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ARTIGOS

Measuring fecal glucocorticoid metabolites of an endangered Neotropical primate: technical details of a physiological validation

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Abstract: Measuring fecal glucocorticoid metabolites (FGM) has become a potent non-invasive tool in ethological studies and conservation biology of threatened species, including primates. However, species differences (among other factors) in excreted metabolites require an experimental validation of the applied method to prove that the enzyme immunoassay (EIA) protocol actually quantifies relevant hormone metabolites. Here, we performed such a physiological validation of an EIA to measure FGM of black lion tamarins (*Leontopithecus chrysopygus*) through an ACTH challenge. We used six black lion tamarins (4 males and 2 females) kept in the Primatology Center of Rio de Janeiro (Brazil). We tested two different EIAs, and our results validate the cortisol EIA (measuring FGM with a 21-ol-20-one structure) for black lion tamarins. The time lag between ACTH challenge and detection of FGM peak was between 20 and 25 hours, and response duration lasted between 6 and 9 hours. This is the first physiological validation of an EIA protocol for the black lion tamarin. Our research shows how a physiological validation can be adapted to an endangered primate species, dealing with a low availability of individuals and ethical considerations linked to specific conservation status.

Key-Words: ACTH challenge; Black lion tamarins; *Leontopithecus;* stress.

Resumo: Medindo metabólitos fecais de glicocorticoides de um primata Neotropical ameaçado de extinção: detalhes técnicos da validação fisiológica. A quantificação de metabólitos fecais de glicocorticóides (MFG) tem se tornado uma ferramenta não invasiva importante em estudos etológicos e biologia da conservação de espécies ameaçadas de extinção, incluindo primatas. Dadas possíveis diferenças interespecíficas nos metabólitos excretados, uma validação experimental é necessária para provar que o protocolo de Ensaios Imunoenzimáticos (EIA) está realmente quantificando metabolitos de glicocorticoides. Aqui, foi realizada uma validação fisiológica do protocolo EIA para medir MFG de micos-leões-pretos (*Leontopithecus chrysopygus*) através de um desafio de ACTH. Foram utilizados seis micos-leões-pretos (4 machos e 2 fêmeas) mantidos no Centro de Primatologia do Rio de Janeiro (Brasil). Foram testados dois EIAs diferentes, e os resultados validaram o EIA-cortisol (medindo MFG com a estrutura 21-ol-20-one) para micos-leões pretos. O tempo entre o desafio de ACTH e a detecção de um pico de MFG nas fezes foi entre 20 e 25 horas, e a resposta durou entre 6 e 9 horas. Esta foi a primeira validação fisiológica feita com um protocolo EIA para o mico-leão-preto. Esse trabalho mostra como uma validação fisiológica pode ser adaptada para uma espécie ameaçada de extinção, que apresenta baixa disponibilidade de indivíduos e considerações éticas relacionadas ao status de conservação.

Palavras-Chave: Desafio de ACTH; Mico-leão-preto; *Leontopithecus;* Validação fisiológica; Estresse.

INTRODUCTION

Animals have a suite of behavioral and physiological responses to cope with environmental, physiological, or psychological challenges (Romero, 2002; Romero, 2004; Sapolsky, 1990; Sheriff *et al.,* 2011; Touma & Palme, 2005). Meaningful stress events lead to the activation of the hypothalamic-pituitary-adrenal axis (HPA) in vertebrates and this activation results in the release of glucocorticoid hormones from the adrenal cortex (Sapolsky *et al.,* 2000). Therefore, measurement of glucocorticoids has been used to investigate questions that involve stress, animal welfare, reproductive physiology, behavioral ecology, conservation biology issues and biomedical research in a high number of species, both in captivity and in the wild (Palme, 2012; Sheriff *et al.,* 2001).

Blood samples were initially used to quantify glucocorticoids, but the stress generated by the capture and contention of the subjects can bias the results. Therefore, researchers are now using non-invasive techniques through the measure of glucocorticoids (or their metabolites) from different matrices, such as urine, feces, and hair (Sheriff *et al.,* 2011). After release into the bloodstream, glucocorticoids are metabolized by the liver and kidneys and then excreted via feces and urine (Heistermann *et al.,* 2006; Wheeler *et al.,* 2013). Consequently, hormone metabolites are found in the feces, rather than the native hormone itself (Heistermann *et al.,* 2006). Furthermore, the metabolism and excretion of glucocorticoids can differ between species and even between sexes of the same species (Heistermann *et al.,* 2006; Touma *et al.,* 2003; Wheeler *et al.,* 2013). Differences in ingestion, metabolism, and defecation rate (among others) lead to species differences in the kind of metabolites presented and in the time lag between cortisol release by the adrenal cortex and the appearance of the hormone metabolites in the feces (Anestis, 2010; Wheeler *et al.,* 2013).

