# Fecal cortisol metabolites as indicators of stress in crab-eating-fox (*Cerdocyoun thous*) in captivity[1]

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**ABSTRACT.-** Paz R.C.R., Souza N.P. & Brown J.L. 2015. **Fecal cortisol metabolites as indicators of stress in crab-eating-fox (*Cerdocyoun thous*) in captivity.** *Pesquisa Veterinária Brasileira 35(10):859-862.* Faculdade de Agronomia, Medicina Veterinária e Zootecnica, Universidade Federal de Mato Grosso, Av. Fernando Correa da Costa, Boa Esperança, Cuiabá, MT 78060-900, Brazil. E-mail: reginacrpaz@gmail.com

Blood samples collection is a common method in biological research using domestic animals. However, most blood sampling techniques are complicated and highly invasive and may therefore not be appropriate for wildlife animals in research concerning stress. Thus, a non-invasive method to measure steroid hormones is critically needed. The first goal of this study was to determine how glucocorticoids concentrations are impacted by translocation and reproductive activity in crab-eating-fox (*Cerdocyoun thous*) in captivity. The physiological relevance of fecal glucocorticoid metabolites was further validated by demonstrating: (1) The translocation of a male to a females enclosure resulted in a 3.5-fold increase compared to baseline concentrations, (2) changes in adrenocortical activity, as reflected in concentrations of fecal cortisol metabolites during reproduction, gestation and lactation in females foxes, indicating that social interactions resulted in large increases of fecal glucocorticoids metabolites during the reproductive season. From these findings we conclude that fecal samples can be used for the non-invasive assessment of adrenocortical status in crab-eating-fox.

INDEX TERMS: Glucocorticoids, noninvasive method, enzyme immunoassay, translocation, reproduction.

**RESUMO.-** [**Metabolitos fecais de cortisol como indicadores de estresse em cachorro-do-mato (*Cerdocyoun thous*) em cativeiro.**] Coleta de sangue é um método comumente utilizado na pesquisa com animais domésticos. Entretanto, a técnica de coleta de sangue torna-se complicada e altamente invasiva em animais selvagens devido ao estresse, tornando-a inapropriada para pesquisa. Dessa maneira, métodos não invasivos utilizados na mensuração de hormonios tornam-se necessários. O principal objetivo deste estudo foi determinar como as concentrações de glucocorticoides atuam durante a translocação e a atividade reprodutiva de cachorro-do-mato (*Cerdocyoun thous*) em cativeiro. A relevância fisiológica da análise de metabolitos fecais de glucocorticoides pôde ser validada pela demonstração de que: (1) A translocação de machos para o recinto de fêmeas resultou em um aumento de 3.5 vezes comparado a concentrações basais; (2) mudanças na atividade adrenocortical, como reflexo das concentrações de metabolitos de cortisol fecal durante a reprodução, gestação e lactação em femeas de cachorro-do-mato (*Cerdocyoun thous*), indicaram que interações sociais resultaram em aumento de glucocorticoides fecais durante a época reprodutiva. Com estas constatações podemos concluir que amostras fecais podem ser usadas para acesso não invasivo da atividade adrenocortical em cachorro-do-mato (*Cerdocyoun thous*).

TERMOS DE INDEXAÇÃO: Glicocorticóides, métodos não invasivos, enzima imunoensaio, translocação, reprodução.

## INTRODUCTION

The crab-eating fox (*Cerdocyoun thous*) is a medium-sized canid found in Colombia, Venezuela, Guyana, French Guyana, Surinam, eastern Peru, eastern Bolivia, Paraguay, Uru-

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guay, northem Argentina and in most of Brazil outside the lowlands of the Amazon basin (Pessuti et al. 2001). The species is socially monogomous, had an annual estrous, with the reproductive season lasting from winter to spring, mating from June to September, and pups born from August to November. The duration of gestation ranged from 58 to 60 days (Sousa et al. 2012).

Concentrations of plasma cortisol have been widely used to evaluate stress responses in domestic animals. However, the blood sampling techniques usually applied in stress research are invasive and it is not convenient for the study of wildlife animals. The capture procedure to obtain blood samples by venipuncture causes stress and thus increased peripheral glucocorticoid concentrations within minutes (De Villiers et al. 1997).

