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NON-INVASIVE ASSESSMENT OF CORTISOL LEVEL AND GASTROINTESTINAL PARASITISM IN CAPTIVE PRIMATES IN ROMANIA

EVALUAREA NON-INVAZIVĂ A CORTIZOLULUI ȘI PARAZITISMULUI GASTRO-INTESTINAL LA PRIMATE ÎN CAPTIVITATE DIN ROMÂNIA

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ABSTRACT | REZUMAT

In order to ensure the welfare of captive primates, zoos should ensure optimal environmental conditions for the development of normal species behaviours, assess health, and avoid stress-generating factors. Gastrointestinal parasitic infections can have a direct influence on the health of primates, zookeepers, and visitors. The aim of this study was to investigate by cross-sectional survey the prevalence of gastrointestinal parasites in captive primates and to find out if their presence influences the level of cortisol, considered a predictor of stress. For this, samples of fresh faeces from 77 primates of different sexes, ages, and species were collected and examined. The study was conducted between March and May 2022, and the primates came from three zoos in Romania: Oradea, Cluj-Napoca, and Târgu Mureș. Faecal samples were analysed using flotation methods, and the enzyme-linked immunosorbent assay (ELISA) was used to determine cortisol. The total prevalence of gastrointestinal parasites was 35,1%, and the most frequently identified parasites were *Trichuris* spp. (15.6%), *Capillaria* spp. (10.4%), and *Strongyloides* spp. (14.3%). Furthermore, the prevalence of different species of parasites varied depending on the age of the primates. Cortisol values ranged from 0.05 to 500 ng/ml. The concentration of cortisol had higher values in primates in which no gastrointestinal parasites were identified, the stress being caused by environmental and social factors and not by parasitism. The prevalence of gastrointestinal parasites with zoonotic potential recorded in primates is a potential risk to human health.

Keywords: primate, faeces, cortisol, endoparasites, stress

Pentru a asigura bunăstarea primatelor captive, grădinile zoologice ar trebui să asigure condiții optime de mediu pentru dezvoltarea comportamentelor normale ale speciilor, să evalueze starea de sănătate și să evite factorii generatori de stres. Paraziții gastro-intestinali pot avea o influență directă asupra sănătății primatelor, a grădinii zoologice și a vizitatorilor. Scopul acestui studiu a fost de a evalua prevalența paraziților gastrointestinali la primatele captive și de a investiga influența parazitismului asupra nivelului de cortizol. Cortizolul este considerat un predictor al stresului. Studiul a fost realizat în perioada martie – mai 2022 în trei grădini zoologice din România: Oradea, Cluj-Napoca și Târgu Mureș. Pentru acest studiu au fost recoltate și examinate 77 probe de fecale de la primate de diferite sexe, vârste și specii. Probele de fecale au fost prelucrate prin metoda de flotație pentru identificarea paraziților gastro-intestinali și prin test imunoenzimatic (ELISA) pentru stabilirea concentrației cortizolului. Prevalența totală a paraziților gastrointestinali a fost de 35,1%. Paraziții cei mai frecvent identificați au fost *Trichuris* spp. (15,6 %), *Capillaria* spp. (10,4%) și *Strongyloides* spp. (14,3 %). În plus, prevalența diferitelor specii de paraziți a variat în funcție de vârsta primatelor. Valorile cortizolului au variat de la 0,05 la 500 ng/ml. Concentrația de cortizol a avut valori mai mari la primatele neinfestate cu paraziți gastrointestinali. La aceste primate stresul a fost indus de factori de mediu și sociali. Prezența paraziților gastrointestinali cu potențial zoonotic la primate reprezintă un risc pentru sănătatea umană.