Since the method used to quantify FGM is an enzyme immunoassay based on cross-reactions between an antibody and the hormone metabolites (Möstl *et al.,* 2005), a validation experiment is necessary to ensure that the EIA used is indeed measuring relevant glucocorticoid metabolites. Due to the species differences mentioned above, this validation should be conducted for each species where FGM are measured. In addition, this kind of experiment aids unraveling the time needed by each species to metabolize and excrete cortisol (now as metabolites) into the feces (an important information for data analysis and for planning future experiments). Consequently, validating this method is a major step for an application in numerous fields of knowledge (Wheeler *et al.,* 2013).

There are two types of experiments to validate the EIA protocol: biological and physiological validations (Touma & Palme, 2005). The physiological validation is the most common and reliable method and consists of generating a significant alteration in circulating glucocorticoid levels pharmacologically then performing the EIA being tested and verifying if it can detect this alteration. Three drugs can be used in such an experiment: a) synthetic adrenocorticotropic hormone (ACTH), which induces glucocorticoid release and, therefore, should provoke a peak of FGM levels; b) dexamethasone, a synthetic glucocorticoid that inhibits glucocorticoid release due to negative feedback, causing a decrease of FGM levels; and c) saline solution, used as a control for the experimental procedures (Touma & Palme, 2005). For a most reliable validation, it is suggested to use the three treatments, with several animals in each one (Touma & Palme, 2005). This process requires a large sample size and/or a permission procedure with the same animals, which sometimes is not possible, particularly when working with endangered primates. When the use of all three drugs is not possible, the best alternative is to perform only the ACTH challenge.

When some ethical problems arise and a physiological validation, even in its simplest form, is not possible, a biological validation may be used. This type of validation consists of measuring FGM before and after an unavoidable stressful event such as the contention, transportation or physical examination of the individuals (Touma & Palme, 2005). However, this process is sometimes inconclusive because the animals may be habituated to such procedures and this may not be stressful enough to detect clear peaks of FGM (Fanson *et al.,* 2017).

Both kinds of validations have been performed in numerous taxa of primates, such as chimpanzee (*Pan troglodytes*), gorilla (*Gorilla gorilla*), barbary macaque (*Macacas ylvanus*), long-tailed macaque (*Macaca fascicularis*), common marmoset (*Callithrix jacchus*) (Heistermann *et al.,* 2006), spider monkeys (*Ateles hybridus*), red howler monkeys (*Alouatta seniculus*) (Rimbach *et al.,* 2013), capuchin monkeys (*Sapajus* spp.) (Wheeler *et al.,* 2013), *Callithrix penicillata* (Pizzutto *et al.,* 2015), golden lion tamarins (*Leontopithecus rosalia*), Goeldi's marmoset (*Callimico goeldii*), white-fronted marmoset (*Callithrix geoffroyi*) and pied tamarin (*Saguinus bicolor*) (Wark *et al.,* 2016). One threatened neotropical primate, the black lion tamarin (*Leontopithecus chrysopygus*), has been the focus of both *in situ* and *ex situ* efforts for its conservation. In this light, a validation of the experimental protocol may be helpful to improve the welfare of the individuals kept in captivity and to determine the physiological condition of the *in situ* populations in different environments. In this way, the aim of this work was to validate an EIA experimental protocol (Palme & Möstl, 1997; Palme, 2005) for black lion tamarins.

MATERIAL AND METHODS

Subjects and housing conditions: The subjects of the experiment were six adult black lion-tamarins (four males and two females) (Table 1), previously habituated to the observer and kept in the Primatological Center of Rio de Janeiro (CPRJ), Guapimirim, RJ, Brazil. The CPRJ is located in the Três Picos State Park (Paraíso Center), an Ecological Reserve closed to visitation. The focal individuals were members of different social groups: a male-male pair, a male-female pair with an offspring in adult age (family) and a male-female pair with their twins, of which we only used the female. All the animals used in the experiment were considered healthy by the CPRJ staff.

