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RESEARCH ARTICLE

Assessment of Adrenocortical Activity and Behavior of the Collared Anteater (*Tamandua tetradactyla*) in Response to Food-Based Environmental Enrichment

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One of the current standard approaches to the study of animal welfare is measuring hypothalamic–pituitary–adrenal activity, frequently in association with behavioral assessment. We studied the effects of food-based environmental enrichment on adrenocortical activity and behavior in zoo-housed collared anteaters (*Tamandua tetradactyla*; $n = 5$). We successfully validated measurements of fecal cortisol metabolites (FCMs) using an 11-oxoetiocholanolone enzyme immunoassay by stimulating (ACTH injection) and suppressing (dexamethasone administration) adrenocortical activity. Three months later, we subjected animals to an ABA-type experiment (three 6-week periods): pre-enrichment (routine diet: A), enrichment (modified diet: B), and post-enrichment (routine diet: A) periods. We assessed adrenocortical activity by collecting individual feces three times a week (total number of samples: 228), and evaluated behavior by performing 3 days of behavioral observations per period (with a total of 3,600 behavioral data points for the individuals studied). Statistical analysis revealed changes in FCM concentrations (μg/g) over the periods (3.04 ± 0.68, 2.98 ± 0.66, and 4.04 ± 0.90, respectively). Additionally, it showed that the number of FCM peaks was highly reduced during enrichment; meanwhile active natural behaviors were significantly increased. We consider that these changes in response to food-based environmental enrichment improved the welfare of individual zoo-housed collared anteaters. This research might contribute to in situ and ex situ studies on the physiology and behavior of this endemic South American species. Zoo Biol. 32:632–640, 2013.

Keywords: non-invasive monitoring of glucocorticoid metabolites; activity pattern; diet; xenarthra; stress; welfare

INTRODUCTION

The collared anteater (*Tamandua tetradactyla*) is a species endemic to South America. It belongs to the mammalian superorder Xenarthra (along with armadillos, sloths, and other anteaters), family Myrmecophagidae. Species of this family have specific adaptations for myrmecophagy, that is, feeding on a diet composed of ants and termites: absent dentition, very long and protrusible tongue, acute sense of smell, and powerful claws in the forefeet (Redford & Eisenberg, 1992). In addition, they exhibit a low metabolic rate associated with their low-energy diet (Fernandes & Young, 2008).

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Free living mammals allocate a great amount of time and energy to food search and handling (foraging), territory defense, reproduction, and dispersal. By contrast, zoo-housed mammals have only restricted (if any) opportunities to perform their daily and/or seasonal activities (Goldblatt, 1993; Carlstead, 1996, 1998; Miller et al., 1998; Broom & Johnson, 2000). In addition, zoo-housed animals are subjected to constant presence of visitors, handling by different keepers, veterinary procedures, space restriction, feeding routines, and human-mediated changes in social structure (Hosey, 2004; Morgan & Tromborg, 2007). Particularly, one of the major concerns about zoo-housed anteaters is the preparation of an appropriate artificial diet. In fact, individuals fed on an artificial diet have been known to exhibit nutritional and digestive problems (Diniz et al., 1995; Morales-Sandoval, 2010). Therefore, captive environments offer few opportunities for natural activities such as feeding, and can negatively affect animal welfare (physiological and behavioral functions).

Environmental enrichment is one of the strategies available to meet zoo-housed animals' needs, evaluate stress responses, and improve animal life quality (Carlstead & Shepherdson, 1994; Swaisgood & Shepherdson, 2005; Hosey et al., 2010). Food-based enrichment, an environmental enrichment type, consists of modifying diet composition, feeding schedule, and/or food presentation. Food-based enrichment has been found not only to significantly stimulate animals by improving physical and psychological aspects, but also to improve diet quality and nutritional issues, providing greater benefits to animal welfare than other environmental enrichment types (social, physical, sensorial, etc.) (Resende et al., 2009; Hosey et al., 2010). Studies on wild animals can reveal species-specific types of environmental enrichment. For example, Brown (2011) found that free living *Tamandua mexicana* individuals consumed palm fruits as a seasonal supplement to its diet, and suggested that fruits could be used as an enrichment item at low cost for zoo-housed tamanduas.

