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Physiological Validation of Fecal Glucocorticoids in Male and Female Primates of the Species *Callithrix jacchus*

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Abstract

Techniques for monitoring adrenal activity through non-invasive methods are increasingly being used. However, for the data obtained to be reliable, it is necessary to validate these techniques. Wild animals kept in captivity are constantly subjected to stressful situations. Chronic stress can adversely affect the physiological responses of individuals. The aim of this study was to conduct physiological validation for fecal glucocorticoid metabolites for the species of non-human primates (*Callithrix jacchus*). We used 12 adult animals (seven males and five females). During the experimental period, the subjects were kept in a closed room under artificial light and controlled temperature. For validation we used the physiological challenge of synthetic ACTH (adrenocorticotropic hormone). Possibly due to the high degree of stress experienced by the animals and individual variation, it was not possible to validate the technique.

Keywords

Stress, Physiological Validation, Fecal Cortisol, Marmoset

1. Introduction

In the last decades, several advances have been made in improving methods of quantifying urinary and fecal steroid metabolites so that they are currently used in the investigation of endocrinology and behavioral relationships in many domestic and wild species [1] [2].

Although there is no accurate definition of stress, it usually refers to a variety of responses to stressor stimuli that can originate from external or internal sources and can alter homeostasis.

These stimuli can be physical, behavioral or psychological [3] [4]. The duration and intensity of the stimulus differentiate their impact on the body. If the stimulus is long-standing usually is considered negative (chronic stress); when the duration is short it can be considered positive (acute stress) [5] [6].

Once a reaction to a stressor occurs there is a marked increase in the release of glucocorticoids from the adrenal cortex and the glucocorticoid levels are also increased in the plasma and urine [7]-[11].

In recent years, the alternative techniques for measuring fecal glucocorticoid metabolites have been increasingly used mostly for being suitable to monitor the activity of the adrenal in a large number of vertebrates including primates. Cortisol is the main glucocorticoid in many mammals; numerous studies have therefore used it as an indicator of stress in Callithrix spp. [12]-[18].

The use of assays for monitoring the endocrine function of adrenal through measuring fecal glucocorticoid metabolites may not be entirely reliable; therefore, it is required to conduct physiological and laboratory validation of these techniques [2] [14] [19].

The objective of the present study was to perform the physiological validation of the Enzimeimmunoassay technique (EIA) in order to measure the fecal glucocorticoid metabolites in common marmosets (*Callithrix jacchus*) through the administration of Adrenocorticotropic hormone (ACTH).

2. Material and Methods

This study was conducted in the School of Veterinary Medicine and Animal Sciences—FMVZ at the São Paulo State University "Júlio de Mesquita Filho"—UNESP, Botucatu SP, Brazil, 22˚53'09" South latitude and 48˚26'42" West longitude with an average annual temperature of 22˚C. All procedures were registered on the System for Authorization and Information on Biodiversity according to the Chico Mendes Institute for Biodiversity (SISBIO/ICMBIO Protocol No. #24055-1) The experiment had the approval of the Ethics Committee on Animal Use São Paulo State University "Júlio de Mesquita Filho"—Botucatu, SP, Brazil, warrant number 126/2010.

A total of 12 adult animals of the species *Callithrix jacchus* (seven males and five females) were used. Before the beginning of the study the animals were housed in cages measuring 60 cm in height, 120 cm in width, and 60 cm in depth, and kept in CEMPAS (Center of Medicine and Research in Wild Animals) at the São Paulo State University—UNESP—Botucatu under natural conditions of temperature and a light-dark cycle. Before the experimental period, the structure provided to the animals was inadequate, allowing the marmosets to have olfactory and visual contact with various animals, including predators housed in adjacent enclosures. The marmosets were also exposed to various sounds and the presence of unfamiliar people. CEMPAS (Center for Medicine and Research in Wild Animals) is part of the São Paulo State

University "Júlio de Mesquita Filho"—UNESP—in Botucatu. The site was designed with the aim of bringing undergraduate and graduate students closer to clinical practice and research involving wild animals. CEMPAS receives animals seized in irregular situations, as well as animals in need of specialized veterinary care from Zoological Parks, Screening Centers, and provides care to wild animals that are legally kept as Pets. It currently offers postgraduate programs in wild animals, as well as being the first place in the country to offer a residency course in the field.

During the experimental period the marmosets were removed from CEMPAS and kept in a closed room under artificial conditions of light and temperature. The animals were housed in this room for two months before the experiment to allow for adaptation. Only people accustomed to handling the animals were allowed to enter the area. This precaution was taken to avoid outside interference that could alter the results of this study, although the external sounds of predators could still be heard by the animals. Two meals a day were provided, consisting of fruits, vegetables and eggs in the morning and a protein paste in afternoon (monkey food ALCON® plus banana, honey and sustagem®). As a supplement, mealworms (Tenebrio molitor) were given once a week.