Each group was housed in a 3.0 × 6.0 × 2.5 m enclosure containing tree branches and a sleeping box. The roof was covered with large tiles and the floor was covered by dried leaves. They received two meals in the morning; one at 8 a.m. composed by monkey chow or bread with vitamins dissolved in milk and, a second one at 11 a.m., composed of seasonal fruits (banana, apple, grapes, cucumber, orange, pineapple, and watermelon). Water was available *ad libitum* between 8 a.m. and 4 p.m.

Table 1: List of the individuals included in the validation experiment with their body mass, amount of injected ACTH and time of injection.

Individual	Housing condition	Studbook Number	Sex	Body Mass (g)	Amount of ACTH injection (ml)	Time of injection
F1	Family	#408	Female	600	0.18	11:09
F2	Male-female pair with their twins	#481	Female	650	0.18	11:47
M1	Family	#486	Male	650	0.18	11:15
M2	Family	#312	Male	700	0.21	11:00
M3	Male-male pair	#387	Male	680	0.18	11:24
M4	Male-male pair	#488	Male	710	0.21	11:35

Fecal marker assessment: As the individuals were housed together and could not be isolated because of CPRJ requirements, fecal markers had to be used to identify the feces of each individual. We tested five kinds of treats and seven colors of food coloring in search of the most suited combination. The treats tested were: balls of mashed banana with Neston (mix of cereals), oat flour and Farinha Lactea (in Portuguese – composed of wheat flour, dried milk and sugar), jellybeans, banana candy, dried grapes and larvae. Food colors were: green (gel arcolor/soft gel mix), black (soft gel mix), blue (soft gel mix), purple (gel arcolor), orange (gel arcolor), red (soft gel mix) and pink (Corallum mix/soft gel mix). For this experiment, we used the mashed banana treat and the green and pink food coloring due to their higher performance. Each individual received separately a colorful treat twice a day before their meals.

ACTH challenge and sample collection: As the animals were not removed from their enclosures for this experiment, there was no necessity to habituate them to new cages. The experiment lasted five days (from July 20 to July 24, 2016). Feces were collected during the two days preceding the ACTH injection, on the day of the ACTH injection and during the two days following the injection, to outline a FGM profile with previous and posterior basal levels, and ensure the sampling of FGM peaks. The individuals were observed every day from 6 a.m. (before leaving their sleep box) to 5 p.m. (after entering into their sleep box) and the fecal samples collected every hour from 7 a.m. to 5 p.m. During the time of the experiment, we did not find any sample from the nighttime. We collected 171 samples, an average of 34 samples per individual and placed them in polypropylene tubes, identified and stored in a freezer.

On the day of the ACTH injection (July 22), the individuals were captured and weighed with the help of the animal caretakers and the veterinarians. Based on the body mass of each individual, the veterinarians injected an intramuscular single dose of ~31 IU/kg of Synacthen (Tetracosactidehexaacetate) (Heinstermann *et al.,* 2006).

Steroid Extraction: The samples were transported to the Behavioral Endocrinology Laboratory of the Psychology Institute of the São Paulo University (USP). First, the samples were removed from the freezer and thawed at ambient temperature. All samples of each individual voided in the same 1-hour period were homogenized with a spatula. An aliquot of 0.2 g was weighted by an analytical balance and transferred into a new propylene tube. Then, 2 ml of methanol 80% were added and the samples were vortex-mixed for 30 min. All the samples were centrifuged at 3,000 rpm for 10 min, and the supernatant was removed and stored in a new 2 ml polypropylene tube at -20°C. This extract was dried in a water bath coupled to an airflow and then transported to the Vetmeduni Vienna (Austria), where they were re-suspended in 2 ml of methanol (80%) before EIA analysis. More details about this method are available in Palme *et al.* (2013).

Enzyme immunoassays of fecal glucocorticoid metabolites: Before analyzing all samples, we tested two EIAs (cortisol EIA (Palme & Möstl, 1997) and 11-oxoetiocholanolone EIA (Möstl *et al.,* 2002)) in a subset of samples (all samples from F1 and M3): The cortisol EIA was the one chosen for subsequent analyses. Although cortisol itself is rarely present in the feces (Bahr *et al.,* 2002), this assay shows enough cross-reactivity with some of its metabolites (sharing a 21-ol-20-one structure; Heistermann *et al.,* 2006). We expressed the concentration of FGM in nanograms per gram of fresh fecal matter.