Measuring hypothalamic–pituitary–adrenal (HPA) activity is one of the current standard approaches to the study of animal welfare, and is frequently measured in association with behavioral assessment (Mormède et al., 2007; Hosey et al., 2010; Palme, 2012). The use of feces as sample material to determine glucocorticoid (cortisol/corticosterone) concentrations to evaluate HPA activity in wild animals has several advantages over the use of other biological matrices, such as blood. For example, steroid metabolites measured in feces represent pooled fractions of excreted hormones, providing an integrated measure of steroid concentrations over a longer period and thus better reflect endocrine status. Nevertheless, such a method warrants a solid validation if applied in a species for the first time (Palme et al., 2005; Touma & Palme, 2005; Sheriff et al., 2011). To our best knowledge, this hormone monitoring strategy has not been employed in the collared anteater to study adrenocortical response to stressors; only a few studies have been conducted to test other steroids in collared anteaters (Hay et al., 1994; Kusuda et al., 2011) or other species of the superorder (Mühlbauer et al., 2006; Superina et al., 2009; Howell-Stephens et al., 2012).

The aims of the present study were: (1) to validate the use of fecal cortisol metabolites (FCMs) for monitoring adrenocortical activity in the collared anteater; (2) to analyze changes in FCMs of zoo-housed collared anteaters subjected to food-based environmental enrichment, and (3) to evaluate the variation in activity pattern of zoo-housed collared anteaters subjected to food-based environmental enrichment.

MATERIALS AND METHODS

Animals and Housing Conditions

We studied five adult collared anteaters housed at Córdoba Zoo (31°12, 32′S; 64°16, 84′W; Argentina). We identified individuals by letters A–E, corresponding to the zoo identification number: "A" 00-0698-A8EG (♀); "B" 00-0167-1EE6 (♀); "C" 00-0629-3499 (♀); "D" 00-01FD-793B (♂), and "E" 00-01D7-5672 (♂).

Each animal was individually kept under natural climatic conditions. Enclosure had a wooden shelter, a concrete feeder, and a drinker. Cleaning and feeding routines at the zoo were performed between 10:00 AM and 1:00 PM, from Monday to Saturday. The routine diet offered consisted of a mixed shake containing lactose-reduced whole powdered milk (25% of the total ration weight), baby cereal (Nestum®; 25%), balanced dog feed for puppies (Eukanuba Small Breed; 50%, previously suspended in water for 2 hr) and drinking water reaching a volume of 800 mL. In addition, 2 mL of vitamin K was included in the diet three times a week, following the veterinary recommendations.

Validation of Fecal Cortisol Metabolites Measurements in Collared Anteater

Pharmacological stimulation and suppression of adrenocortical activity

We performed two pharmacological tests to detect functional changes of adrenocortical activity in the collared anteater, according to previous reports (Brousset Hernández-Jáuregui et al., 2005; Palme et al., 2005; Touma & Palme, 2005). In December, an adrenocorticotrophic hormone (ACTH) *stimulation* test was initiated: (1) all fresh feces were collected from each individual for 10 days; (2) on day 10, ACTH (5 IU/kg body weight; gel form, ELEA, Buenos Aires, Argentina) was given intra-muscularly to each individual at 12:00 PM; and (3) feces collection continued for 10 days. After one week without sampling, we performed a dexamethasone *suppression* test: (1) all fresh feces were collected from each individual for 9 days; (2) on day 9, the synthetic glucocorticoid dexamethasone ("Dex"; 0.1 mg/kg body weight; Surar Pharma, Buenos Aires, Argentina) was given intra-muscularly to each individual at 12:00 PM; and (3) feces collection continued for 7 days. All feces were

collected between 8:00 AM and 7:00 PM, frozen immediately and stored (−20°C) until hormonal analysis. In order to administer drugs, animals were gently managed for injections (without the effects of anesthesia and restraint), and we observed no signs of altered behaviors after both injections.