Cuvinte cheie: primate, fecale, cortizol, endoparaziți, stres

Recent research has shown that promoting the welfare of captive primates is becoming a priority and a real challenge for zoos today. The main stress factors that can influence the welfare of primates relate to environmental factors, such as the size of the enclosure, the presence of other animals near the cage, lighting, and feeding methods, as well as certain social factors,

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hierarchy, competition or social support (10). To these stressors are added intercurrent diseases and parasitism (1). Therefore, stress becomes an omnipresent feature of primate life either in the wild or in captive environments, which leads to the development of abnormal behaviours, such as stereotypes (16). Stress, defined as a disturbance of the physiological or behavioural homeostasis of an organism as a result of exposure to certain stressful events or situations, can manifest both at satisfying events, but especially at certain aversive events (15). In our study, we evaluated stress and its relationship to the welfare of captive primates from two complementary perspectives: comparing cortisol levels and determining the degree of infestation with gastrointestinal parasites. Cortisol is released in order to help the body cope with stressors, and parasitism can be a stress factor that causes changes in homeostasis. Cortisol can be determined from serum, plasma, saliva, urine, faeces, and hair. Each of these sampling matrices has advantages and disadvantages, and these must be considered in determining which sampling matrix is best for answering research questions, such as assessing effects in captive environments, identifying the presence of parasites, and determining behavioural consequences (4). Choosing a non-invasive method for evaluating cortisol as a predictor of stress in primates in captivity has multiple advantages, and understanding its determinants is a major goal of zoos, both in the short term, but especially in the long term (18).

MATERIALS AND METHODS

Animals and samples

The study took place between March 2022 and May 2022 in three zoos located in Romania: Oradea, Târgu Mureș, and Turda zoos (Fig. 1).

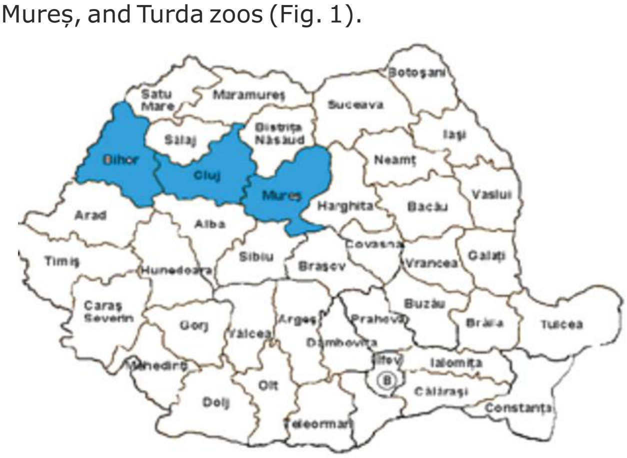

Fig. 1. Sampling areas

There were included in the study 77 primates, females and males, accustomed to visitors throughout the year. The age of the primates varied between 1 year and 29 years. Nine primates were infants (11.2 %), 24 young (31.2%), 27 adults (35.1%), and 17 (22.1%) elders. They belonged to the following species: *Macaca fascicularis* (n=6), *Macaca fuscata* (n= 8), *Macaca luisa* (n=6), *Macaca sylvanus* (n=8), *Mandrillus sphinx* (n=9), *Papio hamadryas* (n=13), *Sanguinus midas* (n=3), *Sapajus apella* (n=2), *Cebus apella* (n=4), *Cercocebus chysogaster* (n=2), *Cercopithecus albogularis kolbi* (n=3), *Erythrocebus patas* (n = 2), *Eulemur macaco* (n=3), *Lemur catta* (n=10).

The participants in the study were accommodated in groups of 2-29 animals, according to the species. The shelters did not have artificial lighting, and the primates had free access to a standard diet. During the collection of faecal samples, they did not show clinical signs of disease. The samples were collected, taking into account the fact that there is a diurnal variation of faecal cortisol levels in some primate species, which could have affected its values (9). Thus, faecal samples were collected in the morning before 12 am, in sterile plastic bags by the zookeepers. The faeces were fresh, and each faecal pellet was collected using sterile gloves and individual sterile spatulas to avoid any cross-contamination, and labelled. For each animal, two faecal samples were collected and assigned with the letter 'C' (cortisol analysis) or 'P' (parasitological analysis) which indicated the type of analysis in the lab. The sample collection was visually monitored. Every effort has been made to avoid collecting parts of biological samples that could have been contaminated with soil or water. No traces of blood or mucus were observed in any of the collected samples. The consistency and colour of the faeces in each sample were not significantly different. In the same day of collection, the samples were transported to the lab in a medical cooler box. The samples for cortisol analysis were stored in the freezer until further analysis. Faeces collected for parasitological analysis were stored in the refrigerator until the next day.