Due to the increasing demand for methods to quantify adrenal activity in zoo and wild animals in response to introduction and transportation stress, after pharmacological and management precautions to minimize stress, and the necessity to investigate stress impacts on animal welfare, noninvasive methods for measuring adrenal activity by measuring fecal glucocorticoid metabolites by enzyme--immunoassays (EIAs) have been developed (Dehnhard et al. 2001).

Alternative noninvasive methods for the determination of fecal cortisol metabolites utilizing an enzyme immunoassay (EIA) have been established in a variety of species: primates (Sousa & Ziegler 1998, Whitten et al. 1997), ruminants (Palme et al. 1997, 2000), several felids (Graham & Brown 1996, 1997, Jurke et al. 1998), and African wild dog (Monfort et al. 1998).

However, it must be taken into account that metabolites are excreted with a species-specific time (Kotrschal et al. 1998, Goyamann et al. 2002, Wasser et al. 2000, Touma et al. 2003) and metabolism and excretion of glucocorticoids differ between species, and sometimes even between sexes and individuals within a given species (Touma et al. 2003). Therefore, it is not possible to draw analogous conclusions from other species. Thus, the aims of this study were to validate an enzyme immunoassay to quantify glucocorticoid metabolites in crab-eating-fox (*Cerdocyoun thous*) using male translocation and female reproduction.

## MATERIALS AND METHODS

**Animals and experimental design.** Permission for the animal experiment was given by SISBIO/IBAMA/Brazil (11167-1) and CEPA/UFMT Bioetical Committee (23108.002900/09-3). We used four females (F1, F2, F4 and F5) and two males (M1 and M2) crab-eating-fox (*Cerdocyoun thous*) housed at UFMT Zoo, Cuiaba, MT, Brazil. Female fecal samples were collected 3 times/week for one year and stored at -22°C until analyses. Individual fecals were identified by visualization with tail tricotomy and feeding dye. The animals were fed a daily with fruits (papaya, banana), fish or meat and commercial dog food dye added to treat. Water provided ad libitum. Before the beginning of the breeding season, in February, one male (M1) was moved to a females' (F1 and F2) enclosure, and two females (F4 and F5) were maintained with a male (M2), remaining two females and one male in each of two enclosures during the reproductive season, with mating occurring from June to September. Two females (F2 and F4) became pregnant and pups born from August to November. Female F5 was removed from its enclosure, while male M2 and female F4 remained together during the study period. Female F2 was maintained alone during parturition and lactation, from August until the end of the study.

**Study area and climate.** The study was conducted between January to December/2008 at Universidade Federal de Mato Grosso Zoo, Pantanal, MT, Brazil, located at -15 latitude and -56 longitude, 186 altitude, with a tropical climate. Minimum and maximum air temperature range from 15 to 23 and from 29 to 36°C, respectively. An annual rainfall presents a typical distribution of rain characterized by wet season (Summer/Fall, 100 to 200mm), and arid season (Winter/Spring, 0 to 50mm). (INPE/CPTEC 2009).

**Fecal steroid analyses.** Extraction of fecal samples followed the procedure described by Schwarzenberger et al. 1998, with minor modifications. Fecal samples were thawed and mixed. For each sample, 0.5g of homogenized wet feces was suspended in 5mL of 80% methanol and homogenized. Tubes were vortexed (30s) and gently homogenized for 15 h. Subsequently, samples were centrifuged (1,300*g* for 15 min) and the supernatant kept in a water bath at 60°C until total evaporation of the 80% methanol. The samples were suspended in 1 mL dilution buffer [$NaH_2PO_4$ (5.4g), $Na_2HPO_4$ (8.66g), NaCl (8.7g), $H_2O$ Mili-Q (1 L), pH (7.0)] and vortexed for about 1 min. An aliquot of the supernatant was diluted with dilution buffer (final dilution: 1:30) and frozen at −22°C until EIA analysis.

For the cortisol EIA, 50µl of cortisol R4866 antibody (Coralie Munro, UC Davis, California) in coating buffer [$Na_2CO_3$ (1.59g), $NaHCO_3$ (2.93g), $H_2O$ Mili-Q (1 L), pH (9.6)] (1:8500) was pipetted onto NUNC microtitre plates and incubated overnight at 5°C. Plates were washed five times with Milli$QH_2O$/Tween 20 (1:500,000), then standards (3.9-1000pg/well), low and high controls, each sample in duplicate and cortisol HRP (Coralie Munro) in assay buffer [Tris (2.42g), NaCl (17.9g), BSA (1g), Tween 80 (1mL) $H_2O$ Mili-Q (1 L), pH (7.5)] (1:20,000) was pipetted into the microtiter plate wells and incubated for 1 h at room temperature. Plates were washed again with MilliQH2O/Tween 20 and 100µl of 40µl 0.5 $H_2O_2$, 125µl 40mM ABTS and 12.5ml substrate buffer was added to each well. The absorbance was measured at 450nm/540nm, using a DYNEX MRX reader (Dynex Technologies, Chantilly, VA, USA).

