explained the seasonal modulations of glucocorticoid concentrations in many species. Washburn and Millspaugh (2002) documented seasonal differences in basal corticosterone levels and responsiveness to stress-inducing procedures, in both captive and free-ranging birds

Huber *et al*. (2002) recorded that faecal glucocorticoid excretion varied seasonally with a peak during winter as a response to cold stress and the parameters such as minimum ambient temperature and snow proved to be the only factors exerting significant effects on fecal glucocorticoid excretion. Oki and Atkinson (2004) observed that mean daily cortisol concentrations were not significantly different between seasons, but cortisol displayed a circadian rhythm only during the summer. The absence of a circadian rhythm of cortisol during the winter might have been a result of the limited amount of daylight as well as the continual need to produce metabolic heat as a by-product of gluconeogenesis. Weingrill *et al.* (2004) reported that seasonal factors had strong effects on fecal cortisol levels and these were found to be independent of the reproductive state in female

Chacma baboons. Touma and Palme (2005) quoted about the significant effects of season or weather conditions such as temperature, humidity and water availability on fecal glucocorticoid metabolites for several species of mammals and birds. Petrauskas and Atkinson (2006) opined that fecal corticosterone concentrations varied with seasons and had individual seasonal patterns in stellar lions.

Dalmau *et al*. (2007) observed clear seasonal changes in fecal cortisol metabolites, which were used as indicators of stress, in a population of Pyrenean Chamois and obtained correlations between fecal cortisol metabolites and monthly mean minimum temperature. Alejandro *et al.* (2008) recorded that the increase in the concentration of fecal cortisol was influenced by the days of high rainfall and lower temperature. While higher temperature, little or no rainfall led decrease in concentrations. Buttoz *et al*. (2009) reported distinct seasonal variations in glucocorticoid levels of male long tailed macaques with concentrations being highest at the beginning of conception period and non conception- period. Rangel-Negrin *et al*. (2009) compared the seasonal variation in glucocorticoid levels and opined that faecal cortisol concentration was higher in the dry season, compared with the wet season. Rehnus *et al*. (2009) however demonstrated the reliable measurement of faecal glucocorticoid metabolites in faeces collected from mountain hares under field condition in various seasons. Sheriff *et al.* (2010) recorded that changes in fecal cortisol metabolite concentrations in autumn and winter reliably tracked changes in plasma free cortisol levels obtained from hormonal challenge-test. The mean minimum ambient temperature and mean temperature humidity index values had a significant positive correlation with mean fecal cortisol values (Smitha *et al*., 2011). Smith *et al*. (2012) suggested that adrenocortical activity might play an important role in the seasonal and daily regulation of their physiological states.

Reproductive stage variations

Saltzman *et al.* (1998) reported that the cortisol levels were increased during the follicular phase, peaked during the late follicular, pre-ovulatory, early luteal phases and declined in the mid to late luteal phase in female marmosets. Lynch *et al.* (2002) reported that the testosterone and cortisol showed a simultaneous and sustained increase at the onset of breeding season in wild adult male Tufted Capuchin monkeys (*Cebus apaella*). Tarlow and Blumstein (2007) quoted that during the reproductive season, glucocorticoid levels could be elevated resulting from a human disturbance and generally this might be associated with reproductive failure in wild animals. Fourie and Bernstein (2011) quoted that the high levels of cortisol might be seen after parturition, which rapidly declined with age in Vervet monkeys and Guinea baboons. Smith *et al.* (2012) reported that pregnancy caused an initial rise in fecal cortisol metabolites (FCM) levels, followed by decrease at the date of pup emergence and during the weaning in marmots (*Marmota flaviventris*).