Cortisol extraction and measure

Before being analysed, the frozen faecal pellets were thawed at room temperature and dried in an oven (55°C), after which they were weighed. The seeds and the fibrous materials were removed by passing the samples through a fine wire mesh. Cortisol was extracted using an adapted version of the method proposed by Wasser et al. (17). Fecal samples (1 g) were placed in a Pyrex screw cap glass tube and 5 ml mixture (1:4, v/v) of phosphate-buffered saline (PBS, pH 7.4) and methanol was added. The tubes were vigorously homogenized for 5 min using an orbital shaker (Heidolph ReaxTop, Schwabach, Germany), placed in an ultrasonic bath (PowerSonic410, Hwashin, Korea) for 60 minutes, and then centrifuged for 15 min, at 2500 RPM. A portion of the supernatant (3.5

Table 1

Frequency and prevalence of endoparasites identified in primates (n=77) from zoological gardens in Romania

	Frequency	Prevalence	95% Confidence interval
Balantidium coli	1	1.3	0.2-7.0
Entamoeba coli	3	3.9	1.3-10.8
Entamoeba histolytica	1	1.3	0.2-7.0
Trichuris spp. [1]	12	15.6***	9.2-25.3
Capillaria spp.	8	10.4***	5.4-19.2
Ancylostoma spp.	2	2.6	7.2-9.0
Strongyloides spp.	11	14.3***	8.2-23.8
Single infection	18	23.4	14.5-34.4
Mixed infection	9	11.7	5.5-21.0
Total	27	35.1	25.4-46.2

* $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$

ml) was transferred in another tube and 3 ml of diethyl ether and 2 ml of PBS were added. This mixture was vigorously shaken and centrifuged. The upper organic layer was transferred in a glass tube. The procedure was repeated and the combined solvent fraction was completely removed by flushing with nitrogen. The residue was resuspended in 100μL of PBS and further used for cortisol measure. Cortisol was determined using a competitive immunoenzymatic colorimetric assay kit for human cortisol (DiaMetra Srl Unipersonale, Boldon, UK). The assay was performed following the kit instruction. The amount of cortisol was expressed in ng/ml extract.

Parasitological examination

Each faecal sample was analysed by the flotation technique using a saturated sodium chloride solution (specific gravity 1.28) and the sedimentation technique. Cysts, oocysts, and eggs were identified based on morphological characteristics using the Olympus BX61 microscope, Japan, using the 10X, 20X, and 40X objectives (8, 12).

Statistical analysis

Frequency and prevalence and its 95% confidence interval of overall, single species, and mixed endoparasite infection were evaluated by total primates, age, gender, and zoological garden. The chi-squared test was used for comparison of the prevalence.

Arithmetic mean and standard error of the mean were calculated for cortisol concentration in faecal samples for different categories (overall, age, gender, zoological garden, and type of endoparasite infection). The data distribution was assessed by the Shapiro-Wilk test for Normal distribution. Because data did not follow a normal distribution, the non-parametric Friedman test was used to evaluate the difference in cortisol concentration by categories.

Also, the correlations between the cortisol concentration and age category, gender, zoo, and the type of endoparasite infection were determined using Spearman's rank correlation coefficient. The strength of correlation was established based on the absolute value of the r(s) coefficient, as follows: 0.00-0.19 "very weak" correlation; 0.20-0.39 "weak" correlation; 0.40-0.59 "moderate" correlation; 0.60-0.79 "strong" correlation; 0.80-1.0 "very strong" correlation. The level of significance was set at 0.05. Statistical analysis was performed with EpiInfo 2000 (5) and MedCalc Statistical Software version 19.0.7.

RESULTS AND DISCUSSIONS

The total prevalence of endoparasites, single and mixed infections is presented in Table 1. The prevalence of intestinal parasites in relation to age, gender, and zoological garden of the primates is presented in Tables 2 and 3. The total prevalence depending on primate species is presented in Table 4.

