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Non-invasive monitoring of steroid hormones in wildlife for conservation and management of endangered species — A review

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Global ecosystems and their constituent flora and fauna are experiencing a decline of biodiversity due to destruction of habitats, climate change, pollution, and invasive species. Of late, the number of species that have become critically endangered has increased extremely, and these species need to be protected from extinction in the wild. The conservation efforts should involve both *in situ*, and *ex situ* conservation and management of populations. Conservation physiology, an emerging multidisciplinary field, helps conservationists understand the physiological responses of endangered species due to the changes in the environment and thereby look for possible options of intervention to save them from extinction. This review summarizes various non-invasive hormone methods and endocrine studies involved in the conservation of endangered animals. The non-invasive hormone method has been successfully used in understanding basic reproductive biology, pregnancy diagnostics and welfare of a wide range of animals in captivity and in free-ranging habitats. This technique would directly or indirectly help in the conservation of endangered animals. This review also sheds light on non-invasive hormone monitoring in effective management and conservation of the endangered species.

Keywords: Biodiversity, Conservation physiology, Faeces, Immunoassays, Reproduction, Stress

Introduction

Global biodiversity is gradually declining owing to the loss and degradation of habitats, poaching, overexploitation, pollution, and climate change[1-3]. The conservation rate of flora and fauna is considerably smaller than the rate of human-induced extinctions of the species and needs immediate attention. By the year 2050, nearly 15 to 37% of the current flora and fauna population in their respective geographical areas are expected to be at the risk of extinction due to the increasing anthropogenic global warming[4]. It is well documented that atmospheric greenhouse gases (methane and nitrous oxide) are increasing in the environment due to human activities, resulting in a proportional rise in temperature.

Conservation physiology is a newly emerging discipline, which addresses the physiological responses of the animal due to changes in the environment[5]. It is an important field in the conservation of endangered species as it helps in understanding endocrine responses to environmental changes, nutrition, metabolism, thermal relationships, etc[6]. One of the earliest studies of conservation physiology was to understand the effect of dichloro-diphenyl-trichloroethane (DDT) on the reproductive biology of predators[7]. Subsequently, the use of DDT was banned in different parts of the world. The DDT incident was a revolution in the field of physiology and highlighted that physiological studies are necessary for the conservation of wild animals. Later, it was shown that environmental pollutants and contaminants have an impact on inhibiting hormonal regulation, reproduction or embryonic development in North American fish, reptiles and mammals[8].

Over the past decades, endocrine monitoring of reproduction and stress has become an important tool in *in situ* and *ex situ* conservation of endangered species. Hormones are measured using a variety of biological samples including faeces, urine, saliva, feather, and hair in wide range species *viz*. big cats[9,10], primates[11], Ungulates[12], Asian elephants[13], birds[14], amphibians[15], Chelonians[16], Rhinoceros[17] and whales[18]. This review briefly summarizes the wildlife endocrine studies on non-invasive methods for the assessment of reproductive and stress physiology of captive and free-ranging animals. It also discusses 'in-the-field' and 'laboratory techniques' which includes sample storage, extraction of hormone metabolites, and validation of immunoassay procedures.

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Non-invasive method

Steroid hormones are essential for reproduction, thus, understanding the endocrine response is crucial for successful breeding programs and management of wildlife populations in captive and wild. Traditionally, steroid hormones are quantified from blood[19,20] but repeated sampling of blood is considered invasive and challenging in the wild animals. Alternatively, assessment of endocrine status is also possible from other biological samples like faeces[17], urine[19], saliva[21], milk[22] and hair[23]. Non-invasive hormone assessment method has several advantages over invasive sampling, as blood collection itself cause stress[24], requires anaesthesia and restraint or handling of animals. In the non-invasive method, sample collection requires minimal or no contact with the animal and samples can be collected without harming the animals for long term monitoring[25]. Further, in faeces, the steroid metabolites are accumulated over a period, thus pooled quantities allow a wider range of assays and assay types[26].