Other Variations

Schwarzenberger *et al*. (1996) opined that the route of excretion of steroid hormone metabolites varied considerably among and within the same species and a similar pattern was exhibited like those in plasma, but had a lag time from twelve hours to more than two days, depending on their intestinal transit time. Wallner *et al.* (1996) concluded that aggressive interactions produced prolonged changes in individuals, which could be monitored in the excreted steroids. Perception, reaction and aggression were related to the individual personality. Ekkel *et al.* (1997) estimated the salivary cortisol in pigs and assessed the circadian parameters of salivary cortisol. Goymann *et al.* (1999) stated that translocation and socially stressful condition were known to enhance glucocorticoid secretion in mammals. Touma *et al.* (2003) investigated about the possible impacts of the animal's activity-rhythm and time of day on metabolism and excretion of corticosterone metabolites. Touma *et al.* (2003) mentioned that faecal samples were to be collected at the same time of the each day or all samples voided over a 24-hr period were to be pooled to avoid fluctuations caused by diurnal variations in faecal glucocorticoid metabolite concentrations. Millspaugh and Washburn (2004) opined that comparison of faecal glucocorticoid metabolite from fecal materials collected at different times of the day would be valid only if faecal glucocorticoid metabolites did not reflect diurnal differences. The detectability of diurnal changes in faecal glucocorticoid metabolite might be related to the lag time of faecal glucocorticoid metabolite excretion which was affected by the gut passage time and this varied according to size and species.

Rubenstein (2007) observed that environmental conditions influenced the relative costs of dominance and subordination, which in turn affected the degree and intensity of social interactions, reproductive decisions and breeding roles. Martinez-Mota *et al*. (2008) observed that faecal cortisol metabolite excretion took 1-3 days after the stressful event, depending on the individual. Further, there was an important individual variability in the concentrations of glucocorticoid metabolites, which might reflect the differences in the stress reactivity or faecal glucocorticoid metabolite-metabolism and excretion. Novakova e*t al.* (2008) recorded that mice adapted to human settlements showed higher faecal cortisol metabolite levels than the outdoor contemporaries and this variation was attributed due to their high densities and predation pressure inside buildings.

Busch and Hayward (2009) stated that environmental factors like predator abundance, food abundance, pollution, exposure to humans and habitat change had considerable effects on the levels of glucocorticoids. Heugten *et al.* (2009) stated that fecal cortisol concentration was high, when the diet consisted of high total sugars and carbohydrates and low fiber. Low percentage of nutritionally complete diet was correlated with the highest cortisol concentration. Behie *et al.* (2010) reported that fruit availability and the presence of tourists influenced cortisol levels in Howler monkeys. Brent *et al.* (2011) concluded that social capital might be an important determinant of cortisol levels in social animals and might influence the cortisol levels on a day to day basis. Kuo *et al*. (2011) demonstrated the biological relevance of faecal cortisol metabolite levels with respect to stress by repeatedly and frequently monitoring the alterations of faecal cortisol metabolite levels before and after experiencing a single fighting interaction. The measurement of the number of faecal pellets indicated time specific increase in two groups after the fight and the measurements of faecal cortisol metabolite levels, further revealed time-specific elevations of faecal cortisol metabolite concentrations in the loser group after stressful defeat. Smith *et al*. (2012) observed that the faecal glucocorticoid levels were 68% lower in captivity than in the wild and suggested that the natural environment in which these animals occurred was generally more challenging or less predictable than in captivity.

Cortisol in Wild Species

Petrauskas *et al.* (2006) reported that invasive procedures under proper veterinary and anesthetic care did not elicit a consistent significant glucocorticoid response in Steller sea lions (*Eumetopias jubatus*). Laws *et al.* (2007) mentioned that the non-invasive monitoring of fecal cortisol metabolites could be used to investigate the adrenal activity in Asian elephants and this might be considered as a safe, practical and accurate welfare indicator. Chelini *et al.* (2010) quoted that Syrian hamsters excreted fecal glucocorticoid metabolites and in males the concentrations were almost four times higher than the females. Hogan *et al.* (2012) reported the distinct annual pattern of the cortisol secretions and was compared with the seasonal reproductive cycle of captive male Numbats. Ugaz *et al.* (2013) reported that the enclosure type influenced the behavior and salivary cortisol concentrations of captive Bottlenose dolphins.

Narayan *et al.* (2013) opined that reliable non-invasive biomarkers of the stress hormone (cortisol) were necessary for assessing the health status and welfare of Tigers in captivity. Fowler and Mikota (2006) opined that the stressor was any stimulus that elicited a biological response in case of wild fauna, when perceived by an animal. The autonomic nervous system dealt with short-term stress responses and any tissue innervated by autonomic nerves might get affected, as happened during the occurrence of increased peristalsis, during occurrence of stress. Janicki *et al.* (2006) suggested that cage breeding induced certain amount of constant stress for adult hares, which was however insufficient to be considered as significant influence on reproduction efficiency and health status. Liu *et al.* (2006) quoted that a correlation was found between the occurrence of stereotypic behavior and elevated fecal cortisol level suggesting that stereotypic behavior might be a response to the increased fecal cortisol level.