## RESULTS

### Male introduction

In female F1 fecal glucocorticoid levels increased after male (M1) introduction returning to the base line after approximately one month, became significantly higher than initial base line levels during reproductive season (June/July). (Fig.1).

Fecal glucocorticoid levels in female F2 increased after a male (M1) introduction and maintained significantly higher than initial base line levels until the end of reproductive season (Fig.1), returning to the base line only after translocation of female F1 and male M1 to another enclosure, one month before parturition. (Fig.2).

### Female reproduction

Both pregnant females (F2 and F4) showed a peak of glucocorticoids levels before parturition (Fig.2) returning to the baseline during lactation period. The glucocorticois levels increased after 28-30 days of lactation.

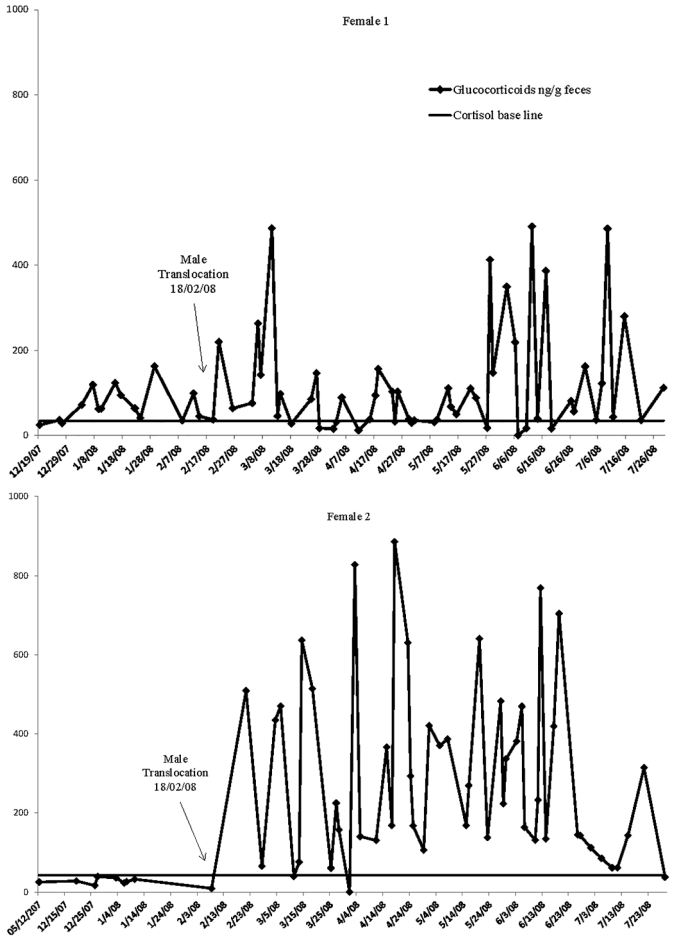


Fig.1. (**A,B**) Fecal glucocorticoid metabolites (ng/g feces) of females crab-eating-fox (*Cerdocyoun thous)* before, during and after male translocation.