Measurements of fecal cortisol metabolites

Fecal cortisol metabolites were extracted by a simple method that is generally applied and proven to yield high recoveries of FCM (Palme et al., 2013). In short, to an aliquot (0.5 g) of each well-homogenized sample 5 mL methanol/water (80%) were added (Palme, 2005). After vortexing (2 min) and centrifugating (15 min, 1,000*G*), an aliquot (0.5 mL) of the supernatant was separated for further use. The content was evaporated at 60°C, sent to the laboratory for analysis, and redissolved in enzyme immunoassay (EIA) buffer there. All measurements were run in duplicate with an 11-oxoetiocholanolone EIA, as described by Möstl et al. (2002). This assay has been found to be useful for monitoring adrenocortical activity in several mammal species (Palme, 2012). The coefficients of variation (CV) of a low and high pool sample were 2.6% and 2.9%, respectively (intraassay CV; $n = 18$ for both) and 8.5% and 4.1%, respectively (interassay CV; $n = 20$ for both). The sensitivity of the EIA for FCM measurement was 4 ng/g feces. In addition, we conducted a parallelism test (Busso et al., 2007) by running serial dilutions of a fecal extract pool (the standard curve was parallel to the dilution curve; $P > 0.05$; $F_{1,8} = 0.39$).

Food-Based Environmental Enrichment: Experimental Design

We analyzed responses of collared anteaters to food-based environmental enrichment using a single-factor (three levels) experiment in which each animal was subjected to diet modification. Due to the low number of animals housed in the zoo, sampling effort was increased by considering prolonged periods (6 weeks each period). This experiment (ABA type) consisted of three periods: pre-enrichment (A: March 21–May 1, routine diet), enrichment (B: May 2–June 12, modified diet), and post-enrichment (A: June 13–July 24, routine diet).

During the period of food-based environmental enrichment, we modified the routine diet by simultaneous changes: (1) addition of novel liquid and solid food items, (2) extension of feeding schedule (9:00 AM to 7:00 PM), (3) fractioned supply of food (up to four times a day), (4) modification of food presentation, and (5) extension of food offer to 7 days per week (from Monday to Sunday). For example, at 13th day of the study, we applied the following feeding schedule for all animals: half ration of mixed shake at 9:00 AM; solid honey on a branch at 12:00 AM; orange juice in a bottle at 4:00 PM, and finally another half of mixed shake at 7:00 PM. We selected diet items according to the literature (Diniz et al., 1995; Morales-Sandoval, 2010) and availability at the zoo: we agreed upon diet modifications with the zoo staff. Novel food items and amounts offered are listed in Table 1. Fresh fruit, vegetables, and meat were provided by the institution, whereas the other items, such as honey (liquid or solid), corn syrup, yoghurt, fruit juice, broth, jelly, cow milk, and crème caramel, were obtained from the market. Worms were purchased and ants were collected from a portion of soil extracted from an ant nest. Food was placed in the animal's enclosure for 24 hr; at the end of each period, we recorded if the food offered was consumed (Table 1). The usual mixed shake was supplied twice a day during this period (400 mL each time), in the concrete feeder (see Animals and Housing Conditions section: routine diet). In addition, we recorded body weight (kg) of animals to assess possible effects of the changes in the diet, and observed no statistical differences. Noticeably, food-based enrichment affected neither feces consistency nor frequency of deposition.

Assessment of Adrenocortical Activity and Behavior of the Collared Anteater in Response to Food-Based Environmental Enrichment

We monitored adrenocortical activity during each experimental period (of 6 weeks each) by collecting feces three times a week on randomly selected days. Feces were collected from each anteater immediately after deposition, and generally obtained between 10:00 and 12:00 in the morning. When we modified the diet (from pre-enrichment to enrichment and from enrichment to post-enrichment), we monitored adrenocortical activity by daily collections of all feces during 1 week. We used FCM measurements from the pre-enrichment period to calculate individual baselines, because during this period animals were exposed to habitual environmental conditions in the zoo facilities. In addition, we used these data to explore possible individual differences in adrenocortical activity (see statistical analysis). Hormonal measurements of each individual were grouped by pre-enrichment (routine diet), enrichment (modified diet), and post-enrichment (routine diet) periods for further statistical analysis of FCM mean and median values.