Mateo (2006) opined that higher cortisol levels just after emergence function to promote rapid acquisition of survival behaviors and additionally facilitate learning and memory. Radostitis *et al.* (2007) opined that stress was a systematic state that developed as a result of the long term application of stressors, which were the environmental factors that stimulated homeostatic, physiological and behavioral responses

in excess of normal. Stress developed, when the animal's mechanism concerned with adaptation of its body to the environment was extended beyond the normal capabilities. Nilsson *et al*. (2008) observed that confinement in individual transport kennels and surgery resulted in elevated serum cortisol levels, throughout the holding period. Bayazit (2009) mentioned that stress caused an increase in the production and release of glucocorticoids and they could be found in blood, urine and feces. It was further stated that the response to an acute stressor could be beneficial, whereas chronic stress caused deleterious effects and could be accurately assessed by estimation of fecal glucocorticoid metabolites.

Bonier *et al*. (2012) concluded that the baseline cortisol could predict the relative fitness of individuals and populations, but the relationship was not always consistent or present. Busch and Hayward (2009) demonstrated that glucocorticoids could be correlated with both environmental changes and fitness-parameters and however, the strength and direction of these relationships did not always fit predictions. Cyr and Romero (2009) opined that hormonal habituation was often used to describe a situation where an individual had learned to perceive a repeated stressor as innocuous and thus, the intensity of hormonal stress mediators reduced over time and ultimately, a habituated animal was not considered stressed. Chelini *et al*. (2010) opined that changes in glucocorticoid concentrations in the blood were well reflected by changes in fecal cortisol metabolite in both sexes. Dickens *et al*. (2010) mentioned that chronic stress in translocated animals enhanced their vulnerability to other environmental factors and thereby, amplified the potential problems encountered during the release, such as succumbing to disease or predation. Sheriff *et al.* (2010) recorded that faecal glucocorticoid metabolites and plasma free cortisol concentrations were concordant, suggesting faecal glucocorticoid metabolites were an excellent predictor of responsiveness to stressors by animals. Poessel *et al.* (2011) observed that faecal glucocorticoid metabolites values were lower in wild living animals when compared to their domestic counterparts. Schmidt *et al*. (2010) demonstrated that transport was stressful for horses, but this transport induced-stress response was found to be decreased the horses that were habituated with such transport-situations. However, an increased cortisol secretion remained detectable. Pifarre *et al.* (2012) indicated that the amount of visitors in zoos influence the behavior and adrenal activity of animals which could be undesirable for *ex-situ* conservation efforts.

Techniques for assessment of cortisol

Wasser *et al.* (1996) described on the extraction process, using either well mixed fecal powder from freeze dried samples obtained from the central or premixed portion of the wet fecal sample and investigated three different available radio immuno assays for estimation of cortisol. Wallner *et al.* (1996) reported about the usage of enzyme immunoassays for determination of the quantities of immuno-reactive cortisol. Palme and Mostl (1997) described the use of immunoassays specially developed for glucocorticoid metabolites. Terio *et al.* (1999) opined about the Radio-immuno assay in the fecal samples. Wasser *et al.* (2000) opined about the usage of vortexing method during the procedures related with assessment of fecal cortisol metabolites.

Dehnhard *et al*. (2001) demonstrated that adrenal activity detected by High-Performance liquid chromatography (HPLC) was found to be a prerequisite because ecologically meaningful levels-imposed stress could be validated. Therefore, non-invasive measurements of faecal metabolites were considered as the promising perspective to monitor stress. Morrow *et al*. (2002) stated that measurement of glucocorticoid metabolites reliably indicated acute adrenal activity in dairy cattle and in combination with other physiological and behavioral measures had potential for monitoring health welfare in dairy cattle. Mostl and Palme (2002) emphasized the possibilities on usage of immunoassays that were specially developed for measurement of glucocorticoid metabolites. Touma *et al*. (2003) stated that measurement of cortisol metabolite in faecal samples proved sensitive enough to detect dosage–dependant effects of Adreno-cortico trophic hormone (ACTH)/dexamethasone treatment. Touma *et al.* (2003) considered Enzyme Immuno Assay (EIA) as a valuable tool, as it enabled frequent sampling of individual animals even over long periods and helped to avoid the blood sample related stress effects.