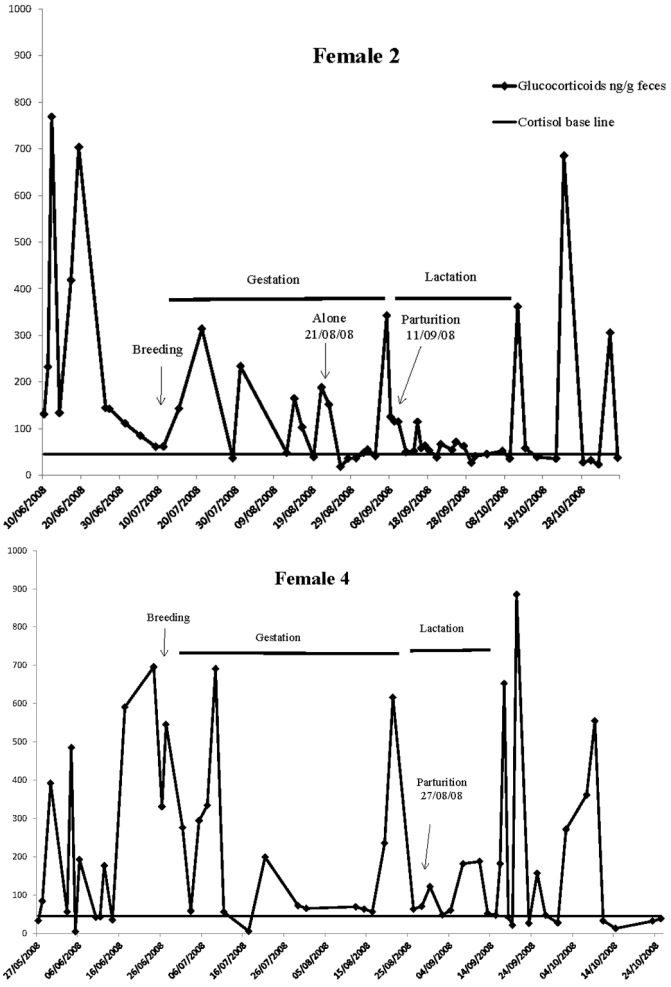


Fig.2. (**A,B**) Fecal glucocorticoid metabolites (ng/g feces) of pregnant females crab-eating-fox (*Cerdocyoun thous)* before, during and after parturition and lactation.

Non-pregnant females (F1 and F5) showed fecal glucocorticoid levels increased during reproductive season (June/July) returned to the baseline after this period.

## DISCUSSION

In this study, we successfully tested and physiologically validated a noninvasive method for evaluating adrenocortical activity in the crab-eating-fox. This method offers the novel possibility of quantifying the level of stress induced in free-ranging and captivity populations of this species, since feces can easily be collected.

Traditionally, glucocorticoids have been measured in blood plasma; however, capture and blood sampling procedures are usually stressful (Sapolsky 1982, Wingfield et al. 1992). Thus, noninvasive methods, such as measuring glucocorticoids in feces, are desirable because animals need not be captured and several samples can be obtained from individuals.

Monitoring stress hormones may also become a useful tool for identifying situations likely to cause stress. This information would be of value both for the conservation management of wild crab-eating-fox populations and for the welfare of animals in captivity.

Concentrations of faecal cortisol metabolites were significant increased following ACTH challenge, indicating that adrenocortical activity can be monitored via fecal sample analyses in crab-eating-fox (Paz et al. 2014).

Together with other findings it is clear that a social conflict induces a stress response, which has a similar impact on behavior and physiology in mammals. In this study we demonstrated a difference in cortisol metabolites secretion in response to female stress after male introduction and during reproductive season.

These data provide the first evidence of an adrenocortical response to a social conflict in crab-eating-fox; at the same time they provide evidence for a physiological basis of reproductive behavior.

Social tension between a male and females crab-eating--fox led to a significant increase in fecal glucocorticoid metabolites in both females after onset of the social tension (Fig.1). The patterns of glucocorticoid clearance were different, levels of female F1 returned to baseline after 30 days, however female F2 maintained significantly higher than initial base line levels until translocation of female F1 and male M1 to another enclosure (Fig.2), suggesting the adrenal activity was highly variable in individual foxes.

Our results suggest that interindividual differences in

sensitivity to stressors occur, indicating that social stress may affect differently individuals. However, no differences were found in reproductive aspects (gestation, parturition and lactation) in crab-eating-fox females. A significant increase in fecal corticoids was observed before parturition and after 28-30 lactation's days in both pregnant females, suggesting a social tension reflected by pup's interaction.

The physiological relevance of fecal glucocorticoid metabolites was further validated by demonstrating that (1) two females display the same reactivity in the adrenocortical response to a male introduction showed increased in fecal glucocorticoids levels, however the intensity showed difference, suggesting the adrenal activity was highly variable in individual foxes, (2) changes in adrenocortical activity, as reflected in concentrations of fecal cortisol metabolites before, during and after the reproductive period in females foxes, indicating that social interactions resulted in large increases of fecal glucocorticoids metabolites during the reproductive season. From these findings we conclude that fecal samples can be used for the non-invasive assessment of adrenocortical status in crab-eating-fox.

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