The ethogram and behavioral categories used are summarized in Table 2. Based on Resende et al. (2009), we conducted ad libitum observations to develop behavioral categories of the ethogram. We assessed individual behavior by performing a day of behavioral observation in weeks 1, 3, and 6 of each period (between 8:00 AM and 7:00 PM). We used the Instantaneous sampling method proposed by Altmann (1974), in which the observer records the individual's current activity at preselected moments in time. On each observation day, we conducted five 30-min observation sessions for each animal across the day, recording its behavior every 2 min (the order of the sessions was randomly determined). All observations were made by one researcher. For statistical analysis we considered all behavioral categories separately. In addition, the sum of the categories *feeding*

TABLE 1. Novel food items and details of food-based environmental enrichment

Food item	Scientific name	Number of times offered	Amount	Consumed
Ant	Not determined	5	[a]	Yes
Cow milk	*Bos taurus taurus*	4	200 mL	Yes
Orange	*Citrus sinensis*	4	200 g	Yes
Corn syrup	*Zea mays*	3	60 g	Yes
Honey	—	3	75 g	Yes
Kiwi	*Actinidia chinensis*	3	100 g	Yes
Orange juice	*Citrus sinensis*	3	300 mL	Yes
Yogurt (strawberry and vanilla)	—	3	300 g	Yes
Apple	*Malus domestica*	2	260 g	Yes
Avocado	*Persea americana*	2	200g	Yes
Banana	*Musa paradisiaca*	2	300 g	Yes
Beef	*Bos taurus taurus*	2	75 g	Yes
Mango	*Mangifera indica*	2	260 g	Yes
Mealworm	*Tenebrio molitor*	2	2 g	Yes
Melon	*Cucumis melo*	2	35 g	Yes
Pineapple	*Ananas comosus*	2	220 g	Yes
Tomato	*Lycopersicum esculentum*	2	500 g	Yes
Beet	*Beta vulgaris*[b]	1	220g	Yes
Bread	—	1	40 g	Yes
Chicken breast	*Gallus* spp.[c]	1	300 g	Yes
Chicken stock	*Gallus* spp.[c]	1	750 mL	Yes
Egg	*Gallus* spp.[c]	1	240 g	Yes
Egg	*Coturnix japonica*	1	110 g	Yes
Flavored custard	—	1	500 g	Yes
Grape	*Vitis vinifera*	1	200 g	Yes
Grape fruit	*Citrus paradise*	1	250 g	Yes
Nectar[d]	—	1	140 mL	Yes
Rice	*Oryza satina*	1	100 g	Yes
Spinach	*Spinacia oleracea*	1	300 g	Yes
Strawberry jelly	—	1	300 g	Yes
Watermelon	*Citrullus lanatus*	1	500 g	Yes
Beef liver	*Bos taurus taurus*	1	130 g	No
Crookneck squash	*Curcubita moschata*	1	800 g	No
Pear	*Pyrus communis*	1	500 g	No
Plains viscacha meat	*Lagostomus maximus*	1	200 g	No
Super worm	*Zophobas morio*	1	14 g	No

We applied the same feeding schedule for all animals, with all food items being offered at the same frequency and amount. [a]Ants were collected from a portion of soil extracted from an ant nest. [b]Variety conditiva. [c]*Gallus gallus domesticus*. [d]Solution of honey (30%) and tap water (70%).

and *exploration* was grouped as *foraging*. Also, we evaluated the activities *feeding*, *exploration*, *locomotion*, and *others* as *natural active behaviors*.

TABLE 2. Ethogram of zoo-housed collared anteater (*Tamandua tetradactyla*) individuals

Behavioral category	Definition
Resting	Lying or sitting, with open or closed eyes, motionless.
Feeding	Eating food, frequently using the claws.
Exploring	Interaction with the environment, frequently sniffing.
Moving	Moving towards a specific direction, either walking or climbing.
Others	Includes interaction with visitors, grooming, defecating.
Stereotyped	Repeated movement, without an apparent aim.

Study Variables

We determined *concentrations of fecal cortisol metabolites* (*FCMs*; expressed as μg/g fresh feces) as described above. We determined *percent of time* for each behavioral category, by employing data obtained during the three behavioral observation days performed in each experimental period.

Statistical Analyses

Validation of fecal cortisol metabolite measurements in collared anteater

We detected that the frequency of defecation was very variable among individuals, for example, female "A" defecated only twice during the first 10 days in the ACTH stimulation test, whereas male "D" defecated seven times during the same period. Therefore, we averaged hormonal measurements per treatment levels (pre-ACTH, post-ACTH,

pre-Dex, post-Dex) on an individual basis for the statistical analysis. We fitted a mixed linear model for the FCM concentrations, with the pharmacological tests as the fixed factor, and the animal effect as random.