Data Analysis: We made a graph showing the FGM profile of each individual during the five days of experiment. To validate the method of FGM measurement, a pronounced peak of the FGM levels must appear (Rangel-Negrín *et al.,* 2014) after the ACTH injection. To detect these peaks, we calculated the following individual metrics: 1) "basal" – the FGM median of all samples two days before the injection; 2) "peak" – the highest FGM value after the ACTH injection (we considered peaks wild-outliers and outliers pointed by the boxplot graphics function in IBM SPSS 20 through an iterative removal process); 3) "increase rate" – rate between "peak" and "basal"; 4) "Latency" – period of time between the ACTH injection and the first (in case of more than one) "peak"; 5) "time to the first sample after the injection" – interval between the injection and the next fecal sample; 6) "Response duration" – time elapsed between the beginning of the first peak and the end of the last peak and; 7) "Peaks mean" – the mean of FGM value of all outliers and wild outliers.

This research complied with protocols approved by the Animal Research Ethics Committee (CEUA) of the Institute of Biosciences of the São Paulo State University (UNESP – Campus of Rio Claro) (protocol number 7733)

Table 2: Results from the ACTH challenges, with five black lion tamarins (*Leontopithecus chrysopygus*) on the CPRJ/Guapimirim, RJ using a cortisol EIA (A1) described by Palme and Möstl (1997) and an 11-oxoetiocholanolone EIA (A2) described by Möstl *et al.* (2002).

	Antibody	Basal	Peak	Increase rate	Latency	Time to the first sample after the injection	Response duration	Peak's mean
F1	A1	390	8565	22	20 h	4 h	9 h	4926
F1	A2	999	3616	4	20 h	4 h	—	3452
F2	A1	305	4620	15	23 h	2 h	4 h	3428
M1	A1	53	1369	26	21 h	1 h	6 h	1417
M2	A1	90	2570	29	22 h	4 h	8 h	1455
M3	A1	349	2983	9	25 h	3 h	6 h	3685
M3*	A2	1428	3947	3	20 h	3 h	—	—
M4*	A1	255	8735	34	23 h	3 h	—	—

FGM values in (ng/g wet feces).
* did not show any outliers or wild outliers.

and the protocols of the System of Biodiversity Authorization and Information (SISBIO) (number 47658-4) from the Ministry of the Environment (MMA) and the Chico Mendes Institute for Biodiversity Conservation (ICMBio) of Brazil.

RESULTS

The individuals voided the first fecal sample on average 3 hours after the injection (Table 2). Only the cortisol EIA was capable of detecting pronounced peaks after the ACTH injection in both individuals (F1 and M3; Fig. 1).

The increase ratio was on average 24-fold higher than the basal values. Latency between the ACTH injection and the peak was in between 20 and 25 hours and the median were 22.5 hours. The ACTH response duration lasted between 6 and 9 hours (Fig. 1A and B, Fig. 2).

According to the boxplot analysis, M4 was the only subject that did not present any peaks.

DISCUSSION

Results show that all animals assessed in this study had elevated FGM within 20 to 25 hours following the ACTH challenge. The comparison between the two EIAs used shows that the cortisol EIA is more sensitive than the 11-oxoaetiocholanolone EIA, being the most suitable for black lion tamarins. This same antibody was the most suitable for another species of the Callitrichidae family, *C. jacchus* (Heistermann *et al.,* 2006).

Some aspects of the FGM response maintained the same pattern across the individuals (latency to the peak and time to the first sample after the injection) while others showed more inter-individual differences (basal and peak). For instance, one individual (Male 4)