Overall, *Trichuris* spp., *Capillaria* spp., and *Strongyloides* spp. were the most prevalent endoparasites ($\chi2 = 27.293$, df = 6, P = 0.0001) (Table 1).

Trichuris spp. was the most prevalent endoparasite in kids ($\chi2 = 13.5$, df = 6, P = 0.04), followed by elders ($\chi2 = 6.375$, df = 6, P = 0.383). *Strongyloides* spp. ($\chi2 = 24.769$, df = 6, P = 0.0004), and *Capillaria* spp. ($\chi2 = 15.839$, df = 6, P = 0.02) were the most prevalent endoparasites in young, and adults (Table 2).

Age was identified as a risk factor in the case of *Strongyloides* spp. by univariate analysis, with young primates being more predisposed to the infection ($\chi2 = 8.68$, df = 3, P = 0.04).

Table 2

Frequency (prevalence) of endoparasites in primates by age, gender, and zoological garden and risk factors identified by univariate analysis

	Age				Gender	
	Kids (*n*=9)	Young (*n*=24)	Adults (*n*=27)	Elder (*n*=17)	Females (*n*=38)	Males (*n*=39)
Balantidium coli	0	0	0	1 (5.9)	1 (2.6)	0
Entamoeba coli	1 (11.1)	0	1 (3.7)	1 (5.9)	3 (7.9)	0
Entamoeba histolytica	0	0	0	1 (5.9)	1 (2.6)	0
Trichuris spp.	4 (44.4)[a]	3 (12.5)	2 (7.4)	3 (17.6)	6 (15.8)	6 (15.4)
Capillaria spp.	1 (11.1)	1 (4.2)	5 (18.5)[a]	1 (5.9)	5 (13.2)	3 (7.7)
Ancylostoma spp.	1 (11.1)	1 (4.2)	0	0	1 (2.6)	1 (2.6)
Strongyloides spp.	0	7 (29.2)[c,*]	4 (14.8)	0	4 (10.5)	7 (17.9)
Total	5 (55.6)	8 (33.3)	10 (37.0)	4 (23.5)	14 (36.8)	13 (33.3)

a/*p<0.05, b/**p<0.01, c/***p<0.001

Table 3

Frequency (prevalence) of endoparasites in primates according to the zoological garden

	Cluj-Napoca (*n*=6)	Oradea (*n*=14)	Târgu Mureș (*n*=57)
Balantidium coli	0	0	1 (1.8)
Entamoeba coli	0	0	3 (5.30
Entamoeba histolytica	0	0	1 (1.8)
Trichuris spp. [1]	0	1 (7.1)	11 (19.3)[b]
Capillaria spp.	0	0	8 (14.0)
Ancylostoma spp.	0	1 (7.1)	1 (1.8)
Strongyloides spp.	0	4 (28.6)[a]	7 (12.3)
Total	0	5 (35.7)	22 (38.6)

Table 4

Frequency and prevalence of total endoparasites and cortisol concentration (mean± std. 159 error) by primate species

Species	Frequency	Prevalence	Cortisol (ng/ml)
Cebus apella (*n*=4)	1	25.0	>500
Cercocebus chysogaster (*n*=2)	0	0	0.6 ± 0.85
Cercopithecus albogularis kolbi (*n*=3)	2	66.7	3.22 ± 1.90
Erythrocebus patas (*n*=2)	2	100.0	1.97 ± 0.66
Eulemur macaco (*n*=3)	0	0	0
Lemur Catta (*n*=10)	2	20.0	0.14 ± 0.35
Macaca fascicularis (*n*=6)	2	33.3	0.05 ± 0.11
Macaca Fuscata (*n*=8)	1	12.5	6.08 ± 9.98
Macaca Luisa (*n*=6)	0	0	20.17 ± 21.27
Macaca sylvanus (*n*=8)	6	75.0	11.54 ± 26.03
Mandrillus sphinx (*n*=9)	2	25.0	0.77 ± 1.38
Papio hamadryas (*n*=13)	9	69.2	55.17 ± 140.96
Sanguinus Midas (*n*=3)	0	0	262.72 ± 232.41
Sapajus apella (*n*=2)	0	0	>500