Assessment of adrenocortical activity of the collared anteater in response to food-based environmental enrichment

We assessed adrenocortical activity by collecting individual feces three times a week (number of samples per period: pre-enrichment = 75, enrichment = 84 and post-enrichment = 68). We detected that the frequency of defecation was less variable among individuals than that observed during pharmacological tests; for example, female "A" defecated an average of 13 times per period, whereas male "D" defecated 17 times. We evaluated the effect of food-based environmental enrichment on FCMs by comparing their concentrations during three periods: pre-enrichment (routine diet), enrichment (modified diet), and post-enrichment (routine diet). We fitted a mixed linear model for the FCM concentrations, with the periods as the fixed factor, and the animal effect as random. Because the concentration of FCMs was measured several times during the periods, a serial correlation—continuous autoregressive (AR) model of order 1—among repeated observations on each animal was included. Additionally, considering that increases in glucocorticoids relative to basal concentrations (undisturbed) are generally associated with the effect of stressors (Landys et al., 2006; Busch & Hayward, 2009; Dickens et al., 2010), we analyzed the number of FCM peaks that reflects when animals experienced stressful circumstances. To analyze these data we fitted a generalized linear mixed model for a binomial variable (number of FCM peaks on the total number of times the FCMs were evaluated). The animal effect was also included as a random term. Finally, we carried out two planned comparisons for FCM mean values and the number of FCM peaks: enrichment versus post- and pre-enrichment, and post- versus pre-enrichment.

Peak identification. We used FCM concentrations during the pre-enrichment period to establish an animal-specific FCM threshold in order to determine the occurrence of a FCM peak. Firstly, we estimated the mean concentration for each animal and then calculated the residuals of FCM concentration as the difference between the observed values and the mean concentration of the corresponding animal. The distribution of residuals reflects the shape and magnitude of the FCM concentration variability. Secondly, we calculated the 75th percentile (0.8) of the empirical distribution of residuals and we added it to the average FCM concentration of each animal. The animal-specific FCM (μg/g) thresholds were: "A" 6.28; "B" 3.56; "C" 2.43; "D" 4.87; and "E"6.78. Therefore, we considered as peaks those FCM values superior to individual thresholds.

Assessment of behavior of the collared anteater in response to food-based environmental enrichment

We analyzed behavior with a Friedman non-parametric test, considering the pre-enrichment, enrichment, and post-enrichment periods as the treatment factor and animals as blocks. We obtained an individual's activity pattern in each period (averaging the percent of time per behavioral category across the 3 days of observation per period).

We performed all the analyses using InfoStat (Di Rienzo et al., 2012) and consulted different bibliographic references (Sokal & Rohlf, 1997; Plowman, 2006; Hosey et al., 2010). Reported values are expressed as mean ± SEM, and the significant level was 5% for all tests.

RESULTS

Validation of Fecal Cortisol Metabolites Measurements in Collared Anteater

The analysis of variance showed that FCM concentrations in zoo-housed collared anteater changed significantly in response to pharmacological tests ($P = 0.0001$). According to the a posteriori test ($P < 0.05$), the post-ACTH group showed the highest value, whereas the pre-ACTH and pre-Dex groups had similar concentrations, and the post-Dex group had the lowest FCM value (Fig. 1). As an example, Figure 2 illustrates an individual profile of adrenocortical activity in response to ACTH and Dex tests, revealing an increase and decrease, respectively. Other individuals exhibited similar FCM profiles (data not shown).