Cortisol enzyme immunoassay was an appropriate tool for the non-invasive assessment of adrenal activity (Ponzio *et al.* 2004). Queyras and Carosi (2004) opined that non invasive techniques allowed physiological imbalances due to stress to be rapidly

assessed, before the appearance of other markers of stress, such as weight loss, poor health, and infertility. Washburn *et al.* (2003) reported that noninvasive fecal glucocorticoid monitoring, used in combination with demographic information might provide an effective tool for examination of the effects of various naturally occurring and human induced stressors on mourning doves. Heistermann *et al.* (2006) stated that the faecal glucocorticoid measurements will provide biologically meaningful data and thus, it could be applied successfully to non invasive assessment of the adrenocortical status in both captive and free-ranging primates, under a variety of conditions. Bauer *et al.* (2008) observed that concentrations of fecal metabolites were significantly increased following adreno corticotrophic hormone-challenge (ACTH), indicating that adrenocortical activity could be monitored via faecal samples. Sink *et al.* (2008) detailed about the methods of estimation of cortisol using enzyme linked immunosorbent assay. Lupica and Turner (2009), Allwin *et al*. (2015) demonstrated that the ELISA was an efficient, sensitive and reliable method for cortisol measurement in faecal extracts.

Gamboa *et al.* (2009) pointed out a linear relationship between baseline plasma cortisol and fecal cortisol metabolites. Palme (2012) addressed analytical issues regarding extraction procedures, and immunoassays and validation for each species and sex.

Cortisol assessment samples

Terio *et al.* (1999) opined about the analysis of fecal cortisol metabolites. Morato *et al.* (2004) suggested that determination of faecal cortisol metabolites could be very useful for a non invasive assessment of animal well-being and as a complement to behavioral, physiological, and pathological studies. Palme (2005) quoted that wet or dry feces were used for analysis of fecal steroids and recorded the merits of collection of wet feces, which were easier to handle and further evaluated the protocols of storage, extraction and immunoassay performance. Rehbinder and Hau (2006) mentioned that the concentration of cortisol and cortisol immune reactive metabolites were low in saliva and significant amounts were present in urine, faeces and serum. Carlsson *et al.* (2007) observed that immunoreactive cortisol metabolites were not evenly distributed in the feces and cautions were required, when using concentration of stress sensitive molecules in random single fecal sample as an indication of animal stress.

Preservatives and storage of samples for cortisol assessment

Palme and Mostl (1997) used methanol as preservative and Wasser *et al.*, (2000) suggested storage of faecal samples at -20°C, prior to the analysis of faecal cortisol. Khan *et al.* (2002) examined the stability of faecal glucocorticoid metabolite samples in 95 per cent ethanol solution at ambient temperature and at -20◦C over six months to determine the effect of storage on steroid concentrations. Mostl and Palme (2002) quoted that samples were to be immediately frozen, to avoid changes after defecation. Palme (2005) quoted that faecal steroids were not always stable and undergo metabolism and emphasized the usage of different preservatives like heat drying or lyophilization, chemical preservatives like alcohol or acids. Bacterial enzymes are the main source for the observed metabolism and hence, water was to be removed by addition of alcohol etc. Pettitt *et al.* (2007) opined that there was no significant difference in steroid concentration between the stored-frozen and stored-dried samples, but a significant difference was found between frozen and ethanol samples. Lupica and Turner (2009) used ethanol as preservative in the dried fecal samples that were subjected to the ELISA technique. Allwin *et al.* (2015) validated the storage of faecal samples in 80 per cent ethanol and demonstrated the extraction of faecal corticosteroids.

Conclusion

Numerous factors govern in for cortisol secretin, excretion, delayed release status, these factors are very important in interpreting conservation and fitness parameters of a population. However, control study units are completely necessary to properly conclude on individual outlaying parameters and functions.

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