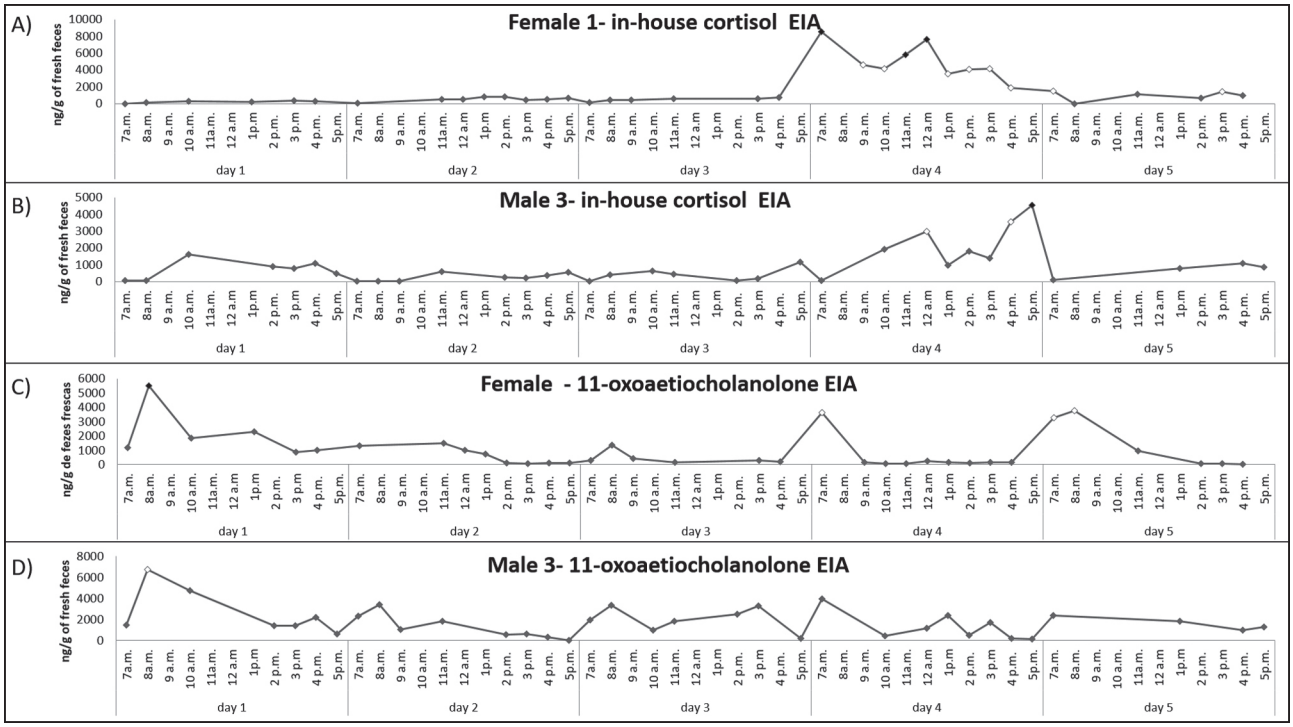

Figure 1: Temporal profile of concentrations of fecal glucocorticoid metabolites of female 1 and male 3 during the validation of the experimental protocol dosed with A) and B) cortisol EIA described by Palme and Möstl (1997) and, C and D) 11-oxoetiocholanolone EIA described by Möstl *et al.* (2002) respectively. The day and time of the injection are indicated by an arrow. Wild outliers are represented by black dots and outliers by white dots.

did not even show outliers or wild outliers in the days after the injection. However, in this specific case, it can be explained by its exacerbated response to the experimental procedure (or by the occurrence of other stressful events during the two days following the ACTH injection), resulting in elevated FGM levels throughout these two days. In addition, our experiment highlighted some differences between sex with regard to baseline levels and increase ratio: females seem to have higher basal levels, but smaller increase rate than males. However, our small sample size hinders any substantial conclusion.

The median value of time lag between the ACTH injection and the first peak was 22.5 hours (20 to 25 hours). Almost the same value was observed for *L. rosalia* after a biological validation (Wark *et al.,* 2016): 27.7 hours (22.3-49.2). In addition, when we compare our results to the ones presented by Wark *et al.* (2016), it seems that the capture added to the ACTH injection produced a prolonged effect in black lion tamarins with individuals presenting several samples with high FGM levels, while in the biological validation with *L. rosalia* most of the individuals had one single FGM elevation. Similarly, an ACTH challenge with *C. penicillata* showed a prolonged effect after the injection with most of the individuals showing several samples with high FGM levels (Pizzutto *et al.,* 2015). However, the latency to the peak was much shorter in this species (females: 8.75 hours, males: 9.25 hours, Pizzutto *et al.,* 2015) than in lion tamarins (Wark *et al.,* 2016; this study).

In relation to the fecal markers, we found that the most reliable colors were green and pink. The colors black, blue, and purple became green after a while (6 or 7 hours) and the orange and red could not be distinguished in the feces. Furthermore, green and pink fecal markers were detected quickly and lasted at least 5 hours in the gut, losing effectiveness with time, which required that the treats were administered twice a day. We also tested several treats and discovered that jellybeans, banana candy, and dried grapes did not call their attention. Insect larvae were a more desired treat than mashed banana balls, being stolen from each other, which could compromise the experiment by the mixture of food coloring between individuals. Mashed banana balls turned out to be a good compromise between being tasteful and healthy (the other treats containing high levels of sugar).