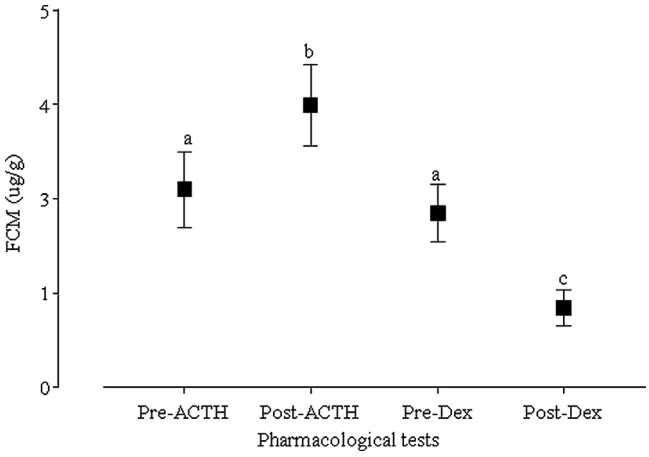

Fig. 1. Concentrations of fecal cortisol metabolites (FCMs) in fresh feces of collared anteater (*Tamandua tetradactyla*; $n = 5$) in response to pharmacological tests (ACTH: 5 IU/kg b.w., i.m.; Dexamethasone: 0.1 mg/kg b.w., i.m.). The means shown represent FCM measurements performed during each experimental period (Pre-ACTH: $n = 20$; Post-ACTH: $n = 20$; Pre-Dex: $n = 23$, and Post-Dex: $n = 20$); different letters indicate significant differences ($P \leq 0.05$). The experimental period spanned 44 days.

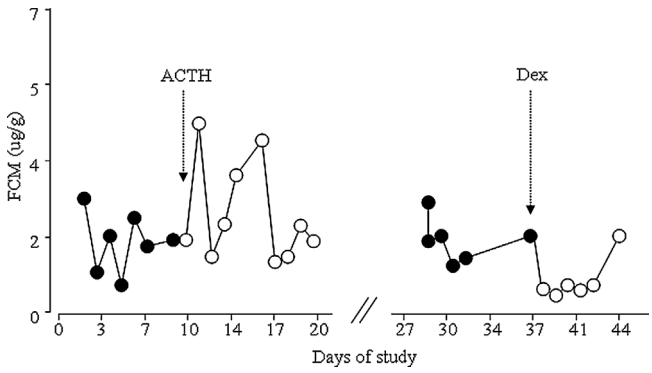

Fig. 2. Profile of fecal cortisol metabolites (FCMs) in fresh feces of a male individual (D) of collared anteater (*Tamandua tetradactyla*) in response to pharmacological tests (ACTH: 5 IU/kg b.w., i.m.; Dexamethasone: 0.1 mg/kg b.w., i.m.). ACTH challenge was performed on day 10 and dexamethasone on day 37 (pre-injection samples: ●; post-injection samples: ○). No fecal samples were collected between tests (8 days). Notice that the first post-ACTH sample was collected 1 hr after injection, and the last pre-Dex sample was collected before injection.

Assessment of Adrenocortical Activity of Collared Anteater in Response to Food-Based Environmental Enrichment

Statistical analysis revealed changes in FCM mean values over the periods ($P = 0.0059$). Decreases in FCM concentrations during enrichment period narrowly reached statistical significance (first comparison). Meanwhile, we detected higher FCM concentrations during post-enrichment compared to pre-enrichment period (Table 3).

Additionally, statistical analysis showed that the number of FCM peaks changed over pre-enrichment, enrichment, and post-enrichment periods (3.60 ± 0.93, 1.60 ± 0.50, 5.80 ± 1.70, respectively; $P < 0.0001$). First comparison (enrichment vs. pre- and post-enrichment) was highly significant, showing that environmental enrichment reduced the probability of the occurrence of FCM peaks. We also detected a higher probability during post-enrichment compared to pre-enrichment period (Table 3).

As an example, we show the hormonal profile of a female individual in Figure 3 (the remaining profiles were qualitatively similar, and they exhibited different individual thresholds: "*A*" = 6.28; "*B*" = 3.56; "*C*" = 2.43; "*D*" = 4.87; and "*E*" = 6.78; FCM μg/g).