Collection, storage and extraction of steroid metabolites

Fresh faecal samples are collected soon after defecation and frozen immediately as it avoids or minimizes bacterial degradation of steroids and other biochemical processes in the sample[27]. In earlier studies, organic solvents like ethanol or methanol were used as a preservative to store faeces in field conditions to prevent the naturally occurring bacterial contamination and decomposition. However, studies have shown that long-term storage of faecal samples in methanol or ethanol at room temperature have resulted in the loss of hormones and species-specific variation in steroid hormone levels[28,29]. On the other hand, short term storage or preservation of faeces in methanol or ethanol (less than three months) has been found to have less effect on original steroid concentrations[13,30]. Alternatively, in field conditions, it is highly recommended that faeces are thoroughly dried to remove the water or moisture appropriately (using hot air oven) in the field, before pulverizing and transporting to the laboratory for further analysis. Moreover, under the field conditions, collection of urine from free-ranging animals is challenging due to unavailability of freezers, liquid nitrogen or dry ice for storage and transport. However, despite difficulties, a practical method for the collection of urine samples, short-term storage and transport is to use ordinary filter paper, which has been successfully demonstrated in a wide range of animals[31].

Before quantification of hormones, steroid hormones and their metabolites must be extracted from the biological samples. The selection of extraction procedure should be appropriate as different biological samples encompass a mixture of many steroid metabolites with different polarities. However, a variety of extraction protocols is available depending on species-specific metabolism, hormone polarities and conjugated or unconjugated route of excretion of the organism.

Steroid hormones present in the faeces are a mixture of polar, non-polar, conjugated and unconjugated forms[14,32]. Most of the extraction procedures involve primary extraction with 90% ethanol as solvent[9]. Some researchers have used the shaking method with various combinations of wet or dry faeces and 80% methanol for the highest recovery of steroid metabolites[25,32]. Studies have also used a combination of organic solvents and buffer, for further purification of faecal extracts including ether[33], dichloromethane[34], solid phase extraction using Sep-Pak C18 cartridge[35], petroleum ether[36] and phosphate buffer[37].

Researchers have used radiolabeled hormones including 3H-labeled and 125I-labeled (P_4, E_2 and C) to estimate the extraction efficiency by adding radiolabeled hormones to faeces prior to extraction[38]. Subsequently, recovery of radiolabeled hormones is used to evaluate the extraction efficiency of endogenous (unlabeled) steroid hormones from faeces[9] and extraction efficiency is calculated as a total percentage of labeled hormones recovered.

Immunoassays and validation

High performance liquid chromatography

High-performance liquid chromatography (HPLC) has been used to identify, separate and quantify the steroid metabolites in non-invasive samples. Immuno-reactivity of steroid metabolites can be assessed by collecting elution fractions at regular intervals with the corresponding antibody[39,40] (Fig. 1). This is used to identify immunogenicity and raising antibodies against the metabolites. Before HPLC, faecal extracts are usually passed through a steroid specific C18 column and eluted with 3 mL of 80% methanol using the solid phase extraction method to purify the extracts[41]. The separation is based on the polarity of the hormone, stationary phase (non-polar C18 column), and mobile phase (polar/non-polar solvent ratio- liquid phase).

Enzyme immunoassays

Selection and development of immunoassays primarily depend on the steroid metabolites present in