The animals were divided into two experimental groups: the first was the Control Group (CG) (without ACTH injection) and the second was the Treated Group (TG) (with ACTH injection). The two groups were housed in separate locations, meaning they were kept apart so that one group did not interfere with the other. The procedures for both groups were conducted on a single day and repeated at an interval of 30 days. A rotation system was applied, meaning the animals that participated in the control group after 30 days were switched to the treatment group and vice versa, so that all animals went through both groups, ensuring that a control group was maintained throughout the experiment.

For physiological validation, 0.33 mL of Synacthen Depot® solution per kg of body weight was administered by intramuscular injection to the animals in the Treated Group. This procedure took place between 6:00 and 6:30 a.m.

The Synacthen Depot® (tetracosactrin) is a synthetic adrenocorticotropic hormone (ACTH) and was the first preparation of corticotrophin to be produced entirely by synthesis, exhibiting all the pharmacological properties of endogenous ACTH. It was acquired from Novartis Pharmaceuticals Canada Inc.

Feces were collected in Eppendorf® tubes with a spatula, homogenized, and stored in a freezer at −20˚C until extraction. All feces produced were collected hourly, between 6:00 a.m. and 6:00 p.m. for three days prior to ACTH administration, on the day of injection, and for three days afterward, ensuring that the cortisol peak was not missed. After each collection, the tray liner was changed to avoid possible contamination of future samples.

A rotation system was applied, meaning that the animals in the Control group after 30 days switched to the Treatment group and vice versa, ensuring that all animals experienced both conditions.

The hormonal extraction of fecal samples was performed at the Laboratory for Research on Acute Phase Proteins and Non-Invasive Monitoring of Animal Reproduction and Welfare at the School of Veterinary Medicine and Animal Science—FMVZ at São Paulo State University "Júlio de Mesquita Filho"—UNESP, Botucatu, SP, Brazil.

The methodology was described by [2]. An aliquot of 0.25 g of wet feces was weighed and homogenized in polystyrene tubes with lids. After weighing, feces were diluted in 2.5 mL of 80% methanol and mixed using a vortex for 30 minutes. Then the extract was centrifuged at 500 g for 20 minutes. After this step, 500 µL of the supernatant was transferred to previously identified Eppendorf® tubes, which were then placed in an incubator overnight to dry.

The dried extract was transported in Eppendorf® tubes to the Veterinary Medicine University of Vienna (Austria). The samples were reconstituted, and the measurement of fecal glucocorticoid metabolites was carried out under the supervision of Dr. Rupert Palme, following the protocol described by [20].

3. Statistical Analysis

For statistical analysis we used the student's t-test to compare the two groups.

4. Results and Discussion

The results of this experiment suggest that some animals that received the ACTH injection did not exhibit the expected physiological response in their feces; that is, there were no significant increases in fecal cortisol metabolite concentrations after ACTH administration (**Table 1**).

Table 1. Mean values of the concentration of fecal glucocorticoid metabolites (ng/g) two days before the application of ACTH, on the day of and two days after application.

	day −2	day −1	day 0	day +1	day +2
Mean c	80 ± 177 *A*	230 ± 634 *B*	81 ± 177 *C*	40 ± 41 *D*	33 ± 21 *E*
Mean t	47 ± 114 *A*	41 ± 80 *B*	216 ± 376 *C*	41 ± 63 *D*	81 ± 133 *E*

Uppercase kind in the same column indicates no statistical difference.

Furthermore, several individual variations were observed in the concentrations of glucocorticoid metabolites (**Figure 1**).

The cortisol and corticosterone are catabolized by the liver and undergo the action of intestinal bacteria prior to being excreted in the feces, resulting in a large number of metabolites and few native hormones present [1] [21]. According [22] the differences in fecal metabolites are due to peculiarities of the intestinal microbiota.

The lack of physiological response to ACTH injection is possibly due to the high degree of stress experienced by the animals during the time they were kept in

CEMPAS. Situations including intense cold, the presence of predators in the next room, sounds and presence of strangers caused intermittent diarrhea in the animals suggesting a degradation of metabolites during the process of excretion, probably caused by the alteration of the intestinal flora. Furthermore, the general literature suggests several possible neuroendocrine alterations underlying cortisol dysfunction: cortisol depletion, insufficient free (unbound) cortisol, impaired cortisol or CRH secretion, glucocorticoid receptor (GR) resistance or downregulation, or hypersensitivity of the negative feedback system [23].

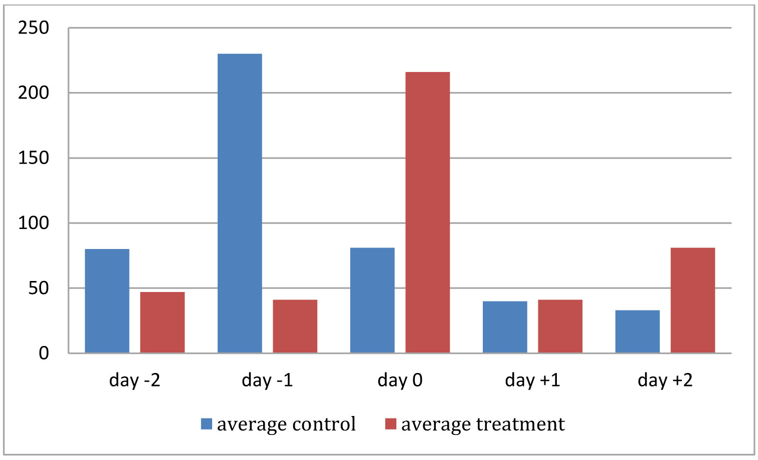

Figure 1. Representation of the average of measures (ng/g) two days before the application of ACTH, on the day of and two days later application.