Assessment of Behavior of Collared Anteater in Response to Food-Based Environmental Enrichment

Food-based environmental enrichment effects on behavior are shown in Table 4, with a statistically significant increase in *exploration* with respect to the other periods. Likewise, *foraging* behavior ($P = 0.002$; $T^2 = 15$) was higher during the enrichment period (pre-enrichment: $6.3 \pm 1.3\%$; enrichment: $11.9 \pm 1.6\%$; and post-enrichment: $7.3 \pm 1.9\%$). In addition, the statistical analysis of the category *natural active behaviors* (which includes *feeding*, *exploring*, *moving*, and *others*) showed significant differences among periods ($P = 0.03$; $T^2 = 6$). We detected a higher percentage during enrichment compared to post-enrichment ($14.6 \pm 3.0\%$ a, b; $18.9 \pm 3.6\%$ a; and $12.4 \pm 3.2\%$ b, respectively; different letters indicate significant differences), meanwhile statistical test did not revealed differences when comparing pre-enrichment versus enrichment or post-enrichment.

DISCUSSION

This study characterized the adrenocortical activity of zoo-housed collared anteater (*T. tetradactyla*) individuals, first exposed to pharmacological and then to environmental (food-based enrichment) stimuli. In addition, we also characterized behavioral response to food-based environmental enrichment.

Validation of Fecal Cortisol Metabolites Measurements in Collared Anteater

For the first time, we validated measurements of fecal cortisol metabolites in order to non-invasively monitor adrenocortical activity in the collared anteater. As expected, adrenocorticotropic hormone (ACTH) administration produced an increase in fecal cortisol metabolite concentrations. We were able to measure highest FCM concentrations between 24 and 72 hr after ACTH stimulation, generally associated with the first individual defecation after the injection. After that, hormone values returned to normal concentrations.

There are only few studies evaluating the effects of pharmacological factors on adrenocortical activity in the superorder Xenarthra. In the Southern three-banded armadillo (*Tolypeutes matacus*), Howell-Stephens et al. (2012) reported peak glucocorticoid values after ACTH challenge (in doses similar to those used in the present study) of up to

TABLE 3. Statistical analyses of fecal cortisol metabolites in collared anteater (*Tamandua tetradactyla*) in response to food-based environmental enrichment

	Periods			Comparisons *P* values	
Parameter	Pre-enrichment	Enrichment	Post-enrichment	Enrichment versus pre- and post-enrichment	Pre- versus post-enrichment
Mean (μg/g)	3.04 ± 0.68	2.98 ± 0.66	4.04 ± 0.90	0.0748	0.0095
Peaks (probability)	0.21 ± 0.07	0.08 ± 0.04	0.44 ± 0.10	0.0002	0.0047

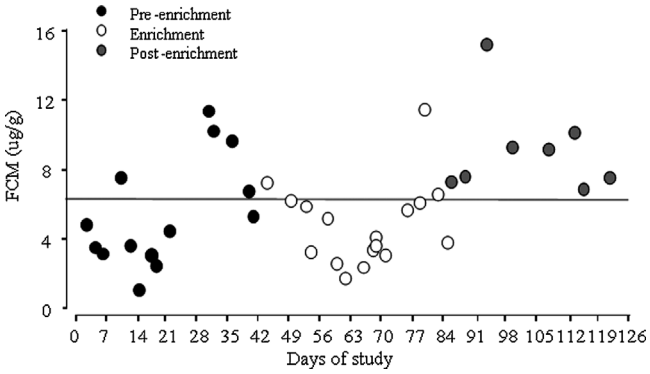

Fig. 3. Concentrations of fecal cortisol metabolites (FCMs) in a female individual (A) collared anteater (*Tamandua tetradactyla*), according to food-based environmental enrichment. A longitudinal analysis was conducted by collecting feces three times a week during 126 days. During the pre- and post-enrichment periods, the animals were fed with the habitual food, whereas in the enrichment period the diet was modified by: (1) adding novel food items, (2) changing the feeding schedule, (3) fractioning supply, (4) changing food presentation, and (5) extending food offer from 6 to 7 days of the week. The horizontal line corresponds to the animal-specific threshold to identify peak values (criterion defined in Materials and Methods section).

1.6 μg/g of dry feces, whereas in the collared anteater a mean peak of 6.7 μg/g was detected after ACTH challenge (5 IU/kg). However, qualitative comparisons among species are still not reliable due to the use of different immunoassays and experimental designs, as pointed out by Möstl et al. (2005).

Dexamethasone evidently inhibited the adrenocortical activity of collared anteater, showing a reduction in FCM values between 7 and 72 hr after challenge. Suppression of adrenocortical activity by dexamethasone was apparently homogeneous among individuals, as observed in other mammal species (Dehnhard et al., 2001; Schatz & Palme, 2001; Touma & Palme, 2005).