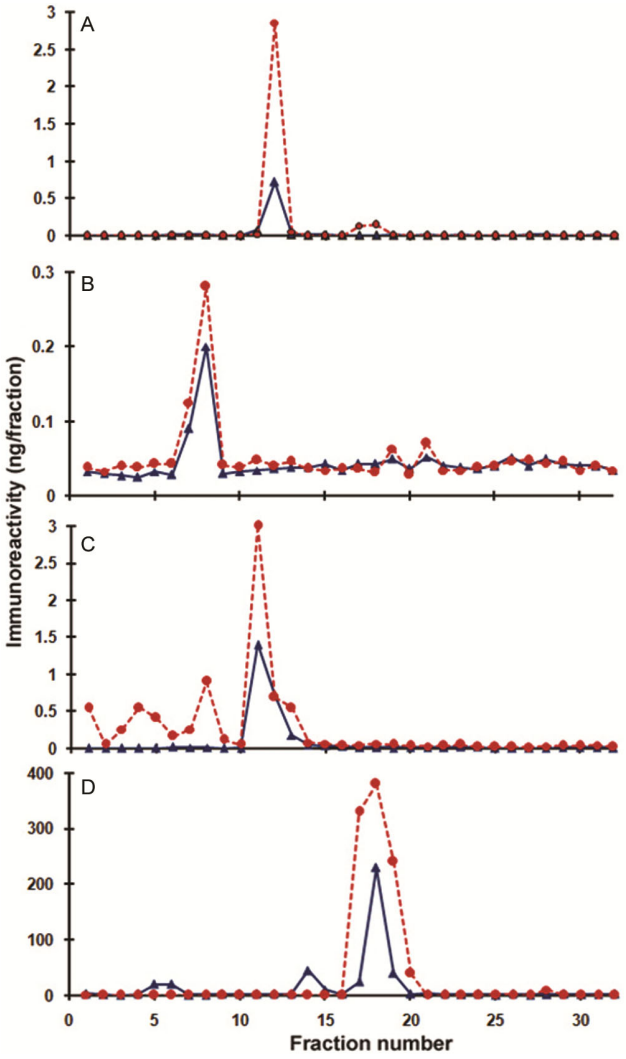

Fig. 1 — High-performance liquid chromatography (HPLC) separation of immunoreactive fractions from (A) testosterone; (B) estradiol; (C) cortisol; and (D) 5α-pregnane standards (triangle) and faecal extracts (circle).

the faeces or urine, and it is recommended to use group-specific antibodies for measurement of steroid hormones[17,32]. However, studies have shown that steroid hormones are metabolized in the liver and gut[25] and excreted as metabolites in faeces and urine. Many reports have demonstrated using radio-metabolism that steroid hormones including progesterone, testosterone, estradiol, glucocorticoid are not present in a native form in the excreta but as metabolites[32]. Radio-immunoassays (RIAs) and Enzyme-immunoassays (EIAs) are commonly used immunoassays to measure steroid hormones accurately and precisely. RIA was the first method of choice to measure steroid hormone metabolites due to high precision, specificity and sensitivity. However, EIA has gained more popularity as RIA requires hazardous radioactive material, expensive equipment, short shelf life and radioactive waste disposal facilities. Alternatively, EIA has more advantages due to its cost-effectiveness and has become the preferred choice as it is devoid of radioisotopes. In the last two decades, a wide range of EIAs have been developed for the assessment and monitoring of reproductive and stress physiology using faecal steroid metabolites in a variety of animals which includes big cats, red panda, elephant, deer, tortoises and turtle[9,13,39,40]. However, EIA must be validated for its cross-reactivity and sensitivity of antibody, parallelism, precision/accuracy, recovery, inter and intra-assay coefficient of variation[42].

Adrenocorticotropic hormone (ACTH) challenge and biological validation

Adrenocorticotrophic hormone (ACTH) challenge test is used for physiological validation of an immunoassay. For this purpose, exogenous ACTH or reversal of the physiological condition by dexamethasone suppression test is normally carried out[26] in animals. Faecal samples are collected on a regular basis before and after injection of ACTH to evaluate the baseline and estimate the significant increase of faecal glucocorticoid levels. Biological validation has been determined based on the response of natural or pharmacologically induced stressor and hormonal challenges. Moreover, hormonal challenges have potential to activate pituitary-gonadal responses by gonadotropin-releasing hormone (GnRH) which helps to study the reproductive hormones including luteinizing hormone (LH), follicle-stimulating hormone (FSH), human chorionic gonadotrophin (HCG) and androgens[43]. Furthermore, biological validation is used to estimate the excretory lag time between stimulation of adrenal/gonadal steroid hormones in blood and their metabolites excretion in the faeces. Most importantly, biological validation demonstrates the naturally occurring events such as behaviour observation (mating, estrus cycle, pregnancy) and stressful events (capture/anaesthesia, translocation or immobilization) that can be used to evaluate the biological relevance of the assay[26].

Radiometabolism study

Radiometabolism study has been used to determine the lag time between the circulatory and excretion profiles of steroid hormones and their relative abundance in the faeces and urine by administration of ^{3}H or ^{14}C radiolabeled steroids. Reports have

shown that lag time varied widely among the species and it ranges from 30 min to 56 h, depending on the species and their metabolic activity[26,32,44]. Moreover, this study also validates excretion routes (urine and faeces) of hormone and type of metabolites (conjugated or free)[45]. However, radio metabolism is not always possible due to animal ethics, economic or welfare restrictions and management issues. The Radio-metabolism study in male African elephants showed that majority of cortisol metabolites are excreted via urine, while testosterone metabolites through faeces[44].