Despite being an endangered species, with few captive individuals and a series of ethical limitations (impossibility to run tests on reproductive females, infants, and juveniles), it was still possible to perform an ACTH challenge (physiological validation). Therefore, even though our small sample size did not allow performing the full physiological test that requires two additional controls (dexamethasone and saline solution injections), we could achieve a reliable validation. Nevertheless, it would have been interesting to conduct a biological challenge to compare the difference between the responses and to evaluate sex differences. This was not possible because it would have implied the use of the same individuals, with an additional capture after a while, which was considered too invasive for an endangered primate species such as the black lion tamarin facing reproduction problems in captivity.

As a closing remark, it is important to highlight that this experiment validates the cortisol EIA presented by Möstl & Palme (1997). However, if one wishes to use another EIA to measure FGM in *Leontopithecus chrysopygus* another validation experiment must be conducted.

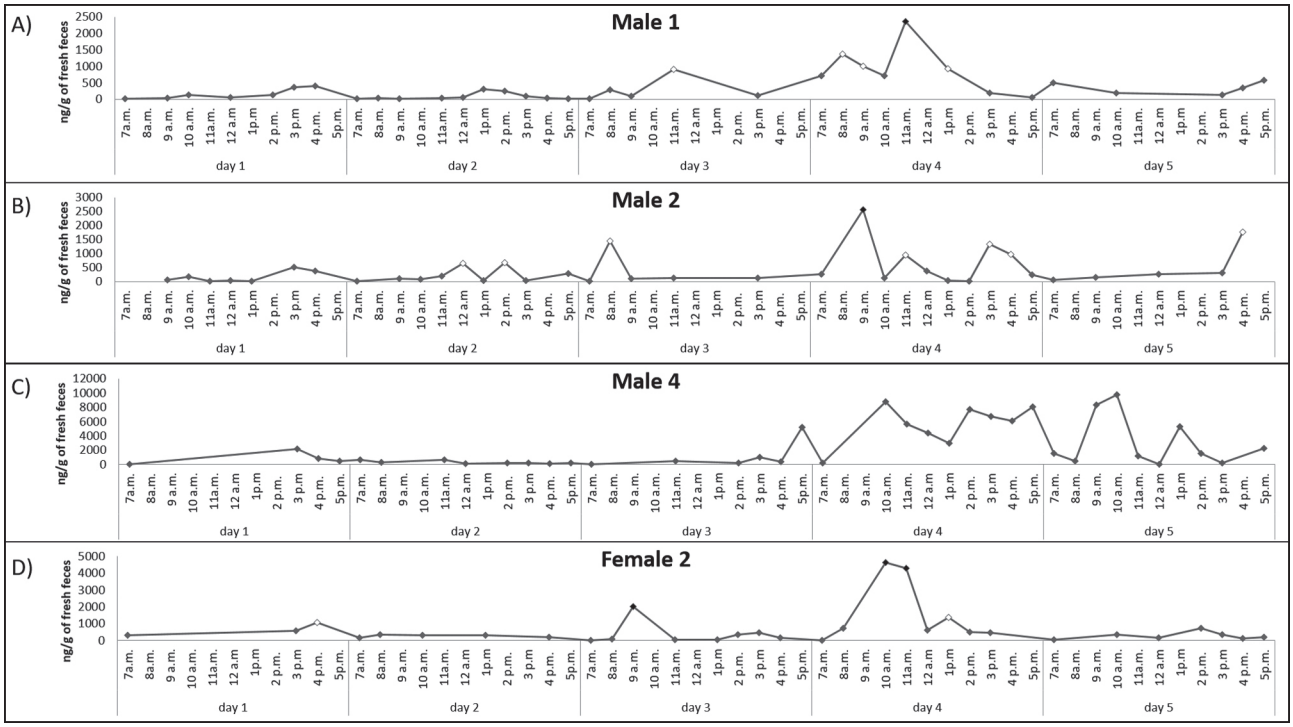

Figure 2: Temporal profile of concentrations of fecal glucocorticoid metabolites during the validation of the experimental protocol referring to A) "Male 1", B) "Male 2", C) "Female 2" and D) "Male 4". The day and time of the injection are indicated by an arrow. Wild outliers are represented by black dots and outliers by white dots.

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