Assessment of Adrenocortical Activity and Behavior of the Collared Anteater in Response to Food-Based Environmental Enrichment

In the present study, we observed that zoo-housed collared anteaters responded positively (i.e., the food was consumed) to food-based environmental enrichment (changes in diet composition, frequency, time and presentation). We also observed that most of the novel food items offered were consumed (except worms *Zophoba morio*, pear, crookneck squash, melon, and mango). Noticeably, food-based enrichment affected neither body weight of individuals nor feces consistency or frequency of deposition; we consider that these are positive aspects supporting the application of this type of enrichment and the study strategy.

When we analyzed adrenocortical responses to food-based environmental enrichment we observed a tendency of decreased FCM mean values and a statistical reduction in the occurrence of FCM peaks. Therefore, collared anteaters' adrenocortical activity was affected by food-based environmental enrichment. However, with only five animals and considering that stress responses vary markedly between individuals, perhaps due to different life histories, we recognize that further studies are necessary to confirm that adrenocortical activity might be reduced by this type of enrichment. Taking into account that significant increases in glucocorticoids from baseline are generally associated with the effect of stressors, and hormone metabolites accumulated in feces are the result of a series of stress responses (Landys et al., 2006; Busch & Hayward, 2009; Dickens et al., 2010), we consider that the number of FCM peaks is a new approach to precisely monitor adrenocortical activity in this species. Moreover, considering that in mammals the mechanisms of stress responses are similar among species (Sapolsky et al., 2000); we are planning to test the usefulness of this variable for non-invasive monitoring adrenocortical activity and its possible direct correlation to stressful circumstances experienced by animals. In addition, when we analyzed behavioral response to food-based environmental enrichment we observed an increase in active natural behaviors during the enrichment period, in which the greatest change was observed for foraging (feeding and exploration) behaviors. Remarkably, pre- and post-enrichment periods exhibited differences in all variables studied, contrary to what was expected. It would be interesting to evaluate if changes observed when enrichment period finished (the highest adrenocortical activity and the reduction of active natural behaviors during post-enrichment) are related to possible

TABLE 4. Behavioral response of zoo-housed collared anteaters subjected to food-based environmental enrichment

	Behavioral response (%)			Statistical analysis	
Behavioral category	Pre-enrichment	Enrichment	Post-enrichment	T^2	*P*-value
Resting	68.0 ± 9.6 a, b	66.1 ± 8.4 a	74.0 ± 9.6 c	3.69	0.07
Feeding	2.1 ± 0.8	5.1 ± 1.0	3.9 ± 1.3	1.81	0.22
Exploring	4.2 ± 1.1 a	6.8 ± 1.1 c	3.4 ± 0.7 a, b	15.00	0.002
Moving	4.8 ± 1.4	4.8 ± 1.7	3.0 ± 1.0	2.15	0.18
Others	3.5 ± 1.1	2.2 ± 0.9	2.1 ± 0.7	0.76	0.50
Stereotyped	17.4 ± 7.7	15.0 ± 5.2	13.6 ± 7.2	1.94	0.21

We assessed individual behavior by performing behavioral observation days in weeks 1, 3, and 6 of each period (pre-enrichment, enrichment, and post-enrichment). On each observation day, we used the Instantaneous sampling method conducting five 30-min observation sessions for each animal, recording its behavior every 2 min. Different letters indicate significant differences within each behavioral category.

seasonal effects and/or stressful circumstances (removing food-based environmental enrichment) that would offer fewer opportunities to meet animals' needs.

Finally, considering changes in adrenocortical activity and behavior during the enrichment period, we think that welfare of collared anteater would be increased by food-based environmental enrichment.

CONCLUSIONS

1. We validated the use of fecal cortisol metabolites for monitoring adrenocortical activity in collared anteater by demonstrating expected changes in response to ACTH and dexamethasone test.
2. Food-based environmental enrichment affected adrenocortical activity of zoo-housed collared anteater. We observed a reduction in adrenocortical activity during the enrichment period.
3. Food-based environmental enrichment affected zoo-housed collared anteater activity pattern, increasing active natural behaviors during the enrichment period.

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