Parallelism

Parallelism experiment has been used to determine the immunological activity between the antigen (steroid) and corresponding antibody with serial dilutions at 50% binding. The parallelism is achieved by the demonstration of parallel displacement curves between the pooled faecal extracts (endogenous antigen) and standards (exogenous antigen; Fig. 2A). However, the slope of the curve between the serially diluted pooled samples and standards should be parallel to validate the immunoassay[13]. A study on spotted hyena showed that non-parallel displacement curves between the faecal extracts and standards (Fig. 2B). This problem was rectified using additional purification steps in the extraction procedure and successful removal of an interfering substance from the sample matrix[38]. However, samples, which show non-parallel displacement curves cannot be used in the assay as they do not have immunological activity with antibody nor having any similarity with the standards (Fig. 2).

Accuracy of assay procedure

This test evaluates the accuracy of the immunoassays for measuring the true concentration (absolute values) of steroid hormone metabolites in the sample. However, interference via substances depends on the purification of sample or standard, matrix and solvent used for extraction all of which may influence the results. The accuracy test usually achieved by adding a known amount of unlabeled steroid hormones (standards) in the faecal extracts and estimated by plotting linear regression analysis of total amount observed by the amount expected in spiked faecal extracts[13].

Scope in wildlife conservation

Endocrine research in wildlife can be classified into three board areas such as theoretical (basic), diagnostic and management.

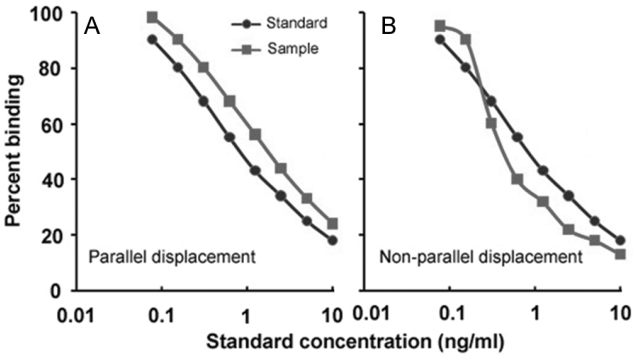

Fig. 2 — (A) Parallel; and (B) non-parallel displacement curves were obtained for each species by comparing serial dilutions of pooled faecal extracts (square) and standard (circle) preparation.

Monitoring of reproduction in free ranging and captive animals

Theoretical

Basic understanding of reproductive physiology is crucial for better management of animals in captivity and wild. Non-invasive hormone monitoring technique has been successfully used to assess gestation length, estrous cycle, ovulation and gonadal activity with reference to seasonality in a variety of wildlife species. For the first time, the gestation period of musk deer was estimated as 189±5 days using non-invasively collected samples[39]. Similarly, the gestation period of many wildlife species including red panda (150 days)[42], Asiatic lion (107 days), Bengal tiger (102 days) and Jaguar (98 days)[9] was estimated. Monitoring estrous cycle in captive animals by measuring faecal hormones has been reported in a variety of species for example in Okapi (14 days)[46], Asian elephant (112-115 days)[47], Eld's deer (18 days)[48], Nile hippopotamus (35 days)[49], and cheetah (13.6 days)[50].

Furthermore, faecal and urinary monitoring helped in understanding seasonal variations in reproduction in wide range of animals that include Black-footed ferret (*Mustela nigripes*), Fijian ground frog (*Platymantis vitiana*)[51], African wild dogs (*Lycaon pictus*)[52], Greylag Geese (Anseranser)[53], Eld's deer (*Rucervus eldii*)[54] and scimitar-horned Oryx (*Oryx dammah*)[55].