Many studies used the evaluation of glucocorticoid levels as a reference for physiological stress in animals, using blood samples. However, it is difficult to collect biological material from wild animals as their capture and handling will rapidly increase glucocorticoid levels. The goal of the initial field applications of faecal steroid analysis was the detection of relatively gross changes in hormonal concentrations or changes in reproductive hormones. This non-invasive method has a huge potential to answer questions regarding the endocrinology of wild animals (9), however, the high variation around mean values in comparison to urine and serum profiles make this method often appear less reliable. Long-term pre-pathological stress in animals is difficult to quantify, but it's well documented that stress of long duration results in immunosu-

Table 5

Cortisol concentration in faecal samples (mean± std. error) by age, gender, zoological garden and endoparasitic infection and Spearman's coefficient of rank correlation

	Cortisol	95% CI	R_s	P
Age				
Kids	57.53 ± 55.32	-70.03 - 185.09	0.07	0.53
Young	63.16 ± 29.73*	1.66 - 124.67		
Adults	59.88 ± 30.65*	-3.11 - 122.88		
Elder	66.44 ± 39.75	-17.82 - 150.70		
Gender				
Females	32.67 ± 17.51	-2.80 - 68.14	0.04	0.76
Males	90.73 ± 29.64	30.73 - 150.74		
Zoological garden				
Cluj-Napoca	20.17 ± 8.68***	-2.15 - 42.49	-0.675	<0.0001
Oradea	250.28 ± 62.30***	115.68 - 384.85		
[illegible]	20.27 ± 12.30	-4.38 - 44.92		
Endoparasite infection				
No	73.58 ± 23.22*	26.91 - 120.25	-0.0606	0.60
Yes	40.78 ± 25.53	-11.69 - 93.26		
Single infection	59.06 ± 37.87	-20.84 - 138.96	-0.0698	0.55
Mixed infection	4.23 ± 2.86	-2.37 - 10.82		
Total	62.08 ± 17.53	27.17 - 96.99		

ppression (7). These kinds of analyses should prove to be a powerful tool to obtain scientific information using a non-invasive method and have important biomedical implications. Recent studies have demonstrated that increased corticoid production can appear following aggressive encounters, which can be particularly the case for animals living in a confined social group, like in zoos, in which escape from attacks is limited (2). More generally, glucocorticoid levels have been reported by precedent studies to be more elevated during periods that include social instability (14). It's interesting to notice the conflicting picture between the dominance status and glucocorticoid levels noticed by several studies (3). Further studies should be realized in order to validate these methods, which could open a new arena of research concerning wild captive and non-captive animals. Parasitic infections play a major role in dog health all over the world, however, it's also the case in wild animals. Parasitic diseases play a principal role in animal health ranging from the negative impact on the host population size to the evolution of host behaviours. Parasitic infections constitute one of the main challenges of wild animals in captivity (6). Concerted efforts should therefore be made to embrace parasitological infections in captive primates. This study, as well as others, also suggests that a program based on the investigation and measures of control and accurate diagnosis, treatment, and prophylaxis would help reversing the hill health conditions in zoo animals (13). Many studies have documented the intestinal parasites of zoo animals. Moreover, there is a link to public health, for both animal handlers and visitors (11), and the maintenance of good hygiene including daily removal of faeces is a necessity.

In the present study, the various frequency rates of intestinal parasites were explicitly isolated and characterized by parasitological and morphometric investigations at the Parasitology Department of FMV (Cluj-Napoca).

Multiple types of parasites were isolated from the examined feces, however, in contrast, the primates examined appeared to be in fairly good health condition but yielded different types of intestinal parasites during examinations. This could be attributed to the subclinical nature of the parasitic conditions.

CONCLUSIONS

The concentration of cortisol had higher values in primates in which no gastrointestinal parasites were identified, which indicates that the stress is caused mainly by environmental and social factors rather than by parasitism.

Different parasitic infections were observed among the examined animals. Identified parasitic infections can be considered threatening to visitors and worker health that have contact with animals or their feces.

Therefore effective preventive strategies should be addressed to determine the risk factors, mechanisms of cross-contamination of parasites, the importance of applying the hygienic practices and adjusting deworming programs for the animals, zoo workers and visitors.

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