Increased cortisol levels have been well documented in primates following stressors such as loud unfamiliar noise and human activity *Callithrix jacchus* [24], and maternal separation [25]. Observing other animals undergoing procedures involving isolation [13] and the death of a social group member *C. jacchus* [24] have also been shown to be physiologically stressful.

The majority of the fecal metabolites are excreted as an unconjugated form, also called the free form. This is due to enzyme activity in feces and the activity of fecal bacteria on the metabolites that were conjugated in the liver and eliminated via bile into the duodenum [19].

According to [26], an imbalance in the intestinal microflora that produces harmful effects is called dysbiosis, which interferes with the integrity of the gut. Toxic agents are bioactivated by enzyme systems of intestinal bacteria, and this process is promoted more rapidly in intestines with an unbalanced population of microorganisms. Stress is one of the factors that can alter the intestinal microbiota [27]. Furthermore, gut microbiota also helps regulate HPA hormones like cortisol which has important implications for host metabolism, stress response, and anti-inflammatory pathways [28].

In addition, many diseases and syndromes cause significant losses of *Callithrix jacchus* in breeding colonies. Among these, Progressive Weight Loss Syndrome occupies a prominent place. According to [29], one of the predominant characteristics observed as indicative of PWL syndrome is colitis and frequent episodes of diarrhea. The Progressive Weight Loss Syndrome is considered a multifactorial syndrome that may be associated with stress.

On their arrival at CEMPAS, none of the marmosets had diarrhea. The symptoms began four months later. At that time, there was no possibility of moving them to another room or improving the structure of the place to reduce stress. During this period, two marmosets died with suspected Progressive Weight Loss Syndrome, and despite preventive measures—such as environmental changes and deworming—diarrhea persisted, even if in a less severe form.

The individual variations observed in the concentrations of cortisol metabolites may be associated with the heterogeneity of the group used. Some animals were rescued from illegal captivity and could have been accustomed to a variety of agents perceived as stressors, while others may not have been. Furthermore, [30] observed a reduced cortisol peak and faster recovery in marmosets subjected to stress; such down-regulation may be an adaptive mechanism to prolonged exposure to high cortisol levels. Overall, these results suggest that cortisol decreases associated with stress may be a common feature across primates.

Under the conditions of this study, we observed that different populations of the same species may have different concentrations of glucocorticoid metabolites when subjected to different degrees of stress or handling. Thus, it is important to consider the individual experiences of animals. [31] wrote a review on the five physiological mechanisms that regulate the release of glucocorticoids during stress. According to him, mechanisms such as previous experiences, physiological acclimation, and stress facilitation must be taken into account when designing and analyzing data on glucocorticoid measurements, since these factors modulate the physiological response and may produce unexpected results.

According to [32], the response to various types of acute stress in humans and animals is well known and is characterized by an increase in HPA axis activity, resulting in higher ACTH and cortisol concentrations [33]. In animals, repeated acute stress episodes cause HPA axis adaptation [34]. However, data on HPA axis responses to prolonged stress are conflicting. In this study, both activation and suppression of the axis were observed. Nevertheless, the HPA response pattern appeared to be dependent on age and the type of stressor [35].

Mobbing alerts other individuals to potential dangers, elicits a group response or attack against the threat source, and buffers the hormonal stress response [36] [37].

According to [38], animals exhibit individual temperament traits and respond differently to the stress of captivity. There is substantial evidence indicating that individuals or entire populations consistently differ in their behavior [39]. These behavioral differences result from variations in temperament traits and can significantly affect how individuals interact with their environment [40].

The literature describes the common marmoset (*Callithrix jacchus*) as an adaptable species in captivity. However, based on the results obtained in this study, we observed that some individuals are less adaptable when exposed to intense stress.

Environmental conditions and characteristics of the captive setting can strongly influence stress levels in animals [41].

5. Conclusions

The physiological validation for fecal glucocorticoid metabolites in the studied species could not be fully achieved, possibly due to the animals' prior exposure to chronic stress and the lack of detailed background information on their history.

Thus, to obtain reliable results in studies using these animals as an experimental model, it is essential to minimize prior chronic stress, provide a stable environment with adequate protection against temperature variations, and shield them from external disturbances such as visitors, loud noises, and other animals. Additionally, using animals from a well-documented strain with a cohesive colony can help improve the consistency of physiological responses.

Ethical Declaration

All procedures were registered in the System for Authorization and Information on Biodiversity, in accordance with the Chico Mendes Institute for Biodiversity (SISBIO/ICMBIO Protocol No. 24055-1). The experiment was approved by the Ethics Committee on Animal Use (CEUA) at São Paulo State University "Júlio de Mesquita Filho"—Botucatu, SP, Brazil (warrant number 126/2010).

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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