Diagnostic

Early pregnancy detection in captive animals would help in better management of post-delivery of mother and young ones in conservation breeding programs, as many big cats are very sensitive to human presence during and immediately after delivery. Therefore, a study was carried out to standardize pregnancy detection method in big cats using non-invasive hormone method[9]. Based on

extensive sample collections and HPLC analysis it was found that a progesterone metabolite (5α-pregnan-3α-ol-20-one) could be used as a potential marker for pregnancy diagnosis for Asiatic Lion, Bengal Tiger and Jaguar. The 5α-pregnan-3α-ol-20-one EIA has shown high sensitivity and low cross-reactivity with other steroids in the faeces and it increased significantly within 10-15 days after successful mating and remained elevated until parturition. A similar study was conducted using faecal prostaglandin F2α metabolite (PGFM) in big cats, which could detect and confirm pregnancy in the last trimester of the gestation period. The major advantage of PGFM assay is that it can be used in pseudo pregnancy, which is common among big cats. Further, the pregnancy could be diagnosed using a single sample collection and could be used in free-ranging animals[10]. The non-invasive method has been successfully used for pregnancy diagnosis in many wild animals including elk (*Cervus elaphus nelsoni*)[56], African elephants (*Loxodonta africana*)[57], baboons (*Papio cynocephalus*)[34] and dwarf mongooses (*Helogale parvula*)[58].

In birds, non-invasive methods could be used to study the relationship between reproductive success, physiological stress, migrant stress and human disturbance. Penfold[59] has successfully assessed the faecal androgen metabolites in male and female Kori Bustards (*Ardeotis kori*) to understand the seasonality, better breeding conditions, promote the reproduction and to check the differences in breeding and non-breeding seasons.

In reptiles, non-invasive faecal steroid analysis has been successfully used for endocrine profiling of free-ranging Cochin cane turtle (*Vijayachelys silvatica*) and Travancore tortoise (*Indotestudo travancorica*) in the southern Western Ghats. This study for the first time revealed that reproductive (progesterone, estradiol, testosterone) and stress hormones (cortisol) were significantly elevated and reflecting the aggressive behaviour among adults during the breeding season than the non-breeding season[16].

Stress assessment in captive and wild animals

Management

Non-invasive measures of physiological stress have tremendous potential to assess the neuroendocrine response in association with the physical stressor, anthropogenic disturbance and environmental effect on reproduction of mammals.

Bhattacharjee *et al*.[30] reported that anthropogenic disturbances, such as cattle grazing, encounters with human and vehicular traffic have significantly increased faecal glucocorticoid metabolites in reintroduced tigers of Sariska tiger reserve. This study further revealed that anthropogenic disturbances have directly affected the reproductive potential of reintroduced tigers (Fig. 3). A similar recent study on captive Asian elephants has shown that a longitudinal faecal hormone monitoring helped in better management and husbandry practices in zoos[13]. Further, this study revealed that elephant participation in public procession or festivals have significantly elevated the stress levels that directly affected the normal estrus cycle in females[13].

Faecal glucocorticoids are used as a biological indicator for activation of hypothalamo–pituitary–adrenal axis that alters the endocrine responses in response to multiple factors including psychological stressors (anthropogenic disturbance, tourism, environment effect)[30], socio-ecological pressure[60] and physical stressor (procession, injury, transport, body condition, restraint)[13,61]. Many studies have used the faecal endocrine monitoring as a tool for assessing factors affecting stress levels in right whales[62], effects of pastoralist activity, tourism, ecological pressure and anthropogenic disturbance in wild spotted hyenas[60], the impacts of long term vehicle exposure on physiology and reproduction of the northern spotted owl[45] and long term pattern of fGCM in white-tailed deer (*Odocoileus virginianus*) and

Fig 3 — Anthropogenic factors influencing fGCM concentrations in tigers of Sariska Tiger reserve

mourning doves (*Zenaida macroura*)[63]. A study on African lions (*Panthera leo*) has demonstrated that higher fGCM level was linked to anthropogenic activities, seasonal variation, human-lion conflict, and livestock movement[64].

Summary

This review showed that non-invasive hormone assay method could be used to address a wide range of problems in the conservation of endangered animals both in captivity and free-ranging wild animals. This technique has been extensively used across all taxa starting from fishes to mammals in both *in situ* and *ex situ* conservation efforts. Once the endocrine parameters are established in *ex-situ* conditions, non-invasive endocrine monitoring can be used inevitably as a potential conservation tool for welfare assessment in *in-situ* conservation. Non-invasive endocrinology is an advanced emerging discipline in conservation biology as it primarily provides valuable information for understanding the reproductive biology and stress physiology of captive and wild animals. It can contribute directly and indirectly to conservation of endangered animals.

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